

DESCRIPTION

OXIDASE, POLYNUCLEOTIDE THAT CODES FOR 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]

An α -amino acid compound has been used for various uses in the field of agriculture, forestry, and fisheries, the field of food, the field of medicine, the field of cosmetics, and the like. For example, methionine 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

Patent Document

[0003]

Patent Document 1: JP 55-102557 A

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Disclosure of the Invention

Problems to be Solved by the Invention

[0004]

The production method mentioned above uses prussic acid
5 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.

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Means for Solving the Problems

[0005]

The present invention provides the followings:

Item 1. A polynucleotide encoding any one of the following
15 amino acid sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,

(A2) an amino acid sequence i) having at least 85% sequence
identity to an amino acid sequence represented by SEQ ID NO:

3, and ii) of a protein having any one or both of the

20 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 and the ability to
oxidize an α -hydroxy-isocaproic acid derivative and convert
the same into a corresponding α -oxo-isocaproic acid

25 derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3
in which one or plural amino acids are deleted, substituted,
or added, and ii) of a protein having any one or both of the
ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid

30 derivative and convert the same into a corresponding 2-oxo-

4-(methylthio)butyric acid derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

5 (A4) 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: 4 or 13, and ii) of a protein having any one or both of the ability to oxidize a 2-

10 hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

15 (A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine,

substitution of the 191st glycine by aspartic acid, and

20 substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-

25 isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the present invented polynucleotide (A));

Item 2. The polynucleotide according to the above item 1,

30 which has an altered base sequence comprising a codon

selected so that the frequency of use of codon of a base sequence encoding the amino acid sequence corresponds to the frequency of use of codon in *E. coli*;

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 (A));

Item 5. A transformant in which the polynucleotide according to any one of the above items 1 to 3 or the recombinant vector according to the above item 4 is introduced into a host cell;

Item 6. The transformant according to the above item 5, wherein the host cell is a microorganism or *E. coli*;

Item 7. A transformant having the polynucleotide according to any one of the above items 1 to 3;

Item 8. 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 9. 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 the above item 4 into a host cell;

Item 10. A polynucleotide encoding any one of the following amino acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,
(B2) an amino acid sequence i) having at least 95% sequence
identity to an amino acid sequence represented by SEQ ID NO:
1, and ii) of a protein having any one or both of the
5 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 and the ability to
oxidize an α -hydroxy-isocaproic acid derivative and convert
the same into a corresponding α -oxo-isocaproic acid
10 derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1
in which one or plural amino acids are deleted, substituted,
or added, and ii) of a protein having any one or both of the
ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid
15 derivative and convert the same into a corresponding 2-oxo-
4-(methylthio)butyric acid derivative and the ability to
oxidize an α -hydroxy-isocaproic acid derivative and convert
the same into a corresponding α -oxo-isocaproic acid
derivative, or

20 (B4) 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, and ii) of a protein having any
one or both of the ability to oxidize a 2-hydroxy-4-
25 (methylthio)butyric acid derivative and convert the same
into a corresponding 2-oxo-4-(methylthio)butyric acid
derivative and the ability to oxidize an α -hydroxy-
isocaproic acid derivative and convert the same into a
corresponding α -oxo-isocaproic acid derivative (hereinafter
30 sometimes referred to as the present invented polynucleotide

(B));

Item 11. A polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide according to the above item 10 so that they can function;

5 Item 12. A recombinant vector comprising the polynucleotide according to the above item 10 or 11 (hereinafter sometimes referred to as the present invented recombinant vector (B));

Item 13. A transformant in which the polynucleotide according to the above item 10 or 11 or the recombinant
10 vector according to the above item 12 is introduced into a host cell;

Item 14. The transformant according to the above item 13, wherein the host cell is a microorganism or E. coli;

Item 15. A transformant having the polynucleotide according
15 to the above item 10 or 11;

Item 16. A method for producing a recombinant vector, which comprises the step of integrating the polynucleotide according to the above item 10 or 11 into a vector which can be replicated in a host cell;

20 Item 17. A method for producing a transformant, which comprises the step of introducing the polynucleotide according to the above item 10 or 11 or the recombinant vector according to the above item 12 into a host cell;

Item 18. A recombinant vector comprising the polynucleotide
25 according to any one of the above items 1 to 3 and the polynucleotide according to the above item 10 or 11 (hereinafter sometimes referred to as the present invented recombinant vector (AB));

Item 19. The recombinant vector according to the above item
30 18, 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
5 a promoter which can function in a host cell so that they
can function (hereinafter sometimes referred to as the
present invented recombinant vector (ABC));

Item 20. The recombinant vector according to the above item
19, wherein the protein having the ability to aminate an α -
10 oxocarboxylic acid compound and convert the same into a
corresponding L- α -amino acid compound is a leucine
dehydrogenase;

Item 21. The recombinant vector according to the above item
19 or 20, wherein the amino acid sequence of the protein
15 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 (C1) to (C3):

(C1) an amino acid sequence represented by SEQ ID NO: 7,

20 (C2) 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 an α -
oxocarboxylic acid compound and convert the same into a
corresponding L- α -amino acid compound, or

25 (C3) 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
an α -oxocarboxylic acid compound and convert the same into a
corresponding L- α -amino acid compound;

30 Item 22. A transformant in which the recombinant vector

according to any one of the above items 18 to 21 is
introduced into a host cell;

Item 23. The transformant according to the above item 22,
wherein the host cell is a microorganism or E. coli;

5 Item 24. 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

10 in which the polynucleotide is connected with a promoter
which can function in a host cell so that they can function;
ii) the polynucleotide according to any one of the above
items 1 to 3; and

15 iii) the polynucleotide according to the above item 10 or
11;

Item 25. A protein having any one of the following amino
acid sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,

(A2) an amino acid sequence i) having at least 85% sequence
20 identity to an amino acid sequence represented by SEQ ID NO:
3, and ii) of a protein having any one or both of 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 and the ability to
25 oxidize an α -hydroxy-isocaproic acid derivative and convert
the same into a corresponding α -oxo-isocaproic acid
derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3
in which one or plural amino acids are deleted, substituted,
30 or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A4) 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: 4 or 13, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the present invented protein (A));

Item 26. A protein having any one of the following amino

acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,

(B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO:

1, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid

derivative, or

(B4) 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, and ii) of a protein having any

one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter

sometimes referred to as the present invented protein (B));

Item 27. A method for producing an L- α -amino acid compound, which comprises (1) the step of reacting any one or both of a protein having any one of the following amino acid

5 sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,

(A2) an amino acid sequence i) having at least 85% sequence identity to an amino acid sequence represented by SEQ ID NO: 3, and ii) of a protein having any one or both of the

10 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid

15 derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid

20 derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

25 (A4) 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: 4 or 13, and ii) of a protein having any one or both of the ability to oxidize a 2-
30 hydroxy-4-(methylthio)butyric acid derivative and convert

the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

5 (A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and
10 substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a
15 corresponding α -oxo-isocaproic acid derivative; and a protein having any one of the following amino acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,

20 (B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, and ii) of a protein having any one or both of 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 and the ability to
25 oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1

30 in which one or plural amino acids are deleted, substituted,

or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

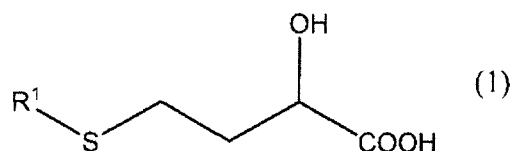
(B4) an amino acid sequence i) encoded by a polynucleotide hybridized under a stringent condition to a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2, and ii) of a protein having any one or both of the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic 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 invented production method);

Item 28. The production method according to the above item 27, wherein the α -hydroxycarboxylic acid compound is a sulfur-containing α -hydroxycarboxylic acid compound, the corresponding α -oxocarboxylic acid compound is a sulfur-

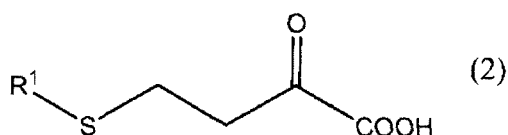
containing α -oxocarboxylic acid compound, the corresponding L- α -amino acid compound is a sulfur-containing L- α -amino acid compound, the protein reacted with an α -hydroxycarboxylic acid compound in the step (1) is a protein at least having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative;

Item 29. The production method according to the above item 28, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



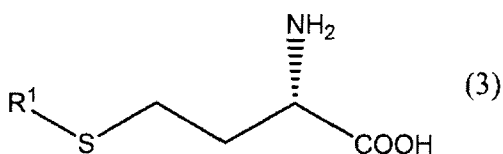
wherein R¹ represents a hydrogen atom or an optionally substituted C1-8 alkyl group;

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 30. The production method according to the above item 27, wherein the α -hydroxycarboxylic acid compound is α -

hydroxy-isocaproic acid, the corresponding α -oxocarboxylic acid compound is α -oxo-isocaproic acid, the corresponding L- α -amino acid compound is L-leucine, and the protein reacted with an α -hydroxycarboxylic acid compound in the step (1) is a protein at least having the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative;

Item 31. The production method according to any one of the above items 27 to 30, 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 32. The production method according to any one of the above items 27 to 31, 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 (C1) to (C3):

(C1) an amino acid sequence represented by SEQ ID NO: 7,

(C2) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or

(C3) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound;

Item 33. The production method according to any one of the

above items of 27 to 32, wherein the protein reacted with an α -hydroxycarboxylic acid compound in the step (1) is provided in a reaction system in the form in which the protein is included in a transformant in which a

5 polynucleotide encoding the protein is introduced into a host cell or in a treated product thereof;

Item 34. The production method according to the above item 33, wherein the transformant is the transformant according to any one of the above items 5, 6, 7, 13, 14, 15, 22, 23, and 24;

Item 35. The production method according to any one of the above items 27 to 34, 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

15 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 36. The production method according to the above item 20 35, wherein the transformant is the transformant according to any one of the above items 22, 23, and 24;

Item 37. The production method according to any one of the above items 27 to 36, wherein the step (1) is performed in the presence of a protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -nicotinamide adenine dinucleotide phosphate into its oxidized form;

Item 38. The production method according to the above item 37, wherein the protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -

nicotinamide adenine dinucleotide phosphate into its oxidized form is a protein further having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound;

5 Item 39. The production method according to the above item 37 or 38, wherein the protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -nicotinamide adenine dinucleotide phosphate into its oxidized form is provided in a reaction system in the form
10 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 27 to 39, wherein the step (2) is performed in
15 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 41. The production method according to the above item
20 40, 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 protein further having the ability to oxidize an α -hydroxycarboxylic acid compound and convert the
25 same into a corresponding α -oxocarboxylic acid compound;

Item 42. The production method according to the above item 40 or 41, wherein the protein having the ability to convert an oxidized β -nicotinamide adenine dinucleotide or an oxidized β -nicotinamide adenine dinucleotide phosphate into
30 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 43. The production method according to any one of the above items 27 to 42, 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 and leucine using these, and the like.

Mode for Carrying Out the Invention

[0007]

In the present invention, 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 including an amino acid to be altered 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 including an amino acid to be altered 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 the above amino acid sequences as a template and using a primer pair which can amplify the full length of the 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.

[0008]

"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).
5 Sequence identity can also be calculated by using commercial
sequence analysis software.

[0009]

In the present invention, microorganisms as a source of
a target polynucleotide may be separated naturally, or may
10 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.

15 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
20 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
25 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).
30 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.

[0010]

Examples of a method for hybridizing a probe to chromosomal DNA or a DNA library for cloning of a target polynucleotide 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.

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 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 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.

[0011]

In any hybridization above, a membrane is placed on the
5 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
example, in the case of plaque hybridization, a
10 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,
phage DNA is eluted on the membrane by immersing the
15 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
neutralizing solution (e.g., 1.5 M sodium chloride and 0.5 M
Tris-hydrochloric acid buffer pH 7.5) for about 5 minutes.
20 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
for about 90 minutes to immobilize the phage DNA on the
25 membrane.

Using the membrane prepared in this way, hybridization
of a probe is performed. Hybridization can be performed in
accordance with, for example, the description such as J. Sam
brook, E.F. Frisch, T. Maniatis "Molecular Cloning: A
30 Laboratory Manual 2nd edition (1989)" Cold Spring Harbor

Laboratory Press.

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 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. can be used.

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, 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 (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 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, a target polynucleotide can be prepared.

[0012]

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.

[0013]

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.

[0014]

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

[0015]

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 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 *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 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 Electroporation: Gene Pulser/E. coli Pulser System" Bio-Rad Laboratories, (1993), and the like.

[0016]

5 A transformant into which a target polynucleotide or a recombinant vector, etc. is introduced can be selected by, for example, using the phenotype of a selection marker gene contained in a vector as mentioned above as an index.

10 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
15 Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, and the like.

