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Co., Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi,
Kanagawa 2108681 (JP).

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(74) Agent: **SAKAI, Hiroaki**; Sakai International Patent Office
Tokyo Club Building, 2-6, Kasumigaseki 3-chome, Chiyo-
oda-ku, Tokyo 1000113 (JP).

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

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **YOKOZEKI,
Kenzo** [JP/JP]; c/o Ajinomoto Co., Inc., 1-1, Suzuki-cho,
Kawasaki-ku, Kawasaki-shi, Kanagawa 2108681 (JP).
OHNO, Ayako [JP/JP]; c/o Ajinomoto Co., Inc., 1-1,
Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa
2108681 (JP). **HARA, Seichi** [JP/JP]; c/o Ajinomoto Co.,
Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kana-
gawa 2108681 (JP). **ABE, Isao** [JP/JP]; c/o Ajinomoto

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(54) Title: METHOD FOR PRODUCING α -L-ASPARTYL-L-PHENYLALANINE- β -ESTER AND METHOD FOR PRODUC-
ING α -L-ASPARTYL-L-PHENYLALANINE- α -METHYL ESTER

(57) Abstract: A method for producing an α -L-aspartyl-L-phenylalanine- β -ester (also named α -L-(β -o-substituted aspartyl)-L-phenylalanine), which is an intermediate of an α -L-aspartyl-L-phenylalanine- α -methyl ester (also named α -L-aspartyl-L-phenylal-
anine methyl ester; product name: aspartame), easily, at high yield and inexpensively without going through a complex synthesis
method is provided. Further, an easy, inexpensive and high-yield production method for an α -L-aspartyl-L-phenylalanine- α -methyl
ester is provided. α -L-aspartyl-L-phenylalanine- α -methyl ester is produced from a L-aspartic acid- α,β -diester and L-phenylalanine
using an enzyme or enzyme-containing substance that has an ability to catalyze a reaction in which L-phenylalanine performs no
nucleophilic attack on a β -ester site of L-aspartic acid- α,β -diester but performs a nucleophilic attack on an α -ester site thereof.



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DESCRIPTION

METHOD FOR PRODUCING α -L-ASPARTYL-L-PHENYLALANINE- β -
ESTER AND METHOD FOR PRODUCING α -L-ASPARTYL-
5 L-PHENYLALANINE- α -METHYL ESTER

Technical Field

The present invention relates to a method for producing an α -L-aspartyl-L-phenylalanine- β -ester (also named as " α -L-(β -o-substituted
10 aspartyl)-L-phenylalanine (abbreviation: α -ARP)) and to a method for producing an α -L-aspartyl-L-phenylalanine- α -methyl ester (also named α -L-aspartyl-L-phenylalanine methyl ester (abbreviation: α -APM). More particularly, the present invention relates to a method for producing an α -L-aspartyl-L-phenylalanine- β -ester, which is an important intermediate for
15 producing an α -L-aspartyl-L-phenylalanine- α -methyl ester (product name: aspartame) that is in great demand as a sweetener, and to a method for producing an α -L-aspartyl-L-phenylalanine- α -methyl ester utilizing the method for producing the α -L-aspartyl-L-phenylalanine- β -ester.

20 Background Art

Conventionally known methods for producing α -L-aspartyl-L-phenylalanine- α -methyl ester (hereinafter, " α -APM" for short in some cases) include a chemical synthesis method and enzymatic synthesis method. As the chemical synthesis method, there has been known a
25 method for condensing an N-protected L-aspartic acid anhydride with L-phenylalanine methyl ester to synthesize an N-protected APM and eliminating the N-protecting group to obtain APM, and as the enzymatic

synthesis method, there has been known a method for condensing an N-protected L-aspartic acid with L-phenylalanine methyl ester to synthesize an N-protected APM and eliminating the N-protecting group to obtain APM. In both of the methods, however, steps of introducing a protecting group and
5 eliminating the protecting group are necessary and the processes are very troublesome. On the other hand, an APM production method in which no N-protecting group is used has been studied (see Japanese Patent Publication No. H02-015196 Gazette). However, this method is not suitable for industrial production due to very low yield of the product. Thus, under
10 such circumstances, development of industrial production methods for aspartame at lower cost has been desired.

Disclosure of Invention

It is an object of the present invention to provide a method for
15 producing an α -L-aspartyl-L-phenylalanine- β -ester, which is an intermediate of an α -L-aspartyl-L-phenylalanine- α -methyl ester, easily, inexpensively and at high yield without going through a complex synthesis method. Further, it is an object of the present invention to provide a method for producing an α -L-aspartyl-L-phenylalanine- α -methyl ester easily, inexpensively, and at high
20 yield.

As a result of conducting extensive research in consideration of the above objects, the inventors of the present invention have found that a newly discovered enzyme or an enzyme-containing substance is capable of selectively producing an α -L-aspartyl-L-phenylalanine- β -ester from an
25 L-aspartic acid- α,β -diester and L-phenylalanine, and have achieved the present invention.

Namely, the present invention is as described below.

[1] A method of producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine), comprising forming the α -L-aspartyl-L-phenylalanine- β -ester from L-aspartic acid- α,β -diester and L-phenylalanine using an enzyme or enzyme-containing substance that has
5 an ability to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[2] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [1] above, wherein the enzyme or enzyme-containing substance is one type or two or
10 more types selected from the group consisting of a culture of a microbe that has an ability to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond, a microbial cell separated from the culture and a treated microbial cell product of the microbe.

[3] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester
15 (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2] above, wherein the microbe is a microbe belonging to a genus selected from the group consisting of *Aeromonas*, *Azotobacter*, *Alcaligenes*, *Brevibacterium*, *Corynebacterium*, *Escherichia*, *Empedobacter*, *Flavobacterium*, *Microbacterium*, *Propionibacterium*, *Brevibacillus*, *Paenibacillus*,
20 *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Sphingobacterium*, *Streptomyces*, *Xanthomonas*, *Williopsis*, *Candida*, *Geotrichum*, *Pichia*, *Saccharomyces*, *Torulaspora*, *Cellulophaga*, *Weeksella*, *Pedobacter*, *Persicobacter*, *Flexithrix*, *Chitinophaga*, *Cyclobacterium*, *Runella*, *Thermonema*, *Psychroserpens*, *Gelidibacter*, *Dyadobacter*, *Flammeovirga*,
25 *Spirosoma*, *Flectobacillus*, *Tenacibaculum*, *Rhodotermus*, *Zobellia*, *Muricauda*, *Salegentibacter*, *Taxeobacter*, *Cytophaga*, *Marinilabilia*, *Lewinella*, *Saprospira*, and *Haliscomenobacter*.

[4] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2] above, wherein the microbe is a transformed microbe that is capable of expressing a protein (A) or (B):

5 (A) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino
10 acids in the amino acid sequence consisting of amino acid residues 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[5] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester
15 (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein the microbe is a transformed microbe that is capable of expressing a protein (C) or (D):

(C) a protein having an amino acid sequence consisting of amino acid residues numbers 21 to 619 of an amino acid sequence described in SEQ ID
20 NO: 12 of the Sequence Listing,

(D) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence
25 Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[6] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester

(i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein the microbe is a transformed microbe that is capable of expressing a protein (E) or (F) below:

(E) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 625 of an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

(F) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 625 of the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[7] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2] above, wherein the microbe is a transformed microbe that is capable of expressing a protein (G) or (H) below:

(G) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 645 of an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

(H) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 645 of the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[8] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein

the microbe is a transformed microbe that is capable of expressing a protein (I) or (J) below:

(I) a protein having an amino acid sequence consisting of amino acid residues numbers 26 to 620 of an amino acid sequence described in SEQ ID

5 NO: 25 of the Sequence Listing,

(J) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 26 to 620 of the amino acid sequence described in SEQ ID NO: 25 of the Sequence

10 Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[9] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2] above, wherein the microbe is a transformed microbe that is capable of expressing a

15 protein (K) or (L) below:

(K) a protein having an amino acid sequence consisting of amino acid residues numbers 18 to 644 of an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,

(L) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 18 to 644 of the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

25 [10] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein the microbe is a transformed microbe that is capable of expressing a protein

(M) or (N) below:

(M) a protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(N) a protein containing a mature protein region, having an amino acid
5 sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

10 [11] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein the microbe is a transformed microbe that is capable of expressing a protein

(O) or (P) below:

(O) a protein having an amino acid sequence described in SEQ ID NO: 12
15 of the Sequence Listing,

(P) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to selectively link
20 L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[12] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a
25 protein (Q) or (R) below:

(Q) a protein containing a mature protein region, having an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

(R) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[13] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [3], wherein the microbe is a transformed microbe that is capable of expressing a protein (S) or (T) below:

10 (S) a protein having an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

(T) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[14] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2], wherein the microbe is a transformed microbe that is capable of expressing a protein (U) or (V) below:

(U) a protein having an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,

(V) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively link

L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[15] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [2] above, wherein the microbe is a transformed microbe that is capable of expressing a protein (W) or (X) below:

(W) a protein having an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,

(X) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[16] The method for producing an α -L-aspartyl-L-phenylalanine- β -ester (i.e., α -L-(β -o-substituted aspartyl)-L-phenylalanine) according to [1] above, wherein the enzyme is at least one selected from the group consisting (A) to (X) below:

(A) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

- (C) a protein having the amino acid sequence consisting of amino acid residue numbers 21 to 619 of an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,
- (D) a protein having an amino acid sequence including substitution,
5 deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residue numbers 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- 10 (E) a protein having the amino acid sequence consisting of amino acid residues numbers 23 to 625 of an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,
- (F) a protein having an amino acid sequence including substitution,
deletion, insertion, addition, and/or inversion of one or a plurality of amino
15 acids in the amino acid sequence consisting of amino acid residues numbers 23 to 625 of the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (G) a protein having an amino acid sequence consisting of amino acid
20 residues numbers 23 to 645 of an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,
- (H) a protein having an amino acid sequence including substitution,
deletion, insertion, addition, and/or inversion of one or a plurality of amino
acids in the amino acid sequence consisting of amino acid residues numbers
25 23 to 645 of the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

- (I) a protein having an amino acid sequence consisting of amino acid residues numbers 26 to 620 of an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,
- (J) a protein having an amino acid sequence including substitution,
5 deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 26 to 620 of the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- 10 (K) a protein having an amino acid sequence consisting of amino acid residues numbers 18 to 644 of an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,
- (L) a protein having an amino acid sequence including substitution,
deletion, insertion, addition, and/or inversion of one or a plurality of amino
15 acids in the amino acid sequence consisting of amino acid residues numbers 18 to 644 of the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (M) a protein having an amino acid sequence described in SEQ ID NO: 6
20 of the Sequence Listing,
- (N) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and activity to selectively linking
25 L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (O) a protein having the amino acid sequence described in SEQ ID NO:

12 of the Sequence Listing,

- (P) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in
5 SEQ ID NO: 12 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (Q) a protein having an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,
- 10 (R) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a
15 peptide bond,
- (S) a protein having an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,
- (T) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion
20 of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (U) a protein having an amino acid sequence described in SEQ ID NO: 25
25 of the Sequence Listing,
- (V) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion

of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

5 (W) a protein having an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and

(X) a protein containing a mature protein region, having an amino acid sequence in the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to
10 an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

[17] The method of producing an α -L-aspartyl-L-phenylalanine- α -methyl ester (i.e., α -L-aspartyl-L-phenylalanine methyl ester), comprising: a reaction step of synthesizing an α -L-aspartyl-L-phenylalanine- β -methyl ester (also named α -L-(β -o-methyl aspartyl)-L-phenylalanine (abbreviation: α -AMP)) by a
15 method of producing an α -L-aspartyl-L-phenylalanine- β -ester according to any one of claims 1 to 16; and a reaction step of converting the α -L-aspartyl-L-phenylalanine- β -methyl ester (i.e., α -L-(β -o-methyl aspartyl)-L-phenylalanine) to α -L-aspartyl-L-phenylalanine- α -methyl ester.

By the present invention, α -L-aspartyl-L-phenylalanine- β -ester can be
20 easily produced. By the method of the present invention, α -L-aspartyl-L-phenylalanine- β -ester can be produced easily and at high yield with reduced use of complicated synthetic methods such as introduction/elimination of protecting groups.

Furthermore, by the present invention,
25 α -L-aspartyl-L-phenylalanine- α -methyl ester can be produced easily, at high yield, and inexpensively.

The other objects, features and advantages of the present invention

are specifically set forth in or will become apparent from the following detailed descriptions of the invention when read in conjunction with the accompanying drawing.

5 Brief Description Of The Drawing

Fig. 1 is a diagram showing amounts of enzymes that exist in a cytoplasm fraction (Cy) and a periplasm fraction (Pe).

Best Mode for Carrying Out the Invention

10 Hereinafter, the present invention will be described in the order of

<1> Method of producing α -L-aspartyl-L-phenylalanine- β -ester

1. Method of producing α -L-aspartyl-L-phenylalanine- β -ester

2. Microbes used in the present invention

3. Enzymes used in the present invention; and

15 <2> Method of producing α -L-aspartyl-L-phenylalanine- α -methyl ester.

<1> Method of producing α -L-aspartyl-L-phenylalanine- β -ester

1. Method of producing α -L-aspartyl-L-phenylalanine- β -ester

In the method of producing α -L-aspartyl-L-phenylalanine- β -ester of the present invention (hereinafter also called "the production method of a peptide of the present invention"), L-phenylalanine and L-aspartic acid- α,β -diester are allowed to react in the presence of an enzyme having a stated peptide forming activity. That is, in the production method of a peptide of the present invention, an α -L-aspartyl-L-phenylalanine- β -ester is formed from an L-aspartic acid- α,β -diester and L-phenylalanine using an enzyme or enzyme-containing substance capable of selectively linking
25 L-phenylalanine to the α -ester site of L-aspartic acid- α,β -diester through a peptide bond. The enzyme or enzyme-containing substance capable of

selectively linking L-phenylalanine to the α -ester site of a L-aspartic acid- α,β -diester refers to an enzyme or enzyme-containing substance having an ability or activity to catalyze a reaction in which substantially, L-phenylalanine is able to perform no nucleophilic attack to a β -ester site of L-aspartic acid- α,β -diester but performs a nucleophilic attack on an α -ester site thereof only. As shown in the reference example hereinbelow, however, an enzyme or enzyme-containing substance has also been obtained that has an ability to catalyze a reaction in which substantially, L-phenylalanine is able to perform no attack on the α -ester site of an L-aspartic acid- α,β -diester but performs nucleophilic attack on the β -ester site thereof only, contrary to the above-mentioned ability, and that produces a β -L-aspartyl-L-phenylalanine- α -ester (also named β -L-(α -o-substituted aspartyl)-L-phenylalanine (abbreviation: β -ARP) from the L-aspartic acid- α,β -diester and L-phenylalanine.

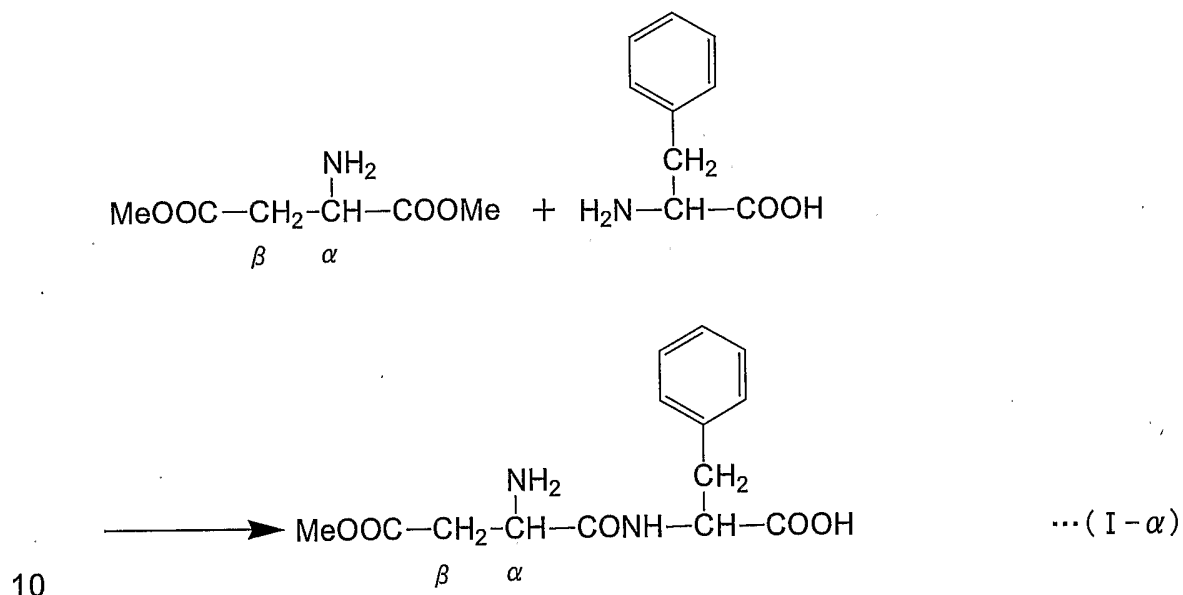
15 The reaction formula in which L-phenylalanine performs nucleophilic attack on the α -ester site of L-aspartic acid- α,β -diester to produce an α -L-aspartyl-L-phenylalanine- β -ester (α -ARP) is shown in the following formula (I- α) (wherein "Me" represents a methyl group) by citing the case where L-aspartic acid- α,β -dimethyl ester is used as the L-aspartic acid- α,β -diester. As shown in the formula (I- α), in the peptide production method of the present invention, the amino group of L-phenylalanine reacts with the α -methyl ester site of the L-aspartic acid- α,β -dimethyl ester to form a peptide bond. On the other hand, the following formula (I- β) indicates a reaction in which the β -methyl ester site of L-aspartic acid- α,β -dimethyl ester undergoes nucleophilic attack to form β -L-aspartyl-L-phenylalanine- α -methyl ester (also named β -L-(α -o-methyl aspartyl)-L-phenylalanine (abbreviation: β -AMP)). The peptide bond in β -AMP is formed at the β -methyl ester site of

20

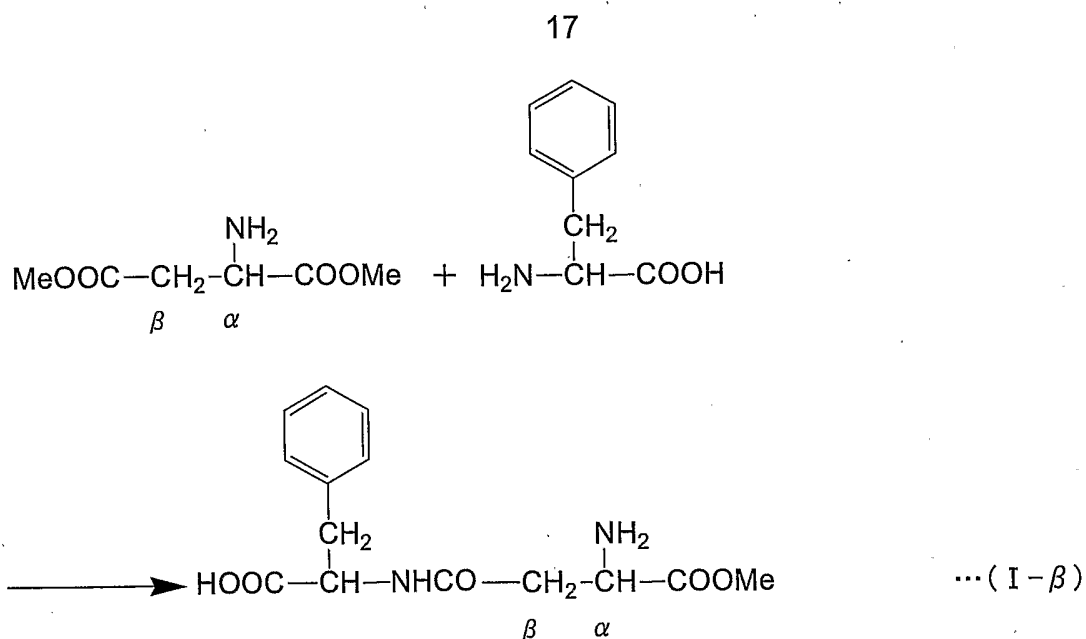
25

the L-aspartic acid- α,β -dimethyl ester. The enzyme or enzyme-containing substance used in the present invention accelerates substantially only a reaction as in the formula (I- α) but causes substantially no reaction as in the formula (I- β). α -APM can be produced from α -AMP through a simple
 5 reaction step (formula (II)), but α -APM cannot be produced directly from β -AMP. That is, the method of the present invention is extremely efficient as a method for producing an intermediate of α -APM and is useful for industrial production.

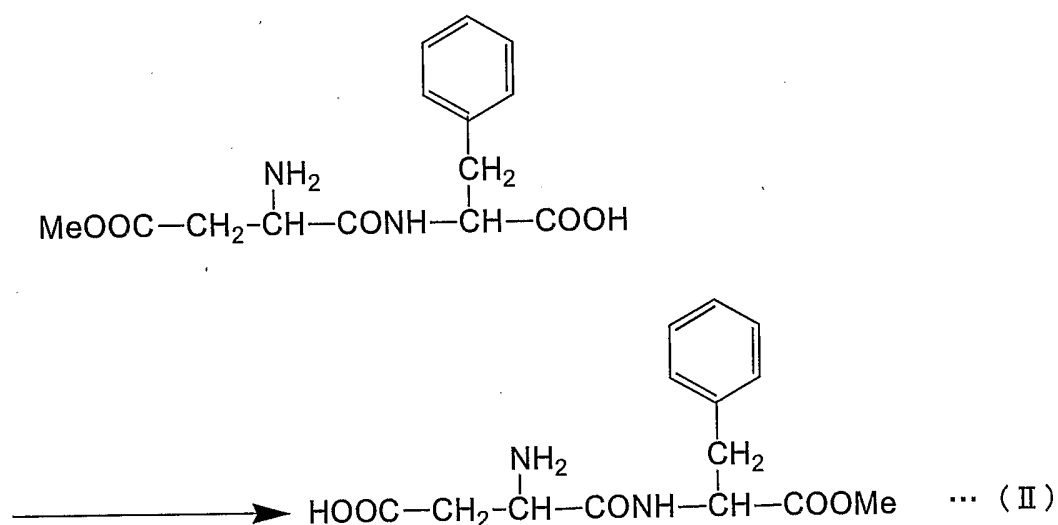
Reaction formula I- α



Reaction formula I- β



Reaction formula II



5

A method for allowing the enzyme or enzyme-containing substance to act on L-aspartic acid- α,β -diester and L-phenylalanine may be performed by mixing the enzyme or enzyme-containing substance with L-aspartic acid- α,β -diester and L-phenylalanine. More specifically, there may be used

10 a method in which the enzyme or enzyme-containing substance is added to a solution containing an L-aspartic acid-diester and L-phenylalanine to effect reaction. When a microbe which produces the enzyme is used as the

enzyme-containing substance, either the reaction may be carried out as described above, or a method which includes culturing a microbe that produces the enzyme to produce and accumulate the enzyme in the microbe or a culture liquid in which the microbe has been cultured, and adding an
5 L-aspartic acid- α,β -diester and L-phenylalanine to the culture liquid, or the like method may be used. The thus produced α -L-aspartyl-L-phenylalanine- β -ester is recovered according to the conventional method and it can be purified, if necessary.

The "enzyme-containing substance" may be any substance so far as it
10 contains the enzyme, and specific modes thereof include a culture of a microbe which produces the enzyme, a microbial cell separated from the culture and a treated microbial cell product of the microbe. The culture of microbe means a substance obtained by culturing a microbe, and specifically means a mixture of microbial cell, a medium used for culturing the microbe
15 and a substance produced by the cultured microbe, and so forth. In addition, the microbial cell may be washed to use as a washed microbial cell. Moreover, the treated microbial cell product includes those obtained by subjecting the microbial cell to crushing, lysis, or freeze-drying, and further a crude enzyme recovered by treating the microbial cell and a purified enzyme
20 obtained by further purification. As the purification-treated enzyme, a partially purified enzyme obtained by various purification methods and so forth may be used. In addition, immobilized enzymes which have been immobilized by a covalent bonding method, an adsorption method, an entrapment method, or the like may be used. Further, for some microbes to
25 be used, a portion of the microbial cells may undergo lysis during culturing and in such a case, the supernatant of the culture liquid may be utilized as the enzyme-containing substance as well.

In addition, as the microbe that contains the enzyme, a wild strain may be used or a gene recombinant strain in which the enzyme has been expressed may be used. Such microbe is not limited to an enzyme microbial cell but the treated microbial cell products such as acetone-treated microbial cell and freeze-dried microbial cell may be used. Further, immobilized microbial cells obtained by immobilizing the treated microbial cell product using a covalent bonding method, an adsorption method, an entrapment method, or the like, or an immobilized treated microbial cell product may be used.

Use of a wild strain which is able to produce a peptide forming enzyme having an activity to form an α -L-aspartyl-L-phenylalanine- β -ester is preferred in that peptide production can be performed more readily without going through a step of making a gene recombinant strain. On the other hand, a gene recombinant strain which has been transformed so as to express a peptide forming enzyme having an activity to produce an α -L-aspartyl-L-phenylalanine- β -ester can be modified such that the peptide forming enzyme is produced in a larger amount. Thus, it is possible to synthesize an α -L-aspartyl-L-phenylalanine- β -ester in a larger amount and at a higher rate. Culturing a microbe of wild strain or gene recombinant strain in a medium to accumulate the peptide forming enzyme in the medium and/or microbe, and mixing the thus accumulated product with an L-aspartic acid- α,β -diester and L-phenylalanine can form an α -L-aspartyl-L-phenylalanine- β -ester.

Note that when cultured products, cultured microbial cells, washed microbial cells and treated microbial cell products obtained by subjecting microbial cells to crushing or lysis are used, it is often the case that an enzyme exists that decomposes the formed

α -L-aspartyl-L-phenylalanine- β -ester without being involved in the formation of the α -L-aspartyl-L-phenylalanine- β -ester. In such a case, it is preferred in some occasions to add a metal protease inhibitor such as ethylenediaminetetraacetic acid (EDTA). The addition amount is in the range of 0.1 millimolar (mM) to 300 mM, preferably 1 mM to 100 mM.

The amount of enzyme or enzyme-containing substance used may be enough if it is an amount at which the target effect is demonstrated (effective amount). While a person with ordinary skill in the art can easily determine this effective amount through simple, preliminary experimentation, the use amount is, for example, about 0.01 to about 100 units ("U") in the case of using enzyme, and about 0.1 to about 500 g/L in the case of using washed microbial cells. Note that 1 U is defined to be an amount of enzyme which allows production of 1 micromole (μ mole) of L- α -aspartyl-L-phenylalanine- β -methyl ester from 100 mM L-aspartic acid- α,β -dimethyl ester and 200 mM L-phenylalanine at 25°C in one minute.

The L-aspartic acid- α,β -diester to be used in the reaction may be any one that is condensed with L-phenylalanine to produce an α -L-aspartyl-L-phenylalanine- β ester. Examples of the L-aspartic acid- α,β -diester include L-aspartic acid- α,β -dimethyl ester and L-aspartic acid- α,β -diethyl ester. When L-aspartic acid- α,β -dimethyl ester and L-phenylalanine are allowed to react, α -L-aspartyl-L-phenylalanine- β -methyl ester (α -AMP) is produced, and when L-aspartic acid- α,β -diethyl ester and L-phenylalanine are reacted, α -L-aspartyl-L-phenylalanine- β -ethyl ester (also named α -L-(β -o-ethyl aspartyl)-L-phenylalanine (abbreviation: α -AEP)) is produced.

While the concentrations of L-aspartic acid- α,β -diester and L-phenylalanine serving as starting materials are each 1 mM to 10 mM, and

preferably 0.05 M to 2 M, there may be cases in which it is preferable to add either one of the substrates in an equimolar amount or more with respect to the other substrate, and selection is made as necessary. In addition, in cases where high concentrations of substrates inhibit the reaction, these can be adjusted to concentrations that do not cause inhibition and successively added during the reaction.

