

DESCRIPTION

OXIDASE, POLYNUCLEOTIDE ENCODING SAME, AND USE THEREOF

5 Technical Field

[0001]

The present invention relates to an oxidase, a polynucleotide encoding the same, and a method for producing an α -amino acid compound, etc. using these.

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Background Art

[0002]

Conventionally, methionine, one of the α -amino acid compounds, has been used as a feed additive for animals. To produce the compound, acrolein is reacted with methyl mercaptan to produce 3-(methylthio)propionaldehyde, and this is further reacted with prussic acid, ammonia, and carbon dioxide to produce 5-(2-methyl-mercaptoethyl)-hydantoin (methionine hydantoin). Finally, this is hydrolyzed with an alkali to produce alkali metal methionate, and then neutralized by using an acid, for example, sulfuric acid or carbonic acid, to release methionine (see, for example, Patent Document 1, etc.).

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Prior Art Document

25 Patent Document

[0003]

Patent Document 1: JP 55-102557 A

Disclosure of the Invention

30 Problems to be Solved by the Invention

[0004]

The production method mentioned above uses prussic acid and acrolein as a C1- or C3-component, and the handling of these raw material compounds requires sufficient control and suitable facilities, etc. Therefore, development of a new method for producing an α -amino acid compound such as methionine is expected.

Means for Solving the Problems

[0005]

The present invention provides the followings:

Item 1. A polynucleotide encoding any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1,

3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative (hereinafter sometimes referred to as the present invented polynucleotide (A));

Item 2. The polynucleotide according to the above item 1, which has a base sequence represented by SEQ ID NO: 2, 4, 6, 15, 16, or 17;

Item 3. A polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide according to the above item 1 or 2 so that they can function;

Item 4. A recombinant vector comprising the polynucleotide according to any one of the above items 1 to 3 (hereinafter sometimes referred to as the present invented recombinant vector);

Item 5. The recombinant vector according to the above item 4, which further comprises a polynucleotide encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function;

Item 6. The recombinant vector according to the above item 5, wherein the amino acid sequence of the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is any one of the following amino acid sequences

(B1) to (B3):

(B1) an amino acid sequence represented by SEQ ID NO: 7,

(B2) an amino acid sequence i) having at least 90% sequence identity to an amino acid sequence represented by SEQ ID NO: 7, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative, or

(B3) an amino acid sequence i) represented by SEQ ID NO: 7 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative;

Item 7. A transformant in which the polynucleotide according to any one of the above item 1 to 3 or the recombinant vector according to the above item 4 is introduced into a host cell;

Item 8. The transformant according to the above item 7, wherein the host cell is a microorganism or E. coli;

Item 9. A transformant in which the recombinant vector according to the above item 5 or 6 is introduced into a host cell;

Item 10. The transformant according to the above item 9, wherein the host cell is a microorganism or E. coli;

Item 11. A transformant having the polynucleotide according to any one of the above items 1 to 3;

Item 12. A transformant having the followings:

i) a polynucleotide having a base sequence encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or a polynucleotide

in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function; and

ii) the polynucleotide according to any one of the above items 1 to 3;

Item 13. A method for producing a recombinant vector, which comprises the step of integrating the polynucleotide according to any one of the above items 1 to 3 into a vector which can be replicated in a host cell;

Item 14. A method for producing a transformant, which comprises the step of introducing the polynucleotide according to any one of the above items 1 to 3 or the recombinant vector according to any one of the above items 4 to 6 into a host cell;

Item 15. A protein having any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-

(methylthio)butyric acid derivative and convert the same

into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative (hereinafter sometimes referred to as the present invented protein (A));

Item 16. A method for producing an α -oxocarboxylic acid compound, which comprises the step of reacting a protein having any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

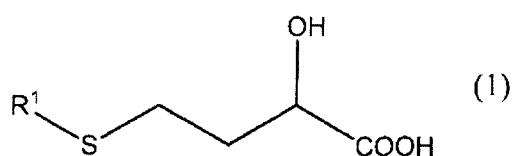
(A4) an amino acid sequence i) represented by SEQ ID NO: 1,

3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative;

with an α -hydroxycarboxylic acid compound (hereinafter sometimes referred to as the present inventive production method 1);

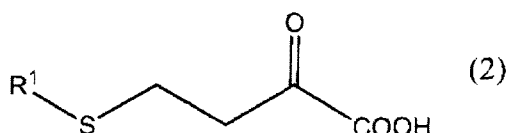
Item 17. The production method according to the above item 16, wherein the α -hydroxycarboxylic acid compound is a sulfur-containing α -hydroxycarboxylic acid compound, and the corresponding α -oxocarboxylic acid compound is a sulfur-containing α -oxocarboxylic acid compound;

Item 18. The production method according to the above item 17, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



wherein R^1 represents a hydrogen atom or an optionally substituted a C1-8 alkyl group;

and the sulfur-containing α -oxocarboxylic acid compound is a compound represented by formula (2):



wherein R^1 is the same as defined above;

Item 19. The production method according to any one of the above items 16 to 18., wherein the protein having any one of the amino acid sequences (A1) to (A4) is provided in a

reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

5 Item 20. The production method according to the above item 19, wherein the transformant is the transformant according to any one of the above items 7, 8, and 11;

Item 21. The production method according to any one of the above items 16 to 20, wherein the step is performed in the
10 presence of a protein having the ability to convert hydrogen peroxide into molecular oxygen;

Item 22. The production method according to the above item 21, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is a catalase;

15 Item 23. The production method according to the above item 21 or 22, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the
20 protein is introduced into a host cell or in a treated product thereof;

Item 24. A method for producing an L- α -amino acid compound, which comprises (1) the step of reacting a protein having any one of the following amino acid sequences (A1) to (A4):

25 (A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to
30 oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative

and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-

(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative;

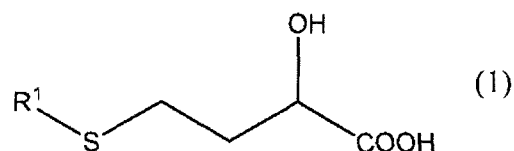
with an α -hydroxycarboxylic acid compound to obtain a corresponding α -oxocarboxylic acid compound, and

(2) the step of reacting a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound with the α -oxocarboxylic acid compound obtained in the step (1) to obtain a corresponding L- α -amino acid compound (hereinafter sometimes referred to as the present inventive production method 2);

Item 25. The production method according to the above item 24, wherein the α -hydroxycarboxylic acid compound is a sulfur-containing α -hydroxycarboxylic acid compound, the corresponding α -oxocarboxylic acid compound is a sulfur-containing α -oxocarboxylic acid compound, and the

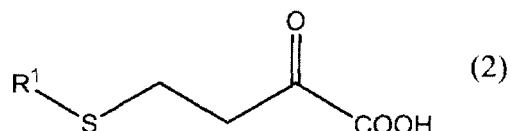
corresponding L- α -amino acid compound is a sulfur-containing L- α -amino acid compound;

Item 26. The production method according to the above item 25, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



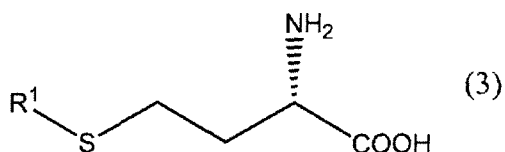
wherein R¹ represents a hydrogen atom or an optionally substituted a C1-8 alkyl group;

the sulfur-containing α -oxocarboxylic acid compound is a compound represented by formula (2):



wherein R¹ is the same as defined above;

and the sulfur-containing L- α -amino acid compound is a compound represented by formula (3):



wherein R¹ is the same as defined above;

Item 27. The production method according to any one of the above items 24 to 26, wherein the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is a leucine dehydrogenase;

Item 28. The production method according to the above item 27, wherein the leucine dehydrogenase is a leucine dehydrogenase derived from *Bacillus sphaericus*;

Item 29. The production method according to any one of the above items 24 to 27, wherein the amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is any one of the following amino acid sequences (B1) to (B3):

(B1) an amino acid sequence represented by SEQ ID NO: 7,

(B2) an amino acid sequence i) having at least 90% sequence identity to an amino acid sequence represented by SEQ ID NO:

7, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative, or

(B3) an amino acid sequence i) represented by SEQ ID NO: 7 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative;

Item 30. The production method according to any one of the above items 24 to 29, wherein the protein having any one of the amino acid sequences (A1) to (A4) is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

Item 31. The production method according to the above item 30, wherein the transformant is the transformant according to any one of the above items 7 to 12;

Item 32. The production method according to any one of the above items 24 to 31, wherein the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the

same into a corresponding L- α -amino acid compound is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

Item 33. The production method according to the above item 32, wherein the transformant is the transformant according to any one of the above items 9, 10, and 12;

Item 34. The production method according to any one of the above items 24 to 33, wherein the step (1) is performed in the presence of a protein having the ability to convert hydrogen peroxide into molecular oxygen;

Item 35. The production method according to the above item 34, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is a catalase;

Item 36. The production method according to the above item 34 or 35, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

Item 37. The production method according to any one of the above items 24 to 36, wherein the step (2) is performed in the presence of a protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form;

Item 38. The production method according to the above item 37, wherein the protein having the ability to convert an

oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form is a formate dehydrogenase;

Item 39. The production method according to the above item 37 or 38, wherein the protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

Item 40. The production method according to any one of the above items 24 to 39, wherein the step (1) and the step (2) are performed in one reaction system; and the like.

Effects of the Invention

[0006]

According to the present invention, it is possible to provide an oxidase, a polynucleotide encoding the same, a method for producing an α -amino acid compound such as methionine using these, and the like.

Mode for Carrying Out the Invention

[0007]

To express a target polynucleotide in a host cell, for example, a polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide so that they can function is prepared, and introduced into a host cell.

As used herein, "connected so that they can function"

means that when a host cell is transformed by introducing a target polynucleotide into the host cell, the polynucleotide is bound to a promoter so that it is expressed under control of the promoter.

5 [0008]

Examples of the promoter which can function in a microorganism include a lactose operon promoter of *E. coli*, a tryptophan operon promoter of *E. coli*, a T7 phage promoter, or a synthetic promoter which can function in *E. coli*, such as a *tac* promoter, a *trc* promoter, or a T7lac promoter.

[0009]

A recombinant vector can be prepared by integrating a target polynucleotide, or a polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide so that they can function into a vector. Examples of the vector to be used can include a vector which contains genetic information replicable in a host cell, can proliferate autonomously, can be isolated and purified from a host cell, and encodes a detectable marker. Examples of a vector available when the host cell is *E. coli* include pUC119 (manufactured by Takara Bio), pTV118N (manufactured by Takara Bio), pBluescriptII (manufactured by Toyobo), pCR2.1-TOPO (Invitrogen), pTrc99A (manufactured by GE Healthcare Japan), pKK22 3-3 (manufactured by GE Healthcare Japan), pET-22b (manufactured by Novagen), and pET-15b (manufactured by Novagen). When a vector containing a selection marker gene (e.g., an antibiotic resistance-imparting gene such as a kanamycin resistance gene and a neomycin resistance gene) is used as the vector, a

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transformant into which the vector is introduced can be selected using the phenotype, etc. of the selection marker gene as an index.

[0010]

5 A transformant to be used in the present invention can be produced by introducing a target polynucleotide, a polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide so that they can function, or a recombinant vector containing these
10 polynucleotides into a host cell.

 Examples of the host cell include a microorganism belonging to the genus *Escherichia*, *Bacillus*, *Corynebacterium*, *Staphylococcus*, *Streptomyces*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Rhodococcus*, or
15 *Aspergillus*.

 As a method for introducing a polynucleotide or a recombinant vector into a host cell, a usually used introduction method can be applied depending on a host cell to be used, and examples thereof include the calcium
20 chloride method mentioned in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN0-471-50338-X, and the like, and electroporation mentioned in "Methods in
25 Electroporation: Gene Pulser/*E. coli* Pulser System" Bio-Rad Laboratories, (1993), and the like.

[0011]

 A transformant into which a target polynucleotide or a recombinant vector, etc. is introduced can be selected by,
30 for example, using the phenotype of a selection marker gene

contained in a vector as mentioned above as an index.

The fact that the obtained transformant has the target polynucleotide can be confirmed by, for example, performing confirmation of a restriction enzyme site, analysis of a base sequence, Southern hybridization, Western hybridization, and the like, in accordance with a usual method mentioned in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, and the like.

[0012]

As a medium for culture of the transformant to be used in the present invention, for example, various media appropriately containing a carbon source, a nitrogen source, an organic salt, an inorganic salt, and the like which are usually used for culture of host cells of microorganisms, etc. can be used.

Examples of the carbon source include saccharides such as glucose, dextrin, and sucrose; sugar alcohols such as glycerol; organic acids such as fumaric acid, citric acid, and pyruvic acid; animal oil; vegetable oil; and molasses. The amount of these carbon sources added to a medium is usually within a range of about 0.1 to 30% (w/v) based on the amount of a culture solution.

Examples of the nitrogen source include natural organic sources of nitrogen such as meat extract, peptone, yeast extract, malt extract, soy flour, corn steep liquor, cottonseed flour, dried yeast, and casamino acid; amino acids; sodium salts of inorganic acids such as sodium nitrate; ammonium salts of inorganic acids such as ammonium chloride, ammonium sulfate, and ammonium phosphate; ammonium

salts of organic acids such as ammonium fumarate and ammonium citrate; and urea. Of these, ammonium salts of organic acids, natural organic sources of nitrogen, amino acids, and the like can also be often used as a carbon
5 source. The amount of these nitrogen sources added to a medium is usually within a range of about 0.1 to 30% (w/v) based on the amount of a culture solution.

Examples of the organic salt and the inorganic salt can include chlorides, sulfates, acetates, carbonates, and
10 phosphates of potassium, sodium, magnesium, iron, manganese, cobalt, zinc, and the like. Specific examples thereof include sodium chloride, potassium chloride, magnesium sulfate, ferrous sulfate, manganese sulfate, cobalt chloride, zinc sulfate, copper sulfate, sodium acetate,
15 calcium carbonate, monopotassium hydrogenphosphate, and dipotassium hydrogenphosphate. The amount of these organic salts and/or inorganic salts added to a medium is usually within a range of about 0.0001 to 5% (w/v) based on the amount of a culture solution.

20 In the case of a transformant into which a polynucleotide is introduced in which a promoter induced by allolactose such as a tac promoter, a trc promoter, a T7lac promoter, and a lac promoter is connected with a polynucleotide encoding a target protein so that they can
25 function, for example, a small amount of isopropyl thio- β -D-galactoside (IPTG) may be added to a medium as an inducer for inducing the production of the target protein. Also, in the case of culture of a transformant in which a polynucleotide in which a T7 phage promoter is connected
30 with a polynucleotide encoding a target protein so that they

can function is introduced into a lysogen of bacteriophage DE3 (λ DE3 lysogen) in which a T7 RNA polymerase gene is integrated under control of an acUV5 promoter, a small amount of IPTG may be added to a medium as an inducer for inducing the production of the target protein.

[0013]

Culture of the transformant can be performed in accordance with a method usually used for culture of host cells such as microorganisms, and examples thereof include liquid culture and solid culture such as test tube-shaking culture, reciprocal shaking culture, jar fermenter culture, and tank culture.

The culture temperature can be appropriately changed in a range so that the transformant can grow, and is usually about 15°C to about 40°C. The pH of the medium is preferably within a range of about 6 to about 8. The culture time varies depending on the culture condition, and is usually preferably about one day to about 5 days.

[0014]

As a method for purifying a target protein from a cultured product of a transformant producing the target protein having a polynucleotide encoding the target protein, for example, a transformant in which a polynucleotide encoding the target protein is introduced into a host cell, a usual method used for purification of proteins can be applied, and examples thereof can include the following methods:

Cells are collected by centrifugation, etc. from a cultured product of the transformant, and then the cells are disrupted by physical disruption such as sonication, Dyno-

Mill treatment, or French press treatment or by chemical disruption using surfactants or lytic enzymes such as lysozyme, etc. From the disruption liquid thus obtained, impurities are removed by centrifugation, membrane filter filtration, and the like to prepare a cell-free extract. The extract is fractionated by appropriately using a separation and purification method such as cation exchange chromatography, anion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, metal chelate chromatography, and affinity chromatography, and thereby the target protein can be purified.

Examples of a carrier to be used in the chromatography include an insoluble macromolecular carrier such as cellulose, dextrin, or agarose into which a carboxymethyl (CM) group, a diethylaminoethyl (DEAE) group, a phenyl group, or a butyl group is introduced. A commercial carrier-filled column can be used, and examples of the commercial carrier-filled column include Q-Sepharose FF (trade name, manufactured by GE Healthcare Japan), Phenyl-Sepharose HP (trade name, manufactured by GE Healthcare Japan), and TSK-gel G3000SW (trade name, manufactured by Tosoh Corporation).

When the target protein is a protein in which consecutive several residues of histidine are added to its amino-terminal or carboxy-terminal domain, the protein can be purified by using a metal chelate affinity column. When the target protein is produced as a protein fused with a glutathione S-transferase, the protein can be purified by using a glutathione S-transferase monoclonal antibody column.