[0017]

 As a medium for culture of the transformant to be used in the present invention, for example, various media
20 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
25 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
30 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 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 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, 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.

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 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 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 lacUV5 promoter, a small amount of IPTG may be added to a medium as an inducer for inducing the production of the target protein.

[0018]

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.

[0019]

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:

5 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
10 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
15 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
20 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
25 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).

30 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.

[0020]

Examples of "treated product of transformant" as used herein include a freeze-dried transformant, an organic solvent-treated transformant, a dried transformant, a triturated 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.

[0021]

The present invented polynucleotide (A) encodes any one of the following amino acid sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,
(A2) an amino acid sequence i) having at least 85% sequence identity to an amino acid sequence represented by SEQ ID NO: 3, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to

oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A4) 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: 4 or 13, and ii) of a protein

having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine,

substitution of the 191st glycine by aspartic acid, and

substitution of the 246th glutamine by arginine, and ii) of

a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

The present invented protein (A) has any one of the above amino acid sequences (A1) to (A5).

[0022]

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: 3 is deletion, substitution, or addition, etc.
of some amino acids (hereinafter sometimes generally
5 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
10 protein having an amino acid sequence represented by SEQ ID
NO: 3, (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,
15 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
20 same ability as that of a protein before alteration, namely
any one or both of 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 and the ability to oxidize an α -hydroxy-
25 isocaproic acid derivative and convert the same into a
corresponding α -oxo-isocaproic acid derivative.

Examples of "plural amino acids" in the amino acid
sequence (A3) encoded by the present invented polynucleotide
(A) or the amino acid sequence (A3) of the present invented
30 protein (A) include 2, 3, 4, 5, 6, 7, 10, 15, 20, 25, 30,

35, 40, 45, or 48 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 7 to 25 residues of amino acid including about consecutive 6 residues of histidine to the amino terminus or carboxy terminus of an amino acid sequence of a protein. More specific examples can include addition of an amino acid sequence represented by SEQ ID NO: 44 to the amino terminus of an amino acid sequence of a protein, and addition of an amino acid sequence represented by SEQ ID NO: 45 to the carboxy terminus of an amino acid sequence of a protein.

[0023]

Examples of "at least 85% 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 85, 90, 95, 98, or 99% sequence identity.

[0024]

In an amino acid sequence encoded by the present 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: 4 or 13" means a polynucleotide (1) which form a hybrid by base pairing with a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 4 or 13 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 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 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: 4 or 13, a polynucleotide having a base sequence represented by SEQ ID NO: 4 or 13 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: 4 or 13.

[0025]

Specific examples of the amino acid sequence (A5) encoded by the present invented polynucleotide (A) or the amino acid sequence (A5) of the present invented protein (A) will be described below.

(A5-1) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution

of the 109th valine by isoleucine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence V109I);

(A5-2) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 191st glycine by aspartic acid, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence G191D);

(A5-3) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence

Q246R);

(A5-4) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 109th valine by isoleucine and substitution of the 191st glycine by aspartic acid, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence V109I/G191D);

(A5-5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 109th valine by isoleucine and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence V109I/Q246R);

(A5-6) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 191st glycine by aspartic acid and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence G191D/Q246R); and

(A5-7) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative (hereinafter sometimes referred to as the altered amino acid sequence V109I/G191D/Q246R).

Specific examples of the altered amino acid sequence V109I/G191D/Q246R can include an amino acid sequence represented by SEQ ID NO: 5. Examples of a base sequence encoding an amino acid sequence represented by SEQ ID NO: 5 can include a base sequence represented by SEQ ID NO: 6.

[0026]

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.

[0027]

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.

For example, 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: 3, a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, or a polynucleotide having a base sequence represented by SEQ ID NO: 4, 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.

[0028]

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: 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.

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.
[0029]

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

[0030]

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
10 having a partial base sequence selected from a base sequence encoding an amino acid sequence represented by SEQ ID NO: 3 to a DNA library into which a vector derived from a microorganism or a phage is inserted under the condition mentioned above to detect DNA to which the probe
15 specifically binds.

[0031]

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
20 method such as, for example, the phosphite-triester method (Hunkapiller, M. et al., Nature, 310, 105, 1984), based on its base sequence.

[0032]

The present invented polynucleotide (A) can also be
25 prepared by selecting, as a codon encoding any one of the above amino acid sequences (A1) to (A5), 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.

30 Specifically, for example, a codon corresponding to

each amino acid contained in an amino acid sequence represented by SEQ ID NO: 3 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.

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).

[0033]

Specific procedures 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 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 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 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

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
5 operations.

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
10 for Plants", p95-100, supervised by Takumi Shimamoto and
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
15 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.
20 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
25 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
30 by, for example, artificially synthesizing a polynucleotide

having a base sequence represented by SEQ ID NO: 13.

[0034]

The present invented polynucleotide (B) encodes any one of the following amino acid sequences (B1) to (B4):

5 (B1) an amino acid sequence represented by SEQ ID NO: 1,

(B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

15 (B3) an amino acid sequence represented by i) SEQ ID NO: 1 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

25 (B4) 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, and ii) of a protein having any one or both of 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

30

derivative and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

The present invented protein (B) has any one of the above amino acid sequences (B1) to (B4).

[0035]

A difference which is sometimes observed between an amino acid sequence encoded by the present invented polynucleotide (B) or an amino acid sequence of the present invented protein (B) and an amino acid sequence represented by SEQ ID NO: 1 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, (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 same ability as that of a protein before alteration, namely any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

5 Examples of "plural amino acids" in the amino acid sequence (B3) encoded by the present invented polynucleotide (B) or the amino acid sequence (B3) of the present invented protein (B) include 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, or 17 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.

 Examples of the addition of an amino acid can include
20 addition of about 7 to 25 residues of amino acid including about consecutive 6 residues of histidine to the amino terminus or carboxy terminus of an amino acid sequence of a protein. More specific examples can include addition of an amino acid sequence represented by SEQ ID NO: 44 to the
25 amino terminus of an amino acid sequence of a protein, and addition of an amino acid sequence represented by SEQ ID NO: 45 to the carboxy terminus of an amino acid sequence of a protein.

[0036]

30 Examples of "at least 95% sequence identity" in the

amino acid sequence (B2) encoded by the present invented polynucleotide (B) or the amino acid sequence (B2) of the present invented protein (B) include at least 95, 98, or 99% sequence identity.

5 [0037]

In an amino acid sequence encoded by the present invented polynucleotide (B) or an amino acid sequence of the present invented protein (B), "polynucleotide hybridized under a stringent condition to a polynucleotide composed of
10 a sequence complementary to a base sequence represented by SEQ ID NO: 2" means a polynucleotide (1) which form a hybrid by base pairing with a polynucleotide composed of a sequence complementary to a base sequence represented by SEQ ID NO: 2 by being hybridized at 65°C under a high ionic concentration
15 [for example, 6XSSC (900 mM sodium chloride and 90 mM sodium citrate)] and (2) in which the 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
20 hybridization mentioned in, for example, "Cloning and Sequence" (supervised by Itaru Watanabe, edited 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
25 NO: 2, a polynucleotide having a base sequence represented by SEQ ID NO: 2 in which some bases are deleted, substituted, or added, or a polynucleotide having a base sequence having at least 95%, 98%, or 99% sequence identity to a base sequence represented by SEQ ID NO: 2.

30 [0038]

The present invented polynucleotide (B) 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
5 polynucleotide is artificially introduced, or a chemically synthesized polynucleotide.

[0039]

The present invented polynucleotide (B) can be obtained from, for example, a microorganism having the ability to
10 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.

For example, a DNA library is prepared from a
15 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
20 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, a polynucleotide encoding an amino acid sequence represented
25 by SEQ ID NO: 1 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, etc. is amplified, and thereby the present invented polynucleotide (B) can be prepared.

30 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.

[0040]

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 can be amplified.

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.

[0041]

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

[0042]

The present invented polynucleotide (B) can also be 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 to a DNA library into which a vector derived from a microorganism or a phage is inserted under the condition mentioned above to detect DNA to which the probe specifically binds.

[0043]

The present invented 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.

[0044]

The present invented polynucleotide (B) can also be prepared by selecting, as a codon encoding any one of the above amino acid sequences (B1) to (B4), 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 each amino acid contained in an amino acid sequence represented by SEQ ID NO: 1 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.

[0045]

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 ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit by PerkinElmer Inc. may be used.

[0046]

The fact that the polynucleotide prepared as mentioned above encodes an amino acid sequence of a protein having the ability to oxidize α -hydroxycarboxylic acid (e.g., 2-hydroxy-4-(methylthio)butyric acid or α -hydroxy-isocaproic acid) and convert the same into corresponding α -

oxocarboxylic acid (e.g., 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid) 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 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 -hydroxycarboxylic acid (e.g., 2-hydroxy-4-(methylthio)butyric acid or α -hydroxy-isocaproic acid). By analyzing the amount of corresponding α -oxocarboxylic acid in the reaction product (e.g., 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid), the fact that the polynucleotide thus obtained encodes an amino acid sequence of a protein having target ability can be confirmed.

[0047]

To express the present invented polynucleotide (A) or the present invented 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 invented polynucleotide (A) or the present invented 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 invented polynucleotide (A) or the present invented polynucleotide (B) in *Achromobacter denitrificans* may be used.

[0048]

The present invented recombinant (A) 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.

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

The present invented recombinant vector (AB) can be prepared by integrating:

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

[0049]

The present invented recombinant vector (AB) can also further include 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 protein (C)), or a polynucleotide in which the polynucleotide is connected with a promoter

which can function in a host cell so that they can function.

For example, the present invented recombinant vector (ABC) can be prepared by integrating

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

5 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;

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

c) a polynucleotide encoding an amino acid sequence of the present protein (C), or a polynucleotide in which the polynucleotide is connected with a promoter which can
15 function in a host cell so that they can function; into a vector.

[0050]

Examples of the protein having the ability to aminate an α -oxocarboxylic acid compound and convert the same into
20 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,
25 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
30 *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.

5 An amino acid sequence represented by SEQ ID NO: 42 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.

10 An amino acid sequence represented by SEQ ID NO: 7 is an amino acid sequence represented by SEQ ID NO: 42 in which the 113th alanine is converted to glycine.

[0051]

15 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 (C1) to (C3):

(C1) an amino acid sequence represented by SEQ ID NO: 7,
20 (C2) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or

25 (C3) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound.

30 [0052]

A difference which is sometimes observed between the amino acid sequence of the present protein (C) 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 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 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 aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound. Examples of "plural amino acids" in the above amino acid sequence (C3) of the present protein (C) include 2, 3, 4, 5, 6, 7, 10, 15, 18, 20, 25, 30, 35, 36, 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.

5 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. More specific examples can include addition of an amino acid sequence
10 represented by SEQ ID NO: 44 to the amino terminus of an amino acid sequence of a protein, and addition of an amino acid sequence represented by SEQ ID NO: 45 to the carboxy terminus of an amino acid sequence of a protein.

15 Examples of "at least 90% sequence identity" in the above amino acid sequence (C2) of the present protein (C) include at least 90, 95, 98, or 99% sequence identity.
[0053]

20 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 (C)) can be obtained from, for example, a microorganism having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a
25 corresponding L- α -amino acid compound, for example, a microorganism belonging to the genus *Bacillus* such as a *Bacillus sphaericus* IF03525 strain.

[0054]

30 A DNA library is prepared from a microorganism belonging to the genus *Bacillus* such as a *Bacillus*

sphaericus IFO3525 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: 42, or a polynucleotide encoding an amino acid sequence represented by SEQ ID NO: 42 in which one or plural amino acids are deleted, substituted, or added, etc. is amplified, and thereby the present polynucleotide (C) can be prepared.

For example, as a primer for amplification of a polynucleotide having a base sequence represented by SEQ ID NO: 43 encoding an amino acid sequence represented by SEQ ID NO: 42, an oligonucleotide having a base sequence represented by SEQ ID NO: 14 and an oligonucleotide having a base sequence represented by SEQ ID NO: 15 are synthesized. As a primer for mutation introduction for converting the 113th alanine in an amino acid sequence represented by SEQ ID NO: 42 to glycine, an oligonucleotide having a base sequence represented by SEQ ID NO: 16 and an oligonucleotide having a base sequence represented by SEQ ID NO: 17 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: 14 and an oligonucleotide having a base sequence represented by SEQ ID NO: 15 as a primer, a polynucleotide having a base sequence represented by SEQ ID NO: 43 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: 43. 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: 16 and an oligonucleotide having a base sequence represented by SEQ ID NO: 17 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: 42 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.

[0055]

The present polynucleotide (C) 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 (C) can also be prepared by selecting, as a codon encoding any one of the above amino acid sequences (C1) to (C3), 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.

[0056]

To express the present polynucleotide (C) 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 (C) so that they can function is prepared, and introduced into a host cell.