The reaction temperature that allows production of α -L-aspartyl-L-phenylalanine- β -ester is 0 to 60°C, and preferably 5 to 40°C. In addition, the reaction pH that allows production of α -L-aspartyl-L-phenylalanine- β -ester is 6.5 to 10.5, and preferably 7.0 to 10.0.

2. Microbes used in the present invention

As the microbes to be used in the present invention, those microbes which have an ability to produce α -L-aspartyl-L-phenylalanine- β -ester from an L-aspartic acid- α,β -diester and L-phenylalanine may be used without particular limitation. The microbes that have an ability to produce α -L-aspartyl-L-phenylalanine- β -ester from an L-aspartic acid- α,β -diester and L-phenylalanine include, for example, microbes belonging to the genera *Aeromonas*, *Azotobacter*, *Alcaligenes*, *Brevibacterium*, *Corynebacterium*, *Escherichia*, *Empedobacter*, *Flavobacterium*, *Microbacterium*, *Propionibacterium*, *Brevibacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Sphingobacterium*, *Streptomyces*, *Xanthomonas*, *Williopsis*, *Candida*, *Geotrichum*, *Pichia*, *Saccharomyces*, *Torulaspora*, *Cellulophaga*, *Weeksella*, *Pedobacter*, *Persicobacter*, *Flexithrix*, *Chitinophaga*, *Cyclobacterium*, *Runella*, *Thermonema*, *Psychroserpens*, *Gelidibacter*, *Dyadobacter*, *Flammeovirga*, *Spirosoma*, *Flectobacillus*, *Tenacibaculum*, *Rhodotermus*, *Zobellia*, *Muricauda*, *Salegentibacter*, *Taxeobacter*, *Cytophaga*,

Marinilabilia, *Lewinella*, *Saprospira*, and *Haliscomenobacter*.

Specifically, the following may be exemplified.

- Aeromonas hydrophila* ATCC 13136
- Azotobacter vinelandii* IFO 3741
- 5 *Alcaligenes faecalis* FERM P-8460
- Brevibacterium minutiferuna* FERM BP-8277
- Corynebacterium flavescens* ATCC 10340
- Escherichia coli* FERM BP-8276
- Empedobacter brevis* ATCC 14234
- 10 *Flavobacterium resinovorum* ATCC 14231
- Microbacterium arborescens* ATCC 4348
- Propionibacterium shermanii* FERM BP-8100
- Brevibacillus parabrevis* ATCC 8185
- Paenibacillus alvei* IFO 14175
- 15 *Pseudomonas fragi* IFO 3458
- Serratia grimesii* ATCC 14460
- Stenotrophomonas maltophilia* ATCC 13270
- Sphingobacterium* sp. FERM BP-8124
- Streptomyces griseolus* NRRL B-1305
- 20 (Streptomyces lavendulae)
- Xanthomonas maltophilia* FERM BP-5568
- Williopsis saturnus* IFO 0895
- Candida magnoliae* IFO 0705
- Geotrichum fragrance* CBS 152.25
- 25 (Geotrichum amycelium)
- Geotrichum amycelium* IFO 0905
- Pichia ciferrii* IFO 0905

- Saccharomyces unisporus* IFO 0724
- Torulaspora delbrueckii* IFO 0422
- Cellulophaga lytica* NBRC 14961
- Weeksella virosa* NBRC 16016
- 5 *Pedobacter heparinus* NBRC 12017
- Persicobacter diffluens* NBRC 15940
- Flexithrix dorotheae* NBRC 15987
- Chitinophaga pinensis* NBRC 15968
- Cyclobacterium marinum* ATCC 25205
- 10 *Runella slithyformis* ATCC 29530
- Thermonema lapsum* ATCC 43542
- Psychroserpens burtonensis* ATCC 700359
- Gelidibacter algens* ATCC 700364
- Dyadobacter fermentans* ATCC 700827
- 15 *Flammeovirga aprica* NBRC 15941
- Spirosoma linguale* DSMZ 74
- Flectobacillus major* DSMZ 103
- Tenacibaculum maritimum* ATCC 43398
- Rhodotermus marinus* DSMZ 4252
- 20 *Zobellia galactanivorans* DSMZ 12802
- Muricauda ruestringensis* DSMZ 13258
- Salegentibacter salegens* DSMZ 5424
- Taxeobacter gelupurpurascens* DSMZ 11116
- Cytophaga hutchinsonii* NBRC 15051
- 25 *Marinilabilia salmonicolor* NBRC 15948
- Lewinella cohaerens* ATCC 23123
- Saprospira grandis* ATCC 23119

Haliscomenobacter hydrossis ATCC 27775

Among the aforementioned strains of microbes, those microbes described with FERM numbers have been deposited at the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan), and can be furnished by referring to each number.

Among the aforementioned strains of microbes, those microbes described with ATCC numbers have been deposited at the American Type Culture Collection (P.O. Box 1549, Manassas, VA 20110, the United States of America), and can be furnished by referring to each number.

Among the aforementioned strains of microbes, those microbes described with IFO numbers have been deposited at the Institute of Fermentation, Osaka (2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan), and can be furnished by referring to each number.

Among the aforementioned strains of microbes, those microbes described with NBRC numbers have been deposited at the NITE Biological Resource Center of the National Institute of Technology and Evaluation (5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan), and can be furnished by referring to each number.

Among the aforementioned strains of microbes, those microbes described with DSMZ numbers have been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures) (Mascheroder Weg 1b, 38124 Braunschweig, Germany), and can be furnished by referring to each number.

Like the aforementioned strains, those microbes described with FERM numbers are microbes that were deposited at the independent

administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan). *Alcaligenes faecalis* FERM P-8460 is a microbe that was deposited on September 30, 5 1985 and assigned the deposit number FERM P-8460. *Propionibacterium shermanii* FERM P-9737 is a microbe that was originally deposited on December 4, 1987 and control of this organism was subsequently transferred to international deposition under the provisions of the Budapest Treaty on July 1, 2002 and was assigned the deposit number of FERM BP-8100.

10 *Xanthomonas maltophilia* FERM BP-5568 is a microbe that was originally deposited on June 14, 1995 and control of this organism was subsequently transferred to international deposition under the provisions of the Budapest Treaty on June 14, 1996. *Brevibacterium minutiferuna* FERM BP-8277 was internationally deposited under the provisions of Budapest Treaty on January 15 20, 2002. *Escherichia coli* FERM BP-8276 was deposited at an international depositary institution under the provisions of Budapest Treaty on January 20, 2002.

Empedobacter brevis strain ATCC 14234 (strain FERM P-18545, strain FERM BP-8113) was deposited at the International Patent Organism 20 Depositary of the independent administrative corporation, National Institute of Advanced Industrial Science and Technology (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on October 1, 2001 and assigned the deposit number of FERM P-18545. Control of this organism was subsequently transferred to deposition under the provisions of the Budapest 25 Treaty at the International Patent Organism Depositary of the independent administrative corporation, National Institute of Advanced Industrial Science and Technology on July 8, 2002 and was assigned the deposit number of

FERM BP-8113 (indication of microbe: *Empedobacter brevis* strain AJ 13933).

Sphingobacterium sp. strain AJ 110003 was deposited at the International Patent Organism Depositary of the independent administrative
5 corporation, National Institute of Advanced Industrial Science and Technology (Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on July 22, 2002, and was assigned the deposit number of FERM BP-8124.

Note that the strain AJ 110003 (FERM BP-8124) was identified to be
10 the aforementioned *Sphingobacterium* sp. by the identification experiment described below. The strain FERM BP-8124 is a Gram-negative rod (0.7 to 0.8×1.5 to $2.0 \mu\text{m}$) that forms no spore and is not motile. Its colonies are round with a completely smooth border, contain low protrusions and have a glossy, light yellow color. The organism grows at 30°C and is catalase
15 positive, oxidase positive and negative for the OF test (glucose), and was identified as a bacterium belonging to the genus *Sphingobacterium* based on these properties. Moreover, because of the properties that it is negative for nitrate reduction, negative for indole production, negative for acid production from glucose, arginine dihydrolase negative, urease positive, esculin
20 hydrolysis positive, gelatin hydrolysis negative, β -galactosidase positive, glucose assimilation positive, L-arabinose assimilation negative, D-mannose assimilation positive, D-mannitol assimilation negative, N-acetyl-D-glucosamine assimilation positive, maltose assimilation positive, potassium gluconate assimilation negative, n-capric acid assimilation
25 negative, adipic acid assimilation negative, dl-malic acid assimilation negative, sodium citrate assimilation negative, phenyl acetate assimilation negative and cytochrome oxidase positive, it was determined to have

properties that are similar to those of *Sphingobacterium multivorum* or *Sphingobacterium spiritivorum*. Moreover, although results of analyzing analyses on the homology of the base sequence of the 16S rRNA gene indicate the highest degree of homology was exhibited with

5 *Sphingobacterium multivorum* (98.8%), there were was no strain with which the bacterial strain matched completely. Accordingly, this bacterial strain was therefore identified as *Sphingobacterium* sp.

As these microbes, either wild strains or mutant strains can be used or recombinant strains induced by cell fusion or genetic techniques such as
10 genetic manipulation can be used.

To obtain microbial cells of such microbes, the microbes can be cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it allows the microbes to grow. This medium may be an ordinary medium containing ordinary carbon sources,
15 nitrogen sources, phosphorus sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

For example, any carbon source may be used so far as the microbes can utilize it. Specific examples of the carbon source that can be used include sugars such as glucose, fructose, maltose and amylose, alcohols
20 such as sorbitol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid and their salts, hydrocarbons such as paraffin as well as mixtures thereof.

Examples of nitrogen sources that can be used include ammonium salts of inorganic acids such as ammonium sulfate and ammonium chloride,
25 ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptones, yeast extract, meat extract

and corn steep liquor as well as mixtures thereof.

In addition, nutrient sources used in ordinary media, such as inorganic salts, trace metal salts and vitamins, can also be suitably mixed and used.

5 There is no particular restriction on culturing conditions, and culturing can be carried out, for example, for about 12 to about 48 hours while properly controlling the pH and temperature within a pH range of 5 to 8 and a temperature range of 15 to 40°C, respectively, under aerobic conditions.

3. Enzymes used in the present invention

10 In the method for producing peptide according to the present invention described above, an enzyme which has an ability to selectively link L-phenylalanine to the α -ester site of an L-aspartic acid- α,β -diester through a peptide bond is used. In the method for producing peptide according to the present invention, the enzyme is not limited by its origination and procuring
15 method so far as it has such an activity. Hereinafter, purification of enzymes used in the present invention and utilization of techniques of genetic engineering will be explained.

(3-1) Microbes having an enzyme which can be used for the production method of the present invention

20 As microbes which produce an enzyme of the present invention, all the microbes that have an ability to produce an α -L-aspartyl-L-phenylalanine- β -ester from an L-aspartic acid- α,β -diester and L-phenylalanine can be used. The microbes include bacteria and the like that belong to genera selected from the group consisting of *Aeromonas*,
25 *Azotobacter*, *Alcaligenes*, *Brevibacterium*, *Corynebacterium*, *Escherichia*, *Empedobacter*, *Flavobacterium*, *Microbacterium*, *Propionibacterium*, *Brevibacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*,

- Sphingobacterium*, *Streptomyces*, *Xanthomonas*, *Williopsis*, *Candida*,
Geotrichum, *Pichia*, *Saccharomyces*, *Torulaspora*, *Cellulophaga*, *Weeksella*,
Pedobacter, *Persicobacter*, *Flexithrix*, *Chitinophaga*, *Cyclobacterium*, *Runella*,
Thermonema, *Psychroserpens*, *Gelidibacter*, *Dyadobacter*, *Flammeovirga*,
5 *Spirosoma*, *Flectobacillus*, *Tenacibaculum*, *Rhodotermus*, *Zobellia*,
Muricauda, *Salegentibacter*, *Taxeobacter*, *Cytophaga*, *Marinilabilia*, *Lewinella*,
Saprospira, and *Haliscomenobacter*. More specifically, the microbes include
Empedobacter brevis ATCC 14234 (FERM P-18545 strain, FERM BP-8113
strain(Depositary institution: the independent administrative corporation,
10 National Institute of Advanced Industrial Science and Technology,
International Patent Organism Depositary, Address of depositary institution:
Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,
International deposit transfer date: July 8, 2002)), *Sphingobacterium* sp.
FERM BP-8124 strain(Depositary institution: the independent administrative
15 corporation, National Institute of Advanced Industrial Science and Technology,
International Patent Organism Depositary, Address of depositary institution:
Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,
International deposit date: July 22, 2002), *Pedobacter heparinus* IFO
12017(Depositary institution: the Institute of Fermentation, Osaka; 2-17-85
20 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan), *Taxeobacter*
gelupurpurascens DSMZ 11116(Depositary institution; the Deutsche
Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection
of Microbes and Cell Cultures, Address of Depositary institution;
Mascheroder Weg 1b, 38124 Braunschweig, Germany), *Cyclobacterium*
25 *marinum* ATCC 25205(Depositary institution; the American Type Culture
Collection, address of depositary institution; P.O. Box 1549, Manassas, VA
20110, the United States of America), and *Psychroserpens burtonensis* ATCC

700359(Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) and so forth. *Empedobacter brevis* ATCC 14234 strain (FERM P-18545 strain, FERM BP-8113 strain (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002)) and *Sphingobacterium* sp. FERM BP-8124 strain(Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002), *Pedobacter heparinus* IFO 12017 strain(Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan), *Taxeobacter gelupurpurascens* DSMZ 11116 strain(Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany), *Cyclobacterium marinum* ATCC 25205 strain(Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), and *Psychroserpens burtonensis* ATCC 700359 strain(Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) and the like are microbes selected by the present inventors as a result of search of enzyme producing microbes which produce an

α -L-aspartyl-L-phenylalanine- β -ester from an L-aspartic acid- α,β -diester and L-phenylalanine at high yield.

(3-2) Purification of Enzyme

As was previously mentioned, the peptide-forming enzyme used in
5 the present invention can be purified from bacteria belonging to, for example, the genus *Empedobacter*. A method for isolating and purifying a peptide-forming enzyme from *Empedobacter brevis* is explained as an example of purification of the enzyme.

First, a microbial cell extract is prepared from microbial cells of
10 *Empedobacter brevis*, for example, the strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer
15 date: July 8, 2002) by disrupting the cells using a physical method such as ultrasonic crushing or an enzymatic method using a cell wall-dissolving enzyme and removing the insoluble fraction by centrifugal separation and so forth. The peptide-producing enzyme can then be purified by fractionating the cell extract obtained in the above manner by combining ordinary protein
20 purification methods such as anion exchange chromatography, cation exchange chromatography or gel filtration chromatography.

An example of a carrier for use in anion exchange chromatography is Q-Sepharose HP (manufactured by Amersham). The enzyme is recovered in the non-adsorbed fraction under conditions of pH 8.5 when the cell extract
25 containing the enzyme is allowed to pass through a column packed with the carrier.

An example of a carrier for use in cation exchange chromatography is

MonoS HR (manufactured by Amersham). After adsorbing the enzyme onto the column by allowing the cell extract containing the enzyme to pass through a column packed with the carrier and then washing the column, the enzyme is eluted with a buffer solution having a high salt concentration. At that time, 5 the salt concentration may be sequentially increased or a concentration gradient may be applied. For example, in the case of using MonoS HR, the enzyme adsorbed onto the column is eluted at an NaCl concentration of about 0.2 to about 0.5 M.

The enzyme purified in the manner described above can then be 10 further uniformly purified by gel filtration chromatography and so forth. An example of the carrier for use in gel filtration chromatography is Sephadex 200pg (manufactured by Amersham).

In the aforementioned purification procedure, the fraction containing the enzyme can be verified by assaying the peptide-forming activity of each 15 fraction according to the method indicated in the examples to be described later. The internal amino acid sequence of the enzyme purified in the manner described above is shown in SEQ ID NO: 1 and SEQ ID NO: 2 of the Sequence Listing.

(3-3) Isolation DNA, Production of Transformant and Purification of 20 Peptide-forming Enzyme

(3-3-1) Isolation of DNA

The inventors of the present invention first succeeded in isolating one type of DNA of a peptide-forming enzyme that can be used in the peptide production method of the present invention from *Empedobacter brevis* strain 25 FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution:

Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,
International deposit transfer date: July 8, 2002).

A DNA having a base sequence consisting of bases numbers 61 to 1908 of the base sequence described in SEQ ID NO: 5, which is a DNA of the present invention, was isolated from *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002). The DNA having the base sequence consisting of bases numbers 61 to 1908 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 61 to 1908 is contained a signal sequence region and a mature protein region. The signal sequence region is a region that consists of bases numbers 61 to 126, while the mature protein region is a region that consists of bases numbers 127 to 1908. Namely, the present invention provides both a gene for a peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein. The signal sequence contained in the sequence described in SEQ ID NO: 5 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases numbers 127 to 1908, namely the-ester site excluding the leader peptide, is presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

The DNA consisting of the base sequence that consists of bases numbers 61 to 1917 described in SEQ ID NO: 11, which is also a DNA of the

- present invention, was isolated from *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: 5 Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). The DNA consisting of the base sequence that consists of bases numbers 61 to 1917 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 61 to 1917, a signal sequence region and a mature protein region are contained.
- 10 The signal sequence region is a region that consists of bases numbers 61 to 120, while the mature protein region is a region that consists of bases numbers 121 to 1917. Namely, the present invention provides both a gene for a peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein.
- 15 The signal sequence contained in the sequence described in SEQ ID NO: 11 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases numbers 121 to 1917, namely the portion excluding the leader peptide, is
- 20 presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

- The DNA consisting of the base sequence that consists of bases numbers 61 to 1935 described in SEQ ID NO: 17, which is also a DNA of the present invention, was isolated from *Pedobacter heparinus* strain IFO 12017
- 25 (Depositary institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan). The DNA consisting of the base sequence that consists of bases numbers 61 to 1935 described in SEQ

ID NO:17 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 61 to 1935, a signal sequence region and a mature protein region are contained. The signal sequence region is a region that consists of bases numbers 61 to 126, while the mature protein region is a region that consists of bases numbers 127 to 1935. Namely, the present invention provides both a gene for a peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein. The signal sequence contained in the sequence described in SEQ ID NO: 17 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases numbers 127 to 1935, namely the portion excluding the leader peptide, is presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

The DNA consisting of the base sequence that consists of bases numbers 61 to 1995 described in SEQ ID NO: 22, which is also a DNA of the present invention, was isolated from *Taxeobacter gelupurpurascens* DSMZ 11116 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). The DNA consisting of the base sequence that consists of bases numbers 61 to 1995 described in SEQ ID NO:22 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 61 to 1995, a signal sequence region and a mature protein region are contained. The signal sequence region is a region that consists of bases numbers 61 to 126, while the mature protein region is a region that consists of bases numbers 127 to 1995. Namely, the present invention provides both a gene for a

peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein. The signal sequence contained in the sequence described in SEQ ID NO: 22 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases numbers 127 to 1995, namely the portion excluding the leader peptide, is presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

The DNA consisting of the base sequence that consists of bases numbers 29 to 1888 described in SEQ ID NO: 24, which is also a DNA of the present invention, was isolated from *Cyclobacterium marinum* ATCC 25205 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). The DNA consisting of the base sequence that consists of bases numbers 29 to 1888 described in SEQ ID NO:24 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 29 to 1888, a signal sequence region and a mature protein region are contained. The signal sequence region is a region that consists of bases numbers 29 to 103, while the mature protein region is a region that consists of bases numbers 104 to 1888. Namely, the present invention provides both a gene for a peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein. The signal sequence contained in the sequence described in SEQ ID NO: 24 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases

numbers 104 to 1888, namely the portion excluding the leader peptide, is presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

The DNA consisting of the base sequence that consists of bases
5 numbers 61 to 1992 described in SEQ ID NO: 26, which is also a DNA of the present invention, was isolated from *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). The DNA consisting of the base sequence that
10 consists of bases numbers 61 to 1992 described in SEQ ID NO:26 is a code sequence (CDS) portion. In the base sequence consisting of bases numbers 61 to 1992, a signal sequence region and a mature protein region are contained. The signal sequence region is a region that consists of bases numbers 61 to 111, while the mature protein region is a region that
15 consists of bases numbers 112 to 1992. Namely, the present invention provides both a gene for a peptide-forming enzyme protein that contains a signal sequence, and a gene for a peptide-forming enzyme protein in the form of a mature protein. The signal sequence contained in the sequence described in SEQ ID NO: 26 is a kind of leader sequence. The main function
20 of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane. The protein encoded by bases numbers 112 to 1992, namely the portion excluding the leader peptide, is presumed to be a mature protein and exhibit a high degree of peptide-forming activity.

25 Furthermore, the various gene recombination techniques indicated below can be carried out in accordance with the descriptions in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989) and other publications.

A DNA encoding an enzyme that can be used in the present invention can be acquired by polymerase chain reaction (PCR, refer to White, T.J. et al., Trends Genet., 5, 185 (1989)) or hybridization from a chromosomal DNA or a DNA library of *Empedobacter brevis*, *Sphingobacterium* sp., *Pedobacter*
5 *heparinus*, *Taxeobacter gelupurpurascens*, *Cyclobacterium marinum*, or *Psychroserpens burtonensis*. Primers used in PCR can be designed based on the internal amino acid base sequences determined on the basis of purified peptide-forming enzyme as explained in the previous section (3). In addition, since the base sequences of the peptide-forming enzyme genes
10 (SEQ ID NO: 5, SEQ ID NO: 11, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:24, and SEQ ID NO:26) have been identified by the present invention, primers or hybridization probes can be designed on the basis of these base sequences, and the gene can be isolated using the probes. If primers having sequences corresponding to the 5'-nontranslated region and
15 3'-nontranslated region, respectively, are used as PCR primers, the full-length encoded region of the enzyme can be amplified. Taking as an example the case of amplifying a region containing both the leader sequence and a mature protein encoding region as described in SEQ ID NO: 5, specific examples of primers include a primer having a base sequence of the region
20 upstream of base number 61 in SEQ ID NO: 5 for the 5'-side primer, and a primer having a sequence complementary to the base sequence of the region downstream of base number 1908 for the 3'-side primer.

Primers can be synthesized, for example, according to ordinary methods using the phosphoamidite method (refer to Tetrahedron Letters
25 (1981), 22, 1859) by use of the Model 380B DNA Synthesizer manufactured by Applied Biosystems. The PCR reaction can be carried out, for example, by using the Gene Amp PCR System 9600 (Perkin-Elmer) and the Takara LA

PCR In Vitro Cloning Kit (Takara Shuzo) in accordance with the method specified by the supplier such as the manufacturer.

A DNA that encodes an enzyme that can be used in the peptide production method of the present invention, regardless of whether the DNA contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 5 of the Sequence Listing. Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA that hybridizes a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 5 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 11 of the Sequence Listing. Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA that hybridizes a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 11 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 17 of the Sequence Listing. Namely, a DNA substantially identical to the DNA of the present

invention can be obtained by isolating a DNA that hybridizes with a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 17 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having
5 peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 22 of the Sequence
10 Listing. Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA that hybridizes with a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 22 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having
15 peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 24 of the Sequence
20 Listing. Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA that hybridizes with a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 24 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having
25 peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a

leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 26 of the Sequence Listing. Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA that hybridizes with a DNA
5 consisting of a base sequence complementary to the CDS described in SEQ ID NO: 26 of the Sequence Listing or with a probe prepared from the base sequence under stringent conditions and encodes a protein having peptide-forming activity from a DNA encoding the mutant enzyme or cells that possess that DNA.

10 A probe can be produced, for example, in accordance with established methods based on, for example, the base sequence described in SEQ ID NO: 5 of the Sequence Listing. In addition, a method for isolating a target DNA by using a probe to find a DNA that hybridizes with the probe may also be carried out in accordance with established methods. For example, a
15 DNA probe can be produced by amplifying a base sequence cloned in a plasmid or phage vector, cleaving the base sequence desired to be used as a probe with a restriction enzyme and then extracting the desired base sequence. The portion to be cleaved out can be adjusted depending on the target DNA.

20 The term "under a stringent condition" as used herein refers to a condition under which a so-called specific hybrid is formed but no non-specific hybrid is formed. It is difficult to precisely express this condition in numerical values. For example, mention may be made of a condition under which DNAs having high homologies, for example, 50% or
25 more, preferably 80% or more, more preferably 90% or more, hybridize with each other and DNAs having lower homologies than these do not hybridize with each other, or ordinary conditions for rinse in Southern hybridization

under which hybridization is performed at 60°C in a salt concentration corresponding 1×SSC and 0.1% SDS, preferably 0.1×SSC and 0.1% SDS. Although the genes that hybridize under such conditions include those genes in which stop codons have occurred at certain locations along their
5 sequences or which have lost activity due to a mutation in the active center, these can be easily removed by ligating them to a commercially available expression vector, expressing them in a suitable host, and assaying the enzyme activity of the expression product using a method to be described later.

10 However, in the case of a base sequence that hybridizes under stringent conditions as described above, it is preferable that the protein encoded by that base sequence retains about a half or more, preferably 80% or more, and more preferably 90% or more, of the enzyme activity of the protein having the amino acid sequence encoded by the original base
15 sequence serving as the base be retained under conditions of 50°C and pH 8. For example, when explained for on the case of, for example, a base sequence that hybridizes under stringent conditions with a DNA that has a base sequence complementary to the base sequence consisting of bases numbers 127 to 1908 of the base sequence described in SEQ ID NO: 5, it is
20 preferable that the protein encoded by that base sequence retains about a half or more, preferably 80% or more, and more preferably 90% or more, of the enzyme activity of the protein having an amino acid sequence that consists of amino acid residues numbers 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 under conditions of 50°C and pH 8.

25 An amino acid sequence encoded by the CDS described in SEQ ID NO: 5 of the Sequence Listing is shown in SEQ ID NO: 6 of the Sequence Listing. An amino acid sequence encoded by the CDS described in SEQ ID

NO: 11 of the Sequence Listing is shown in SEQ ID NO: 12 of the Sequence Listing. An amino acid sequence encoded by the CDS described in SEQ ID NO.: 17 of the Sequence Listing is shown in SEQ ID NO: 18 of the Sequence Listing. An amino acid sequence encoded by the CDS described in SEQ ID NO: 22 of the Sequence Listing is shown in SEQ ID NO: 23 of the Sequence Listing. An amino acid sequence encoded by the CDS described in SEQ ID NO: 24 of the Sequence Listing is shown in SEQ ID NO: 25 of the Sequence Listing. An amino acid sequence encoded by the CDS described in SEQ ID NO: 26 of the Sequence Listing is shown in SEQ ID NO: 27 of the Sequence Listing.

The entire amino acid sequence described in SEQ ID NO: 6 contains a leader peptide and a mature protein region, with amino acid residues numbers 1 to 22 constituting the leader peptide, and amino acid residues numbers 23 to 616 constituting the mature protein region.

The entire amino acid sequence described in SEQ ID NO: 11 includes a leader peptide and a mature protein region, with amino acid residues numbers 1 to 20 constituting the leader peptide, and amino acid residues numbers 21 to 619 constituting the mature protein region.

The entire amino acid sequence described in SEQ ID NO: 18 contains a leader peptide and a mature protein region, with amino acid residues numbers 1 to 22 constituting the leader peptide, and amino acid residues numbers 23 to 625 constituting the mature protein region.

The entire amino acid sequence described in SEQ ID NO: 23 contains a leader peptide and a mature protein region, with amino acid residues numbers 1 to 22 constituting the leader peptide, and amino acid residues numbers 23 to 645 constituting the mature protein region.

The entire amino acid sequence described in SEQ ID NO: 25 contains

a leader peptide and a mature protein region, with amino acid residues numbers 1 to 25 constituting the leader peptide, and amino acid residues numbers 26 to 620 constituting the mature protein region.