[0015]

Examples of "treated product of transformant" as used herein include a freeze-dried transformant, an organic solvent-treated transformant, a dried transformant, a triturerated transformant, an autolysate of a transformant, a sonicate of a transformant, a transformant extract, and an alkali-treated product of a transformant. Examples of the transformant extract include a cell-free extract, a partially purified protein or a purified protein prepared from a transformant, and an immobilized product thereof. Examples of a method for obtaining the immobilized product include the carrier binding method (a method for adsorbing a target protein, etc. to an inorganic carrier such as a silica gel and a ceramic, cellulose, or an ion exchange resin, etc.) and the entrapment method (a method for trapping a target protein, etc. in a macromolecular meshwork such as polyacrylamide, a sulfur-containing polysaccharide gel (e.g., a carrageenan gel), an alginic acid gel, or an agar gel, etc.).

Taking account of industrial production using a transformant, rather than use of a living transformant, use of a treated product obtained by killing the transformant is preferable in terms of less limitation on a production facility. Examples of a method for killing a transformant include physical sterilization (heating, drying, freezing, light, sonication, filtration, electrification) and chemical sterilization (alkali, acid, halogen, an oxidizing agent, sulfur, boron, arsenic, metal, alcohol, phenol, amine, sulfide, ether, aldehyde, ketone, cyanogen, and an antibiotic). Generally, of these sterilization methods, it

is desirable to select a treatment method which does not inactivate the enzyme activity of the target protein as possible and has less effects on the reaction system such as residue and contamination.

5 [0016]

The present invented polynucleotide (A) encodes any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

10 (A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-

15 (methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein
20 having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the
25 ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative.

30 [0017]

The present invented protein (A) has any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-

(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative.

[0018]

A difference which is sometimes observed between an amino acid sequence encoded by the present invented polynucleotide (A) or an amino acid sequence of the present invented protein (A) and an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 is deletion, substitution, or

addition, etc. of some amino acids (hereinafter sometimes generally referred to as alteration of an amino acid). The "addition" includes not only addition of an amino acid to the end of a sequence but also insertion of an amino acid into a sequence. Examples of the alteration of an amino acid can include (a) deletion by intracellular processing of a protein having an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, (b) deletion, substitution, or addition of an amino acid as a result of a naturally occurring gene mutation due to the species difference or individual difference of an organism from which the protein is derived, or (c) deletion, substitution, or addition of an amino acid occurring due to a mutation of an artificially introduced gene, etc.

The number of amino acids to be altered is not limited as long as the number is within a range so that a protein having the above altered amino acid sequence can exert the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative. Examples of "plural amino acids" in the amino acid sequence (A4) encoded by the present invented polynucleotide (A) or the amino acid sequence (A4) of the present invented protein (A) include 2, 3, 4, 5, 6, 7, 10, 15, 20, 25, 30, 35, or 40 amino acids.

Examples of the substitution of an amino acid include conservative substitution to an amino acid having similar hydrophobicity, electric charge, pK, conformational characteristics, or the like. Specific examples of such substitution include substitution of (1) glycine, alanine; (2) valine, isoleucine, leucine; (3) aspartic acid, glutamic

acid, asparagine, glutamine, (4) serine, threonine; (5) lysine, arginine; (6) phenylalanine, tyrosine; and the like in the group.

Examples of the addition of an amino acid can include addition of about 20 residues of amino acid including about consecutive 6 residues of histidine to the amino terminus or carboxy terminus of an amino acid sequence.

Examples of a method for artificially altering an amino acid include a method in which a site-specific mutation is introduced into a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 and then this polynucleotide is expressed by a conventional method.

Examples of a method for introducing a site-specific mutation can include the methods by Olfert Landt et al.

(Gene 96 125-128 1990), Smith et al. (Genetic Engineering 3 1 Setlow, J. and Hollaender, A Plenum: New York), Vlasuk et al. (Experimental Manipulation of Gene Expression, Inouye, M.: Academic Press, New York), Hos. N. Hunt et al. (Gene 77 51 1989), and the like, and a method for using commercial kits such as Mutan-Express Km (manufactured by Takara Bio), TaKaRa La PCR in vitro Mutagenesis Kit (manufactured by Takara Bio), and QuickChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

Examples of the method for artificially altering an amino acid also include a method in which a mutation is randomly introduced into a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 and then this polynucleotide is expressed by a conventional method.

Examples of a method for randomly introducing a mutation

include a method in which PCR is performed using a

polynucleotide encoding any one of the above amino acid sequences as a template and using a primer pair which can amplify the full length of each polynucleotide, under a reaction condition in which the concentration of each of dATP, dTTP, dGTP, and dCTP added which is used as a substrate is changed from the normal concentration, or a reaction condition in which the concentration of Mg^{2+} , which accelerates the polymerase reaction, is increased compared with the normal concentration. Examples of such PCR method include the method mentioned in Method in Molecular Biology, (31), 1994, 97-112.

[0019]

"Sequence identity" means the identity between two amino acid sequences or base sequences. The "sequence identity" is determined by comparing two sequences aligned to an optimal state over all regions of sequences to be compared. In optimal alignment of amino acid sequences or base sequences to be compared, addition or deletion (e.g., gap, etc.) may be allowed. Such sequence identity can be calculated by using, for example, sequence analysis tools such as the BESTFIT program (Devereux et al. (1984) Nucleic Acids Research 12, p387-395) provided by UWGCG Package, PILEUP, and the BLAST algorithm (Altschul S.F. (1993) J Mol Evol 36:290-300; Altschul S.F. (1990) J Mol Biol 215:403-10). Sequence identity can also be calculated by using commercial sequence analysis software.

Examples of "at least 45% sequence identity" in the amino acid sequence (A2) encoded by the present invented polynucleotide (A) or the amino acid sequence (A2) of the present invented protein (A) include at least 45, 50, 55,

60, 65, 70, 75, 80, 85, 90, 95, 98, or 99% sequence identity.

[0020]

In an amino acid sequence encoded by the present
5 invented polynucleotide (A) or an amino acid sequence of the
present invented protein (A), "polynucleotide hybridized
under a stringent condition to a polynucleotide composed of
a sequence complementary to a base sequence represented by
SEQ ID NO: 2, 4, or 6" means a polynucleotide (1) which form
10 a hybrid by base pairing with a polynucleotide composed of a
sequence complementary to a base sequence represented by SEQ
ID NO: 2, 4, or 6 by being hybridized at 65°C under a high
ionic concentration [for example, 6XSSC (900 mM sodium
chloride and 90 mM sodium citrate)] and (2) in which the
15 hybrid is maintained even after being incubated at 65°C for
30 minutes under a low ionic concentration [for example, 0.1
X SSC (15 mM sodium chloride and 1.5 mM sodium citrate)] in
the Southern hybridization mentioned in, for example,
"Cloning and Sequence" (supervised by Itaru Watanabe, edited
20 by Masahiro Sugiura, 1989, published by Nosonbunka-sha),
etc.

Specific examples of the above polynucleotide include a
polynucleotide having a base sequence represented by SEQ ID
NO: 2, 4, 6, 15, 16, or 17, a polynucleotide having a base
25 sequence represented by SEQ ID NO: 2, 4, 6, 15, 16, or 17 in
which some bases are deleted, substituted, or added, or a
polynucleotide having a base sequence having at least 90%,
95%, 98%, or 99% sequence identity to a base sequence
represented by SEQ ID NO: 2, 4, 6, 15, 16, or 17.

[0021]

The present invented polynucleotide (A) may be a polynucleotide cloned from DNAs existing in the natural world, a polynucleotide into which deletion, substitution, or addition of some bases in a base sequence of this cloned polynucleotide is artificially introduced, or a chemically synthesized polynucleotide.

[0022]

The present invented polynucleotide (A) can be obtained from, for example, a microorganism having the ability to oxidize α -hydroxycarboxylic acid to corresponding α -oxocarboxylic acid, and specifically, a microorganism belonging to the genus *Achromobacter* such as an *Achromobacter denitrificans* ATCC55564 strain. These microorganisms may be separated naturally, or may be obtained through purchase from culture collection institutes.

Examples of the culture collection institutes from which such microorganisms can be obtained can include the following culture collection institutes.

[0023]

1. Institute for Fermentation, Osaka (IFO) Collection

At present, it is transferred to the NITE Biological Resource Center (NBRC). For obtaining microorganisms, it is only necessary to apply purchase to the NBRC. For purchase application, for example, it is only necessary to access the website of the NBRC.

2. American Type Culture Collection (ATCC)

Microorganisms can be obtained through the ATCC Business Group of Summit Pharmaceuticals International Corporation. For purchasing microorganisms, for example, it

is only necessary to access the website of the Group.
Microorganisms may be purchased directly from the ATCC.

3. Japan Collection of Microorganisms (JCM)

At present, it is transferred to the Japan Collection
of Microorganisms of RIKEN BioResource Center (RIKEN BRC).
For obtaining microorganisms, it is only necessary to apply
purchase to the institute, and, for example, to access
websites related to culture collection on the website of the
institute.

4. IAM Culture Collection

At present, among the IAM Culture Collection preserved
strains, bacteria, yeasts, filamentous fungi are transferred
to the RIKEN BRC-JCM, and microalgae are transferred to the
Microbial Culture Collection at the National Institute for
Environmental Studies (NIES). For obtaining microorganisms,
it is only necessary to apply purchase to these institutes,
and, for example, to access websites related to culture
collection on the websites of these institutes.

[0024]

The present invented polynucleotide (A) can be prepared
by, for example, the following procedures.

A DNA library is prepared from a microorganism
belonging to the genus *Achromobacter* such as *Achromobacter*
denitrificans, etc. in accordance with a usual genetic
engineering method (e.g., the method mentioned in "New Cell
Engineering Experimental Protocol" (edited by Department of
Oncology, Institute of Medical Science, the University of
Tokyo, Shujunsha Co., Ltd., 1993)). Then, by performing PCR
using the DNA library thus prepared as a template and using
an appropriate primer, a polynucleotide encoding an amino

acid sequence represented by SEQ ID NO: 1, 3, or 5, a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, or a polynucleotide having a base sequence represented by SEQ ID NO: 2, 4, or 6, etc. is amplified, and thereby the present invented polynucleotide (A) can be prepared.

A restriction enzyme recognition sequence, etc. may be added to the 5' end side, the 3' end side, or both of a primer used for the above PCR.

[0025]

For example, by performing PCR using the above DNA library as a template and using an oligonucleotide having a base sequence represented by SEQ ID NO: 9 and an oligonucleotide having a base sequence represented by SEQ ID NO: 10 as a primer, a polynucleotide composed of a base sequence represented by SEQ ID NO: 2 is amplified, and thereby the present invented polynucleotide (A) can be prepared.

By performing PCR using the above DNA library as a template and using an oligonucleotide having a base sequence represented by SEQ ID NO: 11 and an oligonucleotide having a base sequence represented by SEQ ID NO: 12 as a primer, a polynucleotide composed of a base sequence represented by SEQ ID NO: 4 can be amplified.

By performing PCR using the above DNA library as a template and using an oligonucleotide having a base sequence represented by SEQ ID NO: 13 and an oligonucleotide having a base sequence represented by SEQ ID NO: 14 as a primer, a polynucleotide composed of a base sequence represented by

SEQ ID NO: 6 can be amplified.

[0026]

Examples of a condition for the above PCR include a condition in which a reaction solution prepared by mixing 20 μ M each of 4 dNTPs, 15 pmol each of 2 oligonucleotide primers, 1.3 U of a Taq polymerase, and a DNA library as a template is incubated at 94°C for 2 minutes, and then an incubation cycle consisting of incubation at 94°C for 10 seconds, followed by 65°C for 30 seconds, followed by 72°C for 90 seconds is performed 10 times, subsequently an incubation cycle consisting of incubation at 94°C for 10 seconds, followed by 65°C for 30 seconds, followed by 72°C for one minute and 5 seconds is performed 20 times, and further the solution is maintained at 72°C for 7 minutes.

[0027]

Also by performing PCR using the above DNA library as a template and using an oligonucleotide having a partial base sequence selected from a base sequence encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 (e.g., an oligonucleotide composed of a base sequence of at least about 14 bases at the 5' end of a base sequence encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5) and an oligonucleotide of at least about 14 bases composed of a base sequence complementary to a base sequence near the DNA insertion site of the vector used for the DNA library construction as a primer, a polynucleotide having a base sequence encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, or a polynucleotide having a base sequence encoding an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are

deleted, substituted, or added, etc. is amplified, and thereby the present invented polynucleotide (A) can be prepared.

[0028]

5 The present invented polynucleotide (A) can also obtained by, for example, hybridizing, as a probe, DNA composed of a base sequence of at least about 15 bases having a partial base sequence selected from a base sequence encoding an amino acid sequence represented by SEQ ID NO: 1, 10 3, or 5 to a DNA library into which a vector derived from a microorganism or a phage is inserted under the condition mentioned below to detect DNA to which the probe specifically binds.

[0029]

15 Examples of a method for hybridizing a probe to chromosomal DNA or a DNA library include colony hybridization and plaque hybridization, and a method can be selected according to the type of the vector used for preparation of the library.

20 When a library to be used has been prepared by using a plasmid vector, it is better to use colony hybridization. Specifically, the DNA of the library is introduced into a host microorganism to obtain a transformant, and the transformant thus obtained is diluted, and then the dilution 25 is seeded on an agar medium and cultured until a colony appears.

 When a library to be used has been prepared by using a phage vector, it is better to use plaque hybridization. Specifically, a host microorganism and a phage of the 30 library are mixed under an infectible condition, and further

mixed with a soft agar medium, and then the mixture is seeded on an agar medium and cultured until a plaque appears.

[0030]

5 In any hybridization above, a membrane is placed on the above cultured agar medium, a transformant or a phage is adsorbed/transcribed on the membrane. After this membrane is treated with an alkali, it is neutralized, and then DNA is immobilized on the membrane. More specifically, for
10 example, in the case of plaque hybridization, a nitrocellulose membrane or a nylon membrane (e.g., Hybond-N+ (GE Healthcare Japan, trade mark)) is placed on the agar medium, and allowed to stand for about one minute to adsorb/transcribe a phage particle to the membrane. Next,
15 phage DNA is eluted on the membrane by immersing the membrane in an alkali solution (e.g., 1.5 M sodium chloride and 0.5 M sodium hydroxide) for about 3 minutes to dissolve the phage particle, and the membrane is immersed in a
20 neutralizing solution (e.g., 1.5 M sodium chloride and 0.5 M Tris-hydrochloric acid buffer pH 7.5) for about 5 minutes. Subsequently, the membrane is washed with a rinsing fluid (e.g., 0.3 M sodium chloride, 30 mM citric acid, and 0.2 M Tris-hydrochloric acid buffer pH 7.5) for about 5 minutes, and then, for example, the membrane is heated at about 80°C
25 for about 90 minutes to immobilize the phage DNA on the membrane.

[0031]

 Using the membrane prepared in this way, hybridization is performed using the above DNA as a probe. Hybridization
30 can be performed in accordance with, for example, the

description such as J. Sambrook, E.F. Frisch, T. Maniatis
"Molecular Cloning: A Laboratory Manual 2nd edition (1989)"
Cold Spring Harbor Laboratory Press.

5 DNA to be used as a probe may be labeled by a
radioisotope or labeled by a fluorochrome.

Examples of a method for labeling DNA to be used as a
probe by a radioisotope include a method in which PCR is
performed using DNA to be used as a probe as a template by
substituting dCTP in a PCR reaction solution by (α -³²P)dCTP
10 by using the Random Primer DNA Labeling Kit (manufactured by
Takara Bio), etc.

When DNA used as a probe is labeled by a fluorochrome,
for example, the ECL Direct Nucleic Acid Labeling and
Detection System (manufactured by GE Healthcare Japan), etc.
15 can be used.

[0032]

Hybridization can be performed, for example, as
follows. A prehybridization solution containing 450 to 900
mM sodium chloride and 45 to 90 mM sodium citrate,
20 containing sodium dodecyl sulfate (SDS) at a concentration
of 0.1 to 1.0% by weight, containing denatured non-specific
DNA at a concentration of 0 to 200 μ l/ml, and optionally
containing albumin, Ficoll, polyvinylpyrrolidone, and the
like at a concentration of 0 to 0.2% by weight each
25 (preferably, a prehybridization solution containing 900 mM
sodium chloride, 90 mM sodium citrate, 1.0% by weight of
SDS, and 100 μ l/ml of denatured calf-thymus DNA) is prepared
in the proportion within a range of 50 to 200 μ l based on 1
cm² of the membrane prepared as mentioned above, and the
30 membrane is immersed in the prehybridization solution and

incubated at 42 to 65°C for 1 to 4 hours.

Next, a solution prepared by mixing, for example, a hybridization solution containing 450 to 900 mM sodium chloride and 45 to 90 mM sodium citrate, containing SDS at a concentration of 0.1 to 1.0% by weight, containing denatured non-specific DNA at a concentration of 0 to 200 µg/ml, and optionally containing albumin, Ficoll, polyvinylpyrrolidone, and the like at a concentration of 0 to 0.2% by weight each (preferably, a hybridization solution containing 900 mM sodium chloride, 90 mM sodium citrate, 1.0% by weight of SDS, and 100 µg/ml of denatured calf-thymus DNA) with a probe obtained by preparation using the method mentioned above (the amount corresponds to 1.0×10^4 to 2.0×10^6 cpm based on 1 cm² of the membrane) is prepared in the proportion within a range of 50 to 200 µl based on 1 cm² of the membrane, and the membrane is immersed in the hybridization solution and incubated at 42 to 65°C for 12 to 20 hours.