5 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 (C) in a microorganism belonging to the genus *Bacillus* such as
10 *Bacillus sphaericus* may be used.

[0057]

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.,
15 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid) and convert the same into a corresponding L- α -amino acid compound (e.g., L-methionine or L-leucine) can be confirmed by, for example, the following procedures.

The polynucleotide obtained as mentioned above is
20 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 cultured product of the transformant thus obtained is
25 reacted with α -oxocarboxylic acid (e.g., 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid). By analyzing the amount of corresponding L- α -amino acid in the reaction product (e.g., L-methionine or L-leucine), the fact that the polynucleotide thus obtained encodes an amino acid
30 sequence of a protein having target ability can be

confirmed.

[0058]

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

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

[0059]

As mentioned above, by introducing the present invented
15 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
20 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
25 cell, namely a transformant having the above exogenous polynucleotide on a chromosome.

By introducing the present invented polynucleotide (B), or a polynucleotide in which a promoter which can function
in a host cell is connected with the present invented
30 polynucleotide (B) so that they can function into a host

cell, a transformant having the present invented polynucleotide (B), or a polynucleotide in which a promoter which can function in a host cell is connected with the present invented polynucleotide (B) 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.

[0060]

As mentioned above, by introducing

a) 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;

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

c) a polynucleotide encoding an amino acid sequence of the present protein (C), or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function; into a host cell, a transformant having:

a) 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;

b) the present invented polynucleotide (B), or a polynucleotide in which a promoter which can function in a

host cell is connected with the present invented polynucleotide (B) so that they can function; and c) a polynucleotide encoding an amino acid sequence of the present protein (C), or a polynucleotide in which the polynucleotide is connected with a promoter which can function in a host cell so that they can function; can be obtained.

The above a) polynucleotide, b) polynucleotide, and c) polynucleotide may be separately integrated into a different vector and introduced into a host cell, or one or more of these may be integrated into the same vector and introduced into a host cell. When one or more polynucleotides are integrated into a single vector, for example, the polynucleotide(s) 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 target polynucleotides, or the polynucleotide(s) may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that target polynucleotides are expressed. Any one or one or more of the above a) polynucleotide, b) polynucleotide, and c) polynucleotide may be on a chromosome of a host cell.

[0061]

The present invented protein (A) can be produced by, 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 invented polynucleotide (B) to express the present invented

protein (B).

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

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

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

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

[0062]

25 The present invented production method is a method for producing an L- α -amino acid compound, which includes:

(1) the step of reacting any one or both of the present invented protein (A) and the present invented protein (B) with an α -hydroxycarboxylic acid compound to obtain a
30 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.

[0063]

Examples of the above α -hydroxycarboxylic acid include α -hydroxycarboxylic acid represented by formula (4):



wherein R^2 represents a hydrogen atom, a C1-10 linear, branched-chain, or cyclic alkyl group, or a C1-10 linear or branched-chain alkyl group having a substituent of amide, amino, monoalkylamino, dialkylamino, monoalkylamide, dialkylamide, alkoxy, alkylthio, hydroxy, mercapto, carboxyl, or alkoxycarbonyl, or a 1H-imidazolyl group, a phenyl group, a 3'-indolyl group, a p-hydroxyphenyl group, or a p-alkoxyphenyl group, and the number of carbon atoms in alkyl and alkoxy included in the substituent is 1-3 each.

Examples of the above L- α -amino acid compound include L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, L-histidine, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-alanine, glycine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, and L-valine, and preferably L-alanine, glycine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, or L-valine.

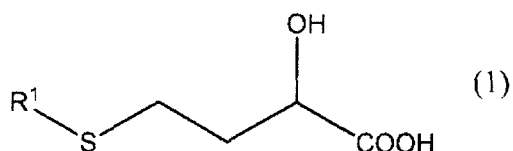
[0064]

Examples of the above α -hydroxycarboxylic acid compound can preferably include α -hydroxy-isocaproic acid, examples of the corresponding α -oxocarboxylic acid compound can include α -oxo-isocaproic acid, and examples of the

corresponding L- α -amino acid compound can include L-leucine.
[0065]

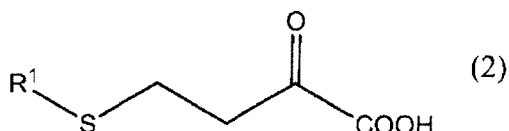
Examples of the above α -hydroxycarboxylic acid compound
can also preferably include a sulfur-containing α -
5 α -hydroxycarboxylic acid compound, examples of the
corresponding α -oxocarboxylic acid compound can include a
sulfur-containing α -oxocarboxylic acid compound, and
examples of the corresponding L- α -amino acid compound can
include a sulfur-containing L- α -amino acid compound.

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



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

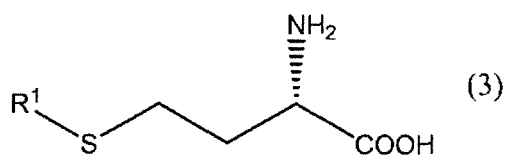
Examples of a sulfur-containing α -oxocarboxylic acid
compound obtained by reacting the above sulfur-containing α -
hydroxycarboxylic acid represented by formula (1) with any
one or both of the present invented protein (A) and the
20 present invented protein (B) include sulfur-containing α -
oxocarboxylic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



wherein R¹ is the same as defined above.

Examples of a sulfur-containing L- α -amino acid compound
25 obtained by reacting the above sulfur-containing α -
oxocarboxylic acid represented by formula (2) with the

present protein (C) include sulfur-containing L- α -amino acid represented by formula (3):



[0066]

5 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 C1-8 alkyl group which
10 is optionally substituted 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 -
15 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
20 acid, 2-hydroxy-4-(heptylthio)butyric acid, and 2-hydroxy-4-(octylthio)butyric acid.

 Specific examples of the sulfur-containing α -oxo
carboxylic acid represented by formula (2) can include 2-
oxo-4-(methylthio)butyric acid, 2-oxo-4-(ethylthio)butyric
25 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.

[0067]

As the C1-8 alkyl group which is optionally substituted represented by R¹, 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.

[0068]

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

In the step (1) of the present invented production method, 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).

[0069]

In the step (1) of the present invented production method, each of the present invented protein (A) and the present invented protein (B) can be provided to a reaction system for reaction with an α -hydroxycarboxylic acid compound, in various forms. Each of the present invented protein (A) and the present invented protein (B) may be

provided to a reaction system in the step (1) of the present
invented production method 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
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". Each of the present
invented protein (A) and the present invented protein (B)
10 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 -
15 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
20 (A) is introduced into a host cell, or a treated product of
the transformant can be provided to a reaction system in the
step (1) of the present invented production method.

To react the present invented protein (B) with an α -
hydroxycarboxylic acid compound, specifically, for example,
25 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
30 the transformant can be provided to a reaction system in the

step (1) of the present invented production method.

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 a reaction system in the step (1) of the present invented production method. 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.

[0070]

The step (1) of the present invented production method

is usually performed in the presence of water and a coenzyme such as an oxidized β -nicotinamide adenine dinucleotide (hereinafter sometimes referred to as NAD⁺). Water used in this case may be a buffered aqueous solution. Examples of a
5 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.

10 [0071]

In the step (1) of the present invented production method, 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
15 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-
20 propanol, butanol, and t-butyl alcohol; organic sulfur compounds such as dimethyl sulfoxide; ketones such as acetone; nitriles such as acetonitrile; and mixtures thereof.

[0072]

25 In the step (1) of the present invented production method, 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 oxidized β -nicotinamide adenine dinucleotide (NAD⁺) and oxidized β -
30 nicotinamide adenine dinucleotide phosphate (hereinafter

sometimes referred to as NADP+). The amount of a cofactor in the reaction system is usually equimolar to or more than the amount of an α -hydroxycarboxylic acid compound as a substrate, and the cofactor is preferably added at the start of reaction.

In the step (1) of the present invented production method, 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 or α -hydroxy-isocaproic acid) proceeds, NAD⁺ in the reaction solution is converted into reduced β -nicotinamide adenine dinucleotide (hereinafter sometimes referred to as NADH). Since NADH occurred as a result of the conversion can return to the original NAD⁺ by being reacted with a protein having the ability to convert NADH into its oxidized form (NAD⁺), a protein having the ability to convert NADH into NAD⁺ may further exist in a reaction system in the above step (1). When a protein having the ability to convert NADH into NAD⁺ further exists in a reaction system in the above step (1), 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 -hydroxycarboxylic acid compound as a substrate.

Examples of the protein having the ability to convert NADH into NAD⁺ include organic acid dehydrogenases such as a malate dehydrogenase; a reductase, an alcohol dehydrogenase, an aldehyde dehydrogenase, an amino acid dehydrogenase, or an NADH oxidase. Examples of the amino acid dehydrogenase can include the present protein (C), more specifically, a leucine dehydrogenase of a microorganism belonging to the

genus *Bacillus*.

When the protein having the ability to convert NADH into NAD⁺ is an NADH oxidase, the activity of the protein is sometimes enhanced by coexistence of FAD, etc. in the reaction system, and, for example, FAD, etc. may be added to the reaction solution. When the protein having the ability to convert NADH into NAD⁺ is an NADH oxidase, 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 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 coexist in a reaction system in the above step. Examples of the protein having the ability to convert hydrogen peroxide into molecular oxygen include a catalase.

[0073]

Each of a protein having the ability to convert NADH into NAD⁺ and a protein having the ability to convert hydrogen peroxide into molecular oxygen may be provided to a reaction system in the step (1) of the present invented production method 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". For example, a transformant in which a polynucleotide encoding a protein having the ability to convert NADH into NAD⁺ is

introduced into a host cell or a treated product thereof may be provided to the above reaction system. 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 further provided to the above reaction system.

A transformant in which a polynucleotide encoding a protein having the ability to convert NADH into NAD⁺ 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 hydrogen peroxide into molecular oxygen 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 a protein having the ability to convert NADH into NAD⁺ and a polynucleotide encoding a protein having the ability to convert hydrogen peroxide into molecular oxygen are introduced in the same host cell or a treated product thereof may be provided to the above reaction system. A polynucleotide encoding a protein having the ability to convert NADH into NAD⁺ 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 a protein having the ability to
5 convert NADH into NAD⁺ 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.

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

[0074]

A reaction in the step (1) of the present invented
production method is performed by, for example, mixing
20 water, an α -hydroxycarboxylic acid compound (e.g., a sulfur-
containing α -hydroxycarboxylic acid compound such as 2-
hydroxy-4-(methylthio)butyric acid or α -hydroxy-isocaproic
acid), NAD⁺, any one or both of the present invented protein
(A) and the present invented protein (B) or a transformant
25 producing them 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.

[0075]

The pH at the time of reaction in the above method can
30 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.

5 [0076]

As the oxidation reaction of an α -hydroxycarboxylic acid compound (e.g., sulfur-containing α -hydroxycarboxylic acid such 2-hydroxy-4-(methylthio)butyric acid or α -hydroxy-isocaproic acid) proceeds, NAD⁺ in the reaction solution is
10 converted into NADH. Therefore, when a protein having the ability to convert NADH into NAD⁺ is not included in a reaction system in the above step (1), the progression of the oxidation reaction can be monitored by, for example, measuring the amount of increase of NADH in the reaction
15 solution per unit time, namely the rate of increase.

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 or α -
20 hydroxy-isocaproic 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.

[0077]

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

30 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.

5 [0078]

An α -oxocarboxylic acid (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid) obtained in the step (1) of the present invented production method is
10 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 reaction solution in the step (2), an α -oxocarboxylic acid (e.g., a sulfur-containing α -oxocarboxylic acid compound such as 2-oxo-4-
15 (methylthio)butyric acid or α -oxo-isocaproic acid) obtained in the step (1) can also be subjected to the step (2).

[0079]

In the step (2) of the present invented production method, the present protein (C) can be provided to a
20 reaction system for reaction with an α -oxocarboxylic acid compound, in various forms. The present protein (C) 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
25 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 protein (C) may be provided to the above reaction
30 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 protein (C) with an α -oxocarboxylic acid compound, specifically, for example, the present protein (C), an immobilized product of the present protein (C), a cultured product of a transformant producing the present protein (C) in which a polynucleotide encoding the present protein (C) 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).

[0080]

The step (2) of the present invented production method is usually performed in the presence of water, an ammonium ion, and a coenzyme such as NADH.

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, 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.

5 [0081]

In the step (2) of the present invented production method, 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
10 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 -
15 oxocarboxylic acid compound as a substrate, and the ammonium ion is preferably added at the start of reaction.

[0082]

In the step (2) of the present invented production method, since a cofactor is used as a conjugated system,
20 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 and reduced β -nicotinamide adenine dinucleotide phosphate (hereinafter sometimes referred to as NADPH). The amount of a cofactor in the
25 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.