The entire amino acid sequence described in SEQ ID NO: 27 contains
5 a leader peptide and a mature protein region, with amino acid residues numbers 1 to 17 constituting the leader peptide, and amino acid residues numbers 18 to 644 constituting the mature protein region.

The protein encoded by the DNA of the present invention is a protein in which the mature protein has peptide-forming activity, and a DNA that
10 encodes a protein substantially identical to a protein having the amino acid sequence described in SEQ ID NO: 6, SEQ ID NO: 12, SEQ ID NO: 18, SEQ ID NO: 23, SEQ ID NO: 25, or SEQ ID NO: 27 of the Sequence Listing, regardless of whether it contains a leader peptide or not, is also included in the DNA of the present invention. (Note that, base sequences are specified
15 from amino acid sequences in accordance with the codes of the universal codons.) Namely, the present invention provides DNAs that encode proteins indicated in (A) to (X) below:

(A) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID
20 NO: 6 of the Sequence Listing,

(B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the
25 Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(C) a protein having the amino acid sequence consisting of amino acid

residue numbers 21 to 619 of an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(D) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residue numbers 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(E) a protein having the amino acid sequence consisting of amino acid residues numbers 23 to 625 of an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

(F) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 625 of the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(G) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 645 of an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

(H) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 645 of the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(I) a protein having an amino acid sequence consisting of amino acid

residues numbers 26 to 620 of an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,

(J) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 26 to 620 of the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(K) a protein having an amino acid sequence consisting of amino acid residues numbers 18 to 644 of an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,

(L) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 18 to 644 of the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(M) a protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(N) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(O) a protein having the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

- (P) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and activity to selectively linking
- 5 L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (Q) a protein having an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,
- (R) a protein containing a mature protein region, having an amino acid
- 10 sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- 15 (S) a protein having an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,
- (T) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in
- 20 SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,
- (U) a protein having an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,
- 25 (V) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in

SEQ ID NO: 25 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(W) a protein having an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and

(X) a protein containing a mature protein region, having an amino acid sequence in the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

Here, although the meaning of the term "a plurality of" varies depending on the locations and types of the amino acid residues in the three-dimensional structure of the protein, it is within a range that does not significantly impair the three-dimensional structure and activity of the protein of the amino acid residues, and is specifically 2 to 50, preferably 2 to 30, and more preferably 2 to 10. However, in the case of amino acid sequences including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in amino acid sequences of the proteins of (B), (D), (F), (H), (J), (L), (N), (P), (R), (T), (V) or (X), it is preferable that the proteins retain about half or more, more preferably 80% or more, and even more preferably 90% or more of the enzyme activity of the proteins in the state where no mutation is included, under conditions of 50°C and pH 8. For example, explanation will be made in the case of (B); in the case of the amino acid sequence (B) including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, it is preferable that this protein retains about half or more, more preferably 80% or more, and even more preferably 90% or more of the enzyme activity of the protein

having the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, under conditions of 50°C and pH 8.

A mutation of an amino acid like that indicated in the aforementioned (B) and so forth is obtained by modifying the base sequence so that an amino acid of a specific-ester site in the present enzyme gene is substituted, deleted, inserted or added by, for example, -ester site-directed mutagenesis. In addition, a modified DNA like that described above can also be acquired by mutagenesis treatment known in the art. Mutagenesis treatment refers to, for example, a method in which a DNA encoding the present enzyme is treated in vitro with hydroxylamine and so forth, as well as a method in which *Escherichia* bacteria that possess a DNA encoding the present enzyme are treated by a mutagen normally used in artificial mutagenesis, such as ultraviolet irradiation, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

In addition, naturally-occurring mutations such as differences attributable to a microbe species or strain are also included in the base substitution, deletion, insertion, addition and/or inversion described above. By expressing a DNA having such a mutation in suitable cells and investigating the enzyme activity of the expression product, a DNA can be obtained that encodes a protein substantially identical to the protein described in SEQ ID NO: 6, 12, 18, 23, 25 or 27 of the Sequence Listing.

(3-3-2) Preparation of Transformants and Production of Peptide-Forming Enzymes

Peptide-forming enzymes that can be used in the method of producing a peptide according to the present invention can be produced by introducing the DNA explained in (3-3-1) above into a suitable host and expressing the DNA in that host.

With respect to hosts for expressing a protein specified by the DNA, examples of the hosts that can be used include various prokaryotic cells including *Escherichia* bacteria such as *Escherichia coli*, *Empedobacter* bacteria, *Sphingobacterium* bacteria, *Flavobacterium* bacteria and *Bacillus subtilis*, as well as various eukaryotic cells including *Saccharomyces cerevisiae*, *Pichia stipitis* and *Aspergillus oryzae*.

A recombinant DNA used to introduce a DNA into a host can be prepared by inserting the DNA to be introduced into a vector corresponding to the type of host in which the DNA is to be expressed, in such a form that the protein encoded by that DNA can be expressed. In the case where a promoter unique to a peptide-forming enzyme gene of *Empedobacter brevis* and so forth functions in the host cells, the promoter can be used as a promoter for expressing the DNA of the present invention. In addition, another promoter that acts in the host cells may be ligated to the DNA of the present invention, and the DNA may be expressed under the control of the promoter as necessary.

Examples of transformation methods for introducing a recombinant DNA into host cells include the method of D.M. Morrison (see Methods in Enzymology, 68, 326 (1979)) or the method in which DNA permeability is increased by treating receptor microbial cells with calcium chloride (see Mandel, H. and Higa, A., J. Mol. Biol., 53, 159 (1970)).

In the case of mass production of a protein using recombinant DNA technology, conjugating the protein within a transformant that produces the protein to form an inclusion body of protein is also a preferable mode for carrying out the present invention. Advantages of this expression and production method include protection of the target protein from digestion by proteases present within the microbial cells, and simple and easy purification

of the target protein by disrupting the microbial cells followed by centrifugal separation and so forth.

The inclusion body of protein obtained in this manner is solubilized with a protein denaturant and the protein is converted to a properly folded,
5 physiologically active protein through an activity regeneration procedure that consists primarily of removal of the denaturant. There are numerous examples of this, including regeneration of the activity of human interleukin-2 (see Japanese Patent Application Laid-open Publication No. S61-257931).

To obtain an active protein from inclusion bodies of, a series of
10 procedures including solubilization and activity regeneration are required, and the procedure is more complex than in the case of producing the active protein directly. However, in the case of producing a protein that has a detrimental effect on microbial growth in large volumes within microbial cells, that effect can be suppressed by accumulating the proteins in the form of
15 inclusion bodies of inactive protein within the microbial cells.

Examples of mass production methods for producing a target protein in the form of inclusion bodies include a method in which a target protein is expressed independently under the control of a powerful promoter, and a method in which a target protein is expressed in the form of a fused protein
20 with a protein that is known to be expressed in a large volume.

Hereinafter, the present invention will be explained more specifically taking as an example a method for producing transformed *Escherichia coli* and using that transformed microbe to produce a peptide-forming enzyme. Furthermore, in the case of producing peptide-forming enzyme in a microbe
25 such as *Escherichia coli*, a DNA that encodes a precursor protein containing a leader sequence may be incorporated or a DNA that consists only of a mature protein region that does not contain a leader sequence may be

incorporated, and the DNA can be suitably selected for the protein encoding sequence depending on the production conditions, form, usage conditions and so forth of the enzyme to be produced.

Promoters normally used in the production of heterogeneous proteins
5 in *Escherichia coli* can be used as a promoter for expressing a DNA
encoding a peptide-forming enzyme. Examples of such promoters include
T7 promoter, lac promoter, trp promoter, trc promoter, tac promoter, lambda
phage PR promoter, PL promoter and other powerful promoters. In addition,
examples of vectors that can be used include pUC19, pUC18, pBR322,
10 pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118,
pMW219, and pMW218. Besides, vectors of phage DNA can also be used.
Moreover, expression vectors that contain promoters and are capable of
expressing an inserted DNA sequence can be used.

To produce peptide-forming enzyme in the form of a fused protein
15 inclusion body, a gene that encodes another protein, and preferably a
hydrophilic peptide, is ligated upstream or downstream of the peptide-forming
enzyme gene to obtain a fused protein gene. The gene that encodes
another protein in this manner may be any gene that increases the amount of
the fused protein accumulated and enhances the solubility of the fused
20 protein after the denaturation and regeneration steps. Examples of
candidates for such genes include T7 gene 10, β -galactosidase gene,
dehydrofolate reductase gene, γ -interferon gene, interleukin-2 gene and
prochymosin gene.

When these genes are ligated to the genes that encode
25 peptide-forming enzymes, the genes are ligated so that reading frames of
codons are consistent. It is recommended that the genes be ligated at a
proper restriction enzyme-ester site or a synthetic DNA having a proper

sequence be utilized.

Further, to increase a production amount of the peptide-forming enzyme, it is preferable in some cases that a terminator, which is a transcription terminating sequence, be ligated to downstream of the fusion
5 protein gene. The terminator includes, for example, a T7 terminator, an fd phage terminator, a T4 terminator, a tetracycline resistant gene terminator, and an *Escherichia coli* trpA gene terminator.

As the vectors for introducing a gene that encodes a peptide-forming enzyme or a fused protein between the peptide-forming enzyme and another
10 protein in *Escherichia coli* are preferred so-called multi-copy type vectors, examples of which include a plasmid having a replicator derived from ColE1, for example, a pUC-based plasmid, and a pBR322-based plasmid or derivatives thereof. The "derivatives" as used herein refer to those plasmids that are subjected modification by substitution, deletion, insertion, addition
15 and/or inversion of bases. Note that the modification as used herein includes modifications by a mutation treatment with a mutagen or UV irradiation, or modifications by spontaneous mutation.

To screen transformants, it is preferable that the vectors have markers such as an ampicillin resistant gene. As such plasmids are
20 commercially available expression vectors having potent promoters (a pUC-based vector (manufactured by Takara Shuzo, Co., Ltd.), pRROK-based vector (manufactured by Clontech Laboratories, Inc.), pKK233-2 (manufactured by Clontech Laboratories, Inc.) and so forth.

A recombinant DNA is obtained by ligating a DNA fragment to a vector
25 DNA. In this case, a promoter, a gene encoding L-amino acid amide hydrolase or a fused protein consisting of an L-amino acid amide hydrolase and another protein, and depending on the case, a terminator are ligated in

that order.

When *Escherichia coli* is transformed using the recombinant DNA and the resulting *Escherichia coli* is cultured, a peptide-forming enzyme or a fused protein consisting of the peptide-forming enzyme and another protein is expressed and produced. Although a strain that is normally used in the expression of a heterogeneous gene can be used as a host to be transformed, *Escherichia coli* strain JM109, for example, is preferable. Methods for carrying out transformation and methods for screening out transformants are described in Molecular Cloning, 2nd Edition, Cold Spring Harbor Press (1989) and other publications.

In the case of expressing a peptide-forming enzyme in the form of a fusion protein, the peptide-forming enzyme may be cleaved out using a restriction protease that uses a sequence not present in the peptide-forming enzyme, such as blood coagulation factor Xa or kallikrein, as the recognition sequence.

A medium normally used for culturing *Escherichia coli*, such as M9-casamino acid medium or LB medium, may be used as a production medium. In addition, culturing conditions and production induction conditions are suitably selected according to the marker of the vector used, promoter, type of host microbe and so forth.

The following method can be used to recover the peptide-forming enzyme or fused protein consisting of the peptide-forming enzyme and another protein. If the peptide-forming enzyme or its fused protein has been solubilized within the microbial cells, after recovering the microbial cells, the microbial cells are crushed or lysed so that they can be used as a crude enzyme liquid. Moreover, the peptide-forming enzyme or its fused protein can be purified prior to use by ordinary techniques such as precipitation,

filtration or column chromatography as necessary. In this case, a purification method can also be used that uses an antibody of the peptide-forming enzyme or its fused protein.

In the case where protein inclusion bodies are formed, the inclusion
5 bodies are solubilized with a denaturant. They may be solubilized together with the microbial cell protein. However, in consideration of the following purification procedure, the inclusion bodies are preferably taken out and then solubilized. Conventionally known methods may be used to recover the inclusion bodies from the microbial cells. For example, inclusion bodies can
10 be recovered by crushing the microbial cells followed by centrifugal separation. Examples of denaturants capable of solubilizing inclusion bodies include guanidine hydrochloride (for example, 6 M, pH 5 to 8) and urea (for example, 8 M).

A protein having activity is regenerated by removing these
15 denaturants by dialysis. A Tris-HCl buffer solution or a phosphate buffer solution and so forth may be used as the dialysis solution to be used in dialysis, and the concentration may be, for example, 20 mM to 0.5 M, while the pH may be, for example, 5 to 8.

The protein concentration during the regeneration step is preferably
20 held to about 500 μ g/ml or less. The dialysis temperature is preferably 5°C or lower to inhibit the regenerated peptide-forming enzyme from undergoing self-crosslinking. Moreover, in addition to dialysis, dilution or ultrafiltration may be used to remove the denaturants, and it is expected that the activity can be regenerated regardless of whichever denaturant is used.

25 <2> Method of producing α -L-aspartyl-L-phenylalanine- α -methyl ester

The method of producing α -APM according to the present invention includes a first step of synthesizing α -L-aspartyl-L-phenylalanine- β -methyl

ester according to the "<1> Method of producing α -L-aspartyl-L-phenylalanine- β -ester" and a second step of converting α -L-aspartyl-L-phenylalanine- β -methyl ester to α -L-aspartyl-L-phenylalanine- α -methyl ester.

- 5 Preferred conditions in the first step and the like are as described in the "<1> Method of producing α -L-aspartyl-L-phenylalanine- β -ester". In addition, the second step can be carried out according to the known method and reference may be made to the method and preferred conditions described in, for example, Japanese Patent Publication No. H4-41155, etc.
- 10 By the production method of α -APM according to the present invention, α -APM, which is important as a sweetener and the like, can be inexpensively produced at high yield.

Examples

- 15 Hereinafter, the present invention will be explained by examples. However, the present invention is not limited to these examples. In addition to confirmation by ninhydrin coloring of thin-film chromatograms (qualitative), quantitative determinations were made by the following high-performance liquid chromatography in order to assay products. Column: Inertsil ODS-2
- 20 (manufactured by GL Science, Inc.), eluate: aqueous phosphate solution containing 5.0 mM sodium 1-octanesulfonate (pH 2.1) : methanol = 100:15 to 50, flow rate: 1.0 mL/min, detection: 210 nanometers (nm).

Example 1

Microbes that produce α -L-aspartyl-L-phenylalanine- β -methyl ester

- 25 50 milliliters ("mL" or "ml") of a medium (pH 7.0) containing 20 grams ("g") of glycerol, 5 g of ammonium sulfate, 1 g of potassium dihydrogen phosphate, 3 g of dipotassium hydrogen phosphate, 0.5 g of magnesium

sulfate, 10 g of yeast extract and 10 g of peptone in 1 liter (L) that was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes (medium 1) was used to culture bacteria and actinomycetes shown in Table 1-1. A slant agar medium (pH 7.0) containing 5 g/L of glucose, 10 g/L of yeast extract, 10 g/L of peptone, 5 g/L of NaCl and 20 g/L of agar in the medium 1 was prepared and a microbe shown in Table 1 was cultured on this slant agar medium at 30°C for 24 hours. Then, one loopful of the microbe was cultured in the medium 1 at 30°C for 24 hours, followed by shake culturing at 30°C and 120 strokes/min for 17 hours. After completion of the culturing, the microbial cells were separated from these culture liquids by centrifugation, and suspended in 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells.

To culture yeast shown in Table 1-1, 50 mL of a medium (pH 6.0) containing 10 g of glucose, 10 g of glycerol, 5 g of ammonium sulfate, 1 g of potassium dihydrogen phosphate, 3 g of dipotassium hydrogen phosphate, 0.5 g of magnesium sulfate, 5 g of yeast extract, 5 g of malt extract and 10 g of peptone in 1 L transferred to a 500-mL Sakaguchi flask and sterilized at 115°C for 15 minutes (medium 2) was used. A slant agar medium (pH 6.0) containing 5 g/L of glucose, 5 g/L of yeast extract, 5 g/L of malt extract, 10 g/L of peptone, 5 g/L of NaCl and 20 g/L agar in the medium 2 was prepared and a yeast shown in Table 1 was cultured on the slant agar medium at 30°C for 24 hours. Then, one loopful of the yeast was shake cultured at 30°C for 24 hours in the medium 2 at 25°C and 120 strokes/min for 17 hours. After completion of the culturing, the microbial cells were separated from these culture liquids by centrifugation, and suspended in 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells.

The microbes shown in Table 1-2 were cultured as follows. An agar

solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 1 g of tryptone, 1 g of yeast extract and 15 g of agar in 1 L of Daigo artificial sea water SP was used to culture *Cellulophaga lytica* NBRC 14961 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) or *Flexithrix dorotheae* NBRC 15987 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Cellulophaga lytica* NBRC 14961 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) or *Flexithrix dorotheae* NBRC 15987 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured on this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

20 A sheep blood agar medium (Nissui Plate, Nissui Pharmaceutical) was used to culture *Weeksellia virosa* NBRC 16016 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Weeksellia virosa* NBRC 16016 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan)

which was seed cultured on this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 10 g of peptone, 2 g of yeast extract, 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 15 g of agar in 1 L of distilled water was used to culture *Pedobacter heparinus* NBRC 12017 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Pedobacter heparinus* NBRC 12017 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured on this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 0.5 g of KNO_3 , 0.1 g of sodium glycerophosphate, 1 g of trishydroxymethylaminomethane, 5 g of tryptone, 5 g of yeast extract, 15 g of agar and 1 ml of a trace element solution in 1L of Daigo artificial sea water SP was used to culture *Persicobacter diffluens* NBRC 15940 ((Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) Note that the trace element solution contained 2.85 g of H_3BO_4 , 1.8 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.36 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 26.9 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 20.8 mg of ZnCl_2 , 40.4 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 25.2 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 1.77 g of sodium tartrate). Microbial cells of *Persicobacter diffluens* NBRC 15940 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology

and Evaluation, address of depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured on this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

- 5 An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 3 g of bactocasitone, 1 g of yeast extract, 1.36 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 15 g of agar in 1 L of distilled water was used to culture *Chitinophaga pinensis* NBRC 15968 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of
10 depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Chitinophaga pinensis* NBRC 15968 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depositary
15 institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured on this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

- An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 5 g of peptone, 1 g of yeast extract, 0.2 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 15 g of agar in 1 L of Daigo artificial sea water SP was used to culture
20 *Cyclobacterium marinum* ATCC 25205 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). Microbial cells of *Cyclobacterium marinum* ATCC 25205 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549,
25 Manassas, VA 20110, the United States of America) which was seed cultured on this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 1 g of peptone, 1 g of yeast extract, 1 g of glucose and 15 g of agar in 1 L of distilled water was used to culture *Runella slithyformis* ATCC 29530 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). Microbial cells of *Runella slithyformis* ATCC 29530 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured in this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 8.2, sterilized at 120°C for 15 minutes) containing 0.2 g of nitrilotriacetic acid, 2 ml of a 0.03% FeCl₃ solution, 0.12 g of CaSO₄·2H₂O, 0.2 g of MgSO₄·7H₂O, 0.016 g of NaCl, 0.21 g of KNO₃, 1.4 g of NaNO₃, 0.22 g of Na₂HPO₄, 2 ml of trace element solution and 15 g of agar in 1 L of distilled water was used to culture *Thermonema lapsum* ATCC 43542 ((Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) it should be noted that the trace element solution contained 0.5 ml of H₂SO₄, 2.2 g of MnSO₄, 0.5 g of ZnSO₄, 0.5 g of H₃BO₃, 0.016 g of CuSO₄, 0.025 g of Na₂MoO₄ and 0.046 g of CoCl₂). Microbial cells of *Thermonema lapsum* ATCC 43542 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured on this medium at 60°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

Marine Agar 2216 (manufactured by Difco) was used to culture

Gelidibacter algens ATCC 700364 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), *Lewinella cohaerens* ATCC 23123 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), or *Salegentibacter salegens* DSMZ 5424 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany)).

In the case of *Gelidibacter algens* ATCC 700364 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) or *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), microbial cells of *Gelidibacter algens* ATCC 700364, or *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured on this medium at 10°C for 72 hours were applied, followed by main culturing at 10°C for 72 hours. In the case of *Lewinella cohaerens* ATCC 23123 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America), microbial cells of *Lewinella cohaerens* ATCC 23123 (Depository institution; the American Type

Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours. In the case of

- 5 *Salagentibacter salegens* DSMZ 5424 (Depositary institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany), microbial cells of *Salagentibacter salegens* DSMZ 5424 (Depositary institution; the Deutsche
- 10 Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) which was seed cultured in this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.
- 15 An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 0.8 g of NH₄Cl, 0.25 g of KH₂PO₄, 0.4 g of K₂HPO₄, 0.505 g of KNO₃, 15 mg of CaCl₂·2H₂O, 20 mg of MgCl₂·6H₂O, 7 mg of FeSO₄·7H₂O, 5 mg of Na₂SO₄, 5 mg of MnCl₂·4H₂O, 0.5 mg of H₃BO₃, 0.5 mg of ZnCl₂, 0.5 mg of CoCl₂·6H₂O, 0.5 mg of NiSO₄·6H₂O, 0.3 mg of CuCl₂·2H₂O, 10 mg of
- 20 Na₂MoO₄·2H₂O, 0.5 g of yeast extract, 0.5g of peptone, 0.5 g of casamino acid, 0.5 g of dextrose, 0.5 g of soluble starch, 0.5 g of sodium pyruvate, and 15 g of agar in 1. L of distilled water was used to culture *Dyadobacter fermentans* ATCC 700827 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA
- 25 20110, the United States of America). Microbial cells of *Dyadobacter fermentans* ATCC 700827 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA

20110, the United States of America) which was seed cultured in this medium at 25°C for 48 hours, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 2 g of tryptone, 0.5 g of meat extract, 0.5 g of yeast extract, 0.2 g of sodium acetate and 15 g of agar in 1 L of Daigo artificial sea water SP was used to culture *Flammeovirga aprica* NBRC 15941 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Flammeovirga aprica* NBRC 15941 (Depository institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depository institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured in this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 1 g of glucose, 1 g of peptone, 1 g of yeast extract, and 15 g of agar in 1 L of distilled water was used to culture *Spirosoma linguale* DSMZ 74 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) or *Flectobacillus major* DSMZ 103 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). Microbial cells of *Spirosoma linguale* DSMZ 74 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b,

38124 Braunschweig, Germany) or *Flectobacillus major* DSMZ

103(Depositary institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures,

Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig,

5 Germany) which was seed cultured on this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 0.5 g of tryptone, 0.5 g of yeast extract, 0.2 g of meat extract, 0.2
10 g of sodium acetate and 15 g of agar in 300 ml of distilled water and 700 ml of Daigo artificial sea water SP was used to culture *Tenacibaculum maritimum* ATCC 43398(Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). Microbial cells of *Tenacibaculum*
15 *maritimum* ATCC 43398(Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured in this medium at 25°C for 48 hours, followed by main culturing at 25°C for 48 hours.

An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes)
20 containing 2.5 g of yeast extract, 2.5 g of tryptone, 100 mg of nitrilotriacetic acid, 40 mg of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 200 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 ml of 0.01M Fe citrate, 0.5 ml of a trace element solution, 100 ml of phosphate buffer, 900 ml of distilled water, and 28 g of agar in 1 L was used to culture *Rhodothermus marinus* DSMZ 4252 (Depositary institution; the Deutsche Sammlung von
25 Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). Note that the trace element solution

- contained 12.8 g of nitrilotriacetic acid, 1 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 g of $\text{CoCl}_2 \cdot 4\text{H}_2\text{O}$, 50 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 20 mg of H_3BO_3 and 20 mg of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$). Microbial cells of *Rhodothermus marinus* DSMZ 4252 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) which was seed cultured in this medium at 60°C for 48 hours were applied on the same medium, followed by main culturing at 60°C for 48 hours.
- 10 An agar solid medium (1.5% agar, pH 7.6, sterilized at 120°C for 15 minutes) containing BACTO MARINE BROTH (DIFCO 2216) was used to culture *Zobellia galactanivorans* DSMZ 12802 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). This medium was applied with microbial cells of *Zobellia galactanivorans* DSMZ 12802 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

- 25 An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 1.5 g of yeast extract, 2.5 g of peptone, 2 g of hexadecane, 17.7 g of NaCl, 0.48 g of KCl, 3.4 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4.46 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.98 g of CaCl_2 and 15 g of agar in 1 L of distilled water was used to culture *Muricauda ruestringensis* DSMZ 13258 (Depository institution; the Deutsche Sammlung

von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). Microbial cells of *Muricauda*

ruestringensis DSMZ 13258 (Depositary institution; the Deutsche Sammlung

- 5 von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

- 10 An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 3 g of casitone, 1 g of yeast extract, 1.36 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 15 g of agar in 1 L of distilled water was used to culture *Taxeobacter* *gelupurpurascens* DSMZ 11116 (Depositary institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection
- 15 of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany). Microbial cells of *Taxeobacter gelupurpurascens* DSMZ 11116 (Depositary institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution;
- 20 Mascheroder Weg 1b, 38124 Braunschweig, Germany) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

- An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 3 g of casitone, 1 g of yeast extract, 1.36 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 g of
- 25 cellobiose and 15 g of agar in 1 L of distilled water was used to culture *Cytophaga hutchinsonii* NBRC 15051 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and

Evaluation, address of depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Cytophaga hutchinsonii* NBRC 15051 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

An agar solid medium (pH 7.2, sterilized at 120°C for 15 minutes) containing 10 g of peptone, 2 g of yeast extract, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 15 g of agar in 250 ml of distilled water and 750 ml of Daigo artificial sea water SP was used to culture *Marinilabilia salmonicolor* NBRC 15948 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan). Microbial cells of *Marinilabilia salmonicolor* NBRC 15948 (Depositary institution; the NITE Biological Resource Center of the National Institute of Technology and Evaluation, address of depositary institution; 5-8 Kazusa-Kamaashi 2-Chome, Kisarazu-shi, Chiba-ken, Japan) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

An agar solid medium (pH 7.0, sterilized at 120°C for 15 minutes) containing 0.5 g of KNO_3 , 0.1 g of sodium glycerophosphate, 1 g of trishydroxymethylaminomethane, 2 g of tryptone, 2 g of yeast extract, 15 g of agar and 1 ml of a trace element solution in 1 L of Daigo artificial sea water SP was used to culture *Saprospira grandis* ATCC 23119 ((Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of

America). Note that the trace element solution contained 2.85 g of H_3BO_4 , 1.8 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.36 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 26.9 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 20.8 mg of ZnCl_2 , 40.4 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 25.2 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 1.77 g of sodium tartrate). Microbial cells of *Saprospira grandis* ATCC

- 5 23119 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed cultured in this medium at 30°C for 48 hours were applied on the same medium, followed by main culturing at 30°C for 48 hours.

- 10 An agar solid medium (pH 7.5, sterilized at 120°C for 15 minutes) containing 27 mg of KH_2PO_4 , 40 mg of K_2HPO_4 , 40 mg of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 75 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.31 g of glutamic acid, 2.5 mg of Trypticase Soy Broth without glucose, 0.4 mg of thiamine, 0.01 mg of vitamin B12, 2 g of glucose, and 1 ml
- 15 of a trace element solution in 1 L of distilled water was used to culture *Haliscomenobacter hydrossis* ATCC 27775 ((Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). Note that the trace element solution contained 0.1 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$,
- 20 0.3 g of H_3BO_3 , 0.2 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.03 g of $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$). Microbial cells of *Haliscomenobacter hydrossis* ATCC 27775 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) which was seed
- 25 cultured in this medium at 25°C for 48 hours were applied on the same medium, followed by main culturing at 25°C for 48 hours.

The thus obtained microbial cells were each collected from the agar

medium, and suspended in 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells.