After the hybridization, the membrane is removed, and washed with a rinsing fluid at 42 to 65°C containing 15 to 300 mM sodium chloride, 1.5 to 30 mM sodium citrate, 0.1 to 1.0% by weight of SDS, and the like (preferably, a rinsing fluid at 65°C containing 15 mM sodium chloride, 1.5 mM sodium citrate, and 1.0% by weight of SDS), etc. The washed membrane is gently washed with 2xSSC (300 mM sodium chloride and 30 mM sodium citrate), and then dried. By subjecting this membrane to, for example, autoradiography, etc. to detect the position of the probe on the membrane, a clone at the position on the membrane of DNA to be hybridized to the probe used is identified on the original agar medium, and fishing of this is performed to isolate a clone having the

DNA.

From a cultured cell obtained by culture of the clone thus obtained, the present invented polynucleotide (A) can be prepared.

5 [0033]

The present invented polynucleotide (A) can also be prepared by performing chemical synthesis of a nucleic acid having a target base sequence in accordance with a usual method such as, for example, the phosphite-triester method
10 (Hunkapiller, M. et al., Nature, 310, 105, 1984), based on its base sequence.

[0034]

The present invented polynucleotide (A) can also be prepared by selecting, as a codon encoding any one of the
15 above amino acid sequences (A1) to (A4), a codon so that the frequency of use of codon corresponds to that in E. coli to design a base sequence, and by chemically synthesizing a polynucleotide composed of the base sequence thus designed.

Specifically, for example, a codon corresponding to
20 each amino acid contained in an amino acid sequence represented by SEQ ID NO: 1, 3, or 5 is selected so that the frequency of use of codon is close to that in a microbial cell to be expressed (e.g., E. coli) to design a base sequence encoding a target amino acid sequence.

25 Information on the frequency of use of codon in E. coli, etc. can be obtained by, for example, using the DNA database well known for a person skilled in the art (GenBank, EMBL, DDBJ, and the like).

[0035]

30 Specific examples will be described below.

The number of respective amino acids contained in a target amino acid sequence is calculated. Codons to be used are assigned to the amino acids with the number calculated above so that the frequency is closest to the mean

5 appearance frequency of codon in a microbial cell in which a polynucleotide is expressed. The use order of each codon is assigned so that the same codon is not consecutive as possible. From an amino acid at the N-terminal side in

10 determined order, a codon is selected for each amino acid in the determined order, and is tentatively determined as a codon of its amino acid residue. By repeating these procedures, codons of all amino acids up to the C terminus are

tentatively determined, and finally a termination codon is placed. With respect to a base sequence composed of the

15 tentatively determined codons, the fact that a base sequence inhibiting the transcription of genes in a microbial cell and a base sequence recognized by restriction enzymes to be used in the subsequent operations do not exist is confirmed.

If such base sequence exists, the codon involved in this

20 base sequence is replaced by a codon used in other parts.

In such base sequence design, it is preferable to add a base sequence recognized by appropriate restriction enzymes to the 5' end side and the 3' end side for the subsequent operations.

25 Synthesis of a polynucleotide having a base sequence designed in this way can be performed by the long-chain DNA synthesis method using PCR (Cell Engineering Supplement, Plant Cell Engineering Series 7 "PCR Experimental Protocol for Plants", p95-100, supervised by Takumi Shimamoto and
30 Takuji Sasaki, Shujunsha Co., Ltd., published on July 1,

1997) (hereinafter this method is sometimes referred to as the assembly PCR method). In the method, DNA is synthesized using only a long synthetic oligonucleotide primer. A primer pair is synthesized so that the 3' end of each primer has a complementary strand or an overlap of about 10 bp to about 12 bp, and DNA synthesis is performed using mutual primers as a template. Examples of the full length of the primer can include about 60 mer to about 100 mer. Preferably, examples thereof include about 80 mer to about 100 mer.

By binding these oligonucleotide primers in order by PCR reaction, DNA having a target base sequence is obtained. The DNA thus obtained is introduced into a cloning vector and cloned in accordance with a conventional method. The base sequence of the clone thus obtained is confirmed with a DNA sequencer, and the fact that a polynucleotide having the target base sequence was obtained is confirmed. In this way, the present invented polynucleotide (A) can be obtained by, for example, artificially synthesizing a polynucleotide having a base sequence represented by SEQ ID NO: 15, 16, or 17 of the present invention.

[0036]

The polynucleotide prepared as mentioned above can be cloned into a vector in accordance with the method mentioned in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN0-471-50338-X, and the like.

The base sequence of the polynucleotide prepared as mentioned above can be analyzed by the dideoxy terminator

method, etc. mentioned in F.Sanger, S .Nicklen, A.R.
Coulson, Proceeding of Natural Academy of Science U.S.A.
(1977) 74: 5463-5467, etc. For sample preparation for base
sequence analysis, for example, a commercial reagent such as
5 ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit
by PerkinElmer Inc. may be used.

[0037]

The fact that the polynucleotide prepared as mentioned
above encodes an amino acid sequence of a protein having the
10 ability to oxidize α -hydroxycarboxylic acid (e.g., 2-
hydroxy-4-(methylthio)butyric acid) and convert the same
into corresponding α -oxocarboxylic acid (e.g., 2-oxo-4-
(methylthio)butyric acid) can be confirmed by, for example,
the following procedures.

15 The polynucleotide obtained as mentioned above is
inserted into a vector so that the polynucleotide is
connected downstream of a promoter which can function in a
host cell, and the recombinant vector thus obtained is
introduced into a host cell to obtain a transformant. A
20 cultured product of the transformant thus obtained is
reacted with α -hydroxycarboxylic acid (e.g., sulfur-
containing α -hydroxycarboxylic acid, more specifically, 2-
hydroxy-4-(methylthio)butyric acid). By analyzing the
amount of corresponding α -oxocarboxylic acid in the reaction
25 product (e.g., sulfur-containing α -oxocarboxylic acid, more
specifically, 2-oxo-4-(methylthio)butyric acid), the fact
that the polynucleotide thus obtained encodes an amino acid
sequence of a protein having target ability can be
confirmed.

30 [0038]

To express the present invented polynucleotide (A) in a host cell, for example, a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function is prepared, and introduced into a host cell.

Examples of the promoter which can function in a microorganism include a synthetic promoter which can function in *E. coli* as mentioned above. A promoter which controls the expression of the present invented polynucleotide (A) in *Achromobacter denitrificans* may be used.

[0039]

The present invented recombinant vector can be prepared by integrating the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function into a vector.

[0040]

The present invented recombinant vector can also include the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function, as well as a polynucleotide encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound (hereinafter sometimes referred to as the present invented protein (B)), or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function.

[0041]

Examples of the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound include an amino acid dehydrogenase and an aminotransferase. Specific examples of the amino acid dehydrogenase can include an alanine dehydrogenase, a glutamic acid dehydrogenase, a leucine dehydrogenase, and a phenylalanine dehydrogenase, and preferably a leucine dehydrogenase.

More specific examples of the above protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound can include a leucine dehydrogenase derived from a *Bacillus sphaericus* IF03525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic 23 (2003) 239-247, and a protein having an amino acid sequence of the leucine dehydrogenase in which the 113th alanine is converted to glycine.

An amino acid sequence represented by SEQ ID NO: 39 is an amino acid sequence of a leucine dehydrogenase derived from a *Bacillus sphaericus* IF03525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic 23 (2003) 239-247.

An amino acid sequence represented by SEQ ID NO: 7 is an amino acid sequence represented by SEQ ID NO: 39 in which the 113th alanine is converted to glycine.

[0042]

Specific examples of the amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -

amino acid compound can also include any one of the following amino acid sequences (B1) to (B3):

(B1) an amino acid sequence represented by SEQ ID NO: 7,

(B2) an amino acid sequence i) represented by SEQ ID NO: 7

5 and having at least 90% sequence identity, and ii) of a

protein having the ability to aminate a 2-oxo-4-

(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative, or

(B3) an amino acid sequence i) represented by SEQ ID NO: 7

10 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative.

[0043]

15 A difference which is sometimes observed between the amino acid sequence of the present invented protein (B) and an amino acid sequence represented by SEQ ID NO: 7 is deletion, substitution, or addition, etc. of some amino acids. The "addition" includes not only addition of an
20 amino acid to the end of a sequence but also insertion of an amino acid into a sequence. Examples of the alteration of an amino acid can include (a) deletion by intracellular processing of a protein having an amino acid sequence represented by SEQ ID NO: 7, (b) deletion, substitution, or
25 addition of an amino acid as a result of a naturally occurring gene mutation due to the species difference or individual difference of an organism from which the protein is derived, or (c) deletion, substitution, or addition of an amino acid occurring due to a mutation of an artificially
30 introduced gene, etc.

The number of amino acids to be altered is not limited as long as the number is within a range so that a protein having the above altered amino acid sequence can exert the ability to aminate a 2-oxo-4-(methylthio)butyric acid

5 derivative and convert the same into a corresponding L-methionine derivative. Examples of "plural amino acids" in the above amino acid sequence (B3) of the present invented protein (B) include 2, 3, 4, 5, 6, 7, 10, 15, 18, 20, 25, 30, 35, 36, or 40 amino acids.

10 Examples of the substitution of an amino acid include conservative substitution to an amino acid having similar hydrophobicity, electric charge, pK, conformational characteristics, or the like. Specific examples of such substitution include substitution of (1) glycine, alanine;
15 (2) valine, isoleucine, leucine; (3) aspartic acid, glutamic acid, asparagine, glutamine, (4) serine, threonine; (5) lysine, arginine; (6) phenylalanine, tyrosine; and the like in the group.

20 Examples of the addition of an amino acid can include addition of about 20 residues of amino acid including about consecutive 6 residues of histidine to the amino terminus or carboxy terminus of an amino acid sequence.

25 Examples of a method for artificially altering an amino acid include a method in which a site-specific mutation is introduced into a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 7 and then this polynucleotide is expressed by a conventional method.

30 Examples of the method for artificially altering an amino acid also include a method in which a mutation is randomly introduced into a polynucleotide encoding an amino

acid sequence represented by SEQ ID NO: 7 and then this polynucleotide is expressed by a conventional method.

Examples of "at least 90% sequence identity" in the above amino acid sequence (B2) of the present invented protein (B) include at least 90, 95, 98, or 99% sequence identity.

[0044]

A polynucleotide encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound (hereinafter sometimes referred to as the present polynucleotide (B)) can be obtained from, for example, a microorganism having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, for example, a microorganism belonging to the genus *Bacillus* such as a *Bacillus sphaericus* IF03525 strain.

[0045]

A DNA library is prepared from a microorganism belonging to the genus *Bacillus* such as a *Bacillus sphaericus* IF03525 strain, etc. in accordance with a usual genetic engineering method. Then, by performing PCR using the DNA library thus prepared as a template and using an appropriate primer, a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 39, or a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 39 in which one or plural amino acids are deleted, substituted, or added, etc. is amplified, and thereby the present polynucleotide (B) can be prepared.

For example, as a primer for amplification of a

polynucleotide having a base sequence represented by SEQ ID NO: 40 encoding an amino acid sequence represented by SEQ ID NO: 39, an oligonucleotide having a base sequence represented by SEQ ID NO: 18 and an oligonucleotide having a base sequence represented by SEQ ID NO: 19 are synthesized. As a primer for mutation introduction for converting the 113th alanine in an amino acid sequence represented by SEQ ID NO: 39 to glycine, an oligonucleotide having a base sequence represented by SEQ ID NO: 20 and an oligonucleotide having a base sequence represented by SEQ ID NO: 21 are synthesized.

By performing PCR using the above DNA library as a template and using an oligonucleotide having a base sequence represented by SEQ ID NO: 18 and an oligonucleotide having a base sequence represented by SEQ ID NO: 19 as a primer, a polynucleotide having a base sequence represented by SEQ ID NO: 40 is amplified. The polynucleotide thus amplified is integrated into a vector to obtain a recombinant vector containing a polynucleotide having a base sequence represented by SEQ ID NO: 40. PCR is performed using the recombinant vector thus obtained as a template and using an oligonucleotide having a base sequence represented by SEQ ID NO: 20 and an oligonucleotide having a base sequence represented by SEQ ID NO: 21 as a primer, and the PCR product thus obtained is processed with Dpn I, and then introduced into E. coli. The base sequence of the recombinant vector of the transformant thus obtained is analyzed to obtain a polynucleotide encoding an amino acid sequence into which the target amino acid mutation is introduced, namely an amino acid sequence represented by SEQ

ID NO: 39 in which the 113th alanine is substituted by glycine (an amino acid sequence represented by SEQ ID NO: 7). Examples of a base sequence encoding an amino acid sequence represented by SEQ ID NO: 7 include a base sequence represented by SEQ ID NO: 8.

[0046]

The present polynucleotide (B) can also be prepared by performing chemical synthesis of a nucleic acid having a target base sequence in accordance with a usual method such as, for example, the phosphite-triester method (Hunkapiller, M. et al., Nature, 310, 105, 1984), based on its base sequence.

The present polynucleotide (B) can also be prepared by selecting, as a codon encoding any one of the above amino acid sequences (B1) to (B3), a codon so that the frequency of use of codon corresponds to that in E. coli to design a base sequence, and by chemically synthesizing a polynucleotide composed of the base sequence thus designed.

[0047]

To express the present polynucleotide (B) in a host cell, for example, a polynucleotide in which a promoter which can function in a host cell is connected with the present polynucleotide (B) so that they can function is prepared, and introduced into a host cell.

Examples of the promoter which can function in a microorganism include a synthetic promoter which can function in E. coli as mentioned above. A promoter which controls the expression of the present polynucleotide (B) in a microorganism belonging to the genus Bacillus such as Bacillus sphaericus may be used.

[0048]

The fact that the polynucleotide prepared as mentioned above encodes an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound (e.g.,
5 2-oxo-4-(methylthio)butyric acid) and convert the same into a corresponding L- α -amino acid compound (e.g., L-methionine) can be confirmed by, for example, the following procedures.

The polynucleotide obtained as mentioned above is inserted into a vector so that the polynucleotide is
10 connected downstream of a promoter which can function in a host cell, and the recombinant vector thus obtained is introduced into a host cell to obtain a transformant. A cultured product of the transformant thus obtained is reacted with α -oxocarboxylic acid (e.g., sulfur-containing
15 α -oxocarboxylic acid, more specifically, 2-oxo-4-(methylthio)butyric acid). By analyzing the amount of corresponding L- α -amino acid in the reaction product (e.g., sulfur-containing L- α -amino acid, more specifically, L-methionine), the fact that the polynucleotide thus obtained
20 encodes an amino acid sequence of a protein having target ability can be confirmed.

[0049]

A transformant can be produced by introducing the present invented polynucleotide (A), the present
25 polynucleotide (B), or a recombinant vector containing any one or both of these polynucleotides into a host cell.

Examples of the host cell include a microorganism belonging to the genus *Escherichia*, *Bacillus*, *Corynebacterium*, *Staphylococcus*, *Streptomyces*,
30 *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Rhodococcus*, or

Aspergillus.

[0050]

As mentioned above, by introducing the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function into a host cell, a transformant having the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function can be obtained. Examples of the transformant can also include a transformant in which the above exogenous polynucleotide is introduced into a chromosome of a host cell, namely a transformant having the above exogenous polynucleotide on a chromosome.

[0051]

The transformant having the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function can also further have the present polynucleotide (B), or a polynucleotide in which a promoter which can function in a host cell is connected with the present polynucleotide (B) so that they can function.

As mentioned above, by introducing:

i) the present polynucleotide (B), or a polynucleotide in which a promoter which can function in a host cell is connected with the present polynucleotide (B) so that they can function; and

ii) the present invented polynucleotide (A), or a

polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function; into a host cell, a transformant having:

- 5 i) the present polynucleotide (B), or a polynucleotide in which a promoter which can function in a host cell is connected with the present polynucleotide (B) so that they can function; and
- 10 ii) the present invented polynucleotide (A), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (A) so that they can function; can be obtained.

The above i) polynucleotide and ii) polynucleotide may
15 be separately integrated into a different vector and introduced into a host cell, or may be integrated into the same vector and introduced into a host cell. When both polynucleotides are integrated into a single vector, for example, the polynucleotides may be integrated into a vector
20 by linking a region involved in the expression control such as a promoter and a terminator to each of both polynucleotides, or the polynucleotides may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that both polynucleotides are
25 expressed. Any one or both of the above i) polynucleotide and ii) polynucleotide may be on a chromosome of a host cell.

[0052]

The present invented protein (A) can be produced by,
30 for example, culturing a transformant having the present

invented polynucleotide (A) to express the present invented protein (A).

The present invented protein (B) can be produced by, for example, culturing a transformant having the present polynucleotide (B) to express the present invented protein (B).

As a method for purifying the present invented protein (A) or the present invented protein (B) from a cultured product of a transformant having the present invented polynucleotide (A) or the present polynucleotide (B), a usual method used for purification of proteins can be applied.