[0083]

30 In the step (2) of the present invented production

method, as the reductive amination reaction of α -oxocarboxylic acid (e.g., sulfur-containing α -oxocarboxylic acid such as 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic acid) proceeds, NADH in the reaction solution is converted into oxidized β -nicotinamide adenine dinucleotide (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.

[0084]

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 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.

[0085]

In the step (2) of the present invented production method, a transformant in which a polynucleotide encoding the present protein (C) 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.

In the step (2) of the present invented production method, a transformant in which both of a polynucleotide encoding the present protein (C) and a polynucleotide encoding a protein having the ability to convert NAD⁺ into NADH are introduced in the same host cell or a treated product thereof may be provided to the reaction system. A polynucleotide encoding the present protein (C) 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 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 protein (C) and a
5 polynucleotide encoding a protein having the ability to
convert NAD⁺ into NADH may be on a chromosome of a host
cell.

[0086]

A reaction in the step (2) of the present invented
10 production method 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 or α -
oxo-isocaproic acid), the present protein (C) or a
15 transformant producing it 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.

20 [0087]

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
25 about 0°C to about 60°C in terms of the stability and the
reaction rate of raw materials and products.

[0088]

The end point of the reaction can be determined by, for
example, analyzing the amount of an α -oxocarboxylic acid
30 compound (e.g., a sulfur-containing α -oxocarboxylic acid

compound such as 2-oxo-4-(methylthio)butyric acid or α -oxo-isocaproic 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.
[0089]

Recovery of L- α -amino acid (e.g., sulfur-containing L- α -amino acid such as L-methionine or L-leucine) 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 filtration, crystallization, extraction with an organic solvent, and concentration in combination with column chromatography, distillation, or the like as needed.
[0090]

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

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

The present invented protein (A), the present invented protein (B), and the present protein (C) 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 proteins or in a treated product of the microorganism.

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

For example, any one or both of a transformant in which a polynucleotide encoding the present invented protein (A) is introduced into a host cell or a treated product thereof,
10 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 the present protein (C) is introduced into a host cell or a treated product thereof may
15 be provided to the above reaction system. A transformant in which any one or both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding the present invented protein (B) and a polynucleotide encoding the present protein (C) are introduced into the
20 same host cell or a treated product thereof may be provided to the above reaction system. Any one or both of a polynucleotide encoding the present invented protein (A) and a polynucleotide encoding the present invented protein (B) and a polynucleotide encoding the present protein (C) may be
25 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 plural polynucleotides are integrated into a single vector, for example, the polynucleotides may be integrated into a vector
30 by linking a region involved in the expression control such

as a promoter and a terminator to each of the above polynucleotides, or the polynucleotides may be integrated into a vector as an operon containing plural cistrons such as a lactose operon so that the above polynucleotides are expressed. Any one or two or more of a polynucleotide encoding the present invented protein (A), the present invented protein (B), and a polynucleotide encoding the present protein (C) may be on a chromosome of a host cell. [0091]

A reaction when the step (1) and the step (2) of the present invented production method 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 mentioned above.

In the step (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 or α -hydroxy-isocaproic acid) proceeds, NAD⁺ in the reaction solution is converted into NADH. In the step (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 or α -oxo-isocaproic acid) proceeds, NADH in the reaction solution is converted into NAD⁺. Therefore, when the step (1) and the step (2) of the present invented production method are performed in one reaction system, coenzymes (NAD⁺ and NADH) can be recycled at a catalytic amount.

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 or α -hydroxy-isocaproic acid) in the reaction solution by liquid chromatography, etc.

5 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 or L-leucine) from the reaction solution may be performed in the same manner as in
10 the step (2) of the present invented production method mentioned above.

Examples

[0092]

15 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.

[0093]

Reference Example 1 (Preparation of Chromosomal DNA)

20 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
25 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
30 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).

[0094]

Example 1 (Preparation of Present Invented Polynucleotide (A) and Present Invented Polynucleotide (B), Recombinant Vector Containing Each Thereof, and Transformant Having the Vector

(1) Primer Synthesis

Oligonucleotide primers each having a base sequence represented by any one of SEQ ID NO: 9 to 12 are synthesized.

[0095]

[Table 1]

Sense primer	Antisense primer
SEQ ID NO: 9 CCATATGAAAAAGCTCTCCATCGCCCAAG	SEQ ID NO: 10 ACTCGAGGCCTTCGTGCGGCAGGGCTTC
SEQ ID NO: 11 CCATATGAGCCAAAAACCGAAAATCATCG	SEQ ID NO: 12 ACTCGAGGCCGCGGGCTTCCCACACCTGC GGG

[0096]

(2) Recombinant Vector Containing Present Invented Polynucleotide (B)

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.

[0097]

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

[0098]

15 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.

20 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 NdeI and XhoI to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.1 kb was purified.

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

30 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.

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 restriction enzymes NdeI and XhoI, 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. When the base sequence of the

15 inserted DNA of about 1.1 kb 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 pET774. A base sequence represented by SEQ ID NO: 2 encodes

an amino acid sequence represented by SEQ ID NO: 1. The 20 plasmid pET774 is designed so that it can express a protein having an amino acid sequence 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 25 of an amino acid sequence represented by SEQ ID NO: 1.

[0099]

(3) Recombinant Vector Containing Present Invented Polynucleotide (A)

Using an oligonucleotide primer having a base sequence 30 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 and with the following reaction solution composition using the Expand High Fidelity PCR System (manufactured by Roche

5 Diagnostics).

[0100]

[Reaction Solution Composition]

Chromosomal DNA (A) solution:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
10 Primer (20 pmol/ μ l):	0.4 μ l each
5xbuffer (with $MgCl_2$):	10 μ l
enz.expandHiFi (3.5×10^3 U/ml):	0.5 μ l
Ultrapure water:	36.7 μ l

[0101]

15 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 65°C for 30 seconds,

20 followed by 72°C for one minute was performed 10 times, and then an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 70°C for 30 seconds, followed by 72°C for one minute was performed 20 times, and further the solution was maintained at 72°C for 7 minutes.

25 Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.0 kb was detected.

 By adding restriction enzymes NdeI and XhoI to the remaining PCR reaction solution, DNA was double-digested,

30 and enzymatically digested DNA of about 1.0 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 restriction enzymes NdeI and XhoI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.0 kb was confirmed to be inserted into the above vector. When the base sequence of the inserted DNA of about 1.0 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 pET43. A base sequence represented by SEQ ID NO: 4 encodes an amino acid sequence represented by SEQ ID NO: 3. The plasmid pET43 is designed so that it can express a protein having an amino acid sequence 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 an amino acid sequence represented by SEQ ID NO: 3.

[0102]

Example 2 (Preparation of Present Invented Protein (A) and Present Invented Protein (B), and Preparation of L- α -Amino Acid Compound Using the Proteins)

5 (1) Present Invented Protein (B)

An E. coli BL21(DE3) strain was transformed using the plasmid pET774. The transformant thus obtained was inoculated into 800 ml of a sterilized LB medium containing 0.1 mM IPTG and 50 μ g/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 4.9 g of the wet cells were suspended in 40 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
15 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 28 ml of centrifuged
20 supernatant liquid.

About 10 ml of the centrifuged supernatant liquid thus obtained was applied to an affinity column (HisTrap HP, gel bed 5 ml, GE Healthcare Japan) with a flow rate of 5 ml/min. By passing about 25 ml of the binding buffer through this
25 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
30 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 120 mM to 270 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 2 ml of a purified enzyme solution (40 g protein/L). With 0.31 ml of a 2-fold diluent of the purified enzyme solution thus obtained, 50 mg of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 10 mg of NAD⁺, 0.04 ml of 0.5 M Tris-HCl buffer (pH 9.0), 40 mg of ammonium sulfate, and 60 U of a leucine dehydrogenase (manufactured by Wako Pure Chemical Industries) were mixed, and the solution was shaken at 30°C for 24 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 35.3% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0103]

(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

[0104]

(2) Present Invented Protein (A)

5 An E. coli BL21(DE3) strain was transformed using the
plasmid pET43. The transformant thus obtained was
inoculated into 800 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
10 solution thus obtained was centrifuged to obtain wet cells.
About 5.2 g of the wet cells were suspended in 40 ml of 20
mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM
imidazole (namely the binding buffer), and disrupted at
2,500 rpm for 20 minutes using the Multi-beads shocker
15 (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 28 ml of centrifuged supernatant liquid.

20 About 10 ml of the centrifuged supernatant liquid thus
obtained was applied to an affinity column (HisTrap HP, gel
bed 5 ml, 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
25 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.

30 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 120 mM to 270 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 2 ml of a purified enzyme solution (9 g protein/L). Then, 0.62 ml of the purified enzyme solution thus obtained was concentrated 2-fold with the Amicon Ultra-15 (manufactured by Merck Millipore). With 0.31 ml of the concentrated solution thus obtained, 50 mg of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 10 mg of NAD⁺, 0.04 ml of 0.5 M Tris-HCl buffer (pH 9.0), 40 mg of ammonium sulfate, and 60 U of a leucine dehydrogenase (manufactured by Wako Pure Chemical Industries) were mixed, and the solution was shaken at 30°C for 24 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 36.9% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0105]

(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

[0106]

(3) Present Invented Protein (A) and Present Invented

5 Protein (B)

After 0.31 ml of a 2-fold diluent of the purified enzyme solution prepared in Example 2 (1) (40 g protein/L) was mixed with 0.31 ml of a 2-fold concentrated solution of the purified enzyme solution prepared in Example 2 (2) (9 g
10 protein/L), the mixture was concentrated 2-fold with the Amicon Ultra-15 (manufactured by Merck Millipore). With 0.31 ml of the enzyme solution thus obtained, 50 mg of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 10 mg of NAD⁺,
15 0.04 ml of 0.5 M Tris-HCl buffer (pH 9.0), 40 mg of ammonium sulfate, and 60 U of a leucine dehydrogenase (manufactured by Wako Pure Chemical Industries) were mixed, and the solution was shaken at 30°C for 3 hours. This reaction solution was subjected to content analysis by liquid
20 chromatography under the following condition. It was found that L-methionine was produced in a proportion of 47.0% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0107]

25 (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

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

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

[0108]

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

[0109]

(1) Preparation of Recombinant Vector Containing Present
10 Polynucleotide (C)

 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
15 Genomic Tip (manufactured by Qiagen) in accordance with the
method mentioned in the manual attached thereto.

 Based on a base sequence encoding a leucine
dehydrogenase derived from a *Bacillus sphaericus* IFO3525
strain mentioned in Journal of Molecular Catalysis B:
20 Enzymatic, 23 (2003) 239-247, an oligonucleotide primer
having a base sequence represented by SEQ ID NO: 14
(GCCATGGAAATCTTCAAGTATATGG) and an oligonucleotide primer
having a base sequence represented by SEQ ID NO: 15
(GGGCCCCGGTTAACGGCCGTTCAAAATATT) are synthesized.

25 Using an oligonucleotide primer having a base sequence
represented by SEQ ID NO: 14 and an oligonucleotide primer
having a base sequence represented by SEQ ID NO: 15, PCR was
performed using the above chromosomal DNA (B) as a template
and with the following reaction solution composition using
30 the Expand High Fidelity PCR System (Roche Diagnostics).

[0110]

[Reaction Solution Composition]

Chromosomal DNA (B) solution:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
5 Primer (20 pmol/ μ l):	0.4 μ l
Primer (4 pmol/ μ l):	2 μ l
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

10 [0111]

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 15 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 20 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 25 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.

30 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 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.

[0112]

(2) Recombinant Vector Containing Present Polynucleotide (C)

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: 16 (GTCGCTATATTACCGGTGAAGATGTTG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 17 (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: 16 and an oligonucleotide having a base sequence represented by SEQ ID NO: 17 as a primer, PCR was performed using the recombinant vector pTrcLD mentioned in Example 3 (1) as a template and with the following reaction

solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

	DNA solution of pTrcLD:	0.4 μ l
5	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
10	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 15 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 μ l of a DpnI restriction enzyme (contained in the above Kit) was 20 added, and then the solution was incubated at 37°C for 1 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 25 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. 30 [0113]

(3) Recombinant Vector Containing Present Polynucleotide (C)

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

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: 18 (GATAGTATTCCAACCTATGTTGCGGC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 19 (GCCGCAACATAGGGTTGGAATACTATC) (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: 18 and an oligonucleotide having a base sequence represented by SEQ ID NO: 19 as a primer, PCR was performed using the recombinant vector pTrcLD(A113G) mentioned in Example 3 (2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by 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,
5 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 µl of a
DpnI restriction enzyme (contained in the above Kit) was
added, and then the solution was incubated at 37°C for 1
10 hour. Using the incubated solution thus obtained, E. coli
DH5α was transformed.

From 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
15 designed mutation was confirmed to be introduced was
designated as pTrcLD(A113G)nd.