To 0.1 mL each of the microbial cell suspensions of these microbes was added 0.1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA,
5 100 mM L-aspartic acid- α,β -dimethyl ester hydrochloride and 200 mM L-phenylalanine to make the total amount 0.2 mL. Then, reaction was carried out at 20°C for 3 hours when the microbe shown in Table 1-1 was used or for 1 hour when the microbe shown in Table 1-2 was used. The amount (mM) of α -L-aspartyl-L-phenylalanine- β -methyl ester (α -AMP)
10 produced is shown in Tables 1-1 and 1-2. Note that no β -AMP was detected in all the cases where the microbes were used.

Table 1-1

Microbe	α -AMP (mM)
<i>Aeromonas hydrophila</i> ATCC 13136	1.55
<i>Azotobacter vinelandii</i> IFO 3741	0.15
<i>Alcaligenes faecalis</i> FERM P-8460	0.37
<i>Brevibacterium minutiferuna</i> FERM BP-8277	0.10
<i>Corynebacterium flavescens</i> ATCC 10340	0.26
<i>Escherichia coli</i> FERM BP-8276	3.68
<i>Empedobacter brevis</i> ATCC 14234	6.31
<i>Flavobacterium resinovorum</i> ATCC 14231	0.62
<i>Microbacterium arborescens</i> ATCC 4348	0.08
<i>Propionibacterium shermanii</i> BERM BP-8100	3.41
<i>Brevibacillus parabrevis</i> ATCC 8185	0.08
<i>Paenibacillus alvei</i> IFO 14175	0.09
<i>Pseudomonas fragi</i> IFO 3458	0.84
<i>Serratia grimesii</i> ATCC 14460	0.47
<i>Stenotrophomonas maltophilia</i> ATCC 13270	0.18
<i>Sphingobacterium</i> sp. FERM BP-8124	5.97
<i>Streptomyces lavendulae</i> NRRL B-1305	0.89
<i>Xanthomonas maltophilia</i> FERM BP-5568	0.40
<i>Williopsis saturnus</i> IFO 0895	0.05
<i>Candida magnoliae</i> IFO 0705	0.26
<i>Geotrichum amycelium</i> CBS 152.25	0.19
<i>Geotrichum amycelium</i> IFO 0905	0.06
<i>Saccharomyces unisporus</i> IFO 0724	0.07
<i>Torulaspora delbrueckii</i> IFO 0422	0.04
<i>Pichia ciferrii</i> IFO 0905	0.06

Table 1-2

Microbe	α -AMP (mM)	Microbe	α -AMP (mM)
<i>Cellulophaga lytica</i> NBRC 14961	tr	<i>Spirosoma linguale</i> DSMZ 74	0.15
<i>Weeksella virosa</i> NBRC 16016	tr	<i>Flectobacillus major</i> DSMZ 103	0.68
<i>Pedobacter heparinus</i> NBRC 12017	0.07	<i>Tenacibaculum maritimum</i> ATCC 43398	tr
<i>Persicobacter diffluens</i> NBRC 15940	tr	<i>Rhodotermus marinus</i> DSMZ 4252	0.06
<i>Flexithrix dorotheae</i> NBRC 15987	2.47	<i>Zobellia galactanivorans</i> DSMZ 12802	0.42
<i>Chitinophaga pinensis</i> NBRC 15968	0.08	<i>Muricauda ruestringensis</i> DSMZ 13258	0.51
<i>Cyclobacterium marinum</i> ATCC 25205	0.91	<i>Salegentibacter salegens</i> DSMZ 5424	tr
<i>Runella slithyformis</i> ATCC 29530	0.07	<i>Taxeobacter gelupurpurascens</i> DSMZ 11116	0.02
<i>Thermonema lapsum</i> ATCC 43542	tr	<i>Cytophaga hutchinsonii</i> NBRC 15051	tr
<i>Psychroserpens burtonensis</i> ATCC 700359	0.09	<i>Marinilabilia salmonicolor</i> NBRC 15948	0.02
<i>Gelidibacter algens</i> ATCC 700364	0.07	<i>Lewinella cohaerens</i> ATCC 23123	0.33
<i>Dyadobacter fermentans</i> ATCC 700827	0.04	<i>Saprospira grandis</i> ATCC 23119	0.03
<i>Flammeovirga aprica</i> NBRC 15941	0.08	<i>Haliscomenobacter hydrossis</i> ATCC 27775	tr

Reference Example 1

5 Microbe that produces β -L-aspartyl-L-phenyl- alanine- α -methyl ester

Microbes shown in Table 2 were cultured similarly to the procedure in bacteria in Table 1 of Example 1. After completion of the culturing, the microbial cells were separated from these culture broths by centrifugation, and suspended in 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 10 100 g/L as wet microbial cells. To 0.1 mL each of the microbial cell suspensions of these microbes was added 0.1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 100 mM L-aspartic acid- α,β -dimethyl ester

hydrochloride and 200 mM L-phenylalanine to make the total amount 0.2 ml, followed by reaction at 30°C for 2 hours. The amount (mM) of β -L-aspartyl-L-phenylalanine- α -methyl ester (β -AMP) produced in this case is indicated in Table 2. Note that no α -AMP was detected in all the microbes.

5

Table 2

Microbe	β -AMP (mM)
<i>Hafnia alvei</i> ATCC 9760	0.30
<i>Klebsiella pneumoniae</i> ATCC 8308	0.26

Example 2

Purification of Enzyme from *Empedobacter brevis*

10 A 50 mL medium (pH 6.2) containing 5 grams (g) of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 liter (L) was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes. This medium was then inoculated with 2
15 milliliters (ml or mL) of *Empedobacter brevis* strain FERM BP-8113 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit
20 transfer date: July 8, 2002) that had been cultured at 30°C for 16 hours in the same medium, followed by shake culturing at 30°C and 120 strokes/min for 16 hours.

Subsequently, the procedure after centrifugal separation was carried out either on ice or at 4°C. The obtained culture broth was centrifuged to
25 collect microbial cells. After washing 16 g of the microbial cells with 50 mM

Tris-HCl buffer (pH 8.0), they were suspended in 40 milliliters (ml or mL) of the same buffer and subjected to ultrasonic crushing treatment for 45 minutes at 195 watts. This ultrasonically crushed liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain an

5 ultrasonic crushed liquid supernatant. This ultrasonic crushed liquid supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH 8.0) followed by removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP

10 column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH 8.0), and the active fraction was collected from the non-adsorbed fraction. This active fraction was dialyzed overnight against 50 mM acetate buffer (pH 4.5) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the

15 supernatant liquid. This dialyzed fraction was then applied to a Mono S column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) to elute enzyme at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl. The fraction that had the lowest level of contaminating protein among the active fractions was applied to a Superdex

20 200pg column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was performed by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to obtain an active fraction solution. As a result of performing these procedures, the peptide-forming enzyme used in the present invention was

25 confirmed to have been uniformly purified based on the experimental results of electrophoresis. The enzyme recovery rate in the aforementioned purification process was 12.2% and the degree of purification was 707 times.

Example 3

Production of α -L-aspartyl-L-phenylalanine- β -methyl ester using enzyme fraction of *Empedobacter brevis*

10 microliters (μ l) of Mono S fraction enzyme (about 20 U/ml)
5 obtained in Example 2 was added to 190 μ l of borate buffer (pH 9.0) containing 105.3 mM L-aspartic acid- α,β -dimethyl ester hydrochloride, 210.5 mM L-phenylalanine and 10.51 mM EDTA and reaction was carried out at 20°C. The course of production of α -L-aspartyl-L-phenylalanine- β -methyl ester (α -AMP) is shown in Table 3. Note that almost no formation of
10 α -L-aspartyl-L-phenylalanine- β -methyl ester was confirmed in the enzyme-not-added lot.

Further, 10 μ l of Mono S fraction enzyme (about 20 U/ml) obtained in Example 2 was added to 190 μ l of borate buffer (pH 9.0) containing each of 105.3 mM L-aspartic acid- α -methyl ester hydrochloride and L-aspartic
15 acid- β -methyl ester hydrochloride, 210.5 mM L-phenylalanine and 10.51 mM EDTA was added and reaction was carried out at 20°C. As a result, no formation of the corresponding peptides was observed.

Table 3

Reaction time (minute)	Produced α -AMP (mM)
30	23.0
60	42.1
120	61.7

20 Example 4

Purification of Enzyme from *Sphingobacterium* sp.

Sphingobacterium sp. strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository,
25 Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome,

Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) was cultured in the same manner as that in Example 2 using the medium shown in Example 2. The following procedure after centrifugal separation was carried out either on ice or at 4°C. The obtained culture broth was centrifuged (10,000 rpm, 15 minutes) to collect microbial cells. After washing 2 g of the microbial cells with 20 mM Tris-HCl buffer (pH 7.6), they were suspended in 8 ml of the same buffer and subjected to ultrasonic crushing treatment for 45 minutes at 195 W. This ultrasonically crushed liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments to obtain an ultrasonically crushed liquid supernatant. This ultrasonically crushed liquid supernatant was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.6) followed by removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH 7.6), and the active fraction was collected from the non-adsorbed fraction. This active fraction was dialyzed overnight against 20 mM acetate buffer (pH 5.0) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the supernatant liquid. This dialyzed fraction was then applied to an SP-Sepharose HP column (manufactured by Amersham) pre-equilibrated with 20 mM acetate buffer (pH 5.0) to obtain the active fraction in which enzyme was eluted at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl.

Example 5

Production of α -L-aspartyl-L-phenylalanine- β -methyl ester and α -L-aspartyl-L-phenylalanine- β -ethyl ester using enzyme fraction of

Sphingobacterium sp.

In the case of production of α -L-aspartyl-L-phenylalanine- β -methyl ester (α -AMP), 15 μ l of concentrated solution of SP-Sepharose HP fraction (about 15 U/ml) obtained in Example 4 was added to 185 μ l of borate buffer (pH 9.0) containing 108.1 mM L-aspartic acid- α,β -dimethyl ester hydrochloride, 216.2 mM L-phenylalanine and 10.8 mM EDTA and reaction was carried out at 20°C. Similarly, in the case of production of α -L-aspartyl-L-phenylalanine- β -ethyl ester (α -AEP), 10 μ l of a concentrated solution of SP-Sepharose HP fraction (about 15 U/ml) obtained in Example 4 was added to 190 μ l of borate buffer (pH 9.0) containing 52.6 mM L-aspartic acid- α,β -diethyl ester hydrochloride, 105.2 mM L-phenylalanine and 10.8 mM EDTA and reaction was carried out at 20°C. The course of formation of AMP or AEP is shown in Table 4. Note that almost no formation of AMP or AEP was confirmed in the enzyme-not-added lot. For formation of AEP, numerical values obtained by using a standard product of AMP are described.

Table 4

Reaction time (minute)	Produced α -AMP (mM)	Produced α -AEP (mM)
30	25.8	7.5
60	40.7	13.3
120	56.0	20.6
180	61.8	-

Example 6

5 Isolation of Peptide-forming enzyme Gene Derived from *Empedobacter brevis*

Hereinafter, isolation of a peptide-forming enzyme gene will be explained. *Empedobacter brevis* strain FERM BP-8113(Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism
10 Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) was used as the microbe. In isolating the gene, *Escherichia coli* JM-109 was used as a host while pUC118 was used as a vector.

15 (1) Production of PCR Primer Based on Determined Internal Amino Acid Sequence

A mixed primer having the base sequences indicated in SEQ ID NO.: 3 and SEQ ID NO: 4, respectively, was produced based on the amino acid sequences (SEQ ID NOs: 1 and 2) determined according to the Edman's
20 decomposition method from the digestion product of lysyl endopeptidase of a peptide-forming enzyme derived from the *Empedobacter brevis* strain FERM BP-8113(Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution:

Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,
International deposit transfer date: July 8, 2002).

(2) Acquisition of Microbial Cells

Empedobacter brevis strain FERM BP-8113(Depository institution: the
5 independent administrative corporation, National Institute of Advanced
Industrial Science and Technology, International Patent Organism Depository,
Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome,
Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8,
2002) was cultured at 30°C for 24 hours on a CM2G agar medium (containing
10 glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l, sodium chloride at
5 g/l, and agar at 20 g/l, pH 7.0). One loopful of the resulting microbial cells
was inoculated into a 500 ml Sakaguchi flask containing 50 ml of a CM2G
liquid medium (the aforementioned medium excluding agar) followed by
shake culturing at 30°C.

15 (3) Acquisition of Chromosomal DNA from Microbial Cells

50 ml of culture broth was centrifuged (12,000 rpm, 4°C, 15 minutes)
to collect the microbial cells. Then, a chromosomal DNA was acquired from
the microbial cells using the QIAGEN Genomic-Tip System (Qiagen) based
on the procedure described in the manual therefor.

20 (4) Acquisition of DNA Fragment Containing Part of Peptide-forming
Enzyme Gene by PCR

A DNA fragment containing a portion of the peptide-forming enzyme
gene derived from *Empedobacter brevis* strain FERM BP-8113(Depository
institution: the independent administrative corporation, National Institute of
25 Advanced Industrial Science and Technology, International Patent Organism
Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi
1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer

date: July 8, 2002) was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out on a chromosomal DNA acquired from *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) using the primers having the base sequences of SEQ ID NOs: 3 and 4.

10 The PCR reaction was carried out for 30 cycles under the following conditions using the Takara PCR Thermal Cycler PERSONAL (manufactured by Takara Shuzo).

94°C 30 seconds

52°C 1 minute

15 72°C 1 minute

After completion of the reaction, 3 μ l of the reaction liquid was applied to 0.8% agarose electrophoresis. As a result, it was verified that a DNA fragment of about 1.5 kilobases (kb) was amplified.

(5) Cloning of Peptide-Forming Enzyme Gene from Gene Library

20 In order to acquire the entire length of peptide-forming enzyme gene in full-length, Southern hybridization was carried out using the DNA fragment amplified in the PCR procedure as a probe. The procedure for Southern hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

25 The approximately 1.5 kb DNA fragment amplified by the PCR procedure was isolated by 0.8% agarose electrophoresis. The target band was then cut out and purified. This The DNA fragment was labeled with

probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor using DIG High Prime (manufactured by Boehringer-Mannheim).

After completely digesting the chromosomal DNA of *Empedobacter brevis* acquired in the step (3) of the present Example 6 by reacting at 37°C for 16 hours with restriction enzyme HindIII, the resultant was electrophoresed with on 0.8% agarose gel. The electrophoresed chromosomal DNA was blotted onto a positively charged Nylon membrane filter (manufactured by Roche Diagnostics) from the agarose gel after the electrophoresis, followed by treatments consisting of alkaline denaturation, neutralization and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 50°C for 1 hour, the probe labeled with digoxinigen prepared as described above was added and hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed for 20 minutes at room temperature with 2 × SSC containing 0.1% SDS. Moreover, the filter was additionally washed twice at 65°C for 15 minutes with 0.1 × SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim). As a result, a roughly 4 kb band was able to be detected that hybridized with the probe.

Then, 5 µg of the chromosomal DNA prepared in the step (3) of the present Example 6 was completely digested with HindIII. A roughly 4 kb of DNA was separated by 0.8% agarose gel electrophoresis, followed by

purification of the DNA using the Gene Clean II Kit (manufactured by Funakoshi) and dissolving the DNA in 10 μ l of TE. 4 μ l of this product was then mixed with pUC118 HindIII/BAP (manufactured by Takara Shuzo) and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2

- 5 (manufactured by Takara Shuzo). 5 μ l of the ligation reaction mixture and 100 μ l of competent cells of *Escherichia coli* JM109 (manufactured by Toyobo) were mixed to transform the *Escherichia coli*. This was then applied to a suitable solid medium to produce a chromosomal DNA library.

- To acquire the entire full-length of peptide-forming enzyme gene, the
10 chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

- The colonies of the chromosomal DNA library were transferred to a Nylon membrane filter (Nylon Membrane for Colony and Plaque Hybridization,
15 (manufactured by Roche Diagnostics) followed by treatments consisting of alkali denaturation, neutralization and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned probe labeled with digoxinigen was added, followed by hybridization at 50°C for 16
20 hours. Subsequently, the filter was washed for 20 minutes at room temperature with 2 $\times$ SSC containing 0.1% SDS. Moreover, the filter was additionally washed twice at 65°C for 15 minutes with 0.1 $\times$ SSC containing 0.1% SDS.

- Detection of colonies that hybridized with the labeled probe was
25 carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor using the DIG Nucleotide Detection Kit (manufactured by

Boehringer-Mannheim). As a result, two strains of colonies were verified to hybridize with the labeled probe.

(6) Base Sequence of Peptide-Forming Enzyme Gene Derived from *Empedobacter brevis*

5 Plasmids possessed by *Escherichia coli* JM109 were prepared from the aforementioned two strains of microbial cells that were verified to hybridize with the labeled probe using the Wizard Plus Minipreps DNA Purification System (manufactured by Promega) to and the base sequence of a portion where hybridization with the probe occurred and nearby was
10 determined. The sequencing reaction was carried out using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor. In addition, electrophoresis was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it was verified that an open reading frame that encodes a
15 protein containing the internal amino acid sequences of the peptide-forming enzyme (SEQ ID NOs: 1 and 2) did exist, thereby confirming that the open reading frame was a gene encoding the peptide-forming enzyme. The base sequence of the full-length of the peptide-forming enzyme genes along with the corresponding amino acid sequences is shown in SEQ ID NO: 5 of the
20 Sequence Listing. As a result of analysis on the homology of the resulting open reading frame with the BLASTP program, homology was discovered between the two enzymes; it showed with a homology of 34% as at the amino acid sequence level exhibited with the α -amino acid ester hydrolase of *Acetobacter pasteurianus* (see Appl. Environ. Microbiol., 68(1), 211-218
25 (2002), and a homology of 26% at the amino acid sequence level exhibited with the glutaryl-7ACA acylase of *Brevibacillus laterosporum* (see J. Bacteriol., 173(24), 7848-7855 (1991).

(7) Expression of Peptide-forming enzyme Gene Derived from
Empedobacter brevis in *Escherichia coli*

A target gene region on the promoter region of the trp operon on the chromosomal DNA of *Escherichia coli* W3110 was amplified by carrying out
5 PCR using the oligonucleotides indicated in SEQ ID NOs: 7 and 8 as primers, and the resulting DNA fragments were ligated to a pGEM-Teasy vector (manufactured by Promega). *E. coli* JM109 was then transformed in this ligation solution, and those strains having the target plasmid in which the direction of the inserted trp promoter is inserted in the opposite to the
10 orientation from of the lac promoter were selected from ampicillin-resistant strains. Next, a DNA fragment containing the trp promoter obtained by treating this plasmid with EcoO109I/EcoRI was ligated to an EcoO109I/EcoRI treatment product of pUC19 (manufactured by Takara). *Escherichia coli* JM109 was then transformed with this ligation solution and those strains
15 having the target plasmid were selected from ampicillin-resistant strains. Next, a DNA fragment obtained by treating this plasmid with HindIII/PvuII was ligated with to a DNA fragment containing an rrnB terminator obtained by treating pKK223-3 (manufactured by Amersham Pharmacia) with HindIII/HincII. *E. coli* JM109 was then transformed with this ligation solution,
20 strains having the target plasmid were selected from ampicillin-resistant strains, and the plasmid was designated as pTrpT.

The target gene was amplified by PCR using the chromosomal DNA of *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced
25 Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8,

2002) as a template and the oligonucleotides indicated in SEQ ID NO: 9 and 10 as primers. This DNA fragment was then treated with NdeI/PstI, and the resulting DNA fragment was ligated with the NdeI/PstI treatment product of pTrpT. *Escherichia coli* JM109 was then transformed with this ligation

5 solution, those strains having the target plasmid were selected from ampicillin-resistant strains, and this plasmid was designated as pTrpT_Gtg2.

Escherichia coli JM109 having pTrpT_Gtg2 was seed cultured at 30°C for 24 hours in LB medium containing 100 mg/l of ampicillin. 1 ml of the resulting culture broth was seeded in a 500 ml Sakaguchi flask containing 50
10 ml of a medium (D glucose at 2 g/l, yeast extract at 10 g/l, casamino acids at 10 g/l, ammonium sulfate at 5 g/l, potassium dihydrogen phosphate at 3 g/l, dipotassium hydrogen phosphate at 1 g/l, magnesium sulfate heptahydrate at 0.5 g/l, and ampicillin at 100 mg/l), followed by culturing at 25°C for 24 hours. The culture broth had an α -L-aspartyl-phenylalanine- β -methyl ester forming
15 activity of 0.11 U per 1 ml of culture broth and it was verified that the cloned gene was expressed by *E. coli*. Furthermore, no activity was detected for a transformant in which only pTrpT had been introduced as a control.

Prediction of Signal Sequence

When the amino acid sequence of SEQ ID NO: 6 described in the
20 Sequence Listing was analyzed with the Signal P v 1.1 program (see Protein Engineering, Vol. 12, No. 1, pp. 3-9, 1999), it was predicted that amino acids numbers 1 to 22 function as a signal that is secreted into the periplasm, while the mature protein was estimated to be downstream of amino acid number 23.

25 Verification of Secretion

Escherichia coli JM109, having pTrpT_Gtg2, was seed cultured at 30°C for 24 hours in LB medium containing 100 mg/l of ampicillin. 1 ml of

the resulting culture broth was seeded into a 500 ml Sakaguchi flask containing 50 ml of medium (glucose at 2 g/l, yeast extract at 10 g/l, casamino acids at 10 g/l, ammonium sulfate at 5 g/l, potassium dihydrogen phosphate at 3 g/l, dipotassium hydrogen phosphate at 1 g/l, magnesium sulfate heptahydrate at 0.5 g/l, and ampicillin at 100 mg/l), followed by final culturing at 25°C for 24 hours to obtain cultured microbial cells.

The cultured microbial cells were fractionated into a periplasm fraction and a cytoplasm fraction by an osmotic pressure shock method using a 20 grams/deciliter (g/dl) sucrose solution. The microbial cells immersed in the 20 g/dl sucrose solution were immersed in a 5 mM aqueous MgSO_4 solution. The centrifuged supernatant was named a periplasm fraction ("Pe"). In addition, the centrifuged sediment was re-suspended and subjected to ultrasonic crushing. The resultant was named a cytoplasm fraction ("Cy"). The activity of glucose 6-phosphate dehydrogenase, which is known to be present in the cytoplasm, was used as an indicator to verify that the cytoplasm had been separated. This measurement was carried out by adding a suitable amount of enzyme to a reaction solution at 30°C containing 1 mM glucose 6-phosphate, 0.4 mM NADP, 10 mM MgSO_4 , and 50 mM Tris-Cl (pH 8), followed by measurement of absorbance at 340 nm to measure production of NADPH.

The amounts of enzymes of in the periplasm fraction and the cytoplasm fraction when the activity of a separately prepared cell-free extract was assigned a value of 100% are shown in Fig. 1. That glucose 6-phosphate dehydrogenase activity did not mix in the periplasm fraction indicates that the periplasm fraction did not mix in the cytoplasm fraction. About 60% of the α -L-aspartyl-L-phenylalanine- β -methyl ester (α -AMP) forming activity was recovered in the periplasm fraction, and it was verified

that the Ala-Gln-forming enzyme was secreted into the periplasm as predicted from the amino acid sequence using the Signal P v 1.1 program.

Example 7

Isolation of Peptide-forming enzyme Gene Derived from *Sphingobacterium*
5 sp.

Hereinafter, isolation of a peptide-forming enzyme gene is described.

The microbe used was *Sphingobacterium* sp. strain FERM

BP-8124 (Depositary institution: the independent administrative corporation,
National Institute of Advanced Industrial Science and Technology,

10 International Patent Organism Depositary, Address of depositary institution:
Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,
International deposit date: July 22, 2002). For the isolation of the gene,
Escherichia coli DH5 α was used as a host, and pUC118 was used as a
vector.

15 (1) Acquisition of Microbial Cells

Sphingobacterium sp. strain FERM BP-8124 (Depositary institution:
the independent administrative corporation, National Institute of Advanced
Industrial Science and Technology, International Patent Organism Depositary,
Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome,
20 Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002)
was cultured for 24 hours at 25°C on a CM2G agar medium (containing
glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l, sodium chloride at
5 g/l, and agar at 20 g/l, pH 7.0). One loopful of the resulting microbial cells
was inoculated into a 500 ml Sakaguchi flask containing 50 ml of CM2G liquid
25 medium (the aforementioned medium excluding agar) followed by shake
culturing at 25°C.

(2) Acquisition of Chromosomal DNA from Microbial Cells

50 ml of culture broth was centrifuged (12,000 rpm, 4°C, 15 minutes) to collect the microbial cells. A chromosomal DNA was then acquired from the microbial cells using the Qiagen Genomic-Tip System (Qiagen) based on the procedure described in the manual therefor.

5 (3) Acquisition of Probe DNA Fragment by PCR

A DNA fragment containing a portion of the peptide-forming enzyme gene derived from *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism
10 Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out on the chromosomal DNA acquired from *Empedobacter brevis* strain FERM
15 BP-8113 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) using primers having the
20 base sequences of SEQ ID NOs: 3 and 4.

The PCR reaction was carried out using the Takara PCR Thermal Cycler – PERSONAL (Takara Shuzo) for 30 cycles under the following conditions.

94°C 30 seconds
25 52°C 1 minute
72°C 1 minute

After completion of the reaction, 3 µl of reaction liquid was applied to

0.8% agarose electrophoresis. As a result, it was verified that a DNA fragment of about 1.5 kb was amplified.

(4) Cloning of Peptide-forming enzyme Gene from Gene Library

In order to acquire the full-length peptide-forming enzyme gene,

5 Southern hybridization was carried out using the DNA fragment amplified in the aforementioned PCR procedure as a probe. The operations of Southern hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The approximately 1.5 kb DNA fragment amplified by the
10 aforementioned PCR procedure was separated by 0.8% agarose electrophoresis. The target band was then cut out and purified. This DNA fragment was labeled with probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

15 After allowing the chromosomal DNA of *Sphingobacterium* sp. acquired in the step (2) of the present Example 7 to react with restriction enzyme *SacI* at 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel. From the agarose gel after the electrophoresis, the electrophoresed chromosomal DNA was blotted onto a
20 positively charged Nylon membrane filter (manufactured by Roche Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the digoxinigen-labeled probe prepared as
25 described above was added and hybridization was carried out at 37°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1 × SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the procedure described in the manual therefor. As a result, a roughly 3 kb band was successfully detected that hybridized with the probe.

5 5 μ g of the chromosomal DNA prepared in the step (2) of the present Example 7 was completely digested with *SacI*. About 3 kb of a DNA was separated by 0.8% agarose gel electrophoresis, the DNA was purified using the Gene Clean II Kit (manufactured by Funakoshi), and dissolved in 10 μ l of TE. 4 μ l of the resulting solution and pUC118 treated with alkaline
10 phosphatase (*E. coli* C75) at 37°C for 30 minutes and at 50°C for 30 minutes, after reaction with *SacI* at 37°C for 16 hours to completely digest, were mixed and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). 5 μ l of this ligation reaction liquid and 100 μ l of competent cells of *Escherichia coli* DH5 α (manufactured by Takara
15 Shuzo) were mixed to transform the *Escherichia coli*. This was then applied to a suitable solid medium to produce a chromosomal DNA library.

To acquire full-length peptide-forming enzyme gene, the chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained in Molecular
20 Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred to a Nylon membrane filter (Nylon Membrane for Colony and Plaque Hybridization, manufactured by Roche Diagnostics), followed by treatments of alkali denaturation, neutralization, and immobilization. Hybridization was carried
25 out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned digoxigenin-labeled probe was added, followed by hybridization at 37°C for

16 hours. Subsequently, the filter was washed twice at 60°C with 1 × SSC containing 0.1% SDS.

Detection of colonies that hybridized with the labeled probe was carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor. As a result, six strains of colonies were verified to have hybridized with the labeled probe.

(5) Base Sequence of Peptide-forming enzyme Gene Derived from *Sphingobacterium* sp.