A fraction containing the present invented protein (A) can be selected by, for example, using the ability to oxidize 2-hydroxy-4-(methylthio)butyric acid and preferentially produce 2-oxo-4-(methylthio)butyric acid as an index.

A fraction containing the present invented protein (B) can be selected by, for example, using the ability to aminate 2-oxo-4-(methylthio)butyric acid and preferentially produce L-methionine as an index.

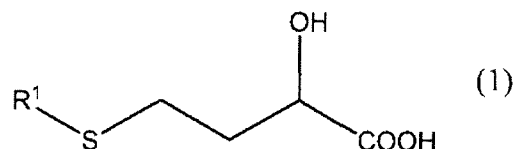
[0053]

The present invented production method 1 is a method for producing an α -oxocarboxylic acid compound, which includes the step of reacting the present invented protein (A) with an α -hydroxycarboxylic acid compound.

Examples of the above α -hydroxycarboxylic acid compound can include a sulfur-containing α -hydroxycarboxylic acid compound, and examples of the corresponding α -oxocarboxylic acid compound can include a sulfur-containing α -

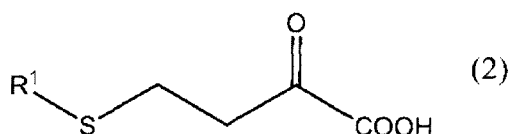
oxocarboxylic acid compound.

Examples of the above sulfur-containing α -hydroxycarboxylic acid compound include sulfur-containing α -hydroxycarboxylic acid represented by formula (1):



wherein R^1 represents a hydrogen atom or an optionally substituted a C1-8 alkyl group.

10 Examples of a sulfur-containing α -oxocarboxylic acid compound obtained by reacting the above sulfur-containing α -hydroxycarboxylic acid represented by formula (1) with the present invented protein (A) include sulfur-containing α -oxocarboxylic acid represented by formula (2):



wherein R^1 is the same as defined above.

15 [0054]

20 In the sulfur-containing α -hydroxycarboxylic acid represented by formula (1) and the sulfur-containing α -oxocarboxylic acid represented by formula (2), examples of a C1-8 alkyl group in the optionally substituted a C1-8 alkyl group represented by R^1 include a methyl group, an ethyl group, a propyl group, an isopropyl group, a butyl group, a pentyl group, a hexyl group, a heptyl group, and an octyl group.

25 Specific examples of the sulfur-containing α -hydroxycarboxylic acid represented by formula (1) can include 2-hydroxy-4-(methylthio)butyric acid, 2-hydroxy-4-

(ethylthio)butyric acid, 2-hydroxy-4-(propylthio)butyric acid, 2-hydroxy-4-(butylthio)butyric acid, 2-hydroxy-4-(pentylthio)butyric acid, 2-hydroxy-4-(hexylthio)butyric acid, 2-hydroxy-4-(heptylthio)butyric acid, and 2-hydroxy-4-(octylthio)butyric acid.

Specific examples of the sulfur-containing α -oxocarboxylic acid represented by formula (2) can include 2-oxo-4-(methylthio)butyric acid, 2-oxo-4-(ethylthio)butyric acid, 2-oxo-4-(propylthio)butyric acid, 2-oxo-4-(butylthio)butyric acid, 2-oxo-4-(pentylthio)butyric acid, 2-oxo-4-(hexylthio)butyric acid, 2-oxo-4-(heptylthio)butyric acid, and 2-oxo-4-(octylthio)butyric acid.

[0055]

As the optionally substituted a C1-8 alkyl group represented by R^1 , a methyl group is preferable, and as the sulfur-containing α -hydroxycarboxylic acid represented by formula (1), 2-hydroxy-4-(methylthio)butyric acid is preferably exemplified.

In the present invented production method 1, when 2-hydroxy-4-(methylthio)butyric acid is used as a substrate, 2-oxo-4-(methylthio)butyric acid is obtained.

[0056]

In the present invented production method 1, the present invented protein (A) can be provided to a reaction system for reaction with an α -hydroxycarboxylic acid compound, in various forms. The present invented protein (A) may be provided to a reaction system in the present invented production method 1 in the form of a purified protein, or may be provided to the reaction system in the form in which the protein is included in a microorganism

producing the protein or in a treated product of the microorganism. "Treated product of microorganism" means that prepared in a similar manner to the above mentioned "treated product of transformant". The present invented protein (A) may be provided to the above reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

To react the present invented protein (A) with an α -hydroxycarboxylic acid compound, specifically, for example, the present invented protein (A), an immobilized product of the present invented protein (A), a cultured product of a transformant producing the present invented protein (A) in which a polynucleotide encoding the present invented protein (A) is introduced into a host cell, or a treated product of the transformant can be provided to a reaction system in the present invented production method 1.

[0057]

The present invented production method 1 is usually performed in the presence of water. Water used in this case may be a buffered aqueous solution. Examples of a buffer used for the buffered aqueous solution include tris(hydroxymethyl)aminomethane, alkali metal phosphates such as sodium phosphate or potassium phosphate, alkali metal acetates such as sodium acetate or potassium acetate, or mixtures thereof.

[0058]

In the present invented production method 1, in addition to water, an organic solvent can also coexist in a reaction system. Examples of the organic solvent to be used

include ethers such as t-butyl methyl ether, diisopropyl ether, and tetrahydrofuran; esters such as ethyl formate, ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, and butyl propionate; hydrocarbons such as toluene, hexane, cyclohexane, heptane, and isooctane; alcohols such as methanol, ethanol, 2-propanol, butanol, t-butyl alcohol; organic sulfur compounds such as dimethyl sulfoxide; ketones such as acetone; nitriles such as acetonitrile; and mixtures thereof.

[0059]

In the present invented production method 1, as the oxidation reaction of an α -hydroxycarboxylic acid compound (e.g., sulfur-containing α -hydroxycarboxylic acid such as 2-hydroxy-4-(methylthio)butyric acid) proceeds, oxygen in the reaction solution is consumed and converted into hydrogen peroxide. Since hydrogen peroxide occurred as a result of the conversion can return to the original molecular oxygen by being reacted with a protein having the ability to convert hydrogen peroxide into molecular oxygen, a protein having the ability to convert hydrogen peroxide into molecular oxygen may further exist in a reaction system in the above method.

Examples of the protein having the ability to convert hydrogen peroxide into molecular oxygen include a catalase. Examples of the catalase can include a catalase of E. coli, more specifically, a catalase having an amino acid sequence represented by SEQ ID NO: 41.

[0060]

A protein having the ability to convert hydrogen peroxide into molecular oxygen may be provided to a reaction

system in the present invented production method 1 in the form of a purified protein or an immobilized product thereof, or may be provided to the reaction system in the form in which the protein is included in a microorganism

5 producing the protein or in a treated product of the microorganism. "Treated product of microorganism" means that prepared in a similar manner to the above mentioned "treated product of transformant". A transformant in which a polynucleotide encoding a protein having the ability to
10 convert hydrogen peroxide into molecular oxygen is introduced into a host cell or a treated product thereof may be provided to the above reaction system. Examples of a base sequence encoding a catalase having an amino acid sequence represented by SEQ ID NO: 41 can include a base
15 sequence represented by SEQ ID NO: 42.

[0061]

In the present invented production method 1, a transformant in which a polynucleotide encoding the present invented protein (A) is introduced into a host cell or a
20 treated product thereof, and a transformant in which a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen is introduced into a host cell or a treated product thereof may be provided to a reaction system for reaction with an
25 α -hydroxycarboxylic acid compound.

In the present invented production method 1, a transformant in which both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into
30 molecular oxygen are introduced in the same host cell or a

treated product thereof may be provided to the reaction system. A polynucleotide encoding the present invented protein (A) and a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen may be separately integrated into a different vector and introduced into a host cell, or may be integrated into the same vector and introduced into a host cell. When both polynucleotides are integrated into a single vector, for example, the polynucleotides may be integrated into a vector by linking a region involved in the expression control such as a promoter and a terminator to each of both polynucleotides, or the polynucleotides may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that both polynucleotides are expressed. Any one or both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen may be on a chromosome of a host cell.

[0062]

A reaction in the present invented production method 1 is performed by, for example, mixing water, an α -hydroxycarboxylic acid compound (e.g., a sulfur-containing α -hydroxycarboxylic acid compound such as 2-hydroxy-4-(methylthio)butyric acid), the present invented protein (A) or a transformant producing it or a treated product thereof, and further, as needed, a reaction solution containing an organic solvent, a catalase, and the like by stirring, shaking, and the like.

[0063]

The pH at the time of reaction in the above method can

be appropriately selected, and is usually within a range of about 3 to about 10. The reaction temperature can be appropriately selected, and is usually within a range of about 0°C to about 60°C in terms of the stability and the reaction rate of raw materials and products.

[0064]

The end point of the reaction can be determined by, for example, analyzing the amount of an α -hydroxycarboxylic acid compound (e.g., a sulfur-containing α -hydroxycarboxylic acid compound such as 2-hydroxy-4-(methylthio)butyric acid) in the reaction solution by liquid chromatography, etc.

The reaction time can be appropriately selected, and is usually within a range of about 0.5 hour to about 10 days.

[0065]

Recovery of α -oxocarboxylic acid (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-(methylthio)butyric acid) from the reaction solution may be performed by a generally known arbitrary method.

Example thereof include a method for purifying a target compound by performing post-treatment operations of the reaction solution such as extraction with an organic solvent and concentration in combination with column chromatography, distillation, or the like as needed.

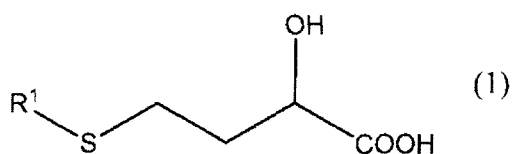
[0066]

The present invented production method 2 is a method for producing an L- α -amino acid compound, which includes: (1) the step of reacting the present invented protein (A) with an α -hydroxycarboxylic acid compound to obtain a corresponding α -oxocarboxylic acid compound, and (2) the step of reacting a protein having the ability to

aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound (the present invented protein (B)) with the α -oxocarboxylic acid compound obtained in the step (1) to obtain a corresponding L- α -amino acid compound.

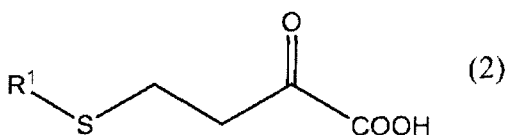
[0067]

Examples of the above α -hydroxycarboxylic acid include sulfur-containing α -hydroxycarboxylic acid represented by formula (1):



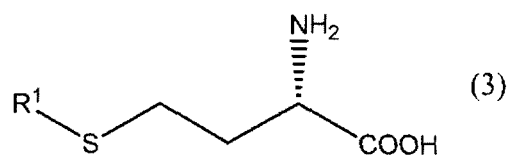
wherein R^1 represents a hydrogen atom or an optionally substituted C1-8 alkyl group.

Examples of a sulfur-containing α -oxocarboxylic acid compound obtained by reacting the above sulfur-containing α -hydroxycarboxylic acid represented by formula (1) with the present invented protein (A) include sulfur-containing α -oxocarboxylic acid represented by formula (2):



wherein R^1 is the same as defined above.

20 Examples of a sulfur-containing L- α -amino acid compound obtained by reacting the above sulfur-containing α -oxocarboxylic acid represented by formula (2) with the present invented protein (B) include sulfur-containing L- α -amino acid represented by formula (3):



[0068]

In the sulfur-containing α -hydroxycarboxylic acid represented by formula (1), the sulfur-containing α -oxocarboxylic acid represented by formula (2), and the sulfur-containing L- α -amino acid represented by formula (3), examples of a C1-8 alkyl group in the optionally substituted C1-8 alkyl group represented by R^1 include a methyl group, an ethyl group, a propyl group, an isopropyl group, a butyl group, a pentyl group, a hexyl group, a heptyl group, and an octyl group.

Specific examples of the sulfur-containing α -hydroxycarboxylic acid represented by formula (1) can include 2-hydroxy-4-(methylthio)butyric acid, 2-hydroxy-4-(ethylthio)butyric acid, 2-hydroxy-4-(propylthio)butyric acid, 2-hydroxy-4-(butylthio)butyric acid, 2-hydroxy-4-(pentylthio)butyric acid, 2-hydroxy-4-(hexylthio)butyric acid, 2-hydroxy-4-(heptylthio)butyric acid, and 2-hydroxy-4-(octylthio)butyric acid.

Specific examples of the sulfur-containing α -oxocarboxylic acid represented by formula (2) can include 2-oxo-4-(methylthio)butyric acid, 2-oxo-4-(ethylthio)butyric acid, 2-oxo-4-(propylthio)butyric acid, 2-oxo-4-(butylthio)butyric acid, 2-oxo-4-(pentylthio)butyric acid, 2-oxo-4-(hexylthio)butyric acid, 2-oxo-4-(heptylthio)butyric acid, and 2-oxo-4-(octylthio)butyric acid.

Specific examples of the sulfur-containing L- α -amino acid represented by formula (3) can include 2-amino-4-

(methylthio)butyric acid, 2-amino-4-(ethylthio)butyric acid, 2-amino-4-(propylthio)butyric acid, 2-amino-4-(butylthio)butyric acid, 2-amino-4-(pentylthio)butyric acid, 2-amino-4-(hexylthio)butyric acid, 2-amino-4-(heptylthio)butyric acid, and 2-amino-4-(octylthio)butyric acid.

[0069]

As the optionally substituted C1-8 alkyl group represented by R^1 , a methyl group is preferable, and as the sulfur-containing α -hydroxycarboxylic acid represented by formula (1), 2-hydroxy-4-(methylthio)butyric acid is preferably exemplified.

In the step (1) of the present invented production method 2, when 2-hydroxy-4-(methylthio)butyric acid is used as a substrate, 2-oxo-4-(methylthio)butyric acid is obtained in the step (1) and L-methionine is obtained in the step (2).

[0070]

The step (1) of the present invented production method 2 can be performed in a similar manner to the present invented production method 1.

An α -oxocarboxylic acid (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-(methylthio)butyric acid) obtained in the step (1) is purified or partially purified from the reaction solution, and then can be subjected to the step (2). By adding the reaction solution in the step (1) to the step (2), an α -oxocarboxylic acid (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-(methylthio)butyric acid) obtained in the step (1) can also

be subjected to the step (2).

[0071]

In the step (2) of the present invented production method 2, the present invented protein (B) can be provided to a reaction system for reaction with an α -oxocarboxylic acid compound, in various forms. The present invented protein (B) may be provided to a reaction system in the above step (2) in the form of a purified protein, or may be provided to the reaction system in the form in which the protein is included in a microorganism producing the protein or in a treated product of the microorganism. "Treated product of microorganism" means that prepared in a similar manner to the above mentioned "treated product of transformant". The present invented protein (B) may be provided to the above reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

To react the present invented protein (B) with an α -oxocarboxylic acid compound, specifically, for example, the present invented protein (B), an immobilized product of the present invented protein (B), a cultured product of a transformant producing the present invented protein (B) in which a polynucleotide encoding the present invented protein (B) is introduced into a host cell, or a treated product of the transformant can be provided to a reaction system in the above step (2).

[0072]

The step (2) of the present invented production method 2 is usually performed in the presence of water, an ammonium

ion, and a coenzyme.

Water used in this case may be a buffered aqueous solution. Examples of a buffer used for the buffered aqueous solution include tris(hydroxymethyl)aminomethane, alkali metal phosphates such as sodium phosphate or potassium phosphate, alkali metal acetates such as sodium acetate or potassium acetate, or mixtures thereof.

In the step (2) of the present invented production method 2, in addition to water, an organic solvent can also coexist in a reaction system. Examples of the organic solvent to be used include ethers such as t-butyl methyl ether, diisopropyl ether, and tetrahydrofuran; esters such as ethyl formate, ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, and butyl propionate; hydrocarbons such as toluene, hexane, cyclohexane, heptane, and isooctane; alcohols such as methanol, ethanol, 2-propanol, butanol, and t-butyl alcohol; organic sulfur compounds such as dimethyl sulfoxide; ketones such as acetone; nitriles such as acetonitrile; and mixtures thereof.

[0073]

In the step (2) of the present invented production method 2, since an ammonium ion is used as an amino group donor, usually an ammonium salt compound is added to a reaction system. Examples of the ammonium salt compound to be added can include ammonium sulfate, ammonium formate, ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium hydroxide, ammonium tartrate, and ammonium acetate. The amount of an ammonium ion in the reaction system is usually equimolar to or more than the amount of an α -

oxocarboxylic acid compound as a substrate, and the ammonium ion is preferably added at the start of reaction.

[0074]

In the step (2) of the present invented production method 2, since a cofactor is used as a conjugated system, usually it is better to add a coenzyme to a reaction system. Examples of the coenzyme to be added can include reduced β -nicotinamide adenine dinucleotide (hereinafter sometimes referred to as NADH) and reduced β -nicotinamide adenine dinucleotide phosphate (hereinafter sometimes referred to as NADPH). The amount of a cofactor in the reaction system is usually equimolar to or more than the amount of an α -oxocarboxylic acid compound as a substrate, and the cofactor is preferably added at the start of reaction.