[0114]

(3-2) Recombinant Vector Containing Present Polynucleotide
(C)

20 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: 20
25 (GGGCATATGGAAATCTTCAAGTATATGG) (sense primer) and an
oligonucleotide primer having a base sequence represented by
SEQ ID NO: 21 (GGATCCTTAACGGCCGTTCAAAATATT) (antisense
primer) are synthesized. Using an oligonucleotide primer
having a base sequence represented by SEQ ID NO: 20 and an
30 oligonucleotide primer having a base sequence represented by

SEQ ID NO: 21 as a primer, PCR was performed using the recombinant vector pTrcLD(A113G)nd mentioned in Example 3 (3-1) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche

[0115]

[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 $MgCl_2$): 10 μ l

enz.expandHiFi (3.5×10^3 U/ml): 0.5 μ l

Ultrapure water: 36.7 μ l

[0116]

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, 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.

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 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 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, 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 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 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).

[0117]

(4) Present Protein (C)

An *E. coli* BL21(DE3) strain was transformed using the recombinant vector pETLD(A113G). The transformant thus

obtained was inoculated into 800 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 6 g of wet cells. About 6 g of the wet cells were suspended in 40 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole (namely 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 25 ml of centrifuged supernatant liquid. About 10 ml of the centrifuged supernatant liquid thus obtained was applied to an affinity column (HisTrap HP, 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 30 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 2 ml of a fraction (32.6 g protein/L). This fraction is hereinafter referred to as the leucine dehydrogenase (A113G) purified enzyme solution.

[0118]

- 5 Example 4 (Preparation of L- α -Amino Acid Compound using Present Invented Protein (A), Present Invented Protein (B), and Present Protein (C))

After 0.31 ml of a 2-fold diluent of the purified enzyme solution prepared in Example 2 (1) (40 g protein/L) was mixed with 0.31 ml of a 2-fold concentrated solution of the purified enzyme solution prepared in Example 2 (2) (9 g protein/L), the mixture was concentrated 2-fold with the Amicon Ultra-15 (manufactured by Merck Millipore). With 0.31 ml of the enzyme solution thus obtained, 50 mg of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid (manufactured by Tokyo Chemical Industry), 10 mg of NAD⁺, 0.04 ml of 0.5 M Tris-HCl buffer (pH 9.0), 40 mg of ammonium sulfate, and 0.15 ml of the leucine dehydrogenase (A113G) purified enzyme solution prepared in Example 3 (4) (32.6 g protein/L) were mixed, and the solution was shaken at 30°C for 1 hour. 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 49.9% based on the amount of a calcium salt of 2-hydroxy-4-(methylthio)butyric acid used for the reaction.

[0119]

(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

5 Detection: 210 nm

[0120]

Example 5 (Preparation of Present Invented Polynucleotide
(A), Present Invented Recombinant Vector (ABC), and
Transformant Having the Vector)

10 (1) Recombinant Vector Containing Present Invented
Polynucleotide (A)

Double-stranded DNA having a base sequence represented
by SEQ ID NO: 13 in which the base sequence ccatggct is
added to its 5' end and the base sequence ggatcc is added to
15 its 3' end is prepared. A base sequence represented by SEQ
ID NO: 13 encodes an amino acid sequence represented by SEQ
ID NO: 3.

The double-stranded DNA of about 1.0 kb thus prepared
was double-digested with restriction enzymes NcoI and BamHI,
20 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.

25 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.
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 restriction enzymes NcoI and BamHI, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.0 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 pTrc43SC.

[0121]

(2) Present Invented Recombinant Vector (ABC)

(2-1) Recombinant Vector Containing Present Polynucleotide (C)

Based on a base 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 oligonucleotide having a base sequence represented by SEQ ID NO: 22 (CGTAGCTTACAAACTTTGCGAGTATTTAC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 23 (GTAAATACTCGCAAAGTTTGTAAGCTACG) (antisense primer) are synthesized as a primer for mutation introduction for substituting the 561st guanine by adenine. 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 plasmid pTrcLD(A113G)nd mentioned in Example 3 (3) as a template and with the following reaction solution composition using the QuikChange II Site-Directed

Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrcLD(A113G)nd:	1 μ l
dNTP mix (contained in the above Kit):	1 μ l
5 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

10 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, 15 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 1 20 hour. Using the incubated solution thus obtained, E. coli DH5 α was transformed.

 From 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 25 designed mutation was confirmed to be introduced was designated as pTrcLD(A113G)ndhd.

[0122]

(2-2) Recombinant Vector Containing Present Invented Polynucleotide (B)

30 Using an oligonucleotide having a base sequence

represented by SEQ ID NO: 24

(CCATGGCTATGAAAAAGCTCTCCATCGCCC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 25 (CAAGCTTGCTAGCCTTCGTGCGGCAGGGCTTC) (antisense primer)

5 as a primer, PCR was performed using the plasmid pET774 mentioned in Example 1 (2) as a template and with the following reaction solution composition.

[0123]

[Reaction Solution Composition]

10	DNA solution of pET774:	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
15	Ultrapure water:	11.9 μ l

[0124]

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
20 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
25 solution was subjected to agarose gel electrophoresis. A DNA band of about 1.1 kb was detected.

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

30 Plasmid vector pTrc99A (manufactured by GE Healthcare

Japan) was double-digested with restriction enzymes NcoI and HindIII, 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.

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 HindIII, 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 pTrc774NH.

[0125]

(2-3) Recombinant Vector Containing Present Invented Polynucleotide (B) and Present Polynucleotide (C)

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 26 (CGGATCCGAGGAAACAGACCATGG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 27 (GCTGCAGCCTAGCCTTCGTGCGGCAGGGCTTC) (antisense primer), PCR was performed using the plasmid pTrc774NH mentioned in Example 5 (2-2) as a template and with the following reaction solution composition.

[0126]

[Reaction Solution Composition]

DNA solution of pTrc774NH:	1.5 μ l
dNTP (a mixture of 2 mM each):	10 μ l
5 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

[0127]

10 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,
15 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 1.1 kb was detected.

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

 The plasmid pTrcLD(A113G)ndhd mentioned in Example 5 (2-1) was double-digested with restriction enzymes BamHI and
25 PstI, and enzymatically digested plasmid 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, 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

5 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 BamHI and PstI, and then subjected to agarose gel electrophoresis. In each of six plasmids,

10 DNA of about 1.1 kb was confirmed to be inserted into the plasmid pTrcLD(A113G)ndhd. One of these plasmids was designated as pTrcLD(A113G)/774.

[0128]

(2-4) Recombinant Vector Containing Present Invented

15 Polynucleotide (A), Present Invented Polynucleotide (B), and Present Polynucleotide (C)

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 28 (GCTGCAGCAGGAAACAGACCATGG)

(sense primer) and an oligonucleotide having a base sequence
20 represented by SEQ ID NO: 29

(CAAGCTTGTTAGCCACGGGCTTCCCACACC) (antisense primer) as a primer, PCR was performed using the plasmid pTrc43SC

mentioned in Example 5 (1) as a template and with the following reaction solution composition using the Expand

25 High Fidelity PCR System (Roche Diagnostics).

[0129]

[Reaction Solution Composition]

DNA solution of pTrc43SC: 1 µl

dNTP (a mixture of 2.5 mM each): 1 µl

30 Primer (20 pmol/µl): 0.4 µl each

5xbuffer (with MgCl₂): 10 µl
enz.expandHiFi (3.5 x 10³ U/ml): 0.5 µl
Ultrapure water: 36.7 µl
[0130]

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 65°C for 30 seconds,
10 followed by 72°C for one minute was performed 10 times, and then an incubation cycle consisting of incubation at 94°C for 15 seconds, followed by 70°C for 30 seconds, followed by 72°C for one minute 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 1.0 kb was detected.

By adding restriction enzymes PstI and HindIII to the remaining PCR reaction solution, DNA was double-digested,
20 and enzymatically digested DNA of about 1.0 kb was purified.

The plasmid pTrcLD(A113G)/774 mentioned in Example 5 (2-3) was double-digested with restriction enzymes PstI and HindIII, and enzymatically digested plasmid DNA 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
30 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 PstI and HindIII, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.0 kb was confirmed to be inserted into the plasmid pTrcLD(A113G)/774. One of these plasmids was designated as pTrcLD(A113G)/774/43SC.

[0131]

Example 6 (Preparation of L-α-Amino Acid Compound Using Treated Product of Transformant Having Present Invented Recombinant Vector (ABC))

[0132]

An E. coli JM109 strain was transformed using the plasmid pTrcLD(A113G)/774/43SC. 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 wet cells. About 0.8 g of the wet cells were suspended in 5 ml of 0.5 M Tris-HCl buffer (pH 9), 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 3.5 ml of centrifuged supernatant liquid (28 g protein/L).

Then, 0.34 ml of the centrifuged supernatant liquid thus obtained was concentrated 3.4-fold with the Amicon Ultra-15 (manufactured by Merck Millipore). With 0.1 ml of the concentrated centrifuged supernatant liquid thus
5 obtained, 0.35 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 0.5 M Tris-HCl buffer (pH 9.0), and 20 mg of ammonium sulfate were mixed, and the solution was
10 shaken at 30°C for 1 hour. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that 93.5 g/L of L-methionine was produced. The yield of L-methionine based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for
15 the reaction was 30.4%.

[0133]

(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
20 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

25 Detection: 210 nm

[0134]

Example 7 (Preparation of Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) and Transformant Having the Vector

30 (1) Recombinant Vector Containing Polynucleotide Encoding

Present Invented Protein (A) Having Amino Acid Sequence
Represented by SEQ ID NO: 3
(1-1)

An oligonucleotide having a base sequence represented
5 by SEQ ID NO: 30 (CCATATGTCTCAAAAACCAAAAATCATCG) (sense
primer) and an oligonucleotide having a base sequence
represented by SEQ ID NO: 31

(ACTCGAGGCCACGGGCTTCCCACACCTGCG) (antisense primer) are

synthesized. Using an oligonucleotide primer having a base
10 sequence represented by SEQ ID NO: 30 and an oligonucleotide
primer having a base sequence represented by SEQ ID NO: 31,
PCR was performed using the plasmid pTrc43SC mentioned in
Example 5 (1) as a template and with the following reaction
solution composition using the Expand High Fidelity PCR
15 System (Roche Diagnostics).

[0135]

[Reaction Solution Composition]

DNA solution of pTrc43SC:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
20 Primer (20 pmol/ μ l):	0.4 μ l each
5xbuffer (with $MgCl_2$):	10 μ l
enz.expandHiFi (3.5×10^3 U/ml):	0.5 μ l
Ultrapure water:	36.7 μ l

[0136]

25 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,
30 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.

5 Subsequently, a part of the above PCR reaction solution was subjected to agarose gel electrophoresis. A DNA band of about 1.0 kb was detected.

 By adding restriction enzymes NdeI and XhoI to the remaining PCR reaction solution, DNA was double-digested,
10 and enzymatically digested DNA of about 1.0 kb was purified.

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

 These purified DNAs were mixed and ligated with a T4
15 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, 10 colonies were randomly selected from the growing colonies. Each of
20 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
25 Qiagen). A part of each of the removed plasmids was double-digested with restriction enzymes NdeI and XhoI, and then subjected to agarose gel electrophoresis. In each of four plasmids, DNA of about 1.0 kb was confirmed to be inserted into the above vector. One of these plasmids was designated
30 as pET43SCcHis. The plasmid pET43SCcHis is designed so that

it can express a protein having an amino acid sequence 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 an amino acid sequence represented by SEQ ID NO: 3.

[0137]

(1-2)

An oligonucleotide having a base sequence represented by SEQ ID NO: 32 (GCCATGGCTATGTCTCAAAAACCAAAATC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 33 (GGATCCTCAGTGGTGGTGGTGGTGGTG) (antisense primer) are synthesized. Using an oligonucleotide primer having a base sequence represented by SEQ ID NO: 32 and an oligonucleotide primer having a base sequence represented by SEQ ID NO: 33, PCR was performed using the plasmid pET43SCcHis mentioned in Example 7 (1-1) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0138]

[Reaction Solution Composition]

DNA solution of pET43SCcHis:	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

[0139]

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,
5 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.

10 Subsequently, a part of the above PCR reaction
solution was subjected to agarose gel electrophoresis. A
DNA band of about 1.0 kb was detected.

By adding restriction enzymes NcoI and BamHI to the
remaining PCR reaction solution, DNA was double-digested,
15 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 DNA was purified.

These purified DNAs were mixed and ligated with a T4
20 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, 10 colonies
were randomly selected from the growing colonies. Each of
25 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
30 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 four plasmids, the DNA of about 1.0 kb was confirmed to be inserted. One of these plasmids was designated as

5 pTrc43SCcHis. The plasmid pTrc43SCcHis is designed so that it can express a protein having an amino acid sequence 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
10 terminus of an amino acid sequence represented by SEQ ID NO: 3.