Plasmids possessed by *Escherichia coli* DH5 α were prepared from the six strains of microbial cells that were verified to have hybridized with the labeled probe using the Wizard Plus Minipreps DNA Purification System (manufactured by Promega) to determine the base sequence of a portion where hybridization with the probe occurred and nearby was determined. The sequencing reaction was carried out using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor. In addition, electrophoresis was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes peptide-forming enzyme did exist. The full-length base sequence of the peptide-forming enzyme gene derived from *Sphingobacterium* sp. along with the corresponding amino acid sequence is shown in SEQ ID NO: 11. Peptide-forming enzyme derived from *Sphingobacterium* sp. exhibited a homology of 63.5% at the amino acid sequence level to the peptide-forming enzyme derived from *Empedobacter brevis* (as determined using the BLASTP program).

(6) Expression of Peptide-forming enzyme Gene Derived from

Sphingobacterium sp. in *Escherichia coli*

The target gene was amplified by PCR using the chromosomal DNA of *Sphingobacterium* sp. FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) as a template and the oligonucleotides shown in SEQ ID NOs: 13 and 14 as primers. This DNA fragment was treated with NdeI/XbaI, and the resulting DNA fragment and an NdeI/XbaI treatment product of pTrpT were ligated. *Escherichia coli* JM109 was then transformed with this ligation solution, and strains having the target plasmid were selected from ampicillin-resistant strains. The plasmid was designated as pTrpT_Sm_aet.

Escherichia coli JM109 having pTrpT_Sm_aet was cultured at 25°C for 20 hours by inoculating one loopful thereof into an ordinary test tube containing 3 ml of a medium (glucose at 2 g/l, yeast extract at 10 g/l, casamino acids at 10 g/l, ammonium sulfate at 5 g/l, potassium dihydrogen phosphate at 3 g/l, dipotassium hydrogen phosphate at 1 g/l, magnesium sulfate heptahydrate at 0.5 g/l and ampicillin at 100 mg/l). It was verified that a cloned gene having an α -AMP production activity of 0.53 U per ml of culture broth was expressed by *Escherichia coli*. Furthermore, no activity was detected for a transformant containing only pTrpT used as a control.

Prediction of Signal Sequence

When the amino acid sequence of SEQ ID NO: 12 described in the Sequence Listing was analyzed with the Signal P v 1.1 program (Protein Engineering, Vol. 12, No. 1, pp. 3-9, 1999), it was predicted that amino acids numbers 1 to 20 function as a signal that is secreted into the periplasm, while

the mature protein was estimated to be downstream of amino acid number 21.

Confirmation of Signal Sequence

One loopful of *Escherichia coli* JM109, having pTrpT_Sm_aet, was
5 inoculated into ordinary test tubes containing 50 ml of a medium (glucose at 2 g/l, yeast extract at 10 g/l, casamino acids at 10 g/l, ammonium sulfate at 5 g/l, potassium dihydrogen phosphate at 3 g/l, dipotassium hydrogen phosphate at 1 g/l, magnesium sulfate heptahydrate at 0.5 g/l and ampicillin at 100 mg/l) and main culturing was performed at 25°C for 20 hours.

10 Hereinafter, procedures after centrifugal separation were carried out either on ice or at 4°C. After completion of the culturing, the microbial cells were separated from the culture broth by centrifugation, washed with 100 mM phosphate buffer (pH 7), and then suspended in the same buffer. The microbial cells were then subjected to ultrasonic crushing treatment for 20
15 minutes at 195 W, the ultrasonic crushed liquid was centrifuged (12,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain a soluble fraction. The resulting soluble fraction was applied to a CHT-II column manufactured by Biorad) pre-equilibrated with 100 mM phosphate buffer (pH 7), and enzyme was eluted at a linear concentration gradient with 500 mM
20 phosphate buffer. A solution obtained by mixing the active fraction with 5 time volumes of 2 M ammonium sulfate and 100 mM phosphate buffer was applied to a Resource-PHE column (manufactured by Amersham) pre-equilibrated with 2 M ammonium sulfate and 100 mM phosphate buffer, and an enzyme was eluted at a linear concentration gradient by 2 to 0 M
25 ammonium sulfate to obtain an active fraction solution. As a result of these procedures, it was verified that the peptide-forming enzyme was electrophoretically uniformly purified.

When the amino acid sequence of the aforementioned peptide-forming enzyme was determined by Edman's decomposition method, the amino acid sequence of SEQ ID NO: 15 was acquired, and the mature protein was verified to be downstream of amino acid number 21 as was
5 predicted by the SignalP v 1.1 program.

Example 8

Isolation of Peptide-forming enzyme Gene Derived from *Pedobacter heparinus* IFO 12017

Hereinafter, isolation of a peptide-forming enzyme gene is described.
10 The microbe used is *Pedobacter heparinus* IFO 12017 (Depository institution; the Institute of Fermentation, Osaka, address of the depository institution; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan). For the isolation of the gene, *Escherichia coli* JM109 was used as a host, and pUC118 was used as a vector.

15 (1) Acquisition of Microbial Cells

Pedobacter heparinus IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) was cultured for 24 hours at 25°C on a CM2G agar medium (containing glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l,
20 sodium chloride at 5 g/l, and agar at 20 g/l, pH 7.0). One loopful of the resulting microbial cells was inoculated into a 500 ml Sakaguchi flask containing 50 ml of CM2G liquid medium (the aforementioned medium excluding agar) followed by shake culturing at 25°C.

(2) Acquisition of Chromosomal DNA from Microbial Cells

25 50 ml of the culture broth was centrifuged (12,000 rpm, 4°C, 15 minutes) to collect the microbial cells. A chromosomal DNA was then acquired from the microbial cells using the Qiagen Genomic-Tip System

(Qiagen) based on the procedure described in the manual therefor.

(3) Acquisition of Probe DNA Fragment by PCR

A DNA fragment containing a portion of the peptide-forming enzyme gene derived from *Pedobacter heparinus* IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out on the chromosomal DNA acquired from *Pedobacter heparinus* IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) using primers having the base sequences of SEQ ID NOs: 15 and 16. The approximately 1 kb DNA fragment amplified by the PCR procedure was isolated by 0.8% agarose electrophoresis. The target band was then cut out and purified. The DNA fragment was labeled with probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

(4) Cloning of Peptide-forming enzyme Gene from Gene Library

To acquire the full-length peptide-forming enzyme gene, Southern hybridization was carried out using the DNA fragment amplified in the aforementioned PCR procedure as a probe. The operations of Southern hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

After allowing the chromosomal DNA of *Pedobacter heparinus* IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) to react with restriction enzyme HindIII at 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel. From the agarose gel

after the electrophoresis, the electrophoresed chromosomal DNA was blotted onto a positively charged Nylon membrane filter (manufactured by Roche Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using

5 EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 50°C for 1 hour, the digoxinigen-labeled probe prepared as described above was added and hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC, containing 0.1% SDS.

10 Detection of bands that hybridized with the probe was carried out using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the procedure described in the manual therefor. As a result, a roughly 5 kb band was successfully detected that hybridized with the probe.

5 µg of the chromosomal DNA of *Pedobacter heparinus* IFO 12017

15 was completely digested with HindIII. About 5 kb of a DNA was separated by 0.8% agarose gel electrophoresis, the DNA was purified using the Gene Clean II Kit (manufactured by Funakoshi), and dissolved in 10 µl of TE. 4 µl of the resulting solution and pUC118 HindIII/BAP were mixed and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2

20 (manufactured by Takara Shuzo). 5 µl of this ligation reaction liquid and 100 µl of competent cells of *Escherichia coli* JM109 (manufactured by Takara Shuzo) were mixed to transform the *Escherichia coli*. This was then applied on a suitable solid medium to produce a chromosomal DNA library.

To acquire a full-length peptide-forming enzyme gene, the

25 chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred to a Nylon membrane filter, Nylon Membrane for Colony and Plaque Hybridization (manufactured by Roche Diagnostics), followed by treatments of alkali denaturation, neutralization, and immobilization. Hybridization was carried
5 out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned digoxinigen-labeled probe was added, followed by hybridization at 37°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

10 Detection of colonies that hybridized with the labeled probe was carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor. As a result, one strain whose colony hybridized with the labeled probe was observed.

15 (5) Base Sequence of Peptide-forming enzyme Gene Derived from *Pedobacter heparinus* IFO 12017

Plasmids possessed by *Escherichia coli* JM109 were prepared from the strain that was verified to have hybridized with the labeled probe and the base sequence of a portion where hybridization with the probe occurred and
20 nearby was determined. The sequencing reaction was carried out using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor. In addition, electrophoresis was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes
25 peptide-forming enzyme did exist. The full-length base sequence of the peptide-forming enzyme gene derived from *Pedobacter heparinus* IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85

Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) along with the corresponding amino acid sequence is shown in SEQ ID NO: 17.

Example 9 Expression of Peptide-forming enzyme Gene Derived from *Pedobacter heparinus* IFO 12017 in *Escherichia coli*

5 The target gene was amplified by PCR using the chromosomal DNA of *Pedobacter heparinus* IFO 12017 (Depository institution: the Institute of Fermentation, Osaka; 2-17-85 Jusanbon-cho, Yodogawa-ku, Osaka-shi, Japan) as a template and the oligonucleotides shown in SEQ ID NOs: 19 and 20 as primers. This DNA fragment was treated with NdeI/HindIII, and the
10 resulting DNA fragment and an NdeI/HindIII treatment product of pTrpT were ligated. *Escherichia coli* JM109 was then transformed with this ligation solution, and strains having the target plasmid were selected from ampicillin-resistant strains. The plasmid was designated as pTrpT_Ph_aet.

One loopful of *Escherichia coli* JM109 having pTrpT_Ph_aet was
15 inoculated in an ordinary test tube containing 3 ml of a medium (glucose at 2 g/l, yeast extract at 10 g/l, casamino acids at 10 g/l, ammonium sulfate at 5 g/l, potassium dihydrogen phosphate at 3 g/l, dipotassium hydrogen phosphate at 1 g/l, magnesium sulfate heptahydrate at 0.5 g/l and ampicillin at 100 mg/l) and main culturing was performed at 25°C for 20 hours. It was
20 verified that the cultured broth had an α -AMP production activity of 0.01 U per ml of culture broth so that it was verified that the cloned gene was expressed in *Escherichia coli*. Furthermore, no activity was detected in the transformant containing only pTrpT used as a control.

Example 10

25 Isolation of Peptide-forming enzyme Gene Derived from *Taxeobacter gelupurpurascens* DSMZ 11116

Hereinafter, isolation of a peptide-forming enzyme gene is described.

The microbe used is *Taxeobacter gelupurpurascens* DSMZ 11116 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures); address of the depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany).

- 5 For the isolation of the gene, *Escherichia coli* JM109 was used as a host, and pUC118 was used as a vector.

(1) Acquisition of Microbial Cells

- Taxeobacter gelupurpurascens* DSMZ 11116 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
10 (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) was cultured for 24 hours at 25°C on a CM2G agar medium (containing glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l, sodium chloride at 5 g/l, and agar at 20 g/l, pH 7.0). One loopful of the resulting microbial cells was
15 inoculated into a 500 ml Sakaguchi flask containing 50 ml of CM2G liquid medium (the aforementioned medium excluding agar) followed by shake culturing at 25°C.

(2) Acquisition of Chromosomal DNA from Microbial Cells

- 50 ml of the culture broth was centrifuged (12,000 rpm, 4°C, 15
20 minutes) to collect the microbial cells. A chromosomal DNA was then acquired from the microbial cells using the Qiagen Genomic-Tip System (Qiagen) based on the procedure described in the manual therefor.

(3) Acquisition of Probe DNA Fragment by PCR

- A DNA fragment containing a portion of the peptide-forming enzyme
25 gene derived from *Taxeobacter gelupurpurascens* DSMZ 11116 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of

Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out on the chromosomal DNA acquired from *Taxeobacter gelupurpurascens* DSMZ 11116 (Depository
5 institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) using primers having the base sequences of SEQ ID NOs: 21 and 16. The approximately 1 kb DNA fragment amplified by the PCR procedure was
10 isolated by 0.8% agarose electrophoresis. The target band was then cut out and purified. The DNA fragment was labeled with probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

(4) Cloning of Peptide-forming enzyme Gene from Gene Library

15 To acquire the full-length peptide-forming enzyme gene, Southern hybridization was carried out using the DNA fragment amplified in the aforementioned PCR procedure as a probe. The operations of Southern hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

20 After allowing the chromosomal DNA of *Taxeobacter gelupurpurascens* DSMZ 11116 (Depository institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depository institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) to react with
25 restriction enzyme PstI at 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel. From the agarose gel after the electrophoresis, the electrophoresed chromosomal DNA was

blotted onto a positively charged Nylon membrane filter (manufactured by Roche Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 50°C for 1 hour, the digoxinigen-labeled probe prepared as described above was added and hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the procedure described in the manual therefor. As a result, a roughly 5 kb band was successfully detected that hybridized with the probe.

5 µg of the chromosomal DNA of *Taxeobacter gelupurpurascens* DSMZ 11116 (Depositary institution; the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microbes and Cell Cultures, Address of Depositary institution; Mascheroder Weg 1b, 38124 Braunschweig, Germany) was completely digested with PstI. About 5 kb of a DNA was separated by 0.8% agarose gel electrophoresis, the DNA was purified using the Gene Clean II Kit (manufactured by Funakoshi), and dissolved in 10 µl of TE. 4 µl of the resulting solution and pUC118 PstI/BAP (manufactured by Takara Shuzo) were mixed and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). 5 µl of this ligation reaction liquid and 100 µl of competent cells of *Escherichia coli* JM109 (manufactured by Takara Shuzo) were mixed to transform the *Escherichia coli*. This was then applied on a suitable solid medium to produce a chromosomal DNA library.

To acquire a full-length peptide-forming enzyme gene, the

chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred to a
5 Nylon membrane filter, Nylon Membrane for Colony and Plaque Hybridization (manufactured by Roche Diagnostics), followed by treatments of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned
10 digoxinigen-labeled probe was added, followed by hybridization at 37°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

Detection of colonies that hybridized with the labeled probe was carried out using the DIG Nucleotide Detection Kit (manufactured by
15 Boehringer-Mannheim) based on the explanation described in the manual therefor. As a result, one strain whose colony hybridized with the labeled probe was observed.

(5) Base Sequence of Peptide-forming enzyme Gene Derived from
Taxeobacter gelupurpurascens DSMZ 11116

20 Plasmids possessed by *Escherichia coli* JM109 were prepared from the strain that was verified to have hybridized with the labeled probe and the base sequence of a portion where hybridization with the probe occurred nearby was determined. The sequencing reaction was carried out using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the
25 procedure described in the manual therefor. In addition, electrophoresis was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes

peptide-forming enzyme did exist. The full-length base sequence of the peptide-forming enzyme gene derived from *Taxeobacter gelupurpurascens* DSMZ 11116 along with the corresponding amino acid sequence is shown in SEQ ID NO: 22.

5 Example 11

Isolation of Peptide-forming enzyme Gene Derived from *Cyclobacterium marinum* ATCC 25205

Hereinafter, isolation of a peptide-forming enzyme gene is described.

The microbe used is *Cyclobacterium marinum* ATCC 25205 (Depository
10 institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). For the isolation of the gene, *Escherichia coli* JM109 was used as a host, and pUC118 was used as a vector.

(1) Acquisition of Microbial Cells

15 *Cyclobacterium marinum* ATCC 25205 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) was cultured for 24 hours at 25°C on a CM2G agar medium (containing glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l, sodium chloride at 5 g/l, and agar at 20 g/l,
20 pH 7.0). One loopful of the resulting microbial cells was inoculated into a 500 ml Sakaguchi flask containing 50 ml of CM2G liquid medium (the aforementioned medium excluding agar) followed by shake culturing at 25°C.

(2) Acquisition of Chromosomal DNA from Microbial Cells

50 ml of the culture broth was centrifuged (12,000 rpm, 4°C, 15
25 minutes) to collect the microbial cells. A chromosomal DNA was then acquired from the microbial cells using the Qiagen Genomic-Tip System (Qiagen) based on the procedure described in the manual therefor.

(3) Acquisition of Probe DNA Fragment by PCR

A DNA fragment containing a portion of the peptide-forming enzyme gene derived from *Cyclobacterium marinum* ATCC 25205 was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR
5 reaction was then carried out on the chromosomal DNA acquired from *Cyclobacterium marinum* ATCC 25205 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) using primers having the base sequences of SEQ ID NOs: 15 and 16. The approximately 1 kb DNA
10 fragment amplified by the PCR procedure was isolated by 0.8% agarose electrophoresis. The target band was then cut out and purified. The DNA fragment was labeled with probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

15 (4) Cloning of Peptide-forming enzyme Gene from Gene Library

To acquire the full-length peptide-forming enzyme gene, Southern hybridization was carried out using the DNA fragment amplified in the aforementioned PCR procedure as a probe. The operations of Southern hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring
20 Harbor Press (1989).

After allowing the chromosomal DNA of *Cyclobacterium marinum* ATCC 25205 (Depositary institution; the American Type Culture Collection, address of depositary institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) to react with restriction enzyme PstI or HincII at
25 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel. From the agarose gel after the electrophoresis, the electrophoresed chromosomal DNA was blotted onto a

positively charged Nylon membrane filter (manufactured by Roche Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing
5 the filter at 50°C for 1 hour, the digoxigenin-labeled probe prepared as described above was added and hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out
10 using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the procedure described in the manual therefor. As a result, a 7 kb band that hybridized with the probe was successfully detected for the PstI-digested product and a 2 kb band that hybridized with the probe was successfully detected for the HincII-digested product.

15 5 µg of the chromosomal DNA of *Cyclobacterium marinum* ATCC 25205 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) was completely digested with PstI or HincII. About 7 kb or 2 kb DNA was separated by 0.8% agarose gel electrophoresis. The DNA
20 was purified using the Gene Clean II Kit (manufactured by Funakoshi) and dissolved in 10 µl of TE. 4 µl of the resulting solution and pUC118 PstI/BAP (manufactured by Takara Shuzo) or pUC118 HincII/BAP (manufactured by Takara Shuzo) were mixed and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). 5 µl of this ligation
25 reaction liquid and 100 µl of competent cells of *Escherichia coli* JM109 (manufactured by Takara Shuzo) were mixed to transform the *Escherichia coli*. This was then applied on a suitable solid medium to produce a

chromosomal DNA library.

To acquire a full-length peptide-forming enzyme gene, the chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained
5 in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred to a Nylon membrane filter, Nylon Membrane for Colony and Plaque Hybridization (manufactured by Roche Diagnostics), followed by treatments of alkali denaturation, neutralization, and immobilization. Hybridization was carried
10 out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned digoxinigen-labeled probe was added, followed by hybridization at 37°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

15 Detection of colonies that hybridized with the labeled probe was carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor. As a result, one strain each whose colony hybridized with the labeled probe was observed.

20 (5) Base Sequence of Peptide-forming enzyme Gene Derived from *Cyclobacterium marinum* ATCC 25205

Plasmids possessed by *Escherichia coli* JM109 were prepared from each strain that was verified to have hybridized with the labeled probe and the base sequence of a portion where hybridization with the probe occurred
25 and nearby was determined. The sequencing reaction was carried out using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor. In addition, electrophoresis

was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes peptide-forming enzyme did exist. The full-length base sequence of the peptide-forming enzyme gene derived from *Cyclobacterium marinum* ATCC 5 25205 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) along with the corresponding amino acid sequence is shown in SEQ ID NO: 24.

Example 12

10 Isolation of Peptide-forming enzyme Gene Derived from *Psychroserpens burtonensis* ATCC 700359

Hereinafter, isolation of a peptide-forming enzyme gene is described. The microbe used is *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository 15 institution; P.O. Box 1549, Manassas, VA 20110, the United States of America). For the isolation of the gene, *Escherichia coli* JM109 was used as a host, and pUC118 was used as a vector.

(1) Acquisition of Microbial Cells

Psychroserpens burtonensis ATCC 700359 (Depository institution; the 20 American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) was cultured for 24 hours at 25°C on a CM2G agar medium (containing glucose at 50 g/l, yeast extract at 10 g/l, peptone at 10 g/l, sodium chloride at 5 g/l, and agar at 20 g/l, pH 7.0). One loopful of the resulting microbial cells was inoculated into a 25 500 ml Sakaguchi flask containing 50 ml of CM2G liquid medium (the aforementioned medium excluding agar) followed by shake culturing at 10°C.

(2) Acquisition of Chromosomal DNA from Microbial Cells

50 ml of the culture broth was centrifuged (12,000 rpm, 4°C, 15 minutes) to collect the microbial cells. A chromosomal DNA was then acquired from the microbial cells using the Qiagen Genomic-Tip System (Qiagen) based on the procedure described in the manual therefor.

5 (3) Acquisition of Probe DNA Fragment by PCR

A DNA fragment containing a portion of the peptide-forming enzyme gene derived from *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) was acquired by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out on the chromosomal DNA acquired from *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) using primers having the base sequences of SEQ ID NOs: 15 and 16. The approximately 1 kb DNA fragment amplified by the PCR procedure was isolated by 0.8% agarose electrophoresis. The target band was then cut out and purified. The DNA fragment was labeled with probe digoxinigen using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

(4) Cloning of Peptide-forming enzyme Gene from Gene Library

To acquire the full-length peptide-forming enzyme gene, Southern hybridization was carried out using the DNA fragment amplified in the aforementioned PCR procedure as a probe. The operations of Southern hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

After allowing the chromosomal DNA of *Psychroserpens burtonensis*

ATCC 700359 to react with restriction enzyme EcoRI at 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel. From the agarose gel after the electrophoresis, the electrophoresed chromosomal DNA was blotted onto a positively charged
5 Nylon membrane filter (manufactured by Roche Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 50°C for 1 hour, the digoxinigen-labeled probe prepared as described above
10 was added and hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the
15 procedure described in the manual therefor. As a result, a roughly 7 kb band was successfully detected that hybridized with the probe.

5 µg of the chromosomal DNA of *Psychroserpens burtonensis* ATCC 700359 was completely digested with EcoRI. About 7 kb of a DNA was separated by 0.8% agarose gel electrophoresis, the DNA was purified using
20 the Gene Clean II Kit (manufactured by Funakoshi), and dissolved in 10 µl of TE. 4 µl of the resulting solution and pUC118 EcoRI/BAP (manufactured by Takara Shuzo) were mixed and a ligation reaction was carried out using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). 5 µl of this ligation reaction liquid and 100 µl of competent cells of *Escherichia coli*
25 JM109 (manufactured by Takara Shuzo) were mixed to transform the *Escherichia coli*. This was then applied on a suitable solid medium to produce a chromosomal DNA library.

To acquire a full-length peptide-forming enzyme gene, the chromosomal DNA library was screened by colony hybridization using the aforementioned probe. The procedure for colony hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

5 The colonies of the chromosomal DNA library were transferred to a Nylon membrane filter, Nylon Membrane for Colony and Plaque Hybridization (manufactured by Roche Diagnostics), followed by treatments of alkali denaturation, neutralization, and immobilization. Hybridization was carried out using EASY HYB (manufactured by Boehringer-Mannheim). After
10 pre-hybridizing the filter at 37°C for 1 hour, the aforementioned digoxinigen-labeled probe was added, followed by hybridization at 37°C for 16 hours. Subsequently, the filter was washed twice at 60°C with 1×SSC containing 0.1% SDS.

Detection of colonies that hybridized with the labeled probe was
15 carried out using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor. As a result, one strain whose colony hybridized with the labeled probe was observed.

(5) Base Sequence of Peptide-forming enzyme Gene Derived from
20 *Psychroserpens burtonensis* ATCC 700359

Plasmids possessed by *Escherichia coli* JM109 were prepared from the strain that was verified to have hybridized with the labeled probe and the base sequence of a portion where hybridization with the probe occurred and nearby was determined. The sequencing reaction was carried out using the
25 CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor. In addition, electrophoresis was carried out using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes peptide-forming enzyme did exist. The full-length base sequence of the peptide-forming enzyme gene derived from *Psychroserpens burtonensis* ATCC 700359 (Depository institution; the American Type Culture Collection, address of depository institution; P.O. Box 1549, Manassas, VA 20110, the United States of America) along with the corresponding amino acid sequence is shown in SEQ ID NO: 26.

Although the invention has been described with respect to a specific embodiment for a complete and clear disclosure, the appended claims are not to be thus limited but are to be construed as embodying all modifications and alternative constructions that may occur to one skilled in the art which fairly fall within the basic teaching herein set forth.

Sequence Listing Free Text

- SEQ ID NO: 3: Synthetic primer 1
- SEQ ID NO: 4: Synthetic primer 2
- SEQ ID NO: 5: Gene encoding a peptide-forming enzyme
- SEQ ID NO: 7: Synthetic primer for preparing pTrpT
- SEQ ID NO: 8: Synthetic primer for preparing pTrpT
- SEQ ID NO: 9: Synthetic primer for preparing pTrpT_Gtg2
- SEQ ID NO: 10: Synthetic primer for preparing pTrpT_Gtg2
- SEQ ID NO: 11: Gene encoding a peptide-forming enzyme
- SEQ ID NO: 13: Synthetic primer for preparing pTrpT_Sm_aet
- SEQ ID NO: 14: Synthetic primer for preparing pTrpT_Sm_aet
- SEQ ID NO: 15: Mix primer 1 for Aet
- SEQ ID NO: 16: Mix primer 2 for Aet
- SEQ ID NO: 19: Primer 1 for constructing aet expression vectors derived

from *pPedobacter*

SEQ ID NO: 20: Primer 2 for constructing aet expression vectors derived
from *pedobacterPedobacter*

SEQ ID NO: 21: Mix primer 3 for Aet

CLAIMS

1. A method of producing an α -L-aspartyl-L-phenylalanine- β -ester,
5 comprising forming the α -L-aspartyl-L-phenylalanine- β -ester from L-aspartic acid- α,β -diester and L-phenylalanine using an enzyme or enzyme-containing substance that has an ability to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.
- 10 2. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 1, wherein the enzyme or enzyme-containing substance is one type or two or more types selected from the group consisting of a culture of a microbe that has an ability to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond, a
15 microbial cell separated from the culture and a treated microbial cell product of the microbe.
3. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a microbe belonging to a genus
20 selected from the group consisting of *Aeromonas*, *Azotobacter*, *Alcaligenes*, *Brevibacterium*, *Corynebacterium*, *Escherichia*, *Empedobacter*, *Flavobacterium*, *Microbacterium*, *Propionibacterium*, *Brevibacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Sphingobacterium*, *Streptomyces*, *Xanthomonas*, *Williopsis*, *Candida*,
25 *Geotrichum*, *Pichia*, *Saccharomyces*, *Torulaspora*, *Cellulophaga*, *Weeksella*, *Pedobacter*, *Persicobacter*, *Flexithrix*, *Chitinophaga*, *Cyclobacterium*, *Runella*, *Thermonema*, *Psychroserpens*, *Gelidibacter*, *Dyadobacter*, *Flammeovirga*,

Spirosoma, Flectobacillus, Tenacibaculum, Rhodotermus, Zobellia, Muricauda, Salegentibacter, Taxeobacter, Cytophaga, Marinilabilia, Lewinella, Saprospira, and Haliscomenobacter.

- 5 4. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (A) or (B) below:

(A) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID
10 NO: 6 of the Sequence Listing,

(B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence
15 Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

5. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is
20 capable of expressing a protein (C) or (D) below:

(C) a protein having an amino acid sequence consisting of amino acid residues numbers 21 to 619 of an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(D) a protein having an amino acid sequence including substitution,
25 deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence

Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

6. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (E) or (F) below:

(E) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 625 of an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

- 10 (F) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 625 of the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

7. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (G) or (H) below:

- 20 (G) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 645 of an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

- (H) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 645 of the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester

site of the L-aspartic acid- α,β -diester through a peptide bond.

8. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is
5 capable of expressing a protein (I) or (J) below:

(I) a protein having an amino acid sequence consisting of amino acid residues numbers 26 to 620 of an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,

- (J) a protein having an amino acid sequence including substitution,
10 deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 26 to 620 of the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

15

9. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (K) or (L) below:

(K) a protein having an amino acid sequence consisting of amino acid
20 residues numbers 18 to 644 of an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,

- (L) a protein having an amino acid sequence including substitution,
deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 18 to 644
25 of the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

10. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (M) or (N) below:

5 (M) a protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(N) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in
10 SEQ ID NO: 6 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

11. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester
15 according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (O) or (P) below:

(O) a protein having an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(P) a protein containing a mature protein region, having an amino acid
20 sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

25

12. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is

capable of expressing a protein (Q) or (R) below:

(Q) a protein containing a mature protein region, having an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

(R) a protein having an amino acid sequence including substitution,
5 deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

10 13. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (S) or (T) below:

(S) a protein having an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

15 (T) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a
20 peptide bond.

14. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (U) or (V) below:

25 (U) a protein having an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,

(V) a protein containing a mature protein region, having an amino acid

sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

15. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 2, wherein the microbe is a transformed microbe that is capable of expressing a protein (W) or (X) below:

10 (W) a protein having an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing,

(X) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively link L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

16. The method for producing an α -L-aspartyl-L-phenylalanine- β -ester according to claim 1, wherein the enzyme is at least one selected from the group consisting (A) to (X) below:

(A) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

25 (B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers

23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(C) a protein having the amino acid sequence consisting of amino acid
5 residue numbers 21 to 619 of an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(D) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residue numbers
10 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(E) a protein having the amino acid sequence consisting of amino acid residues numbers 23 to 625 of an amino acid sequence described in SEQ ID
15 NO: 18 of the Sequence Listing,

(F) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 625 of the amino acid sequence described in SEQ ID NO: 18 of the
20 Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(G) a protein having an amino acid sequence consisting of amino acid residues numbers 23 to 645 of an amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing,

25 (H) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers

23 to 645 of the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(I) a protein having an amino acid sequence consisting of amino acid
5 residues numbers 26 to 620 of an amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing,

(J) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers
10 26 to 620 of the amino acid sequence described in SEQ ID NO: 25 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(K) a protein having an amino acid sequence consisting of amino acid residues numbers 18 to 644 of an amino acid sequence described in SEQ ID
15 NO: 27 of the Sequence Listing,

(L) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 18 to 644 of the amino acid sequence described in SEQ ID NO: 27 of the
20 Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(M) a protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(N) a protein containing a mature protein region, having an amino acid
25 sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and activity to selectively linking

L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(O) a protein having the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

5 (P) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a
10 peptide bond,

(Q) a protein having an amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing,

(R) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion
15 of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 18 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(S) a protein having an amino acid sequence described in SEQ ID NO: 23
20 of the Sequence Listing,

(T) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 23 of the Sequence Listing, and activity to selectively linking
25 L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(U) a protein having an amino acid sequence described in SEQ ID NO: 25

of the Sequence Listing,

(V) a protein containing a mature protein region, having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in

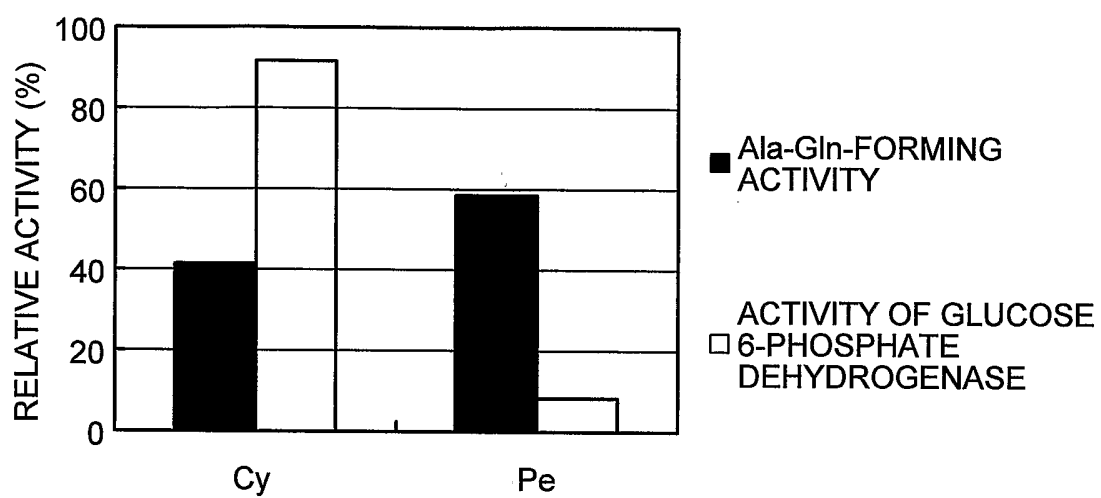
- 5 SEQ ID NO: 25 of the Sequence Listing, and activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond,

(W) a protein having an amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and

- 10 (X) a protein containing a mature protein region, having an amino acid sequence in the amino acid sequence described in SEQ ID NO: 27 of the Sequence Listing, and having activity to selectively linking L-phenylalanine to an α -ester site of the L-aspartic acid- α,β -diester through a peptide bond.

- 15 17. The method of producing an α -L-aspartyl-L-phenylalanine- α -methyl ester, comprising: a reaction step of synthesizing an α -L-aspartyl-L-phenylalanine- β -methyl ester by a method of producing an α -L-aspartyl-L-phenylalanine- β -ester according to any one of claims 1 to 16; and a reaction step of converting the α -L-aspartyl-L-phenylalanine- β -methyl
- 20 ester to α -L-aspartyl-L-phenylalanine- α -methyl ester.

第 1 図



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SEQUENCE LISTING

<110> AJINOMOTO CO., LTD.

<120> METHOD FOR PRODUCING alpha-L-ASPARTYL-L-PHENYLALANINE-beta-ESTER AND METHOD
FOR PRODUCING alpha-L-ASPARTYL-L-PHENYLALANINE-alpha-METHYL ESTER

<130> PAMA-15899

<150> JP2003-016764

<151> 2003-01-24

<150> JP2003-201819

<151> 2003-07-25

<150> US60/491, 546

<151> 2003-08-01

<160> 27

<170> PatentIn Ver. 2.1

<210> 1

<211> 9

<212> PRT

<213> Empedobacter brevis

<220>

<223> Inventor: YOKOZEKI, Kenzo

Inventor: OHNO, Ayako

Inventor: HARA, Seiichi

Inventor: ABE, Isao

<400> 1

Leu Phe Thr Ala Ile Tyr Gln Pro Lys

1

5

<210> 2

<211> 9

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<212> PRT

<213> Empedobacter brevis

<400> 2

Thr Asn Val Thr Tyr Thr Met Pro Asp

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<211> 20

<212> DNA

<213> Artificial Sequence

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<212> DNA

<213> Empedobacter brevis

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<220>

<221> CDS

<222> (61)..(1908)

<223> gene coding peptide-forming enzyme

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Val Lys Lys Leu Thr Leu Lys Val Thr Leu Leu Thr Leu Leu Leu Gly

1

5

10

15

agt aca gtt gga ttt gog caa gat gca aaa gca gat tot got tat gtg 156

Ser Thr Val Gly Phe Ala Gln Asp Ala Lys Ala Asp Ser Ala Tyr Val

20

25

30

cgc gac aat tac gaa aaa ata gaa caa gta att cgc atg cgc gat ggt 204

Arg Asp Asn Tyr Glu Lys Ile Glu Gln Val Ile Pro Met Arg Asp Gly

35

40

45

aca aag tta ttt aca got att tat cag cca aaa gat aaa aca aaa caa 252

Thr Lys Leu Phe Thr Ala Ile Tyr Gln Pro Lys Asp Lys Thr Lys Gln

50

55

60

tat ccc gtt ttg tta aat cgt aog cct tat aca gtt gog cct tat ggt 300

Tyr Pro Val Leu Leu Asn Arg Thr Pro Tyr Thr Val Ala Pro Tyr Gly

65

70

75

80

gta aat gaa tac aag aaa tog tta gga aat ttt cct aca gaa atg cgc 348

Val Asn Glu Tyr Lys Lys Ser Leu Gly Asn Phe Pro Thr Glu Met Arg

85

90

95

gaa ggt ttt att ttt gtt tac caa gat gtg aga gga aaa tgg atg ago 396

Glu Gly Phe Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser

100

105

110

gaa ggc gaa ttt gaa gat gtt cga cct ata aat cct tca aaa agt aaa 444

Glu Gly Glu Phe Glu Asp Val Arg Pro Ile Asn Pro Ser Lys Ser Lys

115

120

125

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aag gca att gac gaa agc aca gat aca ttt gat acg cta gaa tgg ctt 492
 Lys Ala Ile Asp Glu Ser Thr Asp Thr Phe Asp Thr Leu Glu Trp Leu
 130 135 140

ggt aaa aac ttg aag aat tac acg aaa aaa ggt gga att tat gga att 540
 Ala Lys Asn Leu Lys Asn Tyr Thr Lys Lys Ala Gly Ile Tyr Gly Ile
 145 150 155 160

tgg tat cct ggt ttt tat tgg aca atg agt ttg gtt aat tgg cat cca 588
 Ser Tyr Pro Gly Phe Tyr Ser Thr Met Ser Leu Val Asn Ser His Pro
 165 170 175

act cta aaa ggc gtt tgg cca caa ggc ccc gtt acc aat tgg ttt tta 636
 Thr Leu Lys Ala Val Ser Pro Gln Ala Pro Val Thr Asn Trp Phe Leu
 180 185 190

ggt gac gat ttt cat cat aat gga gtt tta ttc ttg aat gat tot ttc 684
 Gly Asp Asp Phe His His Asn Gly Val Leu Phe Leu Asn Asp Ser Phe
 195 200 205

tca ttt atg act ttt ttt ggt gta aaa cgt cgg caa cca att acg cca 732
 Ser Phe Met Thr Phe Phe Gly Val Lys Arg Pro Gln Pro Ile Thr Pro
 210 215 220

gat aaa ggt cgg aaa cgt ttt gaa tat cca ata aaa gat aat tat aga 780
 Asp Lys Gly Pro Lys Arg Phe Glu Tyr Pro Ile Lys Asp Asn Tyr Arg
 225 230 235 240

ttt tat gca agt ggc tct gta aaa gag ttg aaa gat aaa tat ttg caa 828
 Phe Tyr Ala Ser Gly Ser Val Lys Glu Leu Lys Asp Lys Tyr Leu Gln
 245 250 255

gat aat atc aag ttt tac aat gat tta ttt ggg cat cca gat tac gat 876
 Asp Asn Ile Lys Phe Tyr Asn Asp Leu Phe Ala His Pro Asp Tyr Asp
 260 265 270

caa ttt tgg caa gat cgt aat gtt tta cca cat tta act aac gtg caa 924
 Gln Phe Trp Gln Asp Arg Asn Val Leu Pro His Leu Thr Asn Val Gln
 275 280 285

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cct gct gta atg acg gtt gga ggt ttt ttt gat gca gaa gat gtc tac 972
 Pro Ala Val Met Thr Val Gly Gly Phe Phe Asp Ala Glu Asp Val Tyr
 290 295 300

ggc gct ttc gaa acg tat aaa gca att gag aaa caa aat ccg aaa gca 1020
 Gly Ala Phe Glu Thr Tyr Lys Ala Ile Glu Lys Gln Asn Pro Lys Ala
 305 310 315 320

aca aat att atg gtt gcc gga cct tgg ttt cat ggt ggt tgg gtt cgt 1068
 Thr Asn Ile Met Val Ala Gly Pro Trp Phe His Gly Gly Trp Val Arg
 325 330 335

agc aac gga agt act ttt gga gat atg caa ttt gca tog aat aca agt 1116
 Ser Asn Gly Ser Thr Phe Gly Asp Met Gln Phe Ala Ser Asn Thr Ser
 340 345 350

gag cat tat cag caa gaa ata gaa ttg cct ttt ttt aat tat tac tta 1164
 Glu His Tyr Gln Gln Glu Ile Glu Leu Pro Phe Phe Asn Tyr Tyr Leu
 355 360 365

aaa gat aaa ggt aat ttt aaa cca acc gaa gct aca att ttt att acg 1212
 Lys Asp Lys Gly Asn Phe Lys Pro Thr Glu Ala Thr Ile Phe Ile Thr
 370 375 380

gga tct aac gaa tgg aaa caa ttt gat gct tgg cca cca aaa aat gta 1260
 Gly Ser Asn Glu Trp Lys Gln Phe Asp Ala Trp Pro Pro Lys Asn Val
 385 390 395 400

aca aca caa aaa att tat ttg caa caa aat ggt aaa ata gct ttt aat 1308
 Thr Thr Gln Lys Ile Tyr Leu Gln Gln Asn Gly Lys Ile Ala Phe Asn
 405 410 415

aaa acc aat aca aca act act ttt gac gaa tat gtt gca gat cca aat 1356
 Lys Thr Asn Thr Thr Thr Phe Asp Glu Tyr Val Ala Asp Pro Asn
 420 425 430

tct cca gtt cct tat tca gga gga gtt tta gaa act cgt tca aga gaa 1404
 Ser Pro Val Pro Tyr Ser Gly Gly Val Leu Glu Thr Arg Ser Arg Glu
 435 440 445

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tat atg gtc gat gat caa cgc ttt gct tct act cgt cct gat gtt atg 1452
 Tyr Met Val Asp Asp Gln Arg Phe Ala Ser Thr Arg Pro Asp Val Met
 450 455 460

gtg tat caa tct gat att ttg aca gaa gat att acg ctt gct ggt cct 1500
 Val Tyr Gln Ser Asp Ile Leu Thr Glu Asp Ile Thr Leu Ala Gly Pro
 465 470 475 480

gtt atc aat cat tta gtg gtt tct act acg gga aca gac gct gat tat 1548
 Val Ile Asn His Leu Val Val Ser Thr Thr Gly Thr Asp Ala Asp Tyr
 485 490 495

gtt gta aaa ttg att gat gtt tat cct gaa aac acg cca aaa ttt aat 1596
 Val Val Lys Leu Ile Asp Val Tyr Pro Glu Asn Thr Pro Lys Phe Asn
 500 505 510

aac aaa tta atg gct gga tat caa aat ttg att cgt gca gaa att atg 1644
 Asn Lys Leu Met Ala Gly Tyr Gln Asn Leu Ile Arg Ala Glu Ile Met
 515 520 525

cgc gga aaa tat aga aat agt tto tct aac ccc gaa gct atg gtt cgc 1692
 Arg Gly Lys Tyr Arg Asn Ser Phe Ser Asn Pro Glu Ala Met Val Pro
 530 535 540

aat aaa gaa aca aat gta acg tac acg atg cca gat gtt gga cat aca 1740
 Asn Lys Glu Thr Asn Val Thr Tyr Thr Met Pro Asp Val Gly His Thr
 545 550 555 560

ttt aag aaa gga cat cgc att atg att caa gtt cag aac agt tgg ttt 1788
 Phe Lys Lys Gly His Arg Ile Met Ile Gln Val Gln Asn Ser Trp Phe
 565 570 575

cct tta gca gat cgc aat cgc caa caa ttt atg aat gtt tac gaa gca 1836
 Pro Leu Ala Asp Arg Asn Pro Gln Gln Phe Met Asn Val Tyr Glu Ala
 580 585 590

act tct aaa gat tat tta aaa caa acg caa cga att tat cat act tct 1884
 Thr Ser Lys Asp Tyr Leu Lys Gln Thr Gln Arg Ile Tyr His Thr Ser
 595 600 605

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tat atc gaa att ccg gta ttg aaa taacaaaaaa atccagotaa ttagctggat 1938

Tyr Ile Glu Ile Pro Val Leu Lys

610

615

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2024

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<212> PRT

<213> Empedobacter brevis

<400> 6

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1

5

10

15

Ser Thr Val Gly Phe Ala Gln Asp Ala Lys Ala Asp Ser Ala Tyr Val

20

25

30

Arg Asp Asn Tyr Glu Lys Ile Glu Gln Val Ile Pro Met Arg Asp Gly

35

40

45

Thr Lys Leu Phe Thr Ala Ile Tyr Gln Pro Lys Asp Lys Thr Lys Gln

50

55

60

Tyr Pro Val Leu Leu Asn Arg Thr Pro Tyr Thr Val Ala Pro Tyr Gly

65

70

75

80

Val Asn Glu Tyr Lys Lys Ser Leu Gly Asn Phe Pro Thr Glu Met Arg

85

90

95

Glu Gly Phe Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser

100

105

110

Glu Gly Glu Phe Glu Asp Val Arg Pro Ile Asn Pro Ser Lys Ser Lys

115

120

125

Lys Ala Ile Asp Glu Ser Thr Asp Thr Phe Asp Thr Leu Glu Trp Leu

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130	135	140	
Ala Lys Asn Leu Lys Asn Tyr Thr Lys Lys Ala Gly Ile Tyr Gly Ile			
145	150	155	160
Ser Tyr Pro Gly Phe Tyr Ser Thr Met Ser Leu Val Asn Ser His Pro			
	165	170	175
Thr Leu Lys Ala Val Ser Pro Gln Ala Pro Val Thr Asn Trp Phe Leu			
	180	185	190
Gly Asp Asp Phe His His Asn Gly Val Leu Phe Leu Asn Asp Ser Phe			
	195	200	205
Ser Phe Met Thr Phe Phe Gly Val Lys Arg Pro Gln Pro Ile Thr Pro			
	210	215	220
Asp Lys Gly Pro Lys Arg Phe Glu Tyr Pro Ile Lys Asp Asn Tyr Arg			
225	230	235	240
Phe Tyr Ala Ser Gly Ser Val Lys Glu Leu Lys Asp Lys Tyr Leu Gln			
	245	250	255
Asp Asn Ile Lys Phe Tyr Asn Asp Leu Phe Ala His Pro Asp Tyr Asp			
	260	265	270
Gln Phe Trp Gln Asp Arg Asn Val Leu Pro His Leu Thr Asn Val Gln			
	275	280	285
Pro Ala Val Met Thr Val Gly Gly Phe Phe Asp Ala Glu Asp Val Tyr			
	290	295	300
Gly Ala Phe Glu Thr Tyr Lys Ala Ile Glu Lys Gln Asn Pro Lys Ala			
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Thr Asn Ile Met Val Ala Gly Pro Trp Phe His Gly Gly Trp Val Arg			
	325	330	335
Ser Asn Gly Ser Thr Phe Gly Asp Met Gln Phe Ala Ser Asn Thr Ser			
	340	345	350

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Glu His Tyr Gln Gln Glu Ile Glu Leu Pro Phe Phe Asn Tyr Tyr Leu
355 360 365

Lys Asp Lys Gly Asn Phe Lys Pro Thr Glu Ala Thr Ile Phe Ile Thr
370 375 380

Gly Ser Asn Glu Trp Lys Gln Phe Asp Ala Trp Pro Pro Lys Asn Val
385 390 395 400

Thr Thr Gln Lys Ile Tyr Leu Gln Gln Asn Gly Lys Ile Ala Phe Asn
405 410 415

Lys Thr Asn Thr Thr Thr Thr Phe Asp Glu Tyr Val Ala Asp Pro Asn
420 425 430

Ser Pro Val Pro Tyr Ser Gly Gly Val Leu Glu Thr Arg Ser Arg Glu
435 440 445

Tyr Met Val Asp Asp Gln Arg Phe Ala Ser Thr Arg Pro Asp Val Met
450 455 460

Val Tyr Gln Ser Asp Ile Leu Thr Glu Asp Ile Thr Leu Ala Gly Pro
465 470 475 480

Val Ile Asn His Leu Val Val Ser Thr Thr Gly Thr Asp Ala Asp Tyr
485 490 495

Val Val Lys Leu Ile Asp Val Tyr Pro Glu Asn Thr Pro Lys Phe Asn
500 505 510

Asn Lys Leu Met Ala Gly Tyr Gln Asn Leu Ile Arg Ala Glu Ile Met
515 520 525

Arg Gly Lys Tyr Arg Asn Ser Phe Ser Asn Pro Glu Ala Met Val Pro
530 535 540

Asn Lys Glu Thr Asn Val Thr Tyr Thr Met Pro Asp Val Gly His Thr
545 550 555 560

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Phe Lys Lys Gly His Arg Ile Met Ile Gln Val Gln Asn Ser Trp Phe
 565 570 575

Pro Leu Ala Asp Arg Asn Pro Gln Gln Phe Met Asn Val Tyr Glu Ala
 580 585 590

Thr Ser Lys Asp Tyr Leu Lys Gln Thr Gln Arg Ile Tyr His Thr Ser
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Tyr Ile Glu Ile Pro Val Leu Lys
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<211> 40

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:synthesized
 primer for preparation of pTrpT

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<210> 8

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthesized
 primer for preparation of pTrpT

<400> 8

ttcggggatt ccatatgata ccctttttac gtgaacttgc

40

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<210> 9

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthesized
primer for preparation of pTrpT_Gtg2

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gggaattoca tatgaaaaaa ttaacattaa aagtaact

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<210> 10

<211> 36

<212> DNA

<213> Artificial Sequence

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gggggctgca gtacttgtac ggtttcgcc gataaa

36

<210> 11

<211> 1935

<212> DNA

<213> Sphingobacterium sp.

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<222> (61)..(1917)

<223> gene of peptide-forming enzyme

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atg aaa aat aca att tog tgc cta act tta gog ott tta agc gca agc 108
 Met Lys Asn Thr Ile Ser Cys Leu Thr Leu Ala Leu Leu Ser Ala Ser
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cag tta cat gct caa aca gct gcc gac tog gct tat gtt aga gat cat 156
 Gln Leu His Ala Gln Thr Ala Ala Asp Ser Ala Tyr Val Arg Asp His
 20 25 30

tat gaa aag acc gaa gta gca att ccc atg cga gat ggg aaa aaa tta 204
 Tyr Glu Lys Thr Glu Val Ala Ile Pro Met Arg Asp Gly Lys Lys Leu
 35 40 45

ttt act gog atc tac agt cca aaa gac aaa tcc aag aaa tat cca gtt 252
 Phe Thr Ala Ile Tyr Ser Pro Lys Asp Lys Ser Lys Lys Tyr Pro Val
 50 55 60

ttg ctc aat aga acg ccc tac acg gtt toa cct tat ggg cag aac gaa 300
 Leu Leu Asn Arg Thr Pro Tyr Thr Val Ser Pro Tyr Gly Gln Asn Glu
 65 70 75 80

tat aaa aaa agc ttg gga aac ttt ccc caa atg atg cgt gaa ggc tat 348
 Tyr Lys Lys Ser Leu Gly Asn Phe Pro Gln Met Met Arg Glu Gly Tyr
 85 90 95

att ttc gtt tac cag gat gtc cgt ggc aag tgg atg agc gaa ggt gat 396
 Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser Glu Gly Asp
 100 105 110

ttt gaa gat ata cgt ccc acc acg tac agc aaa gat aaa aaa gca atc 444
 Phe Glu Asp Ile Arg Pro Thr Thr Tyr Ser Lys Asp Lys Lys Ala Ile
 115 120 125

gat gaa agt acg gat acc tat gat gog ott gaa tgg tta cag aaa aat 492
 Asp Glu Ser Thr Asp Thr Tyr Asp Ala Leu Glu Trp Leu Gln Lys Asn
 130 135 140

ctc aaa aac tat aat ggc aaa gcc ggg ctc tat ggg att tcc tat cca 540

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145					150				155						160		
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Gly	Phe	Tyr	Ser	Thr	Val	Gly	Leu	Val	Lys	Thr	His	Pro	Ser	Leu	Lys		
				165					170					175			
gca	gtc	toc	cca	cag	gct	ccc	gta	aca	gac	tgg	tat	atc	ggc	gac	gac	636	
Ala	Val	Ser	Pro	Gln	Ala	Pro	Val	Thr	Asp	Trp	Tyr	Ile	Gly	Asp	Asp		
			180					185					190				
tto	cac	cat	aat	ggc	gta	ttg	ttt	ctt	cag	gat	gca	ttt	aca	tto	atg	684	
Phe	His	His	Asn	Gly	Val	Leu	Phe	Leu	Gln	Asp	Ala	Phe	Thr	Phe	Met		
			195				200						205				
tca	acc	ttt	ggg	gtc	oct	cgt	cca	aaa	ccc	att	aca	ccg	gat	caa	ttt	732	
Ser	Thr	Phe	Gly	Val	Pro	Arg	Pro	Lys	Pro	Ile	Thr	Pro	Asp	Gln	Phe		
	210					215					220						
aag	ggc	aaa	att	cag	atc	aaa	gaa	gcc	gat	aaa	tat	aac	ttt	ttt	gca	780	
Lys	Gly	Lys	Ile	Gln	Ile	Lys	Glu	Ala	Asp	Lys	Tyr	Asn	Phe	Phe	Ala		
225				230					235					240			
gaa	gca	gga	aca	gcg	ogg	gaa	ctc	aaa	gaa	aag	tat	ttt	ggg	gac	toc	828	
Glu	Ala	Gly	Thr	Ala	Arg	Glu	Leu	Lys	Glu	Lys	Tyr	Phe	Gly	Asp	Ser		
				245				250					255				
gta	caa	ttt	tgg	aat	gac	ctg	ttt	aag	cat	ccc	gac	tat	gat	gat	ttt	876	
Val	Gln	Phe	Trp	Asn	Asp	Leu	Phe	Lys	His	Pro	Asp	Tyr	Asp	Asp	Phe		
				260				265					270				
tgg	aaa	tcg	cgt	gtg	atc	acg	aat	tct	tta	cag	gag	gta	aaa	cca	gct	924	
Trp	Lys	Ser	Arg	Val	Ile	Thr	Asn	Ser	Leu	Gln	Glu	Val	Lys	Pro	Ala		
			275				280					285					
gtg	atg	gtg	gtt	ggg	ggg	ttc	ttt	gac	gcg	gaa	gat	got	tat	gga	aca	972	
Val	Met	Val	Val	Gly	Gly	Phe	Phe	Asp	Ala	Glu	Asp	Ala	Tyr	Gly	Thr		
	290					295					300						
ttt	aag	acc	tac	caa	tcg	att	gag	gat	aaa	agc	aaa	aaa	aac	aac	tcg	1020	

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Phe Lys Thr Tyr Gln Ser Ile Glu Asp Lys Ser Lys Lys Asn Asn Ser
 305 310 315 320

att tta gtc gcg gga cct tgg tat cat ggc ggt tgg gtt cgt gca gaa 1068
 Ile Leu Val Ala Gly Pro Trp Tyr His Gly Gly Trp Val Arg Ala Glu
 325 330 335

gga aac tat tta ggt gat atc caa ttt gag aaa aaa acc agt att act 1116
 Gly Asn Tyr Leu Gly Asp Ile Gln Phe Glu Lys Lys Thr Ser Ile Thr
 340 345 350

tat cag gaa caa ttt gaa caa cca ttt ttc aaa tat tac cta aaa gat 1164
 Tyr Gln Glu Gln Phe Glu Gln Pro Phe Phe Lys Tyr Tyr Leu Lys Asp
 355 360 365

gaa gga aac ttc gcc cct tcc gaa gct aac att ttt gtt tca ggc agc 1212
 Glu Gly Asn Phe Ala Pro Ser Glu Ala Asn Ile Phe Val Ser Gly Ser
 370 375 380

aac gaa tgg aaa cat ttc gaa cag tgg cca cca aaa aat gta gag aca 1260
 Asn Glu Trp Lys His Phe Glu Gln Trp Pro Pro Lys Asn Val Glu Thr
 385 390 395 400

aaa aaa cta tac ttc caa cct cag ggg aaa ctt gga ttt gac aaa gtt 1308
 Lys Lys Leu Tyr Phe Gln Pro Gln Gly Lys Leu Gly Phe Asp Lys Val
 405 410 415

caa cgt aca gat tcc tgg gat gaa tat gta aca gac cct aat aaa cct 1356
 Gln Arg Thr Asp Ser Trp Asp Glu Tyr Val Thr Asp Pro Asn Lys Pro
 420 425 430

gtt cgg cat caa ggt ggg gta att caa aac cga aca cgg gag tat atg 1404
 Val Pro His Gln Gly Gly Val Ile Gln Asn Arg Thr Arg Glu Tyr Met
 435 440 445

gta gat gat caa cgt ttc gcg gct agt cgc cct gat gtc atg gtt tat 1452
 Val Asp Asp Gln Arg Phe Ala Ala Ser Arg Pro Asp Val Met Val Tyr
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[illegible]