[0075]

In the step (2) of the present invented production method 2, as the reductive amination reaction of α -oxocarboxylic acid (e.g., sulfur-containing α -oxocarboxylic acid such as 2-oxo-4-(methylthio)butyric acid) proceeds, NADH in the reaction solution is converted into oxidized β -nicotinamide adenine dinucleotide (hereinafter sometimes referred to as NAD⁺). Since NAD⁺ occurred as a result of the conversion can return to the original NADH by being reacted with a protein having the ability to convert NAD⁺ into its reduced form (NADH), a protein having the ability to convert NAD⁺ into NADH may further exist in a reaction system in the above step (2). When a protein having the ability to convert NAD⁺ into NADH further exists in a reaction system in the above step (2), the amount of a cofactor in the reaction system may be usually a catalytic

amount, and equimolar to or less than the amount of an α -oxocarboxylic acid compound as a substrate.

Examples of the protein having the ability to convert NAD⁺ into NADH include organic acid dehydrogenases such as a formate dehydrogenase and a malate dehydrogenase; a glucose dehydrogenase, an alcohol dehydrogenase, an aldehyde dehydrogenase, or an amino acid dehydrogenase. Examples of the formate dehydrogenase can include a formate dehydrogenase of a microorganism belonging to the genus *Bacillus*, more specifically, a formate dehydrogenase having an amino acid sequence represented by SEQ ID NO: 43.

[0076]

A protein having the ability to convert NAD⁺ into NADH may be provided to a reaction system in the step (2) of the present invented production method 2 in the form of a purified protein or an immobilized product thereof, or may be provided to the reaction system in the form in which the protein is included in a microorganism producing the protein or in a treated product of the microorganism. "Treated product of microorganism" means that prepared in a similar manner to the above mentioned "treated product of transformant". A transformant in which a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH is introduced into a host cell or a treated product thereof may be provided to the above reaction system.

[0077]

In the step (2) of the present invented production method 2, a transformant in which a polynucleotide encoding the present invented protein (B) is introduced into a host cell or a treated product thereof, and a transformant in

which a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH is introduced into a host cell or a treated product thereof may be provided to a reaction system for reaction with an α -oxocarboxylic acid compound.

5 In the step (2) of the present invented production method 2, a transformant in which both of a polynucleotide encoding the present invented protein (B) and a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH are introduced in the same host cell
10 or a treated product thereof may be provided to the reaction system. A polynucleotide encoding the present invented protein (B) and a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH may be separately integrated into a different vector and introduced into a
15 host cell, or may be integrated into the same vector and introduced into a host cell. When both polynucleotides are integrated into a single vector, for example, the polynucleotides may be integrated into a vector by linking a region involved in the expression control such as a promoter and a terminator to each of both polynucleotides, or the
20 polynucleotides may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that both polynucleotides are expressed. Any one or both of a polynucleotide encoding the present invented protein (B) and
25 a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH may be on a chromosome of a host cell.

[0078]

A reaction in the step (2) of the present invented
30 production method 2 is performed by, for example, mixing

water, an ammonium salt compound, NADH, an α -oxocarboxylic acid compound (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-(methylthio)butyric acid), the present invented protein (B) or a transformant producing it
5 or a treated product thereof, and further, as needed, a reaction solution containing an organic solvent, a protein having the ability to convert NAD⁺ into NADH, and the like by stirring, shaking, and the like.

When the protein having the ability to convert NAD⁺
10 into NADH is a glucose dehydrogenase, the activity of the protein is sometimes enhanced by coexistence of glucose, etc. in the reaction system, and, for example, glucose, etc. may be added to the reaction solution. When the protein having the ability to convert NAD⁺ into NADH is a formate
15 dehydrogenase, the activity of the protein is sometimes enhanced by coexistence of ammonium formate as an amino group donor in the reaction system, and, for example, ammonium formate may be added to the reaction solution.

[0079]

20 The pH at the time of reaction in the above method can be appropriately selected, and is usually within a range of about 3 to about 10. The reaction temperature can be appropriately selected, and is usually within a range of about 0°C to about 60°C in terms of the stability and the
25 reaction rate of raw materials and products.

[0080]

The end point of the reaction can be determined by, for example, analyzing the amount of an α -oxocarboxylic acid compound (e.g., a sulfur-containing α -oxocarboxylic acid
30 compound such as 2-oxo-4-(methylthio)butyric acid) in the

reaction solution by liquid chromatography, etc.

The reaction time can be appropriately selected, and is usually within a range of about 0.5 hour to about 10 days.

[0081]

5 Recovery of L- α -amino acid (e.g., sulfur-containing L- α -amino acid such as L-methionine) from the reaction solution may be performed by a generally known arbitrary method.

10 Example thereof include a method for purifying a target compound by performing post-treatment operations of the reaction solution such as crystallization, extraction with an organic solvent, and concentration in combination with column chromatography, distillation, or the like as needed.

[0082]

15 The step (1) and step (2) of the present invented production method 2 can be performed in one reaction system.

In this case, the present invented protein (A) and the present invented protein (B) can be provided to the above reaction system in different various forms.

20 The present invented protein (A) and the present invented protein (B) may be provided to the above reaction system in the form of a purified protein, or may be provided to the above reaction system in the form in which the proteins are included in a microorganism producing these
25 proteins or in a treated product of the microorganism.

 The present invented protein (A) and the present invented protein (B) may be provided to the above reaction system in the form in which the proteins are included in a transformant in which a polynucleotide encoding these
30 proteins is introduced into a host cell or in a treated

product thereof.

For example, a transformant in which a polynucleotide encoding the present invented protein (A) is introduced into a host cell or a treated product thereof, and a transformant in which a polynucleotide encoding the present invented protein (B) is introduced into a host cell or a treated product thereof may be provided to the above reaction system. A transformant in which both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding the present invented protein (B) are introduced into the same host cell or a treated product thereof may be provided to the above reaction system. A polynucleotide encoding the present invented protein (A) and a polynucleotide encoding the present invented protein (B) may be separately integrated into a different vector and introduced into a host cell, or may be integrated into the same vector and introduced into a host cell. When both polynucleotides are integrated into a single vector, for example, the polynucleotides may be integrated into a vector by linking a region involved in the expression control such as a promoter and a terminator to each of both polynucleotides, or the polynucleotides may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that both polynucleotides are expressed. Any one or both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding the present invented protein (B) may be on a chromosome of a host cell.

[0083]

A protein having the ability to convert hydrogen

peroxide into molecular oxygen or a protein having the ability to convert NAD⁺ into NADH, or both of these proteins may further exist in the above reaction system. A protein having the ability to convert hydrogen peroxide into molecular oxygen and a protein having the ability to convert NAD⁺ into NADH may be provided to the above reaction system in the form of a purified protein or an immobilized product thereof, or may be provided to the above reaction system in the form in which the proteins are included in a microorganism producing these proteins or in a treated product of the microorganism. A transformant in which a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen or a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH is introduced into a host cell or a treated product thereof may be provided to the above reaction system.

A transformant in which two or more polynucleotides selected from the group consisting of a polynucleotide encoding the present invented protein (A), a polynucleotide encoding the present invented protein (B), a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen, and a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH are introduced into the same host cell or a treated product thereof may be provided to the above reaction system.

[0084]

A reaction when the step (1) and the step (2) of the present invented production method 2 are performed in one

reaction system can be performed in a reaction solution and under a reaction condition in accordance with the reaction in the step (2) of the present invented production method 2 mentioned above.

5 The end point of the reaction can be determined by, for example, analyzing the amount of an α -hydroxycarboxylic acid compound (e.g., a sulfur-containing α -hydroxycarboxylic acid compound such as 2-hydroxy-4-(methylthio)butyric acid) in the reaction solution by liquid chromatography, etc.

10 The reaction time can be appropriately selected, and is usually within a range of about 0.5 hour to about 10 days.

 Recovery of L- α -amino acid (e.g., sulfur-containing L- α -amino acid such as L-methionine) from the reaction solution may be performed in the same manner as in the step
15 (2) of the present invented production method 2 mentioned above.

Examples

[0085]

20 The present invention will be described in more detail below by way of Examples, etc., but the present invention is not limited to these Examples.

[0086]

Reference Example 1 (Preparation of Chromosomal DNA)

25 Into each of two 500 ml flasks, 100 ml of a medium (2 g of glucose, 0.5 g of polypeptone, 0.3 g of yeast extract, 0.3 g of meat extract, 0.2 g of ammonium sulfate, 0.1 g of potassium dihydrogenphosphate, 0.05 g of magnesium sulfate heptahydrate were dissolved in 100 ml of water, and the pH
30 was adjusted to 6 with 2 N HCl) was put, and the medium was

sterilized at 121°C for 15 minutes. To each thereof, 0.3 ml of a culture solution of an *Achromobacter denitrificans* ATCC55564 strain which was cultured by shaking in a medium of the same composition at 30°C for 48 hours was added, and the medium was cultured by shaking at 30°C for 24 hours. The culture solution thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes and the precipitate thus produced was collected. The precipitate thus obtained was washed with 50 ml of 0.85% saline to obtain 3.5 g of wet cells.

From the cells thus obtained, chromosomal DNA (hereinafter referred to as the chromosomal DNA (A)) was obtained using the QIAprep Genomic-tip System (manufactured by Qiagen).

[0087]

Example 1 (Preparation of Present Invented Polynucleotide (A), Present Invented Recombinant Vector, and Transformant of Present Invention)

Oligonucleotide primers each having a base sequence represented by any one of SEQ ID NO: 9 to 14 are synthesized.

[0088]

[Table 1]

Sense primer	Antisense primer
SEQ ID NO: 9 CCATATGAGCCGGCTGGACCGCTGCCTGTC	SEQ ID NO: 10 GGATCCCTATGCCGGGCTGGCCGGCCGTATC
SEQ ID NO: 11 CCATATGAACTCAAAGAACTCTTGTGATAG	SEQ ID NO: 12 GGATCCCTAAGGGCGCGACACGATGAAGTCG
SEQ ID NO: 13 CCATATGACATCCATCCTTCCGTCCGTCACC	SEQ ID NO: 14 GGATCCCTAATCTGCCAGGCTCTCGCGGGCC

[0089]

Using an oligonucleotide primer having a base sequence

represented by SEQ ID NO: 9 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 10, PCR was performed using the above chromosomal DNA (A) as a template and with the following reaction solution composition.

5 [Reaction Solution Composition]

Chromosomal DNA (A) solution:	1.5 μ l
dNTP (a mixture of 2 mM each):	10 μ l
Primer (50 pmol/ μ l):	0.3 μ l each
2xbuffer:	25 μ l
10 KOD-FX (1 U/ μ l, Toyobo):	1 μ l
Ultrapure water:	11.9 μ l
[0090]	

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-
15 GeneAmp PCR System 9700) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 98°C for 10 seconds, followed by 60°C for 30 seconds, followed by 68°C for 60 seconds was performed 30 times, and then the solution was maintained at 4°C.

20 Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.2 kb was detected.

By adding restriction enzymes NdeI and BamHI to the remaining PCR reaction solution, DNA was double-digested,
25 and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4
30 DNA ligase, and using the ligation solution thus obtained,

E. coli DH5 α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 μ g/ml of ampicillin, eight colonies were randomly selected from the growing colonies.

5 Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 μ g/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by
10 Qiagen). A part of each of the removed plasmids was double-digested with NdeI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. When the base sequence of the inserted DNA of about 1.2 kb
15 of one of these plasmids was analyzed, it was found that the DNA has a base sequence represented by SEQ ID NO: 2. This plasmid was designated as pET174. A base sequence represented by SEQ ID NO: 2 encodes an amino acid sequence represented by SEQ ID NO: 1.

20 [0091]

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 11 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 12, PCR was performed using the above chromosomal DNA (A) as a template
25 and with the following reaction solution composition.

[Reaction Solution Composition]

Chromosomal DNA (A) solution:	1.5 μ l
dNTP (a mixture of 2 mM each):	10 μ l
Primer (50 pmol/ μ l):	0.3 μ l each
30 2xbuffer:	25 μ l

KOD-FX (1 U/ μ l, Toyobo): 1 μ l
Ultrapure water: 11.9 μ l
[0092]

5 A container containing a reaction solution with the
above composition was set in a thermal cycler (PERKIN ELMER-
GeneAmp PCR System 9700) and incubated at 94°C for 2
minutes, and an incubation cycle consisting of incubation at
98°C for 10 seconds, followed by 60°C for 30 seconds,
followed by 68°C for 60 seconds was performed 30 times, and
10 then the solution was maintained at 4°C.

Subsequently, a part of the above PCR reaction
solution was subjected to agarose gel electrophoresis. A
DNA band of about 1.2 kb was detected.

15 By adding restriction enzymes NdeI and BamHI to the
remaining PCR reaction solution, DNA was double-digested,
and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was
double-digested with restriction enzymes NdeI and BamHI, and
enzymatically digested vector DNA was purified.

20 These purified DNAs were mixed and ligated with a T4
DNA ligase, and using the ligation solution thus obtained,
E. coli DH5 α was transformed.

The transformant thus obtained was cultured in an LB
agar medium containing 50 μ g/ml of ampicillin, eight
25 colonies were randomly selected from the growing colonies.
Each of the selected colonies was inoculated into 2 ml of a
sterilized LB medium containing 50 μ g/ml of ampicillin, and
the medium was cultured by shaking in a test tube at 37°C
for 17 hours. Plasmids were removed from each cultured cell
30 using the QIAprep Spin Miniprep Kit (manufactured by

Qiagen). A part of each of the removed plasmids was double-digested with NdeI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector.

5 When the base sequence of the inserted DNA of about 1.2 kb of one of these plasmids was analyzed, it was found that the DNA has a base sequence represented by SEQ ID NO: 4. This plasmid was designated as pET204. A base sequence represented by SEQ ID NO: 4 encodes an amino acid sequence
10 represented by SEQ ID NO: 3.

[0093]

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 13 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 14, PCR was
15 performed using the above chromosomal DNA (A) as a template and with the following reaction solution composition.

[Reaction Solution Composition]

Chromosomal DNA (A) solution:	1.5 μ l
dNTP (a mixture of 2 mM each):	10 μ l
20 Primer (50 pmol/ μ l):	0.3 μ l each
2xbuffer:	25 μ l
KOD-FX (1 U/ μ l, Toyobo):	1 μ l
Ultrapure water:	11.9 μ l

[0094]

25 A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 9700) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 98°C for 10 seconds, followed by 60°C for 30 seconds,
30 followed by 68°C for 60 seconds was performed 30 times, and

then the solution was maintained at 4°C.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.2 kb was detected.

5 By adding restriction enzymes NdeI and BamHI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and BamHI, and
10 enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, *E. coli* DH5α was transformed.

The transformant thus obtained was cultured in an LB
15 agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C
20 for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NdeI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about
25 1.2 kb was confirmed to be inserted into the above vector. When the base sequence of the inserted DNA of about 1.2 kb of one of these plasmids was analyzed, it was found that the DNA has a base sequence represented by SEQ ID NO: 6. This plasmid was designated as pET436. A base sequence
30 represented by SEQ ID NO: 6 encodes an amino acid sequence

represented by SEQ ID NO: 5.

[0095]

Example 2 (Preparation of L- α -Amino Acid Compound Using
Treated Product of Transformant of Present Invention)

5 An E. coli BL21(DE3) strain was transformed using the
plasmid pET174. The transformant thus obtained was
inoculated into 10 ml of a sterilized LB medium containing
0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium was
cultured by shaking at 37°C for 15 hours. The culture
10 solution thus obtained was centrifuged to obtain wet cells.
About 1 g of the wet cells were suspended in 1 ml of 0.1 M
Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20
minutes using the Multi-beads shocker (manufactured by Yasui
Kikai Corporation) and glass beads (0.1 mm Φ). The
15 disruption liquid thus obtained was centrifuged at 8,000 rpm
and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged
supernatant liquid. With 0.45 ml of the centrifuged
supernatant liquid thus obtained, 1.0 mg of a calcium salt
of 2-hydroxy-4-(methylthio)butyric acid (manufactured by
20 Tokyo Chemical Industry), 2.5 mg of NADH, 0.05 ml of 100 mM
Tris-HCl buffer (pH 8.0), 0.2 mg of ammonium sulfate, and
0.4 U of a leucine dehydrogenase (Wako Pure Chemical
Industries) were mixed, and the solution was shaken at 30°C
for 22 hours. This reaction solution was subjected to
25 content analysis by liquid chromatography under the
following condition. It was found that L-methionine was
produced in a proportion of 1.6% based on the amount of a
calcium salt of 2-hydroxy-4-(methylthio)butyric acid used
for the reaction.