[0140]

(2) Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid
15 Sequence V109I

Based on a base sequence represented by SEQ ID NO: 13, an oligonucleotide having a base sequence represented by SEQ ID NO: 34 (CGCTGTTGCTGATATTACCATCGGCCTG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ
20 ID NO: 35 (CAGGCCGATGGTAATATCAGCAACAGCG) (antisense primer) are synthesized as a primer for mutation introduction for substituting the 325th guanine by adenine. Substitution of the 325th guanine by adenine in a base sequence represented by SEQ ID NO: 13 brings about substitution of the 109th
25 valine by isoleucine in an amino acid sequence represented by SEQ ID NO: 3.

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 34 and an oligonucleotide having a base sequence represented by SEQ ID NO: 35 as a primer, PCR
30 was performed using the plasmid pTrc43SCcHis mentioned in

Example 7 (1-2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

5	DNA solution of pTrc43SCcHis:	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
10	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 1 hour. Using the incubated solution thus obtained, E. coli DH5 α was transformed.

From 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 pTrc43SCcHis-g325a. The plasmid pTrc43SCcHis-g325a is designed so that it can express a protein having an amino acid sequence 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 an altered amino acid sequence having substitution of the 109th valine by isoleucine in an amino acid sequence represented by SEQ ID NO: 3.

[0141]

(3) Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence G191D

Based on a base sequence represented by SEQ ID NO: 13, an oligonucleotide having a base sequence represented by SEQ ID NO: 36 (GGCGGCATACGTCGATCGTGACGAGCTG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 37 (CAGCTCGTCACGATCGACGTATGCCGCC) (antisense primer) are synthesized as a primer for mutation introduction for substituting the 572nd guanine by adenine. Substitution of the 572nd guanine by adenine in a base sequence represented by SEQ ID NO: 13 brings about substitution of the 191st glycine by aspartic acid in an amino acid sequence represented by SEQ ID NO: 3.

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 36 and an oligonucleotide having a base sequence represented by SEQ ID NO: 37 as a primer, PCR was performed using the plasmid pTrc43SCcHis mentioned in Example 7 (1-2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis: 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
5 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
10 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
15 added, and then the solution was incubated at 37°C for 1 hour. Using the incubated solution thus obtained, E. coli DH5 α was transformed.

From the transformants thus obtained, plasmids were extracted, and then a base sequence at the mutation site was
20 determined by the dideoxy method. A plasmid into which the designed mutation was confirmed to be introduced was designated as pTrc43SCcHis-g572a. The plasmid pTrc43SCcHis-g572a is designed so that it can express a protein having an amino acid sequence in which an amino acid sequence composed
25 of 8 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 45: LeuGluHisHisHisHisHisHis) is added to the carboxy terminus of an altered amino acid sequence having substitution of the 191st glycine by aspartic acid in an amino acid sequence represented by SEQ ID NO: 3.

30 [0142]

(4) Recombinant Vector Containing Polynucleotide Encoding
Present Invented Protein (A) Having Altered Amino Acid
Sequence Q246R

Based on a base sequence represented by SEQ ID NO: 13,
an oligonucleotide having a base sequence represented by SEQ
ID NO: 38 (GCAGCACTGGTACGGGCACTGCGCTCTG) (sense primer) and
an oligonucleotide having a base sequence represented by SEQ
ID NO: 39 (CAGAGCGCAGTGCCCGTACCAGTGCTGC) (antisense primer)
are synthesized as a primer for mutation introduction for
substituting the 737th adenine by guanine. Substitution of
the 737th adenine by guanine in a base sequence represented
by SEQ ID NO: 13 brings about substitution of the 246th
glutamine by arginine in an amino acid sequence represented
by SEQ ID NO: 3.

Using an oligonucleotide having a base sequence
represented by SEQ ID NO: 38 and an oligonucleotide having a
base sequence represented by SEQ ID NO: 39 as a primer, PCR
was performed using the plasmid pTrc43SCcHis mentioned in
Example 7 (1-2) as a template and with the following
reaction solution composition using the QuikChange II Site-
Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis:	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,
5 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 µl of a
DpnI restriction enzyme (contained in the above Kit) was
added, and then the solution was incubated at 37°C for 1
10 hour. Using the incubated solution thus obtained, E. coli
DH5α was transformed.

From 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
15 designed mutation was confirmed to be introduced was
designated as pTrc43SCcHis-a737g. The plasmid pTrc43SCcHis-
a737g is designed so that it can express a protein having an
amino acid sequence in which an amino acid sequence composed
of 8 amino acids including consecutive 6 residues of
20 histidine (SEQ ID NO: 45: LeuGluHisHisHisHisHisHis) is added
to the carboxy terminus of an altered amino acid sequence
having substitution of the 246th glutamine by arginine in an
amino acid sequence represented by SEQ ID NO: 3.

[0143]

25 (5) Recombinant Vector Containing Polynucleotide Encoding
Present Invented Protein (A) Having Altered Amino Acid
Sequence V109I/G191D

Using an oligonucleotide having a base sequence
represented by SEQ ID NO: 36 and an oligonucleotide having a
30 base sequence represented by SEQ ID NO: 37, which are

primers for mutation introduction for converting the 572nd
guanine in a base sequence represented by SEQ ID NO: 13 to
adenine, as a primer, PCR was performed using the plasmid
pTrc43SCcHis-g325a mentioned in Example 7 (2) as a template
5 and with the following reaction solution composition using
the QuikChange II Site-Directed Mutagenesis Kit
(manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis-g325a:	1 μ l
10 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
15 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
20 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
25 added, and then the solution was incubated at 37°C for 1
hour. Using the incubated solution thus obtained, E. coli
DH5 α was transformed.

From the transformants thus obtained, plasmids were
extracted, and then a base sequence at the mutation site was
30 determined by the dideoxy method. A plasmid into which the

designed mutation was confirmed to be introduced was designated as pTrc43SCcHis-g325a/g572a. The plasmid pTrc43SCcHis-g325a/g572a is designed so that it can express a protein having an amino acid sequence 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 an altered amino acid sequence having substitution of the 109th valine by isoleucine and substitution of the 191st glycine by aspartic acid in an amino acid sequence represented by SEQ ID NO: 3.

[0144]

(6) Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence V109I/Q246R

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 38 and an oligonucleotide having a base sequence represented by SEQ ID NO: 39, which are primers for mutation introduction for converting the 737th guanine in a base sequence represented by SEQ ID NO: 13 to adenine, as a primer, PCR was performed using the plasmid pTrc43SCcHis-g325a mentioned in Example 7 (2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis-g325a:	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 µl
PfuUltra (contained in the above Kit): 1 µl
Ultrapure water: 41.2 µl

A container containing a reaction solution with the
5 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
10 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 1
hour. Using the incubated solution thus obtained, E. coli
15 DH5α was transformed.

From 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
20 designated as pTrc43SCcHis-g325a/a737g. The plasmid
pTrc43SCcHis-g325a/a737g is designed so that it can express
a protein having an amino acid sequence in which an amino
acid sequence composed of 8 amino acids including
consecutive 6 residues of histidine (SEQ ID NO: 45:
25 LeuGluHisHisHisHisHisHis) is added to the carboxy terminus
of an altered amino acid sequence having substitution of the
109th valine by isoleucine and substitution of the 246th
glutamine by arginine in an amino acid sequence represented
by SEQ ID NO: 3.

30 [0145]

(7) Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence G191D/Q246R

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 38 and an oligonucleotide having a base sequence represented by SEQ ID NO: 39, which are primers for mutation introduction for converting the 737th guanine in a base sequence represented by SEQ ID NO: 13 to adenine, as a primer, PCR was performed using the plasmid pTrc43SCcHis-g572a mentioned in Example 7 (3) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis-g572a:	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 1 hour. Using the incubated solution thus obtained, E. coli DH5α was transformed.

From 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 pTrc43SCcHis-g572a/a737g. The plasmid pTrc43SCcHis-g572a/a737g is designed so that it can express a protein having an amino acid sequence 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 an altered amino acid sequence having substitution of the 191st glycine by aspartic acid and substitution of the 246th glutamine by arginine in an amino acid sequence represented by SEQ ID NO: 3.

[0146]

(8) Recombinant Vector Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence V109I/G191D/Q246R

Using an oligonucleotide having a base sequence represented by SEQ ID NO: 38 and an oligonucleotide having a base sequence represented by SEQ ID NO: 39, which are primers for mutation introduction for converting the 737th adenine in a base sequence represented by SEQ ID NO: 13 to guanine, as a primer, PCR was performed using the plasmid pTrc43SCcHis-g325a/g572a mentioned in Example 7 (5) as a template and with the following reaction solution composition using the QuikChange II Site-Directed

Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pTrc43SCcHis-g325a/g572a: 1 μ l

dNTP mix (contained in the above Kit): 1 μ l

5 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

10 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, 15 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 1 20 hour. Using the incubated solution thus obtained, E. coli DH5 α was transformed.

From 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 25 designed mutation was confirmed to be introduced was designated as pTrc43SCcHis-g325a/g572a/a737g. The plasmid pTrc43SCcHis-g325a/g572a/a737g is designed so that it can express a protein having an amino acid sequence in which an amino acid sequence composed of 8 amino acids including 30 consecutive 6 residues of histidine (SEQ ID NO: 45:

LeuGluHisHisHisHisHisHis) is added to the carboxy terminus of an altered amino acid sequence having substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine in an amino acid sequence represented by SEQ ID NO: 3.

[0147]

Example 8

Thermal Stability Evaluation of Present Invented Protein (A) (1)

An E. coli JM109 strain was transformed using the plasmid pTrc43SCcHis, pTrc43SCcHis-g325a/g572a, pTrc43SCcHis-g325a/a737g, or pTrc43SCcHis-g325a/g572a/a737g. 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. Each of the culture solutions thus obtained was centrifuged to obtain about 0.1 g of wet cells. About 0.1 g of each of the wet cells was suspended in 1 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 5 mM imidazole (namely 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 13,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of each of centrifuged supernatant liquid.

About 0.3 ml of the binding buffer was added to each of the centrifuged supernatant liquid thus obtained to make about 1 ml, and then this solution was applied to an affinity column (HisTrap HP, gel bed 1 ml, GE Healthcare

Japan). By passing about 3 ml of the binding buffer through this column, non-adsorbed proteins were eluted. Then, by passing about 2 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole through this column, each adsorbed protein was eluted, and eluates were collected. About 2 ml of each eluate 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 0.4 ml of each of purified enzyme solutions. The protein concentration of each purified enzyme solution was measured. The protein concentration of the purified enzyme solution from the pTrc43SCcHis-introduced transformant was 3.1 g protein/L, that from the pTrc43SCcHis-g325a/g572a-introduced transformant was 2.5 g protein/L, that from the pTrc43SCcHis-g325a/a737g-introduced transformant was 3.1 g protein/L, and that from the pTrc43SCcHis-g325a/g572a/a737g-introduced transformant was 3.2 g protein/L.

Each of the purified enzyme solutions thus obtained was diluted so that the concentration was 0.45 g protein/L, and 0.06 ml of the diluted enzyme solution thus obtained was heated at 41°C for 30 minutes and then cooled to 4°C. After 0.045 ml of the enzyme solution thus obtained was mixed with 0.055 ml of 0.5 M Tris-HCl buffer (pH 9.0), calcium 2-hydroxy-4-(methylthio)butyrate (manufactured by Tokyo Chemical Industry) and NAD⁺ were added so that the concentration was 47 mM and 3.2 mM, respectively, and the solution was incubated at 30°C. The absorbance of the reaction solution was measured at a wavelength of 340 nm for about 10 minutes at 10-second intervals. The enzyme

activity was calculated by defining the enzyme amount which reduces 1 μ mol of NAD⁺ per minute as 1 U. A reaction was performed in the same manner using a non-heated diluted enzyme solution as a control, and the absorbance of the reaction solution was measured to calculate the enzyme activity. By defining the enzyme activity in a reaction using a non-heated diluted enzyme solution as 100%, the remaining activity of a heated diluted enzyme solution was calculated. The results are shown in Table 2.

[0148]

[Table 2]

Enzyme solution		Remaining activity (%)	
Introduced plasmid	Amino acid sequence	Non-heated	Heated (41°C, 30 min)
pTrc43SCcHis	SEQ ID NO: 3	100	0
pTrc43SCcHis-g325a/g572a	V109I/G191D	100	51.6
pTrc43SCcHis-g325a/a737g	V109I/Q246R	100	56.4
pTrc43SCcHis-g325a/g572a/a737g	V109I/G191D/Q246R	100	59.9

[0149]

(2)

An E. coli JM109 strain was transformed using the pTrc43SCcHis, pTrc43SCcHis-g325a, pTrc43SCcHis-g 572a, pTrc43SCcHis-a737g, pTrc43SCcHis-g325a/g572a, pTrc43SC cHis-g572a/a737g, pTrc43SCcHis-g325a/a737g, or pTrc43SCcHis-g325a/g572a/a737g. 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. Each of the culture solutions thus obtained was centrifuged to obtain about 0.1 g of wet cells. About 0.1 g of each of the wet cells was suspended in 1 ml of 20 mM phosphate buffer (pH

7.4) containing 0.5 M NaCl and 5 mM imidazole (namely 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 12,000 rpm and 4°C for 10 minutes to obtain about 0.7 ml of each of centrifuged supernatant liquid.