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<211> 619

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<400> 12

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20 25 30

Tyr Glu Lys Thr Glu Val Ala Ile Pro Met Arg Asp Gly Lys Lys Leu
35 40 45

Phe Thr Ala Ile Tyr Ser Pro Lys Asp Lys Ser Lys Lys Tyr Pro Val
50 55 60

Leu Leu Asn Arg Thr Pro Tyr Thr Val Ser Pro Tyr Gly Gln Asn Glu
65 70 75 80

Tyr Lys Lys Ser Leu Gly Asn Phe Pro Gln Met Met Arg Glu Gly Tyr
85 90 95

Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser Glu Gly Asp
100 105 110

Phe Glu Asp Ile Arg Pro Thr Thr Tyr Ser Lys Asp Lys Lys Ala Ile
115 120 125

Asp Glu Ser Thr Asp Thr Tyr Asp Ala Leu Glu Trp Leu Gln Lys Asn
130 135 140

Leu Lys Asn Tyr Asn Gly Lys Ala Gly Leu Tyr Gly Ile Ser Tyr Pro
145 150 155 160

Gly Phe Tyr Ser Thr Val Gly Leu Val Lys Thr His Pro Ser Leu Lys
165 170 175

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Ala Val Ser Pro Gln Ala Pro Val Thr Asp Trp Tyr Ile Gly Asp Asp
 180 185 190

Phe His His Asn Gly Val Leu Phe Leu Gln Asp Ala Phe Thr Phe Met
 195 200 205

Ser Thr Phe Gly Val Pro Arg Pro Lys Pro Ile Thr Pro Asp Gln Phe
 210 215 220

Lys Gly Lys Ile Gln Ile Lys Glu Ala Asp Lys Tyr Asn Phe Phe Ala
 225 230 235 240

Glu Ala Gly Thr Ala Arg Glu Leu Lys Glu Lys Tyr Phe Gly Asp Ser
 245 250 255

Val Gln Phe Trp Asn Asp Leu Phe Lys His Pro Asp Tyr Asp Asp Phe
 260 265 270

Trp Lys Ser Arg Val Ile Thr Asn Ser Leu Gln Glu Val Lys Pro Ala
 275 280 285

Val Met Val Val Gly Gly Phe Phe Asp Ala Glu Asp Ala Tyr Gly Thr
 290 295 300

Phe Lys Thr Tyr Gln Ser Ile Glu Asp Lys Ser Lys Lys Asn Asn Ser
 305 310 315 320

Ile Leu Val Ala Gly Pro Trp Tyr His Gly Gly Trp Val Arg Ala Glu
 325 330 335

Gly Asn Tyr Leu Gly Asp Ile Gln Phe Glu Lys Lys Thr Ser Ile Thr
 340 345 350

Tyr Gln Glu Gln Phe Glu Gln Pro Phe Phe Lys Tyr Tyr Leu Lys Asp
 355 360 365

Glu Gly Asn Phe Ala Pro Ser Glu Ala Asn Ile Phe Val Ser Gly Ser
 370 375 380

Asn Glu Trp Lys His Phe Glu Gln Trp Pro Pro Lys Asn Val Glu Thr

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385 390 395 400

Lys Lys Leu Tyr Phe Gln Pro Gln Gly Lys Leu Gly Phe Asp Lys Val

405 410 415

Gln Arg Thr Asp Ser Trp Asp Glu Tyr Val Thr Asp Pro Asn Lys Pro

420 425 430

Val Pro His Gln Gly Gly Val Ile Gln Asn Arg Thr Arg Glu Tyr Met

435 440 445

Val Asp Asp Gln Arg Phe Ala Ala Ser Arg Pro Asp Val Met Val Tyr

450 455 460

Gln Thr Glu Pro Leu Thr Glu Asp Leu Thr Ile Val Gly Pro Ile Lys

465 470 475 480

Asn Phe Leu Lys Val Ser Ser Thr Gly Thr Asp Ala Asp Tyr Val Val

485 490 495

Lys Leu Ile Asp Val Tyr Pro Asn Asp Ala Ala Ser Tyr Gln Gly Lys

500 505 510

Thr Met Ala Gly Tyr Gln Met Met Val Arg Gly Glu Ile Met Ala Gly

515 520 525

Lys Tyr Arg Asn Gly Phe Asp Lys Ala Gln Ala Leu Thr Pro Gly Met

530 535 540

Val Glu Lys Val Asn Phe Glu Met Pro Asp Val Ala His Thr Phe Lys

545 550 555 560

Lys Gly His Arg Ile Met Val Gln Val Gln Asn Ser Trp Phe Pro Leu

565 570 575

Ala Glu Arg Asn Pro Gln Val Phe Leu Ala Pro Tyr Thr Ala Thr Lys

580 585 590

Ala Asp Phe Arg Lys Ala Thr Gln Arg Ile Phe His Asp Val Asn Asn

19/56

595

600

605

Ala Thr Tyr Ile Glu Phe Ser Val Leu Lys Asp

610

615

<210> 13

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthesized
primer for preparation of pTrpT_Sm_aet

<400> 13

gggaattcca tatgaaaaat acaatttogt

30

<210> 14

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthesized
primer for preparation of pTrpT_Sm_aet

<400> 14

gctctagact aatctttgag gacagaaaa

29

<210> 15

<211> 17

<212> DNA

20/56

<213> Artificial

<220>

<223> mix primer 1 for Aet

<400> 15

gaygaytttc aycayaa

17

<210> 16

<211> 20

<212> DNA

<213> Artificial

<220>

<223> mix primer 2 for Aet

<220>

<221> misc_feature

<222> (9).. (9)

<223> any base

<400> 16

tgrtortona ccatrtaytc

20

<210> 17

<211> 1974

<212> DNA

<213> Pedobacter heparinus

<220>

<221> GDS

<222> (61).. (1935)

<223>

<400> 17

aaacctatcc cgtattcagc aatcaattcc atatatttat ccttaaaaaa accttctct

60

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atg act cct ttc aaa tgg ttc tcc ttc att ttt ctc ttt att ttt acc 108
 Met Thr Pro Phe Lys Ser Phe Ser Phe Ile Phe Leu Phe Ile Phe Thr
 1 5 10 15

agt ctt tct gct tct gca caa cag tcc gac tct gct tat ata cgt cag 156
 Ser Leu Ser Ala Ser Ala Gln Gln Ser Asp Ser Ala Tyr Ile Arg Gln
 20 25 30

aac tat acc aaa ata gaa agg ctg atc cct atg cgg gat ggc att aag 204
 Asn Tyr Thr Lys Ile Glu Arg Leu Ile Pro Met Arg Asp Gly Ile Lys
 35 40 45

cta ttt aca gcc att tac atc ccc aaa gac aaa agc aag aag tat cct 252
 Leu Phe Thr Ala Ile Tyr Ile Pro Lys Asp Lys Ser Lys Lys Tyr Pro
 50 55 60

ttt atg ctc aac cgt act cct tat acc gtt tgg cct tat ggc gaa aac 300
 Phe Met Leu Asn Arg Thr Pro Tyr Thr Val Ser Pro Tyr Gly Glu Asn
 65 70 75 80

aat tat aaa aca agc ctt ggc ccc tct ccg ctc ttt ata aaa gaa ggc 348
 Asn Tyr Lys Thr Ser Leu Gly Pro Ser Pro Leu Phe Ile Lys Glu Gly
 85 90 95

ttt atc ttt gtt tat cag gat gta agg ggc aaa tgg atg agt gag gga 396
 Phe Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser Glu Gly
 100 105 110

aaa ttt gaa gac gta agg ccg caa ata gcc agc aag aaa cgc aaa acg 444
 Lys Phe Glu Asp Val Arg Pro Gln Ile Ala Ser Lys Lys Arg Lys Thr
 115 120 125

gat att gat gaa agc tcc gat act tat gat acg atc gac tgg ctg atc 492
 Asp Ile Asp Glu Ser Ser Asp Thr Tyr Asp Thr Ile Asp Trp Leu Ile
 130 135 140

agg aac att cct gga aac aac cgt aaa acc ggt att tac ggt atc tca 540
 Arg Asn Ile Pro Gly Asn Asn Arg Lys Thr Gly Ile Tyr Gly Ile Ser
 145 150 155 160

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tac cca ggc ttt tat got act got gcc cta cca gat gcg cat cca tot 588
Tyr Pro Gly Phe Tyr Ala Thr Ala Ala Leu Pro Asp Ala His Pro Ser

165

170

175

tta aag gca gta tog ccc cag got ccg gtt acc gac tgg ttt ata ggc 636
Leu Lys Ala Val Ser Pro Gln Ala Pro Val Thr Asp Trp Phe Ile Gly

180

185

190

gat gat ttt cat cac aat ggc acc ttg ttc ott goa gat atc ttt agc 684
Asp Asp Phe His His Asn Gly Thr Leu Phe Leu Ala Asp Ile Phe Ser

195

200

205

ttc tat tat acc ttc ggg gta ccg cga cct caa cca att acg ccc gac 732
Phe Tyr Tyr Thr Phe Gly Val Pro Arg Pro Gln Pro Ile Thr Pro Asp

210

215

220

aaa cgt cca aaa ccc ttt gat ttc ccg gtt aaa gac aac tac cgt ttt 780
Lys Arg Pro Lys Pro Phe Asp Phe Pro Val Lys Asp Asn Tyr Arg Phe
225 230 235 240

ttt ott gaa ctg ggc ccc tta aaa aac atc acc aaa aaa tat tat ggc 828
Phe Leu Glu Leu Gly Pro Leu Lys Asn Ile Thr Lys Lys Tyr Tyr Gly

245

250

255

gat acc ata cga ttc tgg aat gat atc aat gcg cat acc aat tat gat 876
Asp Thr Ile Arg Phe Trp Asn Asp Ile Asn Ala His Thr Asn Tyr Asp

260

265

270

gcc ttc tgg aaa gcc cgt aac att acg ccg cat tta att ggt gta aaa 924
Ala Phe Trp Lys Ala Arg Asn Ile Thr Pro His Leu Ile Gly Val Lys

275

280

285

cot gca gtt ttg gta gtt ggc gcc ttc ttt gat goa gaa gac ott tac 972
Pro Ala Val Leu Val Val Gly Gly Phe Phe Asp Ala Glu Asp Leu Tyr

290

295

300

ggt acg ott aaa acc tat cag gcc atc gaa aaa caa aat cca tcc tca 1020
Gly Thr Leu Lys Thr Tyr Gln Ala Ile Glu Lys Gln Asn Pro Ser Ser

23/56

305	310	315	320	
aaa aac aac ctc gtt atg ggc ccc tgg tac cat ggt ggc tgg gca aga				1068
Lys Asn Asn Leu Val Met Gly Pro Trp Tyr His Gly Gly Trp Ala Arg				
	325	330	335	
agt acg gga ago agt ttc ggg gat att aat ttc gga cag cca acc agt				1116
Ser Thr Gly Ser Ser Phe Gly Asp Ile Asn Phe Gly Gln Pro Thr Ser				
	340	345	350	
act tca tac cag caa aat gtt gag ttc cct ttc ttt atg caa tac ctc				1164
Thr Ser Tyr Gln Gln Asn Val Glu Phe Pro Phe Phe Met Gln Tyr Leu				
	355	360	365	
aaa gag gca ccg gat goa aaa att gca gag gca acc att ttt atc act				1212
Lys Glu Ala Pro Asp Ala Lys Ile Ala Glu Ala Thr Ile Phe Ile Thr				
	370	375	380	
ggc ago aat gaa tgg aag aaa ttt agc tcc tgg cca cct cag gat aca				1260
Gly Ser Asn Glu Trp Lys Lys Phe Ser Ser Trp Pro Pro Gln Asp Thr				
	385	390	395	400
gaa gaa aga aca tta tac ctg cag ccc aat ggc aaa ctg ago ttt gag				1308
Glu Glu Arg Thr Leu Tyr Leu Gln Pro Asn Gly Lys Leu Ser Phe Glu				
	405	410	415	
aag gta cag cgg acc gac agc tgg gat gaa tat gta agt gat ccc aat				1356
Lys Val Gln Arg Thr Asp Ser Trp Asp Glu Tyr Val Ser Asp Pro Asn				
	420	425	430	
tca cct gtc cct tat cag gat ggc ata caa acc ago aga acc cgg gaa				1404
Ser Pro Val Pro Tyr Gln Asp Gly Ile Gln Thr Ser Arg Thr Arg Glu				
	435	440	445	
tat atg atc gat gac cag cgt ttt gcc tcg cgc aga ccg gat gta agg				1452
Tyr Met Ile Asp Asp Gln Arg Phe Ala Ser Arg Arg Pro Asp Val Arg				
	450	455	460	
gta ttc caa aca gag ccc ctc agt tcc gac ctt aca ctt acc ggc ccg				1500
Val Phe Gln Thr Glu Pro Leu Ser Ser Asp Leu Thr Leu Thr Gly Pro				

24/56

465	470	475	480	
gta ttg gcc aaa ctg gtg gta tca acc aca ggt acg gat gca gat tat				1548
Val Leu Ala Lys Leu Val Val Ser Thr Thr Gly Thr Asp Ala Asp Tyr				
	485	490	495	
gtg gta aaa ctg ata gat gta tat cgg gaa gat aca cca aat cct gta				1596
Val Val Lys Leu Ile Asp Val Tyr Pro Glu Asp Thr Pro Asn Pro Val				
	500	505	510	
cct aac cct aaa aac ctg atc atg ggt ggt tac cag atg ctg gta cgc				1644
Pro Asn Pro Lys Asn Leu Ile Met Gly Gly Tyr Gln Met Leu Val Arg				
	515	520	525	
ggc gag atc atg cgt gga aaa tac cgt aat agc ttt gaa aaa ccc gag				1692
Gly Glu Ile Met Arg Gly Lys Tyr Arg Asn Ser Phe Glu Lys Pro Glu				
	530	535	540	
cct ttt gtt cct gga aca att aca aaa gta aac tat gcc ctt cgg gat				1740
Pro Phe Val Pro Gly Thr Ile Thr Lys Val Asn Tyr Ala Leu Pro Asp				
545	550	555	560	
gta gcc cat acc ttt aaa aaa ggc cac cgc atc atg atc cag gtc cag				1788
Val Ala His Thr Phe Lys Lys Gly His Arg Ile Met Ile Gln Val Gln				
	565	570	575	
aat tca tgg ttt ccc ctg gcc gac cgg aat cca cag cag ttt atg gac				1836
Asn Ser Trp Phe Pro Leu Ala Asp Arg Asn Pro Gln Gln Phe Met Asp				
	580	585	590	
att tac cag gcc gaa cct ggc gat ttc aga aaa gct acg cat agg atc				1884
Ile Tyr Gln Ala Glu Pro Gly Asp Phe Arg Lys Ala Thr His Arg Ile				
	595	600	605	
ttc cac gat gta cac aat gca tct gca att acg gta aac gta ctg aaa				1932
Phe His Asp Val His Asn Ala Ser Ala Ile Thr Val Asn Val Leu Lys				
	610	615	620	
cct taaaacggat gaaaccagta tattgtgccat tccttaactt				1974
Pro				

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625

<210> 18

<211> 625

<212> PRT

<213> Pedobacter heparinus

<400> 18

Met Thr Pro Phe Lys Ser Phe Ser Phe Ile Phe Leu Phe Ile Phe Thr
1 5 10 15

Ser Leu Ser Ala Ser Ala Gln Gln Ser Asp Ser Ala Tyr Ile Arg Gln
20 25 30

Asn Tyr Thr Lys Ile Glu Arg Leu Ile Pro Met Arg Asp Gly Ile Lys
35 40 45

Leu Phe Thr Ala Ile Tyr Ile Pro Lys Asp Lys Ser Lys Lys Tyr Pro
50 55 60

Phe Met Leu Asn Arg Thr Pro Tyr Thr Val Ser Pro Tyr Gly Glu Asn
65 70 75 80

Asn Tyr Lys Thr Ser Leu Gly Pro Ser Pro Leu Phe Ile Lys Glu Gly
85 90 95

Phe Ile Phe Val Tyr Gln Asp Val Arg Gly Lys Trp Met Ser Glu Gly
100 105 110

Lys Phe Glu Asp Val Arg Pro Gln Ile Ala Ser Lys Lys Arg Lys Thr
115 120 125

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Asp Ile Asp Glu Ser Ser Asp Thr Tyr Asp Thr Ile Asp Trp Leu Ile
130 135 140

Arg Asn Ile Pro Gly Asn Asn Arg Lys Thr Gly Ile Tyr Gly Ile Ser
145 150 155 160

Tyr Pro Gly Phe Tyr Ala Thr Ala Ala Leu Pro Asp Ala His Pro Ser
165 170 175

Leu Lys Ala Val Ser Pro Gln Ala Pro Val Thr Asp Trp Phe Ile Gly
180 185 190

Asp Asp Phe His His Asn Gly Thr Leu Phe Leu Ala Asp Ile Phe Ser
195 200 205

Phe Tyr Tyr Thr Phe Gly Val Pro Arg Pro Gln Pro Ile Thr Pro Asp
210 215 220

Lys Arg Pro Lys Pro Phe Asp Phe Pro Val Lys Asp Asn Tyr Arg Phe
225 230 235 240

Phe Leu Glu Leu Gly Pro Leu Lys Asn Ile Thr Lys Lys Tyr Tyr Gly
245 250 255

Asp Thr Ile Arg Phe Trp Asn Asp Ile Asn Ala His Thr Asn Tyr Asp
260 265 270

Ala Phe Trp Lys Ala Arg Asn Ile Thr Pro His Leu Ile Gly Val Lys
275 280 285

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Pro Ala Val Leu Val Val Gly Gly Phe Phe Asp Ala Glu Asp Leu Tyr
290 295 300

Gly Thr Leu Lys Thr Tyr Gln Ala Ile Glu Lys Gln Asn Pro Ser Ser
305 310 315 320

Lys Asn Asn Leu Val Met Gly Pro Trp Tyr His Gly Gly Trp Ala Arg
325 330 335

Ser Thr Gly Ser Ser Phe Gly Asp Ile Asn Phe Gly Gln Pro Thr Ser
340 345 350

Thr Ser Tyr Gln Gln Asn Val Glu Phe Pro Phe Phe Met Gln Tyr Leu
355 360 365

Lys Glu Ala Pro Asp Ala Lys Ile Ala Glu Ala Thr Ile Phe Ile Thr
370 375 380

Gly Ser Asn Glu Trp Lys Lys Phe Ser Ser Trp Pro Pro Gln Asp Thr
385 390 395 400

Glu Glu Arg Thr Leu Tyr Leu Gln Pro Asn Gly Lys Leu Ser Phe Glu
405 410 415

Lys Val Gln Arg Thr Asp Ser Trp Asp Glu Tyr Val Ser Asp Pro Asn
420 425 430

Ser Pro Val Pro Tyr Gln Asp Gly Ile Gln Thr Ser Arg Thr Arg Glu
435 440 445

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Tyr Met Ile Asp Asp Gln Arg Phe Ala Ser Arg Arg Pro Asp Val Arg
450 455 460

Val Phe Gln Thr Glu Pro Leu Ser Ser Asp Leu Thr Leu Thr Gly Pro
465 470 475 480

Val Leu Ala Lys Leu Val Val Ser Thr Thr Gly Thr Asp Ala Asp Tyr
485 490 495

Val Val Lys Leu Ile Asp Val Tyr Pro Glu Asp Thr Pro Asn Pro Val
500 505 510

Pro Asn Pro Lys Asn Leu Ile Met Gly Gly Tyr Gln Met Leu Val Arg
515 520 525

Gly Glu Ile Met Arg Gly Lys Tyr Arg Asn Ser Phe Glu Lys Pro Glu
530 535 540

Pro Phe Val Pro Gly Thr Ile Thr Lys Val Asn Tyr Ala Leu Pro Asp
545 550 555 560

Val Ala His Thr Phe Lys Lys Gly His Arg Ile Met Ile Gln Val Gln
565 570 575

Asn Ser Trp Phe Pro Leu Ala Asp Arg Asn Pro Gln Gln Phe Met Asp
580 585 590

Ile Tyr Gln Ala Glu Pro Gly Asp Phe Arg Lys Ala Thr His Arg Ile
595 600 605

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Phe His Asp Val His Asn Ala Ser Ala Ile Thr Val Asn Val Leu Lys

610

615

620

Pro

625

<210> 19

<211> 38

<212> DNA

<213> Artificial

<220>

<223> primer 1 for constructing a expression vector for aet derived from Pedobacter

<400> 19

gggaattcca tatgactoct ttcaaatcgt tctccttc

38

<210> 20

<211> 30

<212> DNA

<213> Artificial

<220>

<223> primer 2 for constructing a expression vector for aet derived from Pedobacter

<400> 20

cccaagcttt taaggtttca gtacgtttac

30

<210> 21

<211> 17

<212> DNA

<213> Artificial

30/56

<220>

<223> mix primer 3 for Aet

<220>

<221> misc_feature

<222> (9)..(9)

<223> any base

<400> 21

athhtygtnt aycarga

17

<210> 22

<211> 2018

<212> DNA

<213> Taxeobacter gelupurpurascens

<220>

<221> CDS

<222> (61)..(1995)

<223>

<400> 22

ctgaatgtct gctgaagaat tggaactaca ttaggctcgt tottcacota cccttccact 60

atg ccc tac tct ttc cgg aaa gtt gcc gcc ctg agt ggc cta ctg gtg 108

Met Pro Tyr Ser Phe Pro Lys Val Ala Ala Leu Ser Gly Leu Leu Val

1

5

10

15

gcc ggt tta tcc ggt gcc cac gcc caa act cct gtt acc tat cgg ctg 156

Ala Gly Leu Ser Gly Ala His Ala Gln Thr Pro Val Thr Tyr Pro Leu

20

25

30

gct tct gag gct gaa aaa ggg cag ctg ggg gtg gta cta gcc gat aag 204

Ala Ser Glu Ala Glu Lys Ala Gln Leu Ala Val Val Leu Ala Asp Thr

35

40

45

gct tac atc aag gag cgc tat acc aaa aca gaa tat cag att cgg atg 252

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Ala Tyr Ile Lys Glu Arg Tyr Thr Lys Thr Glu Tyr Gln Ile Pro Met
 50 55 60

cgc gat ggg gtg aag ttg tac acc att gtg tac gcg ccc aac gat gcc 300
 Arg Asp Gly Val Lys Leu Tyr Thr Ile Val Tyr Ala Pro Asn Asp Ala
 65 70 75 80

aac aag gta aag tac cct att ctg ctc aac cgt acc cct tac gct att 348
 Asn Lys Val Lys Tyr Pro Ile Leu Leu Asn Arg Thr Pro Tyr Ala Ile
 85 90 95

ggc ccc tac ggc ccc ggc aaa tac aag ctc aac ctg ggc ccc agc agc 396
 Gly Pro Tyr Gly Pro Gly Lys Tyr Lys Leu Asn Leu Gly Pro Ser Ser
 100 105 110

acg atg atg cat gag gga tac atc ttc gcc tac cag gat gtg cgt ggg 444
 Thr Met Met His Glu Gly Tyr Ile Phe Ala Tyr Gln Asp Val Arg Gly
 115 120 125

cga tat atg tcg gaa gga gag ttt gtg gat gtg cgc ccc gaa aag gac 492
 Arg Tyr Met Ser Glu Gly Glu Phe Val Asp Val Arg Pro Glu Lys Asp
 130 135 140

atg cac aaa ggc aag aac gac atc gat gaa ggc acc gac acc tac gat 540
 Met His Lys Gly Lys Asn Asp Ile Asp Glu Gly Thr Asp Thr Tyr Asp
 145 150 155 160

acc att gag tgg ctt ctg aag cac ggg ccc aag aat aac ggc cgc gta 588
 Thr Ile Glu Trp Leu Leu Lys His Gly Pro Lys Asn Asn Gly Arg Val
 165 170 175

ggc cag tgg ggc atc tcc tac ccc ggc tac tat acc gct act ggc cta 636
 Gly Gln Trp Gly Ile Ser Tyr Pro Gly Tyr Tyr Thr Ala Thr Gly Leu
 180 185 190

ctg agc cgc cac aag gcc cta aag gca tcc tca ccg cag gcc cct att 684
 Leu Ser Arg His Lys Ala Leu Lys Ala Ser Ser Pro Gln Ala Pro Ile
 195 200 205

gcc gac tgg ttc tgg gac gat ttt cac cac aac ggc gcg ttc ttc ctg 732

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Ala Asp Trp Phe Trp Asp Asp Phe His His Asn Gly Ala Phe Phe Leu	
210 215 220	
cgc cac gct ttc aac ttc ctg gcc tcc ttt ggg ctg gcc cgc ccc cag	780
Pro His Ala Phe Asn Phe Leu Ala Ser Phe Gly Leu Ala Arg Pro Gln	
225 230 235 240	
ccc acg cct acc gcc aac ccc gcc ttc aag cac gcc acc ccc gat gcc	828
Pro Thr Pro Thr Gly Asn Pro Gly Phe Lys His Gly Thr Pro Asp Gly	
245 250 255	
tac gat ttt ttc ctg aag atg ggt cgc ctg aaa aac gct gat gcc aac	876
Tyr Asp Phe Phe Leu Lys Met Gly Pro Leu Lys Asn Ala Asp Ala Asn	
260 265 270	
tac tac aaa gcc aaa gtg gcc ttc tgg aac gaa atg gcc agc cac ccc	924
Tyr Tyr Lys Gly Lys Val Ala Phe Trp Asn Glu Met Ala Ser His Pro	
275 280 285	
aac tac gac gaa ttc tgg cag gcc cgt aac cta cgc ccc cac ctc aag	972
Asn Tyr Asp Glu Phe Trp Gln Ala Arg Asn Leu Arg Pro His Leu Lys	
290 295 300	
aac ctc aac aaa gcc acc gcg gtg ctc acg gtt ggt gcc ttc aat gat	1020
Asn Leu Asn Lys Gly Thr Ala Val Leu Thr Val Gly Gly Phe Asn Asp	
305 310 315 320	
gcc gag gac ctg ttt gcc gcc ctg aaa acc tac gaa agc atc gag aag	1068
Ala Glu Asp Leu Phe Gly Ala Leu Lys Thr Tyr Glu Ser Ile Glu Lys	
325 330 335	
caa aac ccc gcc atg cgc aac gcc ctc gtg atg ggg cgc tgg gta cac	1116
Gln Asn Pro Gly Met Arg Asn Gly Leu Val Met Gly Pro Trp Val His	
340 345 350	
ggt gcc tgg gcc cgc gcc act gcc gaa atg gta gcc aat gtg gcc tac	1164
Gly Gly Trp Ala Arg Gly Thr Gly Glu Met Val Gly Asn Val Ala Tyr	
355 360 365	
ggc gag tcg cgc tcg ttg tat tac cag aag cag att gaa gcg cgc ttc	1212