30 [0096]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ $\times$ 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and
5 acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

10 [0097]

An E. coli BL21(DE3) strain was transformed using the plasmid pET204. The transformant thus obtained was inoculated into 10 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium was
15 cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui
20 Kikai Corporation) and glass beads (0.1 mm ϕ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.45 ml of the centrifuged supernatant liquid thus obtained, 1.0 mg of a calcium salt
25 of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 2.5 mg of NADH, 0.05 ml of 100 mM Tris-HCl buffer (pH 8.0), 0.2 mg of ammonium sulfate, and 0.4 U of a leucine dehydrogenase (Wako Pure Chemical Industries) were mixed, and the solution was shaken at 30°C
30 for 22 hours. This reaction solution was subjected to

content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 2.8% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0098]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ $\times$ 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0099]

An E. coli BL21(DE3) strain was transformed using the plasmid pET436. The transformant thus obtained was inoculated into 10 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium was cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm ϕ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.45 ml of the centrifuged supernatant liquid thus obtained, 1.0 mg of a calcium salt

of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 2.5 mg of NADH, 0.05 ml of 100 mM Tris-HCl buffer (pH 8.0), 0.2 mg of ammonium sulfate, and 0.4 U of a leucine dehydrogenase (Wako Pure Chemical Industries) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 3.9% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0100]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0101]

Example 3 (Preparation of Recombinant Vector Containing Polynucleotide Encoding Present Protein (B) and Transformant Having the Vector, and Preparation of Present Protein (B))

(1) Preparation of Recombinant Vector Containing Polynucleotide Encoding Amino Acid Sequence of Present Protein (B) and Transformant Having the Vector (1)

A *Bacillus sphaericus* IFO3525 strain was cultured in 100 ml of a sterilized LB medium to obtain 0.4 g of cells.

From the cells, chromosomal DNA (hereinafter referred to as the chromosomal DNA (B)) was purified using the Qiagen Genomic Tip (manufactured by Qiagen) in accordance with the method mentioned in the manual attached thereto.

5 Based on a base sequence encoding a leucine dehydrogenase derived from a *Bacillus sphaericus* IFO3525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic, 23 (2003) 239-247, an oligonucleotide primer having a base sequence represented by SEQ ID NO: 18

10 (GCCATGGAAATCTTCAAGTATATGG) and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 19 (GGGCCCCGGTTAACGGCCGTTCAAAATATT) are synthesized.

 Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 18 and an oligonucleotide primer
15 having a base sequence represented by SEQ ID NO: 19, PCR was performed using the above chromosomal DNA (B) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0102]

20 [Reaction Solution Composition]

Chromosomal DNA (B) solution:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
Primer (20 pmol/ μ l):	0.4 μ l
Primer (4 pmol/ μ l):	2 μ l
25 5xbuffer (with MgCl ₂):	10 μ l
enz.expandHiFi (3.5 x 10 ³ U/ml):	0.5 μ l
Ultrapure water:	35.1 μ l

[0103]

 A container containing a reaction solution with the
30 above composition was set in a thermal cycler (PERKIN ELMER-

GeneAmp PCR System 2400) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 94°C for 20 seconds, followed by 55°C for 30 seconds, followed by 72°C for 1.5 minutes was performed 25 times, and further the solution was maintained at 72°C for 7 minutes.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.1 kb was detected.

By adding restriction enzymes NcoI and SmaI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.1 kb was purified.

Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and SmaI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, *E. coli* DH5 α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 μ g/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 μ g/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with restriction enzymes NcoI and XbaI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.1 kb was confirmed to be inserted into the above vector. One of these plasmids was designated

as pTrcLD.

[0104]

(2) Preparation of Recombinant Vector Containing
Polynucleotide Encoding Amino Acid Sequence of Present
Protein (B) and Transformant Having the Vector (2)

Based on a base sequence encoding a leucine
dehydrogenase derived from a *Bacillus sphaericus* IFO3525
strain mentioned in Journal of Molecular Catalysis B:
Enzymatic, 23 (2003) 239-247, an oligonucleotide having a
base sequence represented by SEQ ID NO: 20

(GTCGCTATATTACCGGTGAAGATGTTG) (sense primer) and an
oligonucleotide having a base sequence represented by SEQ ID
NO: 21 (CAACATCTTCACCGGTAATATAGCGAC) (antisense primer) are
synthesized as a primer for mutation introduction for
substituting the 113th alanine in the enzyme by glycine.

Using an oligonucleotide having a base sequence represented
by SEQ ID NO: 20 and an oligonucleotide having a base
sequence represented by SEQ ID NO: 21 as a primer, PCR was
performed using the recombinant vector pTrcLD prepared in
the above (1) as a template and with the following reaction
solution composition using the QuikChange II Site-Directed
Mutagenesis Kit (STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrcLD:	0.4 μ l
dNTP mix (contained in the above Kit):	1 μ l
Sense primer (50 μ M):	0.4 μ l
Antisense primer (50 μ M):	0.4 μ l
10xbuffer (contained in the above Kit):	5 μ l
PfuUltra (contained in the above Kit):	1 μ l
Ultrapure water:	41.8 μ l

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 95°C for one minute, and an incubation cycle consisting of incubation at 95°C for 50 seconds, followed by 55°C for one minute, followed by 68°C for 5 minutes was performed 12 times, and then the solution was stored at 4°C.

To the PCR reaction solution thus obtained, 1 µl of a DpnI restriction enzyme (contained in the above Kit) was added, and then the solution was incubated at 37°C for one hour. Using the incubated solution thus obtained, E. coli DH5α was transformed.

From each of the transformants thus obtained, plasmids were extracted, and then a base sequence at the mutation site was determined by the dideoxy method to confirm that the designed mutation was introduced. A plasmid having a base sequence represented by SEQ ID NO: 8 was designated as pTrcLD(A113G). A base sequence represented by SEQ ID NO: 8 encodes an amino acid sequence represented by SEQ ID NO: 7.

[0105]

(3) Preparation of Recombinant Vector Containing Polynucleotide Encoding Amino Acid Sequence of Present Protein (B) and Transformant Having the Vector (3)

(3-1) Introduction of Site-specific Mutation for Base Substitution

Based on a base sequence encoding a leucine dehydrogenase derived from a Bacillus sphaericus IF03525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic, 23 (2003) 239-247, an oligonucleotide having a base sequence represented by SEQ ID NO: 22

(GATAGTATTCCAACCTATGTTGCGGC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 23 (GCCGCAACATAGGTTGGAATACTATC) (antisense primer) are synthesized as a primer for mutation introduction for substituting the 993rd adenine by cytosine. Using an oligonucleotide having a base sequence represented by SEQ ID NO: 22 and an oligonucleotide having a base sequence represented by SEQ ID NO: 23 as a primer, PCR was performed using the recombinant vector pTrcLD(A113G) prepared in the above (2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrcLD(A113G):	1 μ l
dNTP mix (contained in the above Kit):	1 μ l
Sense primer (50 μ M):	0.4 μ l
Antisense primer (50 μ M):	0.4 μ l
10xbuffer (contained in the above Kit):	5 μ l
PfuUltra (contained in the above Kit):	1 μ l
Ultrapure water:	41.2 μ l

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 95°C for one minute, and an incubation cycle consisting of incubation at 95°C for 50 seconds, followed by 60°C for one minute, followed by 68°C for 5.5 minutes was performed 12 times, and then the solution was stored at 4°C.

To the PCR reaction solution thus obtained, 1 μ l of a DpnI restriction enzyme (contained in the above Kit) was added, and then the solution was incubated at 37°C for one

hour. Using the incubated solution thus obtained, *E. coli* DH5 α was transformed.

From each of the transformants thus obtained, plasmids were extracted, and then a base sequence at the mutation site was determined by the dideoxy method. A plasmid into which the designed mutation was confirmed to be introduced was designated as pTrcLD(A113G)nd.

[0106]

(3-2) Preparation of Recombinant Vector Containing Polynucleotide Encoding Amino Acid Sequence of Present Protein (B) and Transformant Having the Vector

Based on a base sequence encoding a leucine dehydrogenase derived from a *Bacillus sphaericus* IF03525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic, 23 (2003) 239-247, an oligonucleotide primer having a base sequence represented by SEQ ID NO: 24 (GGGCATATGGAAATCTTCAAGTATATGG) (sense primer) and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 25 (GGATCCTTAACGGCCGTTCAAAATATT) (antisense primer) are synthesized. Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 24 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 25 as a primer, PCR was performed using the recombinant vector pTrcLD(A113G)nd prepared in the above (3-1) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0107]

[Reaction Solution Composition]

DNA solution of pTrcLD(A113G)nd: 1 μ l

dNTP (a mixture of 2.5 mM each): 1 μ l
Primer (20 pmol/ μ l): 0.4 μ l each
5xbuffer (with MgCl₂): 10 μ l
enz.expandHiFi (3.5 x 10³ U/ml): 0.5 μ l
5 Ultrapure water: 36.7 μ l
[0108]

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 94°C for 2
10 minutes, and an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 55°C for 30 seconds, followed by 72°C for 1.5 minutes was performed 10 times, and then an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 60°C for 30 seconds, followed by
15 72°C for 1.5 minutes was performed 20 times, and further the solution was maintained at 72°C for 7 minutes.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.1 kb was detected.

20 By adding restriction enzymes NdeI and BamHI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.1 kb was purified.

Plasmid vector pET-15b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and BamHI, and
25 enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, E. coli DH5 α was transformed.

The transformant thus obtained was cultured in an LB
30 agar medium containing 50 μ g/ml of ampicillin, 10 colonies

were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with restriction enzymes NdeI and BamHI, and then subjected to agarose gel electrophoresis. In each of four plasmids, DNA of about 1.1 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed of 20 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 44: MetGlySerSerHisHisHisHisHisSerSerGlyLeuValProArgGlySerHis) is added to the amino terminus of a leucine dehydrogenase encoded by the recombinant vector pTrcLD(A113G)nd. One of the plasmids thus obtained was designated as pETLD(A113G). [0109]

(4) Preparation of Present Protein (B)

An E. coli BL21(DE3) strain was transformed using the recombinant vector pETLD(A113G). The transformant thus obtained was inoculated into 100 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain about 0.8 g of wet cells. About 0.8 g of the wet cells were suspended in 10 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole (hereinafter sometimes referred to as the binding buffer), and disrupted

at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 7 ml of centrifuged supernatant liquid.

To about 7 ml of the centrifuged supernatant liquid thus obtained, 3 ml of the binding buffer was added to make about 10 ml, and then this liquid was applied to a HisTrap HP column (gel bed 5 ml) (manufactured by GE Healthcare Japan) with a flow rate of 5 ml/min. By passing about 25 ml of the binding buffer through this column with a flow rate of 5 ml/min, non-adsorbed proteins were eluted. Then, while maintaining the flow rate, by passing about 35 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 29.75 mM imidazole through the column, non-adsorbed proteins and low adsorbed proteins were eluted. Next, adsorbed proteins were eluted by gradient elution in which the imidazole concentration was increased from 29.75 mM to 500 mM while 47.5 ml was passed through, and 25 ml of a fraction with the imidazole concentration of about 160 mM to 443 mM was collected. The fraction thus obtained was subjected to the Amicon Ultra-15 (manufactured by Merck Millipore), and desalting and concentration was performed and the buffer was replaced by 0.5 M Tris-HCl (pH 9) to obtain about 1.5 ml of a fraction. This fraction is hereinafter referred to as the leucine dehydrogenase (A113G) purified enzyme solution.

[0110]

Example 4 (Preparation of L- α -Amino Acid Compound Using Treated Product of Transformant of Present Invention)

An E. coli BL21(DE3) strain was transformed using the

plasmid pET174. The transformant thus obtained was inoculated into 10 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.4 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 2 U of a formate dehydrogenase (Sigma-Aldrich), 2.5 mg of ammonium formate, and 0.1 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 16 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 1.7% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0111]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 µm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and

acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

5 Detection: 210 nm

[0112]

An E. coli BL21(DE3) strain was transformed using the plasmid pET204. The transformant thus obtained was inoculated into 10 ml of a sterilized LB medium containing
10 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20
15 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.4 ml of the centrifuged
20 supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 2 U of a formate dehydrogenase (Sigma-Aldrich), 2.5 mg of ammonium
25 formate, and 0.1 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 16 hours. This reaction solution was subjected to content analysis by liquid chromatography under the
30 following condition. It was found that L-methionine was

produced in a proportion of 9.7% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0113]

(Content Analysis Condition)

5 Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

10 Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0114]

An E. coli BL21(DE3) strain was transformed using the
15 plasmid pET436. The transformant thus obtained was
inoculated into 10 ml of a sterilized LB medium containing
0.1 mM IPTG and 50 μg/ml of ampicillin, and the medium was
cultured by shaking at 37°C for 15 hours. The culture
solution thus obtained was centrifuged to obtain wet cells.
20 About 1 g of the wet cells were suspended in 1 ml of 0.1 M
Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20
minutes using the Multi-beads shocker (manufactured by Yasui
Kikai Corporation) and glass beads (0.1 mmφ). The
disruption liquid thus obtained was centrifuged at 8,000 rpm
25 and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged
supernatant liquid. With 0.4 ml of the centrifuged
supernatant liquid thus obtained, 0.02 ml of a 40% aqueous
solution of 2-hydroxy-4-(methylthio)butyric acid
(manufactured by Tokyo Chemical Industry) whose pH was
30 adjusted to 9 with ammonia water, 10 mg of NAD⁺, 2 U of a

formate dehydrogenase (Sigma-Aldrich), 2.5 mg of ammonium formate, and 0.1 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 16 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 6.7% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0115]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0116]

Example 5 (Preparation of Present Invented Polynucleotide (A), Present Invented Recombinant Vector, and Transformant of Present Invention)

Double-stranded DNA having a base sequence represented by SEQ ID NO: 15 in which the base sequence ccatggct is added to its 5' end and the base sequence ggatcc is added to its 3' end is prepared. A base sequence represented by SEQ ID NO: 15 encodes an amino acid sequence represented by SEQ ID NO: 1.

The above double-stranded DNA (about 1.2 kb) thus

prepared was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested DNA was purified.

Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, an E. coli JM109 strain was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with restriction enzymes NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. Base sequences of the plasmids thus obtained were determined, and a plasmid having the target base sequence was designated as pTrc174.

[0117]

Double-stranded DNA having a base sequence represented by SEQ ID NO: 16 in which the base sequence ccatggct is added to its 5' end side and the base sequence ggatcc is added to its 3' end side is prepared. A base sequence represented by SEQ ID NO: 16 encodes an amino acid sequence represented by SEQ ID NO: 3.

The above double-stranded DNA (about 1.2 kb) thus prepared was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested DNA was purified.

Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, an E. coli JM109 strain was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. Base sequences of the plasmids thus obtained were determined, and a plasmid having the target base sequence was designated as pTrc204.

[0118]

Double-stranded DNA having a base sequence represented by SEQ ID NO: 17 in which the base sequence ccatggct is added to its 5' end side and the base sequence ggatcc is added to its 3' end side is prepared. A base sequence represented by SEQ ID NO: 17 encodes an amino acid sequence

represented by SEQ ID NO: 5.

The above double-stranded DNA (about 1.2 kb) thus prepared was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested DNA was purified.

5 Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained,
10 an E. coli JM109 strain was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a
15 sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-
20 digested with NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, the above DNA of about 1.2 kb was confirmed to be inserted into the above vector. Base sequences of the plasmids thus obtained were determined, and a plasmid having the target base sequence
25 was designated as pTrc436.

[0119]

Example 6 (Preparation of Recombinant Vector Containing a Polynucleotide Encoding Protein Having Ability to Convert Oxidized β-Nicotinamide Adenine Dinucleotide into its
30 Reduced Form and Transformant Having the Vector)

Based on an amino acid sequence of a formate dehydrogenase derived from a *Bacillus* sp. F1(2010) strain mentioned in Journal of Applied Microbiology, 111 (2011) 1075-1085, a base sequence represented by SEQ ID NO: 26 is designed. A base sequence represented by SEQ ID NO: 26 encodes an amino acid sequence represented by SEQ ID NO: 43. Double-stranded DNA having a base sequence represented by SEQ ID NO: 26 in which the base sequence ccatggct is added to its 5' end side and the base sequence ggatcc is added to its 3' end side is prepared.

The above double-stranded DNA (about 1.2 kb) thus prepared was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested DNA was purified.

Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, an *E. coli* JM109 strain was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about

1.2 kb was confirmed to be inserted into the above vector. Base sequences of the plasmids thus obtained were determined, and a plasmid having the target base sequence was designated as pTrcFDH.

5 [0120]

An E. coli JM109 strain was transformed using the plasmid pTrcFDH. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was
10 cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.7 g of the wet cells were suspended in 10 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by
15 Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid.

[0121]

20 Example 7 (Preparation of L-α-Amino Acid Compound Using Treated Product of Transformant of Present Invention)

An E. coli JM109 strain was transformed using the plasmid pTrc174. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing
25 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20
30 minutes using the Multi-beads shocker (manufactured by Yasui

Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.4 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 0.05 ml of the centrifuged supernatant liquid obtained in Example 6, 2.5 mg of ammonium formate, and 0.05 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 17.5 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 5.6% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0122]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm Φ × 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0123]

An E. coli JM109 strain was transformed using the plasmid pTrc204. The transformant thus obtained was

inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells.

5 About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.4 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid

10 (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 0.05 ml of the centrifuged supernatant liquid obtained in Example 6, 2.5 mg of ammonium formate, and 0.05 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 17.5 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 30.2% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

25 [0124]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 µm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

5 [0125]

An E. coli JM109 strain was transformed using the plasmid pTrc436. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was
10 cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui
15 Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.4 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous
20 solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 0.05 ml of the centrifuged supernatant liquid obtained in Example 6, 2.5 mg of ammonium formate, and 0.05 ml of the leucine
25 dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 17.5 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was
30 produced in a proportion of 12.1% based on the amount of 2-

hydroxy-4-(methylthio)butyric acid used for the reaction.