About 0.3 ml of the binding buffer was added to each of the centrifuged supernatant liquid thus obtained to make about 1 ml, and then this solution was applied to an affinity column (HisTrap HP, gel bed 1 ml, GE Healthcare Japan). By passing about 3 ml of the binding buffer through this column, non-adsorbed proteins were eluted. Then, by passing about 2 ml of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole through this column, each adsorbed protein was eluted, and eluates were collected. About 2 ml of each eluate 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 0.4 ml of each of purified enzyme solutions. The protein concentration of each purified enzyme solution was measured. The protein concentration of the purified enzyme solution from the pTrc43SCcHis-introduced transformant was 2.3 g protein/L, that from the pTrc43SCcHis-g325a-introduced transformant was 2.9 g protein/L, that from the pTrc43SCcHis-g572a-introduced transformant was 2.9 g protein/L, that from the pTrc43SCcHis-a737g-introduced transformant was 4.2 g protein/L, that from the pTrc43SCcHis-g325a/g572a-introduced transformant was 2.1 g

protein/L, that from the pTrc43SCcHis-g572a/a737g-introduced transformant was 3.3 g protein/L, that from the pTrc43SCcHis-g325a/a737g-introduced transformant was 2.6 g protein/L, and that from the pTrc43SCcHis-g325a/g572a/a737g-introduced transformant was 2.7 g protein/L.

Each of the purified enzyme solutions thus obtained was diluted so that the concentration was 0.45 g protein/L, and 0.06 ml of the diluted enzyme solution thus obtained was heated at 40.5°C for 30 minutes and then cooled to 4°C.

After 0.045 ml of the enzyme solution thus obtained was mixed with 0.055 ml of 0.5 M Tris-HCl buffer (pH 9.0), calcium 2-hydroxy-4-(methylthio)butyrate (manufactured by Tokyo Chemical Industry) and NAD⁺ were added so that the concentration was 47 mM and 3.2 mM, respectively, and the solution was incubated at 30°C. The absorbance of the reaction solution was measured at a wavelength of 340 nm for about 10 minutes at 10-second intervals. The enzyme activity was calculated by defining the enzyme amount which reduces 1 μmol of NAD⁺ per minute as 1 U. A reaction was performed in the same manner using a non-heated diluted enzyme solution as a control, and the absorbance of the reaction solution was measured to calculate the enzyme activity. By defining the enzyme activity in a reaction using a non-heated diluted enzyme solution as 100%, the remaining activity of a heated diluted enzyme solution was calculated. The results are shown in Table 3.

[0150]

[Table 3]

Enzyme solution		Remaining activity (%)	
Introduced plasmid	Amino acid sequence	Non-heated	Heated (40.5°C, 30 min)
pTrc43SCcHis	SEQ ID NO: 3	100	6.9
pTrc43SCcHis-g325a	V109I	100	16.6
pTrc43SCcHis-g572a	G191D	100	8.1
pTrc43SCcHis-a737g	Q246R	100	22.5
pTrc43SCcHis-g325a/g572a	V109I/G191D	100	10.9
pTrc43SCcHis-g572a/a737g	G191D/Q246R	100	11.9
pTrc43SCcHis-g325a/a737g	V109I/Q246R	100	53.1
pTrc43SCcHis-g325a/g572a/a737g	V109I/G191D/Q246R	100	65.1

[0151]

5 Example 9 (Preparation of Present Invented Recombinant Vector (ABC) and Transformant Having the Vector)

(1) Present Invented Recombinant Vector (ABC) Containing Polynucleotide Encoding Present Invented Protein (A) Having Amino Acid Sequence Represented by SEQ ID NO: 3

10 [0152]

(1-1) Recombinant Vector Containing Present Polynucleotide (C)

Based on a base 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 oligonucleotide having a base sequence represented by SEQ ID NO: 22 (CGTAGCTTACAAACTTTGCGAGTATTTAC) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 23 (GTAAATACTCGCAAAGTTTGTAAGCTACG) (antisense primer) are synthesized as a primer for mutation introduction for converting the 561st guanine to adenine.

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 plasmid pETLD(A113G) mentioned in Example 3 (3-2) as a template and with the following reaction solution composition using the QuikChange II Site-Directed Mutagenesis Kit (manufactured by STRATAGENE).

[Reaction Solution Composition]

DNA solution of pETLD(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

[PCR Reaction Condition]

A container containing a reaction solution with the above reaction solution 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 6.8 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 1 hour. Using the incubated solution thus obtained, E. coli DH5 α was transformed.

From 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 pETLD(A113G)hd.

[0153]

By adding restriction enzymes NcoI and BamHI to the plasmid pETLD(A113G)hd, the plasmid 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 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 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 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 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. One of these plasmids was designated as pTrcLD(A113G)nh. The plasmid pTrcLD(A113G)nh is designed so that it can express a protein having an amino acid sequence in which an amino acid sequence composed of 20 amino acids including consecutive 6 residues of histidine (SEQ ID NO: 44:

MetGlySerSerHisHisHisHisHisSerSerGlyLeuValProArgGlySerHis

solution was subjected to agarose gel electrophoresis. A DNA band of about 1.1 kb was detected.

By adding restriction enzymes NcoI and PstI 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 PstI, 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 PstI, 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 pTrc774chNP. The plasmid pTrc774chNP is designed so that it can express a protein having an amino acid sequence 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 an amino acid sequence represented by SEQ ID NO:

DNA band of about 1.1 kb was detected.

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

5 The plasmid pTrcLD(A113G)nh mentioned in Example 9 (1-1) was double-digested with restriction enzymes BamHI and PstI, and enzymatically digested plasmid DNA was purified.

These purified DNAs were mixed and ligated with a T4 DNA ligase, and using the ligation solution thus obtained,
10 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
15 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
20 with restriction enzymes BamHI and PstI, 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 plasmid pTrcLD(A113G)nh. One of these plasmids was designated as plasmid pTrcLD(A113G)nh/774ch.

25 [0160]

(1-4) Recombinant Vector Containing Present Invented Polynucleotide (A)

An oligonucleotide having a base sequence represented by SEQ ID NO: 32 (GCCATGGCTATGTCTCAAAAACCAAAAATC) (sense
30 primer) and an oligonucleotide having a base sequence

represented by SEQ ID NO: 41 (CAAGCTTGTCAGTGGTGGTGGTGGTGGTG)
(antisense primer) are synthesized. Using an
oligonucleotide primer having a base sequence represented by
SEQ ID NO: 32 and an oligonucleotide primer having a base
5 sequence represented by SEQ ID NO: 41, PCR was performed
using the plasmid pET43SCcHis mentioned in Example 7 (1-1)
as a template and with the following reaction solution
composition using the Expand High Fidelity PCR System (Roche
Diagnostics).

10 [0161]

[Reaction Solution Composition]

DNA solution of pET43SCcHis:	1 μ l
dNTP (a mixture of 2.5 mM each):	1 μ l
Primer (20 pmol/ μ l):	0.4 μ l each
15 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

[0162]

A container containing a reaction solution with the
20 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,
followed by 72°C for 1.5 minutes was performed 10 times, and
25 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.

Subsequently, a part of the above PCR reaction
30 solution was subjected to agarose gel electrophoresis. A

DNA band of about 1.0 kb was detected.

By adding restriction enzymes NcoI and HindIII to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.0 kb was purified.

5 Plasmid vector pTrc99A (manufactured by GE Healthcare Japan) was double-digested with restriction enzymes NcoI and HindIII, 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 E. coli DH5α was transformed.

The transformant thus obtained was cultured in an LB agar medium containing 50 µg/ml of ampicillin, 10 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 restriction enzymes NcoI and HindIII, and then subjected to agarose gel electrophoresis. In each of four plasmids, DNA of about 1.0 kb was confirmed to be inserted into the above vector. One of these plasmids was designated as pTrc43SCchNH. The plasmid pTrc43SCchNH is designed so
25 that it can express a protein having an amino acid sequence 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 an amino acid sequence represented by SEQ ID NO:
30 3.

[0163]

(1-5) Recombinant Vector Containing Present Invented Polynucleotide (A), Present Invented Polynucleotide (B), and Present Polynucleotide (C)

5 Using an oligonucleotide having a base sequence represented by SEQ ID NO: 28 (GCTGCAGCAGGAAACAGACCATGG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 41 (CAAGCTTGTCAGTGGTGGTGGTGGTGGTG) (antisense primer) as a primer, PCR was performed using the
10 plasmid pTrc43SCchNH mentioned in Example 9 (1-4) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0164]

15 [Reaction Solution Composition]

DNA solution of pTrc43SCchNH:	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
20 enz.expandHiFi (3.5 x 10 ³ U/ml):	0.5 μ l
Ultrapure water:	36.7 μ l

[0165]

25 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 65°C for 30 seconds, followed by 72°C for one minute was performed 10 times, and then an incubation cycle consisting of incubation at 94°C
30 for 15 seconds, followed by 70°C for 30 seconds, followed by

72°C for one minute 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 1.0 kb was detected.

By adding restriction enzymes PstI and HindIII to the remaining PCR reaction solution, a DNA fragment was double-digested, and enzymatically digested DNA of about 1.0 kb was purified.

The plasmid pTrcLD(A113G)nh/774ch mentioned in Example 9 (1-3) was double-digested with restriction enzymes PstI and HindIII, and enzymatically digested plasmid 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 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 PstI and HindIII, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.0 kb was confirmed to be inserted into the plasmid pTrcLD(A113G)nh/774ch. One of these plasmids was designated as pTrcLD(A113G)nh/774ch/43SCch.

[0166]

(2) Present Invented Recombinant Vector (ABC) Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence V109I/G191D/Q246R

5 Using an oligonucleotide having a base sequence represented by SEQ ID NO: 28 (GCTGCAGCAGGAAACAGACCATGG) (sense primer) and an oligonucleotide having a base sequence represented by SEQ ID NO: 41 (CAAGCTTGTCAGTGGTGGTGGTGGTGGTG) (antisense primer) as a primer, PCR was performed using the
10 plasmid pTrc43SCcHis-g325a/g572a/a737g mentioned in Example 7 (8) as a template and with the following reaction solution composition using the Expand High Fidelity PCR System (Roche Diagnostics).

[0167]

15 [Reaction Solution Composition]

DNA solution of pTrc43SCcHis-g325a/g572a/a737g: 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

20 enz.expandHiFi (3.5×10^3 U/ml): 0.5 μ l

Ultrapure water: 36.7 μ l

[0168]

A container containing a reaction solution with the above composition was set in a thermal cycler (PERKIN ELMER-
25 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 65°C for 30 seconds, followed by 72°C for one minute was performed 10 times, and then an incubation cycle consisting of incubation at 94°C
30 for 15 seconds, followed by 70°C for 30 seconds, followed by

72°C for one minute 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 1.0 kb was detected.

By adding restriction enzymes PstI and HindIII to the remaining PCR reaction solution, DNA was double-digested, and enzymatically digested DNA of about 1.0 kb was purified.

The plasmid TrcLD(A113G)nh/774ch/43SCch mentioned in Example 9 (1-5) was double-digested with restriction enzymes PstI and HindIII, and enzymatically digested plasmid DNA of about 6.4 kb 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 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 PstI and HindIII, and then subjected to agarose gel electrophoresis. In each of six plasmids, DNA of about 1.0 kb was confirmed to be inserted into the above plasmid DNA of about 6.4 kb. One of these plasmids was designated as pTrcLD(A113G)nh/774ch/43SCMch.

[0169]

Example 10 (Preparation of L- α -Amino Acid Compound Using
Treated Product of Transformant Having Present Invented
Recombinant Vector (ABC))

[0170]

- 5 (1) Transformant Having Present Invented Recombinant Vector
(ABC) Containing Polynucleotide Encoding Present Invented
Protein (A) Having Amino Acid Sequence Represented by SEQ ID
NO: 3

10 An E. coli JM109 strain was transformed using the
plasmid pTrcLD(A113G)nh/774ch/43SCch. 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
15 cells. About 0.1 g of the wet cells were suspended in 1 ml
of 0.5 M Tris-HCl buffer (pH 9), 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
20 and 4°C for 10 minutes to obtain about 0.7 ml of centrifuged
supernatant liquid.

With 0.1 ml of the centrifuged supernatant liquid thus
obtained, 0.4 ml of a 40% aqueous solution of 2-hydroxy-4-
(methylthio)butyric acid (manufactured by Tokyo Chemical
25 Industry) whose pH was adjusted to 9 with ammonia water, 10
mg of NAD⁺, and 20 mg of ammonium sulfate were mixed, and
the solution was shaken at 30°C for 18 hours. This reaction
solution was subjected to content analysis by liquid
chromatography under the following condition. It was found
30 that 85.1 g/L of L-methionine was produced. The yield of L-

methionine based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction was 21.5%.
[0171]

(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
[0172]

(2) Transformant Having Present Invented Recombinant Vector
15 (ABC) Containing Polynucleotide Encoding Present Invented Protein (A) Having Altered Amino Acid Sequence
V109I/G191D/Q246R

An E. coli JM109 strain was transformed using the plasmid pTrcLD(A113G)nh/774ch/43SCMch. The transformant
20 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
25 ml of 0.5 M Tris-HCl buffer (pH 9), 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
30 about 0.7 ml of centrifuged supernatant liquid.

With 0.1 ml of the centrifuged supernatant liquid thus obtained, 0.4 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⁺, and 20 mg of ammonium sulfate were mixed, and the solution was shaken at 30°C for 2 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that 82.5 g/L of L-methionine was produced. The yield of L-methionine based on the amount of 2-hydroxy-4-(methylthio)butyric acid used for the reaction was 20.8%.

[0173]

(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

[0174]

Example 11 (Preparation of L-α-Amino Acid Compound Using Present Invented Protein (A), Present Invented Protein (B), and Present Protein (C))

[0175]

(1) Production of L-α-Amino Acid Compound Using Present Invented Protein (A), Present Invented Protein (B), and Present Protein (C) Having Amino Acid Sequence Represented by SEQ ID NO: 3

After 0.1 ml of a 2-fold diluent of the purified enzyme solution of the present invented protein (B) prepared in Example 2 (1) (40 g protein/L) was mixed with 0.1 ml of a 2-fold concentrated solution of the purified enzyme solution of the present invented protein (A) prepared in Example 2 (2) (9 g protein/L) and 0.1 ml of a 2-fold diluent of the leucine dehydrogenase (A113G) purified enzyme solution prepared in Example 3 (4) (32.6 g protein/L), the mixture was concentrated with the Amicon Ultra-15 (manufactured by Merck Millipore) to obtain 0.1 ml of a purified enzyme mixture. With 0.1 ml of the purified enzyme mixture thus obtained, 0.1 ml of a 40% aqueous solution of DL- α -hydroxy-isocaproic acid (ACROSORGANICS) whose pH was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 0.3 ml of 0.5 M Tris-HCl buffer (pH 9.0), and 20 mg of ammonium sulfate were mixed, and the solution was shaken at 30°C for 18 hours. This reaction solution was subjected to content analysis by liquid chromatography under the following condition. It was found that L-leucine was produced in a proportion of 60.9% based on the amount of DL- α -hydroxy-isocaproic acid used for the reaction.

[0176]

(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

[0177]

(2) Preparation of L- α -Amino Acid Compound Using Present
Invented Protein (A), Present Invented Protein (B), and
5 Present Protein (C) Having Altered Amino Acid Sequence
V109I/G191D/Q246R

After 0.1 ml of a 2.5-fold diluent of the purified
enzyme solution of the present invented protein (B) prepared
in Example 2 (1) (40 g protein/L) was mixed with 0.1 ml of a
10 4.5-fold concentrated solution of the purified enzyme
solution of the present invented protein (A) prepared from
the pTrc43SCcHis-g325a/g572a/a737g transformant in Example 8
(1) (3.2 g protein/L) and 0.1 ml of a 2-fold diluent of the
leucine dehydrogenase (A113G) purified enzyme solution
15 prepared in Example 3 (4) (32.6 g protein/L), the mixture
was concentrated with the Amicon Ultra-15 (manufactured by
Merck Millipore) to obtain 0.1 ml of a purified enzyme
mixture. With 0.1 ml of the purified enzyme mixture thus
obtained, 0.1 ml of a 40% aqueous solution of DL- α -hydroxy-
20 isocaproic acid (manufactured by ACROS ORGANICS) whose pH
was adjusted to 9 with ammonia water, 10 mg of NAD⁺, 0.3 ml
of 0.5 M Tris-HCl buffer (pH 9.0), and 20 mg of ammonium
sulfate were mixed, and the solution was shaken at 30°C for
18 hours. This reaction solution was subjected to content
25 analysis by liquid chromatography under the following
condition. It was found that L-leucine was produced in a
proportion of 88.0% based on the amount of DL- α -hydroxy-
isocaproic acid used for the reaction.

[0178]

30 (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

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

Flow rate: 0.8 ml/min

Column temperature: 37°C

Detection: 210 nm

10 Industrial Applicability

[0179]

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 and leucine using these, and the like.

15

[Sequence Listing Free Text]

[0180]

SEQ ID NO: 9-12

An oligonucleotide primer designed for PCR

20 SEQ ID NO: 13

A polynucleotide encoding a dehydrogenase

SEQ ID NO: 14-41

An oligonucleotide primer designed for PCR

SEQ ID NO: 44-45

25 An amino acid sequence designed

CLAIMS

1. A polynucleotide encoding any one of the following amino acid sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,
(A2) an amino acid sequence i) having at least 85% sequence identity to an amino acid sequence represented by SEQ ID NO: 3, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A4) 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: 4 or 13, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

2. The polynucleotide according to claim 1, which has an altered base sequence comprising a codon selected so that the frequency of use of codon of a base sequence encoding the amino acid sequence corresponds to the frequency of use of codon in *E. coli*.

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. 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.

6. The transformant according to claim 5, wherein the host cell is a microorganism or *E. coli*.

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

8. 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.

9. 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 claim 4 into a host cell.

10. A polynucleotide encoding any one of the following amino acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,

(B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid

derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(B4) 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, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

11. A polynucleotide in which a promoter which can function in a host cell is connected with the polynucleotide according to claim 10 so that they can function.

12. A recombinant vector comprising the polynucleotide according to claim 10 or 11.

13. A transformant in which the polynucleotide according to claim 10 or 11 or the recombinant vector according to claim

12 is introduced into a host cell.

14. The transformant according to claim 13, wherein the host cell is a microorganism or *E. coli*.

15. A transformant having the polynucleotide according to claim 10 or 11.

16. A method for producing a recombinant vector, which comprises the step of integrating the polynucleotide according to claim 10 or 11 into a vector which can be replicated in a host cell.

17. A method for producing a transformant, which comprises the step of introducing the polynucleotide according to claim 10 or 11 or the recombinant vector according to claim 12 into a host cell.

18. A recombinant vector comprising the polynucleotide according to any one of claims 1 to 3 and the polynucleotide according to claim 10 or 11.

19. The recombinant vector according to claim 18, 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.

20. The recombinant vector according to claim 19, 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.

21. The recombinant vector according to claim 19 or 20, 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 (C1) to (C3):

(C1) an amino acid sequence represented by SEQ ID NO: 7,

(C2) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or

(C3) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound.

22. A transformant in which the recombinant vector according to any one of claims 18 to 21 is introduced into a host cell.

23. The transformant according to claim 22, wherein the host cell is a microorganism or E. coli.

24. 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;
- ii) the polynucleotide according to any one of claims 1 to 3; and
- iii) the polynucleotide according to claim 10 or 11.

25. A protein having any one of the following amino acid sequences (A1) to (A5):

- (A1) an amino acid sequence represented SEQ ID NO: 3,
- (A2) an amino acid sequence i) having at least 85% sequence identity to an amino acid sequence represented by SEQ ID NO: 3, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,
- (A3) an amino acid sequence represented by i) SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert

the same into a corresponding α -oxo-isocaproic acid derivative,

(A4) 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: 4 or 13, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

26. A protein having any one of the following amino acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,

(B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO:

1, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(B4) 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, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

27. A method for producing an L- α -amino acid compound, which comprises (1) the step of reacting any one or both of a protein having any one of the following amino acid

sequences (A1) to (A5):

(A1) an amino acid sequence represented SEQ ID NO: 3,
(A2) an amino acid sequence i) having at least 85% sequence identity to an amino acid sequence represented by SEQ ID NO: 3, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A3) an amino acid sequence represented by i) SEQ ID NO: 3 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(A4) 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: 4 or 13, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(A5) an amino acid sequence i) represented by SEQ ID NO: 3 which is an altered amino acid sequence having one or more amino acid substitutions selected from the group consisting of substitution of the 109th valine by isoleucine, substitution of the 191st glycine by aspartic acid, and substitution of the 246th glutamine by arginine, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative; and a protein having any one of the following amino acid sequences (B1) to (B4):

(B1) an amino acid sequence represented by SEQ ID NO: 1,

(B2) an amino acid sequence i) having at least 95% sequence identity to an amino acid sequence represented by SEQ ID NO: 1, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative,

(B3) an amino acid sequence represented by i) SEQ ID NO: 1 in which one or plural amino acids are deleted, substituted, or added, and ii) of a protein having any one or both of 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 and the ability to

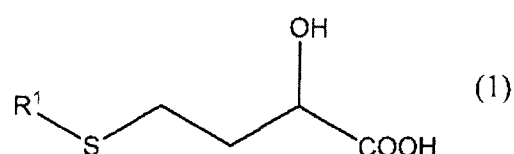
oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative, or

(B4) 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, and ii) of a protein having any one or both of 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 and the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic 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.

28. The production method according to claim 27, 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, the corresponding L- α -amino acid compound is a sulfur-containing L- α -amino acid compound, the protein reacted with an α -hydroxycarboxylic acid compound in the step (1) is a protein at least having the ability to oxidize a 2-hydroxy-4-(methylthio)butyric acid

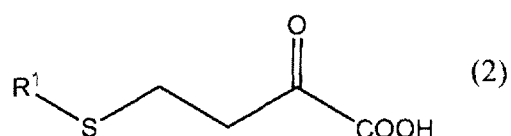
derivative and convert the same into a corresponding 2-oxo-4-(methylthio)butyric acid derivative.

29. The production method according to claim 28, wherein the sulfur-containing α -hydroxycarboxylic acid compound is a compound represented by formula (1):



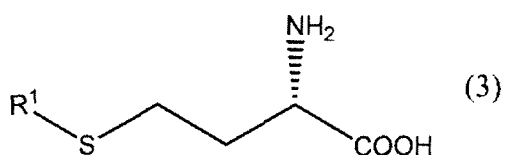
wherein R^1 represents a hydrogen atom or an optionally substituted C1-8 alkyl group;

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.

30. The production method according to claim 27, wherein the α -hydroxycarboxylic acid compound is α -hydroxyisocaproic acid, the corresponding α -oxocarboxylic acid compound is α -oxo-isocaproic acid, the corresponding L- α -amino acid compound is L-leucine, and the protein reacted with an α -hydroxycarboxylic acid compound in the step (1) is

a protein at least having the ability to oxidize an α -hydroxy-isocaproic acid derivative and convert the same into a corresponding α -oxo-isocaproic acid derivative.

31. The production method according to any one of claims 27 to 30, 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.

32. The production method according to any one of claims 27 to 31, 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 (C1) to (C3):

(C1) an amino acid sequence represented by SEQ ID NO: 7,
(C2) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound, or

(C3) 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 an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound.

33. The production method according to any one of claims 27 to 32, wherein the protein reacted with an α -

hydroxycarboxylic acid compound in the step (1) 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.

34. The production method according to claim 33, wherein the transformant is the transformant according to any one of claims 5, 6, 7, 13, 14, 15, 22, 23, and 24.

35. The production method according to any one of claims 27 to 34, 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.

36. The production method according to claim 35, wherein the transformant is the transformant according to any one of claims 22, 23, and 24.

37. The production method according to any one of claims 27 to 36, wherein the step (1) is performed in the presence of a protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -nicotinamide adenine dinucleotide phosphate into its oxidized form.

38. The production method according to claim 37, wherein the protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -nicotinamide adenine dinucleotide phosphate into its oxidized form is a protein further having the ability to aminate an α -oxocarboxylic acid compound and convert the same into a corresponding L- α -amino acid compound.

39. The production method according to claim 37 or 38, wherein the protein having the ability to convert a reduced β -nicotinamide adenine dinucleotide or a reduced β -nicotinamide adenine dinucleotide phosphate into its oxidized 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 27 to 39, 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.

41. The production method according to claim 40, 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 protein further having the ability to oxidize an α -hydroxycarboxylic acid compound and convert the same into

a corresponding α -oxocarboxylic acid compound.

42. The production method according to claim 40 or 41, 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.

43. The production method according to any one of claims 27 to 42, wherein the step (1) and the step (2) are performed in one reaction system.