[0126]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

5 Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A (%) : Solution B (%) = 90:10

Flow rate: 0.8 ml/min

10 Column temperature: 37°C

Detection: 210 nm

[0127]

Example 8 (Preparation of Present Invented Polynucleotide (A), Present Invented Recombinant Vector, and Transformant of Present Invention)

15

Oligonucleotide primers each having a base sequence represented by any one of SEQ ID NO: 27 to 32 are synthesized.

[0128]

20 [Table 2]

Sense primer	Antisense primer
SEQ ID NO: 27 CCATATGTCTCGCCTGGACCGCTGTCTGAG	SEQ ID NO: 28 GGATCCTTAAGCCGGGCTGGCCGGACGG
SEQ ID NO: 29 CCATATGAACTCCAAGAACTGCTGTCTATC	SEQ ID NO: 30 GGATCCTTACGGACGAGAAACGATAAAG
SEQ ID NO: 31 CCATATGACCTCTATTCTGCCTTCTGTTAC	SEQ ID NO: 32 ACTCGAGGTCAGCCAGGGATTACGCGCCAG

[0129]

25 Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 27 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 28, PCR was

performed using the plasmid pTrc174 as a template and with the following reaction solution composition.

[0130]

[Reaction Solution Composition]

5	DNA solution of pTrc174:	1.5 μ l
	dNTP (a mixture of 2 mM each):	10 μ l
	Primer (50 pmol/ μ l):	0.3 μ l each
	2xbuffer:	25 μ l
	KOD-FX (1 U/ μ l, Toyobo):	1 μ l
10	Ultrapure water:	11.9 μ l

[0131]

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 9700) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 98°C for 10 seconds, followed by 60°C for 30 seconds, followed by 68°C for 60 seconds was performed 30 times, and then the solution was maintained at 4°C.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.2 kb was detected.

By adding restriction enzymes NdeI and BamHI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-15b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, E. coli DH5 α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed of 20 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 44: MetGlySerSerHisHisHisHisHisSerSerGlyLeuValProArgGlySerHis) is added to the amino terminus of the present invented protein (A) encoded by the recombinant vector pTrc174. One of the plasmids thus obtained was designated as pET174SC.

[0132]

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 29 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 30, PCR was performed using the plasmid pTrc204 as a template and with the following reaction solution composition.

[0133]

[Reaction Solution Composition]

DNA solution of pTrc204:	1.5 µl
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dNTP (a mixture of 2 mM each): 10 μ l
Primer (50 pmol/ μ l): 0.3 μ l each
2xbuffer: 25 μ l
KOD-FX (1 U/ μ l, Toyobo): 1 μ l
5 Ultrapure water: 11.9 μ l
[0134]

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 9700) and incubated at 94°C for 2
10 minutes, and an incubation cycle consisting of incubation at 98°C for 10 seconds, followed by 60°C for 30 seconds, followed by 68°C for 60 seconds was performed 30 times, and then the solution was maintained at 4°C.

Subsequently, a part of the above PCR reaction
15 solution was subjected to agarose gel electrophoresis. A DNA band of about 1.2 kb was detected.

By adding restriction enzymes NdeI and BamHI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.2 kb was purified.

20 Plasmid vector pET-15b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and BamHI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained,
25 E. coli DH5 α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 μ g/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a
30 sterilized LB medium containing 50 μ g/ml of ampicillin, and

the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-
5 digested with NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed
10 of 20 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 44:
MetGlySerSerHisHisHisHisHisSerSerGlyLeuValProArgGlySerHis) is added to the amino terminus of the present invented protein (A) encoded by the recombinant vector pTrc204. One
15 of the plasmids thus obtained was designated as pET204SC.
[0135]

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 31 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 32, PCR was
20 performed using the plasmid pTrc436 as a template and with the following reaction solution composition.
[0136]

[Reaction Solution Composition]

DNA solution of pTrc436:	1.5 µl
25 dNTP (a mixture of 2 mM each):	10 µl
Primer (50 pmol/µl):	0.3 µl each
2xbuffer:	25 µl
KOD-FX (1 U/µl, Toyobo):	1 µl
Ultrapure water:	11.9 µl
30 [0137]	

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 9700) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 5 98°C for 10 seconds, followed by 60°C for 30 seconds, followed by 68°C for 60 seconds was performed 30 times, and then the solution was maintained at 4°C.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A 10 DNA band of about 1.2 kb was detected.

By adding restriction enzymes NdeI and XhoI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was 15 double-digested with restriction enzymes NdeI and XhoI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, E. coli DH5α was transformed.

20 The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and 25 the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NcoI and XhoI, and then subjected to agarose 30 gel electrophoresis. In each of six plasmids, DNA of about

1.2 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed of 20 amino acids including consecutive 6 residues of

histidine (SEQ ID NO: 44:

MetGlySerSerHisHisHisHisHisSerSerGlyLeuValProArgGlySerHis) is added to the amino terminus of the present invented protein (A) encoded by the recombinant vector pTrc436. One of the plasmids thus obtained was designated as pET436SC.

[0138]

Example 9 (Preparation of Recombinant Vector Containing Polynucleotide Encoding Protein having Ability to Convert Hydrogen Peroxide into Molecular Oxygen and Transformant Having the Vector, and Preparation of the Protein)

(1) Preparation of Recombinant Vector Containing Polynucleotide Encoding Amino Acid Sequence of Protein Having Ability to Convert Hydrogen Peroxide into Molecular Oxygen, and Transformant Having the Vector

An E. coli BL21(DE3) strain was cultured in 100 ml of a sterilized LB medium to obtain about 1.0 g of cells. From the cells, chromosomal DNA (hereinafter referred to as the chromosomal DNA (C)) was purified using the Qiagen Genomic Tip (manufactured by Qiagen) in accordance with the method mentioned in the manual attached thereto.

Based on a base sequence encoding a catalase derived from E. coli mentioned in Journal of Bacteriology, 170(9) (1988) 4415-4419, an oligonucleotide primer having a base sequence represented by SEQ ID NO: 33

(CCATATGAGCACGTCAGACGATATCCATAAC) and an oligonucleotide

primer having a base sequence represented by SEQ ID NO: 34

(ACTCGAGCAGCAGGTCGAAACGGTCGAGGTTC) are synthesized.

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 33 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 34, PCR was performed using the chromosomal DNA (C) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0139]

[Reaction Solution Composition]

10	Chromosomal DNA (C) solution:	1 μ l
	dNTP (a mixture of 2.5 mM each):	1 μ l
	Primer (20 pmol/ μ l):	0.4 μ l each
	5xbuffer (with MgCl ₂):	10 μ l
	enz.expandHiFi (3.5 x 10 ³ U/ml):	0.5 μ l
15	Ultrapure water:	33.7 μ l

[0140]

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 60°C for 30 seconds, followed by 72°C for 2 minutes was performed 10 times, and then an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 65°C for 30 seconds, followed by 72°C for 2 minutes was performed 20 times, and further the solution was maintained at 72°C for 7 minutes.

Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 2.2 kb was detected.

By adding restriction enzymes NdeI and XhoI to the

remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 2.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and XhoI, and enzymatically digested vector DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, *E. coli* DH5 α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 μ g/ml of ampicillin, eight colonies were randomly selected from the growing colonies. Each of the selected colonies was inoculated into 2 ml of a sterilized LB medium containing 50 μ g/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NdeI and XhoI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 2.2 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed of 8 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 45: LeuGluHisHisHisHisHisHis) is added to the carboxy terminus of the above catalase. One of the plasmids thus obtained was designated as pETcatE.

[0141]

(2) Preparation of Protein Having Ability to Convert Hydrogen Peroxide into Molecular Oxygen

An *E. coli* BL21(DE3) strain was transformed using the

recombinant vector pETcatE. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain about 0.8 g of wet cells. About 0.8 g of the wet cells were suspended in 10 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole (i.e., the binding buffer), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 7 ml of centrifuged supernatant liquid.

To about 7 ml of the centrifuged supernatant liquid thus obtained, 3 ml of the binding buffer was added to make about 10 ml, and then this liquid was applied to a HisTrap HP column (gel bed 1 ml) (manufactured by GE Healthcare Japan) with a flow rate of 1 ml/min. By passing about 5 ml of the binding buffer through this column with a flow rate of 1 ml/min, non-adsorbed proteins were eluted. Then, while maintaining the flow rate, by passing about 7 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 29.75 mM imidazole through the column, non-adsorbed proteins and low adsorbed proteins were eluted. Next, adsorbed proteins were eluted by gradient elution in which the imidazole concentration was increased from 29.75 mM to 500 mM while 9.5 ml was passed through, and 5 ml of a fraction with the imidazole concentration of about 30 mM to 180 mM was collected. The fraction thus obtained was subjected to the

Amicon Ultra-15 (manufactured by Merck Millipore), and desalting and concentration was performed and the buffer was replaced by 0.1 M Tris-HCl (pH 8) to obtain about 1 ml of a fraction. This fraction is hereinafter referred to as the catalase purified enzyme solution.

[0142]

Example 10 (Preparation of α -Oxocarboxylic Acid Compound Using Treated Product of Transformant of Present Invention)

An E. coli BL21(DE3) strain was transformed using the plasmid pET174SC. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution obtained in Example 9 (3.3 g protein/l), and 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.0) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following

condition. It was found that 2-oxo-4-(methylthio)butyric acid was produced in a proportion of 18.0% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

5 [0143]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ $\times$ 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and
10 acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

15 [0144]

An E. coli BL21(DE3) strain was transformed using the plasmid pET204SC. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium was
20 cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui
25 Kikai Corporation) and glass beads (0.1 mm ϕ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous
30 solution of 2-hydroxy-4-(methylthio)butyric acid

(manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution obtained in Example 9 (3.3 g protein/l), and 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.0) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that 2-oxo-4-(methylthio)butyric acid was produced in a proportion of 22.8% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0145]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0146]

An E. coli BL21(DE3) strain was transformed using the plasmid pET436SC. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20

minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution obtained in Example 9 (3.3 g protein/l), and 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.0) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that 2-oxo-4-(methylthio)butyric acid was produced in a proportion of 24.2% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0147]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ × 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0148]

Example 11 (Preparation of Recombinant Vector Containing

Polynucleotide Encoding Protein Having Ability to Convert
Oxidized β -Nicotinamide Adenine Dinucleotide into its
Reduced Form and Transformant Having the Vector, and
Preparation of the Protein)

- 5 (1) Preparation of Recombinant Vector Containing
Polynucleotide Encoding Protein Having Ability to Convert
Oxidized β -Nicotinamide Adenine Dinucleotide into its
Reduced Form and Transformant Having the Vector

Based on an amino acid sequence of a formate
10 dehydrogenase derived from a *Bacillus* sp. F1(2010) strain
mentioned in Journal of Applied Microbiology 111 (2011)
1075-1085, a base sequence represented by SEQ ID NO: 26 is
designed so that the frequency of use of codon is close to
that in *E. coli*, and based on the base sequence, an
15 oligonucleotide primer having a base sequence represented by
SEQ ID NO: 35 (CCATATGGCTAAGATCGTTTGC GTTCTGTAC) and an
oligonucleotide primer having a base sequence represented by
SEQ ID NO: 36 (ACTCGAGAGCAGATTTCTTGAAACGTGCAG) are
synthesized.

20 Using an oligonucleotide primer having a base sequence
represented by SEQ ID NO: 35 and an oligonucleotide primer
having a base sequence represented by SEQ ID NO: 36, PCR was
performed using the recombinant vector pTrcFDH as a template
and with the following reaction solution composition using
25 the Expand High Fidelity PCR System (Roche Diagnostics).

[0149]

[Reaction Solution Composition]

DNA solution of pTrcFDH:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
30 Primer (20 pmol/ μ l):	0.4 μ l each

5xbuffer (with MgCl₂): 10 µl
enz.expandHiFi (3.5 x 10³ U/ml): 0.5 µl
Ultrapure water: 33.7 µl
[0150]

5 A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 94°C for 2 minutes, and an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 55°C for 30 seconds,
10 followed by 72°C for 1.5 minutes was performed 10 times, and then an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 60°C for 30 seconds, followed by 72°C for 1.5 minutes was performed 20 times, and further the solution was maintained at 72°C for 7 minutes.

15 Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.2 kb was detected.

By adding restriction enzymes NdeI and XhoI to the remaining PCR reaction solution, DNA was double-digested,
20 and enzymatically digested DNA of about 1.2 kb was purified.

Plasmid vector pET-22b (manufactured by Novagen) was double-digested with restriction enzymes NdeI and XhoI, and an enzymatically digested vector DNA fragment was purified.

25 These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, E. coli DH5α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, eight colonies were randomly selected from the growing colonies.
30 Each of the selected colonies was inoculated into 2 ml of a

sterilized LB medium containing 50 µg/ml of ampicillin, and the medium was cultured by shaking in a test tube at 37°C for 17 hours. Plasmids were removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids was double-digested with NdeI and XhoI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.2 kb was confirmed to be inserted into the above vector. The plasmids thus obtained are designed so that they can express a protein in which an amino acid sequence composed of 8 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 45: LeuGluHisHisHisHisHisHis) is added to the carboxy terminus of the above formate dehydrogenase. One of the plasmids thus obtained was designated as pETFDH.

[0151]

(2) Preparation of Protein Having Ability to Convert Oxidized β-Nicotinamide Adenine Dinucleotide into its Reduced Form

An E. coli BL21(DE3) strain was transformed using the recombinant vector pETFDH. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 37°C for 15 hours. The culture solution thus obtained was centrifuged to obtain about 0.8 g of wet cells. About 0.8 g of the wet cells were suspended in 10 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole (i.e., the binding buffer), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption

liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 7 ml of centrifuged supernatant liquid.

To about 7 ml of the centrifuged supernatant liquid thus obtained, 3 ml of the binding buffer was added to make about 10 ml, and then this liquid was applied to a HisTrap HP column (gel bed 1 ml) (manufactured by GE Healthcare Japan) with a flow rate of 1 ml/min. By passing about 5 ml of the binding buffer through this column with a flow rate of 1 ml/min, non-adsorbed proteins were eluted. Then, while maintaining the flow rate, by passing about 7 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 29.75 mM imidazole through the column, non-adsorbed proteins and low adsorbed proteins were eluted. Next, adsorbed proteins were eluted by gradient elution in which the imidazole concentration was increased from 29.75 mM to 500 mM while 9.5 ml was passed through, and 4 ml of a fraction with the imidazole concentration of about 30 mM to 230 mM was collected. The fraction thus obtained was subjected to the Amicon Ultra-15 (manufactured by Merck Millipore), and desalting and concentration was performed and the buffer was replaced by 0.1 M Tris-HCl (pH 8) to obtain about 1 ml of a fraction. This fraction is hereinafter referred to as the formate dehydrogenase purified enzyme solution.

[0152]

Example 12 (Preparation of L- α -Amino Acid Compound Using Treated Product of Transformant of Present Invention)

An E. coli BL21(DE3) strain was transformed using the plasmid pET174SC. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing

0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution obtained in Example 9 (3.3 g protein/l), 0.05 ml of the formate dehydrogenase purified enzyme solution obtained in Example 11 (5.3 g protein/l), 10 mg of NAD⁺, 2.5 mg of ammonium formate, and 0.05 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 12.0% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0153]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmΦ × 25 cm, 3 µm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate

solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

5 Column temperature: 37°C

Detection: 210 nm

[0154]

An E. coli BL21(DE3) strain was transformed using the plasmid pET204SC. The transformant thus obtained was
10 inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M
15 Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmΦ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged
20 supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase
25 purified enzyme solution obtained in Example 9 (3.3 g protein/l), 0.05 ml of the formate dehydrogenase purified enzyme solution obtained in Example 11 (5.3 g protein/l), 10 mg of NAD⁺, 2.5 mg of ammonium formate, and 0.05 ml of the leucine dehydrogenase (A113G) purified enzyme solution
30 obtained in Example 3 (36 g protein/l) were mixed, and the

solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 7.7% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0155]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0156]

An E. coli BL21(DE3) strain was transformed using the plasmid pET436SC. The transformant thus obtained was inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μg/ml of ampicillin, and the medium was cultured by shaking at 30°C for 15 hours. The culture solution thus obtained was centrifuged to obtain wet cells. About 0.1 g of the wet cells were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mmφ). The disruption liquid thus obtained was centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged

supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH was adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution obtained in Example 9 (3.3 g protein/l), 0.05 ml of the formate dehydrogenase purified enzyme solution obtained in Example 11 (5.3 g protein/l), 10 mg of NAD⁺, 2.5 mg of ammonium formate, and 0.05 ml of the leucine dehydrogenase (A113G) purified enzyme solution obtained in Example 3 (36 g protein/l) were mixed, and the solution was shaken at 30°C for 22 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-methionine was produced in a proportion of 33.9% based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0157]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0158]

Example 13 (Preparation of Present Invented Recombinant Vector)

(1) Preparation of Polynucleotide Encoding Present Protein

(B)

With reference to a sequence near the Shine-Dalgarno sequence of pTrc99A (manufactured by GE Healthcare Japan), an oligonucleotide primer having a base sequence represented by SEQ ID NO: 37 (CGGATCCGAGGAAACAGACCATGG) is synthesized. Based on a sequence of a leucine dehydrogenase derived from a *Bacillus sphaericus* IF 03525 strain mentioned in Journal of Molecular Catalysis B: Enzymatic, 23 (2003) 239-247, an oligonucleotide primer having a base sequence represented by SEQ ID NO: 38 (ctcagagTTAACGGCCGTTCAAAATATT) is synthesized.

Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 37 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 38, PCR is performed using the recombinant vector pTrcLD(A113G) mentioned in Example 3 (2) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (manufactured by Roche Diagnostics).

[0159]

[Reaction Solution Composition]

Plasmid pTrcLD solution:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
Primer (20 pmol/ μ l):	0.4 μ l each
5xbuffer (with MgCl ₂):	10 μ l
enz.expandHiFi (3.5 x 10 ³ U/ml):	0.5 μ l
Ultrapure water:	36.7 μ l

[0160]

A container containing a reaction solution with the above composition is set in a thermal cycler (PERKIN ELMER-GeneAmp PCR System 2400) and incubated at 94°C for 2

minutes, and an incubation cycle consisting of incubation at 94°C for 20 seconds, followed by 55°C for 30 seconds, followed by 72°C for 1.5 minutes is performed 25 times, and further the solution is maintained at 72°C for 7 minutes.

5 Subsequently, a part of the above PCR reaction solution is subjected to agarose gel electrophoresis. A DNA band of about 1.1 kb is detected.

By adding restriction enzymes BamHI and XbaI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.1 kb was purified.
10 [0161]

(2) Preparation of Present Invented Vector

The recombinant vector pTrc174 mentioned in Example 5 is double-digested with restriction enzymes BamHI and XbaI, and enzymatically digested DNA is purified.
15

The DNA thus obtained and the DNA of about 1.1 kbp purified in the above (1) are mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, an E. coli DH5α is transformed.

20 The transformant thus obtained is cultured in an LB agar medium containing 50 µg/ml of ampicillin, colonies are randomly selected from the growing colonies. Each of the selected colonies is inoculated into 2 ml of a sterilized LB medium containing 50 µg/ml of ampicillin, and the medium is cultured by shaking in a test tube at 37°C for 17 hours.
25 Plasmids are removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids is double-digested with BamHI and XbaI, and then subjected to agarose gel
30 electrophoresis. Confirm that DNA of about 1.1 kb is

inserted into the plasmid thus obtained. The plasmid thus obtained is hereinafter referred to as pTrc174LD(A113G).

[0162]

The recombinant vector pTrc204 mentioned in Example 5 is double-digested with restriction enzymes BamHI and XbaI, and enzymatically digested DNA is purified.

The DNA thus obtained and the DNA of about 1.1 kbp purified in the above (1) are mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained, an E. coli DH5 α is transformed.

The transformant thus obtained is cultured in an LB agar medium containing 50 μ g/ml of ampicillin, colonies are randomly selected from the growing colonies. Each of the selected colonies is inoculated into 2 ml of a sterilized LB medium containing 50 μ g/ml of ampicillin, and the medium is cultured by shaking in a test tube at 37°C for 17 hours. Plasmids are removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part of each of the removed plasmids is double-digested with BamHI and XbaI, and then subjected to electrophoresis. Confirm that DNA of about 1.1 kb is inserted into the plasmid thus obtained. The plasmid thus obtained is hereinafter referred to as pTrc204LD(A113G).

[0163]

The recombinant vector pTrc436 mentioned in Example 5 is double-digested with restriction enzymes BamHI and XbaI, and enzymatically digested DNA is purified.

The DNA thus obtained and the DNA of about 1.1 kbp purified in the above (1) are mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained,

an E. coli DH5 α is transformed.

The transformant thus obtained is cultured in an LB agar medium containing 50 μ g/ml of ampicillin, colonies are randomly selected from the growing colonies. Each of the
5 selected colonies is inoculated into 2 ml of a sterilized LB medium containing 50 μ g/ml of ampicillin, and the medium is cultured by shaking in a test tube at 37°C for 17 hours.

Plasmids are removed from each cultured cell using the QIAprep Spin Miniprep Kit (manufactured by Qiagen). A part
10 of each of the removed plasmids is double-digested with BamHI and XbaI, and then subjected to electrophoresis. Confirm that DNA of about 1.1 kb is inserted into the plasmid thus obtained. The plasmid thus obtained is hereinafter referred to as pTrc436LD(A113G).

15 [0164]

Example 14 (Preparation of L- α -Amino Acid Compound Using Treated Product of a Transformant of Present Invention)

An E. coli JM109 strain is transformed using the plasmid pTrc174LD(A113G) mentioned in (2) of Example 13.

20 The transformant thus obtained is inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/ml of ampicillin, and the medium is cultured by shaking at 30°C for 15 hours. The culture solution thus obtained is centrifuged to obtain wet cells. About 0.1 g of the wet
25 cells are suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained is centrifuged at 8,000 rpm and 4°C for
30 10 minutes to obtain about 0.7 ml of centrifuged supernatant

liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH is adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution mentioned in Example 9 (3.3 g protein/l), 0.05 ml of the formate dehydrogenase purified enzyme solution mentioned in Example 11 (5.3 g protein/l), 10 mg of NAD⁺, and 2.5 mg of ammonium formate are mixed, and the solution is shaken at 30°C for 22 hours. This reaction solution is subjected to content analysis by liquid chromatography under the following condition. The yield of L-methionine based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction is confirmed.

[0165]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mmφ × 25 cm, 3 μm)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0166]

An E. coli JM109 strain is transformed using the plasmid pTrc204LD(A113G) mentioned in (2) of Example 13. The transformant thus obtained is inoculated into 20 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μg/ml of ampicillin, and the medium is cultured by shaking at 30°C

for 15 hours. The culture solution thus obtained is centrifuged to obtain wet cells. About 0.1 g of the wet cells are suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0), and disrupted at 2,500 rpm for 20 minutes using the Multi-beads shocker (manufactured by Yasui Kikai Corporation) and glass beads (0.1 mm Φ). The disruption liquid thus obtained is centrifuged at 8,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged supernatant liquid. With 0.35 ml of the centrifuged supernatant liquid thus obtained, 0.02 ml of a 40% aqueous solution of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry) whose pH is adjusted to 9 with ammonia water, 0.05 ml of the catalase purified enzyme solution mentioned in Example 9 (3.3 g protein/l), 0.05 ml of the formate dehydrogenase purified enzyme solution mentioned in Example 11 (5.3 g protein/l), 10 mg of NAD⁺, and 2.5 mg of ammonium formate are mixed, and the solution is shaken at 30°C for 22 hours. This reaction solution is subjected to content analysis by liquid chromatography under the following condition. The yield of L-methionine based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction is confirmed.

[0167]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ × 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate solution containing 50 mM phosphoric acid (Solution A) and acetonitrile (Solution B) in a ratio of Solution A (%) : Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0168]

An E. coli JM109 strain is transformed using the
5 plasmid pTrc436LD(A113G) mentioned in (2) of Example 13.
The transformant thus obtained is inoculated into 20 ml of a
sterilized LB medium containing 0.1 mM IPTG and 50 µg/ml of
ampicillin, and the medium is cultured by shaking at 30°C
for 15 hours. The culture solution thus obtained is
10 centrifuged to obtain wet cells. About 0.1 g of the wet
cells are suspended in 1 ml of 0.1 M Tris-HCl buffer (pH
8.0), and disrupted at 2,500 rpm for 20 minutes using the
Multi-beads shocker (manufactured by Yasui Kikai
Corporation) and glass beads (0.1 mmΦ). The disruption
15 liquid thus obtained is centrifuged at 8,000 rpm and 4°C for
10 minutes to obtain about 0.7 ml of centrifuged supernatant
liquid. With 0.35 ml of the centrifuged supernatant liquid
thus obtained, 0.02 ml of a 40% aqueous solution of 2-
hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo
20 Chemical Industry) whose pH is adjusted to 9 with ammonia
water, 0.05 ml of the catalase purified enzyme solution
mentioned in Example 9 (3.3 g protein/l), 0.05 ml of the
formate dehydrogenase purified enzyme solution mentioned in
Example 11 (5.3 g protein/l), 10 mg of NAD⁺, and 2.5 mg of
25 ammonium formate are mixed, and the solution is shaken at
30°C for 22 hours. This reaction solution is subjected to
content analysis by liquid chromatography under the
following condition. The yield of L-methionine based on the
amount of 2-hydroxy-4-(methylthio)butyric acid used for the
30 reaction is confirmed.

[0169]

(Content Analysis Condition)

Column: UNISON UK-C18 (4.6 mm ϕ $\times$ 25 cm, 3 μ m)

Mobile phase: A mixture of a 12 mM sodium 1-heptanesulfonate

5 solution containing 50 mM phosphoric acid (Solution A) and

acetonitrile (Solution B) in a rate of Solution A

(%):Solution B (%) = 90:10

Flow rate: 0.8 ml/min

Column temperature: 37°C

10 Detection: 210 nm

Industrial Applicability

[0170]

According to the present invention, it is possible to
15 provide an oxidase, a polynucleotide encoding the same, a
method for producing an α -amino acid compound such as
methionine using these, and the like.

[Sequence Listing Free Text]

[0171]

20 SEQ ID NO: 9-14

An oligonucleotide primer designed for PCR

SEQ ID NO: 15-17

A polynucleotide encoding an oxidase

SEQ ID NO: 18-38

25 An oligonucleotide primer designed for PCR

SEQ ID NO: 44-45

An amino acid sequence designed

CLAIMS

1. A polynucleotide encoding any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-

(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative.

2. The polynucleotide according to claim 1, which has a base sequence represented by SEQ ID NO: 2, 4, 6, 15, 16, or 17.

3. A polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide according to claim 1 or 2 so that they can function.

4. A recombinant vector comprising the polynucleotide according to any one of claims 1 to 3.

5. The recombinant vector according to claim 4, which further comprises a polynucleotide encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function.

6. The recombinant vector according to claim 5, wherein the amino acid sequence of the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is any one of the following amino acid sequences (B1) to (B3):

(B1) an amino acid sequence represented by SEQ ID NO: 7,

(B2) an amino acid sequence i) having at least 90% sequence identity to an amino acid sequence represented by SEQ ID NO: 7, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative, or

(B3) an amino acid sequence i) represented by SEQ ID NO: 7 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to aminate

a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative.

7. A transformant in which the polynucleotide according to any one of claims 1 to 3 or the recombinant vector according to claim 4 is introduced into a host cell.

8. The transformant according to claim 7, wherein the host cell is a microorganism or E. coli.

9. A transformant in which the recombinant vector according to claim 5 or 6 is introduced into a host cell.

10. The transformant according to claim 9, wherein the host cell is a microorganism or E. coli.

11. A transformant having the polynucleotide according to any one of claims 1 to 3.

12. A transformant having the followings:

- i) a polynucleotide having a base sequence encoding an amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function; and
- ii) the polynucleotide according to any one of claims 1 to 3.

13. A method for producing a recombinant vector, which comprises the step of integrating the polynucleotide according to any one of claims 1 to 3 into a vector which can be replicated in a host cell.

14. A method for producing a transformant, which comprises the step of introducing the polynucleotide according to any one of claims 1 to 3 or the recombinant vector according to any one of claims 4 to 6 into a host cell.

15. A protein having any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted,

substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative.

16. A method for producing an α -oxocarboxylic acid compound, which comprises the step of reacting a protein having any one of the following amino acid sequences (A1) to (A4):

(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,

(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

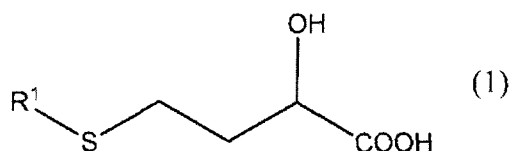
(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-

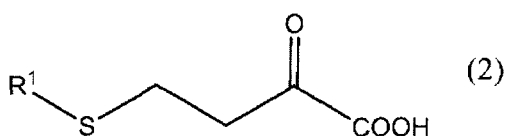
4-(methylthio)butyric acid derivative;
with an α -hydroxycarboxylic acid compound.

17. The production method according to claim 16, wherein the α -hydroxycarboxylic acid compound is a sulfur-containing α -hydroxycarboxylic acid compound, and the corresponding α -oxocarboxylic acid compound is a sulfur-containing α -oxocarboxylic acid compound.

18. The production method according to claim 17, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



wherein R^1 represents a hydrogen atom or an optionally substituted a C1-8 alkyl group;
and the sulfur-containing α -oxocarboxylic acid compound is a compound represented by formula (2):



wherein R^1 is the same as defined above.

19. The production method according to any one of claims 16 to 18, wherein the protein having any one of the amino acid sequences (A1) to (A4) is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

20. The production method according to claim 19, wherein the transformant is the transformant according to any one of claims 7, 8, and 11.

21. The production method according to any one of claims 16 to 20, wherein the step is performed in the presence of a protein having the ability to convert hydrogen peroxide into molecular oxygen.

22. The production method according to claim 21, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is a catalase.

23. The production method according to claim 21 or 22, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

24. A method for producing an L- α -amino acid compound, which comprises (1) the step of reacting a protein having any one of the following amino acid sequences (A1) to (A4):
(A1) an amino acid sequence represented by SEQ ID NO: 1, 3, or 5,
(A2) an amino acid sequence i) having at least 45% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, 3, or 5, and ii) of a protein having the ability to

oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative,

(A3) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, 4, or 6, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative, or

(A4) an amino acid sequence i) represented by SEQ ID NO: 1, 3, or 5 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative;

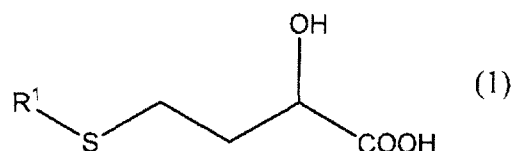
with an α -hydroxycarboxylic acid compound to obtain a corresponding α -oxocarboxylic acid compound, and

(2) the step of reacting a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound with the α -oxocarboxylic acid compound obtained in the step (1) to obtain a corresponding L- α -amino acid compound.

25. The production method according to claim 24, wherein the α -hydroxycarboxylic acid compound is a sulfur-containing α -hydroxycarboxylic acid compound, the corresponding α -oxocarboxylic acid compound is a sulfur-containing α -oxocarboxylic acid compound, and the corresponding L- α -amino

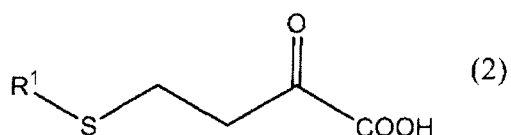
acid compound is a sulfur-containing L- α -amino acid compound.

26. The production method according to claim 23, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



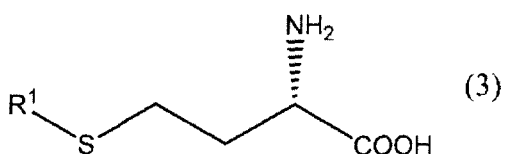
wherein R^1 represents a hydrogen atom or an optionally substituted a C1-8 alkyl group;

the sulfur-containing α -oxocarboxylic acid compound is a compound represented by formula (2):



wherein R^1 is the same as defined above;

and the sulfur-containing L- α -amino acid compound is a compound represented by formula (3):



wherein R^1 is the same as defined above.

27. The production method according to any one of claims 24 to 26, wherein the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is a leucine dehydrogenase.

28. The production method according to claim 27, wherein the leucine dehydrogenase is a leucine dehydrogenase derived from *Bacillus sphaericus*.

29. The production method according to any one of claims 24 to 27, wherein the amino acid sequence of a protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is any one of the following amino acid sequences (B1) to (B3):

(B1) an amino acid sequence represented by SEQ ID NO: 7,
(B2) an amino acid sequence i) having at least 90% sequence identity to an amino acid sequence represented by SEQ ID NO: 7, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative, or
(B3) an amino acid sequence i) represented by SEQ ID NO: 7 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having the ability to aminate a 2-oxo-4-(methylthio)butyric acid derivative and convert the same into a corresponding L-methionine derivative.

30. The production method according to any one of claims 24 to 29, wherein the protein having any one of the amino acid sequences (A1) to (A4) is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

31. The production method according to claim 30, wherein

the transformant is the transformant according to any one of claims 7 to 12.

32. The production method according to any one of claims 24 to 31, wherein the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

33. The production method according to claim 32, wherein the transformant is the transformant according to any one of claims 9, 10, and 12.

34. The production method according to any one of claims 24 to 33, wherein the step (1) is performed in the presence of a protein having the ability to convert hydrogen peroxide into molecular oxygen.

35. The production method according to claim 34, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is a catalase.

36. The production method according to claim 34 or 35, wherein the protein having the ability to convert hydrogen peroxide into molecular oxygen is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein

is introduced into a host cell or in a treated product thereof.

37. The production method according to any one of claims 24 to 36, wherein the step (2) is performed in the presence of a protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form.

38. The production method according to claim 37, wherein the protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form is a formate dehydrogenase.

39. The production method according to claim 37 or 38, wherein the protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into its reduced form is provided in a reaction system in the form in which the protein is included in a transformant in which a polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof.

40. The production method according to any one of claims 24 to 39, wherein the step (1) and the step (2) are performed in one reaction system.