Gly	Glu	Ser	Pro	Ser	Leu	Tyr	Tyr	Gln	Lys	Gln	Ile	Glu	Ala	Pro	Phe		
370						375					380						
ttc	aaa	tca	tat	ctg	aag	gat	ggc	aaa	cct	gcc	gct	acc	ccc	gag	gct	1260	
Phe	Lys	Ser	Tyr	Leu	Lys	Asp	Gly	Lys	Pro	Ala	Ala	Thr	Pro	Glu	Ala		
385					390					395					400		
acc	atc	ttt	gaa	agc	ggc	acc	aac	cgc	tgg	cgc	agc	ttc	gaa	acc	tgg	1308	
Thr	Ile	Phe	Glu	Ser	Gly	Thr	Asn	Arg	Trp	Arg	Ser	Phe	Glu	Thr	Trp		
				405				410					415				
ccg	ccc	aaa	gaa	gcc	aaa	gag	ogc	act	ttg	tac	ttt	cag	tgc	gcc	ggg	1356	
Pro	Pro	Lys	Glu	Ala	Lys	Glu	Arg	Thr	Leu	Tyr	Phe	Gln	Ser	Ala	Gly		
			420					425					430				
aaa	atc	ggc	ttc	gag	aag	cct	gcc	agt	ggc	cta	gag	tac	gac	cag	ttc	1404	
Lys	Ile	Gly	Phe	Glu	Lys	Pro	Ala	Ser	Gly	Leu	Glu	Tyr	Asp	Gln	Phe		
	435						440						445				
ctc	agc	gac	ccg	gct	cac	cca	gtg	cct	ttc	acc	gaa	gct	acg	gct	acg	1452	
Leu	Ser	Asp	Pro	Ala	His	Pro	Val	Pro	Phe	Thr	Glu	Ala	Thr	Ala	Thr		
	450					455						460					
ggc	atg	acc	cgc	gag	tac	atg	acc	gac	gac	cag	cgc	ttc	gcc	agc	cgc	1500	
Gly	Met	Thr	Arg	Glu	Tyr	Met	Thr	Asp	Asp	Gln	Arg	Phe	Ala	Ser	Arg		
465					470					475					480		
cgc	ccc	gac	gtg	ctg	acc	tac	cag	acc	gaa	gog	ctt	acc	gag	gac	atg	1548	
Arg	Pro	Asp	Val	Leu	Thr	Tyr	Gln	Thr	Glu	Ala	Leu	Thr	Glu	Asp	Met		
				485					490					495			
acg	ctg	gct	ggc	cct	atc	gag	gog	ctg	ttg	cag	gta	gcc	acc	acc	ggc	1596	
Thr	Leu	Ala	Gly	Pro	Ile	Glu	Ala	Leu	Leu	Gln	Val	Ala	Thr	Thr	Gly		
			500					505					510				
acc	gat	gcc	gac	tgg	gta	gtg	aag	att	att	gat	gtg	tac	ccc	gac	gat	1644	
Thr	Asp	Ala	Asp	Trp	Val	Val	Lys	Ile	Ile	Asp	Val	Tyr	Pro	Asp	Asp		
			515				520					525					
acg	ccc	aac	aac	ccc	agc	acg	aac	ccc	gcc	gtg	aaa	ctg	ggc	ggc	tac	1692	

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Thr Pro Asn Asn Pro Ser Thr Asn Pro Ala Val Lys Leu Gly Gly Tyr
 530 535 540
 cag cag atg gtt cgc tcc gag gtg atg cgc ggt cgt ttc cgc aac agc 1740
 Gln Gln Met Val Arg Ser Glu Val Met Arg Gly Arg Phe Arg Asn Ser
 545 550 555 560
 ttc tcc aag ccc gaa gcc ttt gta ccg gaa cag gta acg gcc gtg ccc 1788
 Phe Ser Lys Pro Glu Ala Phe Val Pro Glu Gln Val Thr Ala Val Pro
 565 570 575
 ttc acg gtg cag gac ctg tgc cac acc ttc cgg aaa gga cac cgc ctg 1836
 Phe Thr Val Gln Asp Leu Cys His Thr Phe Arg Lys Gly His Arg Leu
 580 585 590
 atg gtg cag gtg caa ago ago tgg ttc ccg att gtt gac cgc aac ccg 1884
 Met Val Gln Val Gln Ser Ser Trp Phe Pro Ile Val Asp Arg Asn Pro
 595 600 605
 cag acc ttc gta ccc aat att ttc gag gcc gat gag aag gat ttc cag 1932
 Gln Thr Phe Val Pro Asn Ile Phe Glu Ala Asp Glu Lys Asp Phe Gln
 610 615 620
 gcc gcc acg cat cgg ctg tac cat tcg ccg gcg cat ago tcg cag ctc 1980
 Ala Ala Thr His Arg Leu Tyr His Ser Pro Ala His Ser Ser Gln Leu
 625 630 635 640
 acg ttg cgc gtt ctg taggcactc taaacaggct cgg 2018
 Thr Leu Arg Val Leu
 645

<210> 23

<211> 645

<212> PRT

<213> *Taxeobacter gelupurpurascens*

<400> 23

Met Pro Tyr Ser Phe Pro Lys Val Ala Ala Leu Ser Gly Leu Leu Val

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1 5 10 15

Ala Gly Leu Ser Gly Ala His Ala Gln Thr Pro Val Thr Tyr Pro Leu
 20 25 30

Ala Ser Glu Ala Glu Lys Ala Gln Leu Ala Val Val Leu Ala Asp Thr
 35 40 45

Ala Tyr Ile Lys Glu Arg Tyr Thr Lys Thr Glu Tyr Gln Ile Pro Met
 50 55 60

Arg Asp Gly Val Lys Leu Tyr Thr Ile Val Tyr Ala Pro Asn Asp Ala
65 70 75 80

Asn Lys Val Lys Tyr Pro Ile Leu Leu Asn Arg Thr Pro Tyr Ala Ile
 85 90 95

Gly Pro Tyr Gly Pro Gly Lys Tyr Lys Leu Asn Leu Gly Pro Ser Ser
 100 105 110

Thr Met Met His Glu Gly Tyr Ile Phe Ala Tyr Gln Asp Val Arg Gly
 115 120 125

Arg Tyr Met Ser Glu Gly Glu Phe Val Asp Val Arg Pro Glu Lys Asp
 130 135 140

Met His Lys Gly Lys Asn Asp Ile Asp Glu Gly Thr Asp Thr Tyr Asp
145 150 155 160

Thr Ile Glu Trp Leu Leu Lys His Gly Pro Lys Asn Asn Gly Arg Val

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165

170

175

Gly Gln Trp Gly Ile Ser Tyr Pro Gly Tyr Tyr Thr Ala Thr Gly Leu

180

185

190

Leu Ser Arg His Lys Ala Leu Lys Ala Ser Ser Pro Gln Ala Pro Ile

195

200

205

Ala Asp Trp Phe Trp Asp Asp Phe His His Asn Gly Ala Phe Phe Leu

210

215

220

Pro His Ala Phe Asn Phe Leu Ala Ser Phe Gly Leu Ala Arg Pro Gln

225

230

235

240

Pro Thr Pro Thr Gly Asn Pro Gly Phe Lys His Gly Thr Pro Asp Gly

245

250

255

Tyr Asp Phe Phe Leu Lys Met Gly Pro Leu Lys Asn Ala Asp Ala Asn

260

265

270

Tyr Tyr Lys Gly Lys Val Ala Phe Trp Asn Glu Met Ala Ser His Pro

275

280

285

Asn Tyr Asp Glu Phe Trp Gln Ala Arg Asn Leu Arg Pro His Leu Lys

290

295

300

Asn Leu Asn Lys Gly Thr Ala Val Leu Thr Val Gly Gly Phe Asn Asp

305

310

315

320

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Ala Glu Asp Leu Phe Gly Ala Leu Lys Thr Tyr Glu Ser Ile Glu Lys
325 330 335

Gln Asn Pro Gly Met Arg Asn Gly Leu Val Met Gly Pro Trp Val His
340 345 350

Gly Gly Trp Ala Arg Gly Thr Gly Glu Met Val Gly Asn Val Ala Tyr
355 360 365

Gly Glu Ser Pro Ser Leu Tyr Tyr Gln Lys Gln Ile Glu Ala Pro Phe
370 375 380

Phe Lys Ser Tyr Leu Lys Asp Gly Lys Pro Ala Ala Thr Pro Glu Ala
385 390 395 400

Thr Ile Phe Glu Ser Gly Thr Asn Arg Trp Arg Ser Phe Glu Thr Trp
405 410 415

Pro Pro Lys Glu Ala Lys Glu Arg Thr Leu Tyr Phe Gln Ser Ala Gly
420 425 430

Lys Ile Gly Phe Glu Lys Pro Ala Ser Gly Leu Glu Tyr Asp Gln Phe
435 440 445

Leu Ser Asp Pro Ala His Pro Val Pro Phe Thr Glu Ala Thr Ala Thr
450 455 460

Gly Met Thr Arg Glu Tyr Met Thr Asp Asp Gln Arg Phe Ala Ser Arg
465 470 475 480

38/56

Arg Pro Asp Val Leu Thr Tyr Gln Thr Glu Ala Leu Thr Glu Asp Met
485 490 495

Thr Leu Ala Gly Pro Ile Glu Ala Leu Leu Gln Val Ala Thr Thr Gly
500 505 510

Thr Asp Ala Asp Trp Val Val Lys Ile Ile Asp Val Tyr Pro Asp Asp
515 520 525

Thr Pro Asn Asn Pro Ser Thr Asn Pro Ala Val Lys Leu Gly Gly Tyr
530 535 540

Gln Gln Met Val Arg Ser Glu Val Met Arg Gly Arg Phe Arg Asn Ser
545 550 555 560

Phe Ser Lys Pro Glu Ala Phe Val Pro Glu Gln Val Thr Ala Val Pro
565 570 575

Phe Thr Val Gln Asp Leu Cys His Thr Phe Arg Lys Gly His Arg Leu
580 585 590

Met Val Gln Val Gln Ser Ser Trp Phe Pro Ile Val Asp Arg Asn Pro
595 600 605

Gln Thr Phe Val Pro Asn Ile Phe Glu Ala Asp Glu Lys Asp Phe Gln
610 615 620

Ala Ala Thr His Arg Leu Tyr His Ser Pro Ala His Ser Ser Gln Leu
625 630 635 640

39/56

Thr Leu Arg Val Leu

645

<210> 24

<211> 1931

<212> DNA

<213> Cyclobacterium marinum

<220>

<221> CDS

<222> (29)..(1888)

<223>

<400> 24

cccaaagcat taacaaaata atttagtc atg aaa cac tgt tac aaa ctt ctg 52

Met Lys His Cys Tyr Lys Leu Leu

1

5

gtc ttt tac aca tta ttt ttg atg acc aca aac tgg got tta toa caa 100

Val Phe Tyr Thr Leu Phe Leu Met Thr Thr Asn Trp Ala Leu Ser Gln

10

15

20

gcc att aat gga tat gat aag goa gcc tat gac att cct atg oga gat 148

Ala Ile Asn Gly Tyr Asp Lys Ala Ala Tyr Asp Ile Pro Met Arg Asp

25

30

35

40

gga gtt cac ctt cac acc atc gtc tat agc ccc aaa gat tta tog cag 196

Gly Val His Leu His Thr Ile Val Tyr Ser Pro Lys Asp Leu Ser Gln

45

50

55

ccc tat cct ata ttg atg caa agg aca cct tac agc gcc ggc cct tat 244

Pro Tyr Pro Ile Leu Met Gln Arg Thr Pro Tyr Ser Ala Gly Pro Tyr

60

65

70

ggt cct gga aat atg aaa aat aag ctt ggc cct tct cag ttt tta atg 292

Gly Pro Gly Asn Met Lys Asn Lys Leu Gly Pro Ser Gln Phe Leu Met

75

80

85

40/56

aac gat ggc tat ata ttt gtt tac cag gat gta aga ggg cgg tgg atg	340
Asn Asp Gly Tyr Ile Phe Val Tyr Gln Asp Val Arg Gly Arg Trp Met	
90 95 100	
tcg gaa gga tcc tat gac aac atg cgc cct acc cta tcc aaa tca gaa	388
Ser Glu Gly Ser Tyr Asp Asn Met Arg Pro Thr Leu Ser Lys Ser Glu	
105 110 115 120	
aga aat tcc aac caa ata gac gaa agc aca gac acc tat gat acc ata	436
Arg Asn Ser Asn Gln Ile Asp Glu Ser Thr Asp Thr Tyr Asp Thr Ile	
125 130 135	
gaa tgg ttg ctc gcc aat atc aaa aat cac aat gaa aaa gta ggc cta	484
Glu Trp Leu Leu Ala Asn Ile Lys Asn His Asn Glu Lys Val Gly Leu	
140 145 150	
tgg gga atc agc tat ccc gga ttt tat agt got gca gcc ctt cct ttt	532
Trp Gly Ile Ser Tyr Pro Gly Phe Tyr Ser Ala Ala Ala Leu Pro Phe	
155 160 165	
gcc cat cca aac ctg aaa gcc gtt tcc cct caa gca ccc ata ggg gat	580
Ala His Pro Asn Leu Lys Ala Val Ser Pro Gln Ala Pro Ile Gly Asp	
170 175 180	
ttt tac ttt gat gat ttt cat cat aac ggt gct tac tta tta agt tat	628
Phe Tyr Phe Asp Asp Phe His His Asn Gly Ala Tyr Leu Leu Ser Tyr	
185 190 195 200	
tgg ttg gcc act tot gtt ttc ggc tac caa aaa gac ggc cct aca cag	676
Trp Leu Ala Thr Ser Val Phe Gly Tyr Gln Lys Asp Gly Pro Thr Gln	
205 210 215	
gaa gca tgg tat ggc atg gtg aat cgc gaa aca aat gac ggc tat cag	724
Glu Ala Trp Tyr Gly Met Val Asn Pro Glu Thr Asn Asp Gly Tyr Gln	
220 225 230	
ttt ttt atg gat atg ggg cca tta aaa aat gcc gat aaa tgg tat ggt	772
Phe Phe Met Asp Met Gly Pro Leu Lys Asn Ala Asp Lys Trp Tyr Gly	

41/56

235	240	245	
gaa gac aat ttt ttc tgg caa caa ctt aaa aac aat cct gat tac aac			820
Glu Asp Asn Phe Phe Trp Gln Gln Leu Lys Asn Asn Pro Asp Tyr Asn			
250	255	260	
gct ttc tgg caa aag aga agt att att cct cac tta aaa gaa gtg aag			868
Ala Phe Trp Gln Lys Arg Ser Ile Ile Pro His Leu Lys Glu Val Lys			
265	270	275	280
cct gca gtt tta acc gtt ggg ggc tgg ttt gat gca gaa gat ctc tat			916
Pro Ala Val Leu Thr Val Gly Gly Trp Phe Asp Ala Glu Asp Leu Tyr			
285	290	295	
gga cca ctt aca att tat aaa acc att gaa aaa aat aat cct gag acc			964
Gly Pro Leu Thr Ile Tyr Lys Thr Ile Glu Lys Asn Asn Pro Glu Thr			
300	305	310	
tac aat acc att gtc atg ggc cct tgg tcc cac gga gat tgg tca agg			1012
Tyr Asn Thr Ile Val Met Gly Pro Trp Ser His Gly Asp Trp Ser Arg			
315	320	325	
gaa cct gga tca cag gtc att tca aat att tat ttt ggt gat tct atc			1060
Glu Pro Gly Ser Gln Val Ile Ser Asn Ile Tyr Phe Gly Asp Ser Ile			
330	335	340	
tcc aca tgg tat caa aaa aat ata gaa cgt gtt ttt ttc aat cat ttt			1108
Ser Thr Trp Tyr Gln Lys Asn Ile Glu Arg Val Phe Phe Asn His Phe			
345	350	355	360
cta aaa gaa tcc gaa aat agc aat cct gcc ctt cct gaa gcc tac atg			1156
Leu Lys Glu Ser Glu Asn Ser Asn Pro Ala Leu Pro Glu Ala Tyr Met			
365	370	375	
ttt gat acc gga aaa cat aaa tgg gaa aaa ttt gac gat tgg cct cct			1204
Phe Asp Thr Gly Lys His Lys Trp Glu Lys Phe Asp Asp Trp Pro Pro			
380	385	390	
aaa gaa ago caa tgg aaa ago ttt tac ttt caa gag aaa gga gag tta			1252
Lys Glu Ser Gln Trp Lys Ser Phe Tyr Phe Gln Glu Lys Gly Glu Leu			

42/56

395	400	405	
act gag gta aca cct gag gga aat agg ttt act acc tat gtc tca gac			1300
Thr Glu Val Thr Pro Glu Gly Asn Arg Phe Thr Thr Tyr Val Ser Asp			
410	415	420	
ccc tot aat cct gtc ccc tat agt caa gat att aaa cta aac ttc act			1348
Pro Ser Asn Pro Val Pro Tyr Ser Gln Asp Ile Lys Leu Asn Phe Thr			
425	430	435	440
ccg aga aaa tac atg gcc gat gac cag oga ttt gca gcc aga aga ccg			1396
Pro Arg Lys Tyr Met Ala Asp Asp Gln Arg Phe Ala Ala Arg Arg Pro			
445	450	455	
gac gta ctg acc ttt acg agc gaa gta tta agt caa gac atg acg ctt			1444
Asp Val Leu Thr Phe Thr Ser Glu Val Leu Ser Gln Asp Met Thr Leu			
460	465	470	
gcg ggg gaa gtc atg gca aac tta aaa gtt gcc act tca caa act gat			1492
Ala Gly Glu Val Met Ala Asn Leu Lys Val Ala Thr Ser Gln Thr Asp			
475	480	485	
gct gat tgg gta gtt aaa atc atc gat ata ttt ccc gga gat cag cca			1540
Ala Asp Trp Val Val Lys Ile Ile Asp Ile Phe Pro Gly Asp Gln Pro			
490	495	500	
aat cat gcc tat gtt tta gat ggg gtg gac atg ggc aat tac cac cta			1588
Asn His Ala Tyr Val Leu Asp Gly Val Asp Met Gly Asn Tyr His Leu			
505	510	515	520
atg gtt cgt tca gag gta att aga ggg agg tat aga gaa agt ttt gag			1636
Met Val Arg Ser Glu Val Ile Arg Gly Arg Tyr Arg Glu Ser Phe Glu			
525	530	535	
ttt cct aaa ccc ttt gtt cct gat caa atc act gct gtt gat ttc agg			1684
Phe Pro Lys Pro Phe Val Pro Asp Gln Ile Thr Ala Val Asp Phe Arg			
540	545	550	
tta caa gat ctt ttc cat act ttc aaa aag ggg cat aaa att caa ata			1732
Leu Gln Asp Leu Phe His Thr Phe Lys Lys Gly His Lys Ile Gln Ile			

43/56

555	560	565	
caa ata caa agt act tgg ttt ccc cta att gat cga aat ccc caa aaa Gln Ile Gln Ser Thr Trp Phe Pro Leu Ile Asp Arg Asn Pro Gln Lys			1780
570	575	580	
tat gta caa aac ata ttt gaa got gag gaa gcc gat ttt gtc aaa gcc Tyr Val Gln Asn Ile Phe Glu Ala Glu Glu Ala Asp Phe Val Lys Ala			1828
585	590	595	600
acc cat agg gtt ttt cat aca gaa aag ttt gcc ago aaa att gaa gta Thr His Arg Val Phe His Thr Glu Lys Phe Ala Ser Lys Ile Glu Val			1876
	605	610	615
atg gtt ctt cct tagaattaga atggtttaaa attactatatt gtagcagaag ata Met Val Leu Pro			1931
	620		

<210> 25

<211> 620

<212> PRT

<213> Cyclobacterium marinum

<400> 25

Met	Lys	His	Cys	Tyr	Lys	Leu	Leu	Val	Phe	Tyr	Thr	Leu	Phe	Leu	Met
1			5					10					15		

Thr	Thr	Asn	Trp	Ala	Leu	Ser	Gln	Ala	Ile	Asn	Gly	Tyr	Asp	Lys	Ala
		20					25						30		

Ala	Tyr	Asp	Ile	Pro	Met	Arg	Asp	Gly	Val	His	Leu	His	Thr	Ile	Val
	35						40				45				

Tyr	Ser	Pro	Lys	Asp	Leu	Ser	Gln	Pro	Tyr	Pro	Ile	Leu	Met	Gln	Arg
	50					55					60				

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Thr Pro Tyr Ser Ala Gly Pro Tyr Gly Pro Gly Asn Met Lys Asn Lys
65 70 75 80

Leu Gly Pro Ser Gln Phe Leu Met Asn Asp Gly Tyr Ile Phe Val Tyr
85 90 95

Gln Asp Val Arg Gly Arg Trp Met Ser Glu Gly Ser Tyr Asp Asn Met
100 105 110

Arg Pro Thr Leu Ser Lys Ser Glu Arg Asn Ser Asn Gln Ile Asp Glu
115 120 125

Ser Thr Asp Thr Tyr Asp Thr Ile Glu Trp Leu Leu Ala Asn Ile Lys
130 135 140

Asn His Asn Glu Lys Val Gly Leu Trp Gly Ile Ser Tyr Pro Gly Phe
145 150 155 160

Tyr Ser Ala Ala Ala Leu Pro Phe Ala His Pro Asn Leu Lys Ala Val
165 170 175

Ser Pro Gln Ala Pro Ile Gly Asp Phe Tyr Phe Asp Asp Phe His His
180 185 190

Asn Gly Ala Tyr Leu Leu Ser Tyr Trp Leu Ala Thr Ser Val Phe Gly
195 200 205

Tyr Gln Lys Asp Gly Pro Thr Gln Glu Ala Trp Tyr Gly Met Val Asn
210 215 220

45/56

Pro Glu Thr Asn Asp Gly Tyr Gln Phe Phe Met Asp Met Gly Pro Leu
225 230 235 240

Lys Asn Ala Asp Lys Trp Tyr Gly Glu Asp Asn Phe Phe Trp Gln Gln
245 250 255

Leu Lys Asn Asn Pro Asp Tyr Asn Ala Phe Trp Gln Lys Arg Ser Ile
260 265 270

Ile Pro His Leu Lys Glu Val Lys Pro Ala Val Leu Thr Val Gly Gly
275 280 285

Trp Phe Asp Ala Glu Asp Leu Tyr Gly Pro Leu Thr Ile Tyr Lys Thr
290 295 300

Ile Glu Lys Asn Asn Pro Glu Thr Tyr Asn Thr Ile Val Met Gly Pro
305 310 315 320

Trp Ser His Gly Asp Trp Ser Arg Glu Pro Gly Ser Gln Val Ile Ser
325 330 335

Asn Ile Tyr Phe Gly Asp Ser Ile Ser Thr Trp Tyr Gln Lys Asn Ile
340 345 350

Glu Arg Val Phe Phe Asn His Phe Leu Lys Glu Ser Glu Asn Ser Asn
355 360 365

Pro Ala Leu Pro Glu Ala Tyr Met Phe Asp Thr Gly Lys His Lys Trp
370 375 380

46/56

Glu Lys Phe Asp Asp Trp Pro Pro Lys Glu Ser Gln Trp Lys Ser Phe
385 390 395 400

Tyr Phe Gln Glu Lys Gly Glu Leu Thr Glu Val Thr Pro Glu Gly Asn
405 410 415

Arg Phe Thr Thr Tyr Val Ser Asp Pro Ser Asn Pro Val Pro Tyr Ser
420 425 430

Gln Asp Ile Lys Leu Asn Phe Thr Pro Arg Lys Tyr Met Ala Asp Asp
435 440 445

Gln Arg Phe Ala Ala Arg Arg Pro Asp Val Leu Thr Phe Thr Ser Glu
450 455 460

Val Leu Ser Gln Asp Met Thr Leu Ala Gly Glu Val Met Ala Asn Leu
465 470 475 480

Lys Val Ala Thr Ser Gln Thr Asp Ala Asp Trp Val Val Lys Ile Ile
485 490 495

Asp Ile Phe Pro Gly Asp Gln Pro Asn His Ala Tyr Val Leu Asp Gly
500 505 510

Val Asp Met Gly Asn Tyr His Leu Met Val Arg Ser Glu Val Ile Arg
515 520 525

Gly Arg Tyr Arg Glu Ser Phe Glu Phe Pro Lys Pro Phe Val Pro Asp
530 535 540

47/56

Gln Ile Thr Ala Val Asp Phe Arg Leu Gln Asp Leu Phe His Thr Phe
 545 550 555 560

Lys Lys Gly His Lys Ile Gln Ile Gln Ile Gln Ser Thr Trp Phe Pro
 565 570 575

Leu Ile Asp Arg Asn Pro Gln Lys Tyr Val Gln Asn Ile Phe Glu Ala
 580 585 590

Glu Glu Ala Asp Phe Val Lys Ala Thr His Arg Val Phe His Thr Glu
 595 600 605

Lys Phe Ala Ser Lys Ile Glu Val Met Val Leu Pro
 610 615 620

<210> 26

<211> 2036

<212> DNA

<213> *Psycloserpens burtonensis*

<220>

<221> CDS

<222> (61).. (1992)

<223>

<400> 26

catattogta aaatagctat aagtttttgt aaatttagtc aatcaaaatt ttaaattgtaa 60

atg aag act ctt ttt aaa ttg ttg ctc cta ttt gta ttt gtt cta acg 108

Met Lys Thr Leu Phe Lys Leu Leu Leu Leu Phe Val Phe Val Leu Thr

1

5

10

15

tct tgt aat aag gcc aac aaa gac gct act gaa att gtg aaa acc gaa 156

48/56

Ser Cys Asn Lys Ala Asn Lys Asp Ala Thr Glu Ile Val Lys Thr Glu	
20 25 30	
gta gaa gat act tac gtt aaa gat aat tat aac aaa caa gag gtg act	204
Val Glu Asp Thr Tyr Val Lys Asp Asn Tyr Asn Lys Gln Glu Val Thr	
35 40 45	
att gaa atg cgc gat ggt ata aaa ctt cac acg acc att tat tca cca	252
Ile Glu Met Arg Asp Gly Ile Lys Leu His Thr Thr Ile Tyr Ser Pro	
50 55 60	
aaa gat gaa agt cag acc tat cct att tta atg atg aga aca cca tat	300
Lys Asp Glu Ser Gln Thr Tyr Pro Ile Leu Met Met Arg Thr Pro Tyr	
65 70 75 80	
agt tct caa cct tat ggt gac aat gag ttt aag acg aaa att ggt cct	348
Ser Ser Gln Pro Tyr Gly Asp Asn Glu Phe Lys Thr Lys Ile Gly Pro	
85 90 95	
aat gtt cat tta atg aaa gaa ggg aat att gtt gtg tat caa gat gta	396
Asn Val His Leu Met Lys Glu Gly Asn Ile Val Val Tyr Gln Asp Val	
100 105 110	
cga ggt cgt tgg atg agt gaa ggt gtc tat gat aat atg cgt got tat	444
Arg Gly Arg Trp Met Ser Glu Gly Val Tyr Asp Asn Met Arg Ala Tyr	
115 120 125	
atc cca aat aaa aca gag gat tct caa att gat gag gca tca gac act	492
Ile Pro Asn Lys Thr Glu Asp Ser Gln Ile Asp Glu Ala Ser Asp Thr	
130 135 140	
tat gac acg att gac tgg ctg gta aat aac gta gaa aat aat aac ggg	540
Tyr Asp Thr Ile Asp Trp Leu Val Asn Asn Val Glu Asn Asn Asn Gly	
145 150 155 160	
aat gtt ggt act tgg gga att tca tat cct ggt ttt tat gct aca tat	588
Asn Val Gly Thr Trp Gly Ile Ser Tyr Pro Gly Phe Tyr Ala Thr Tyr	
165 170 175	
tct act ata gac gca cac cca gct tta aaa gca gca tcg cct caa gcg	636

49/56

Ser Thr Ile Asp Ala His Pro Ala Leu Lys Ala Ala Ser Pro Gln Ala	
180 185 190	
tgt att gga gat ttc ttt ttt gac gat ttt cat cat aat ggt gct ttt	684
Cys Ile Gly Asp Phe Phe Phe Asp Asp Phe His His Asn Gly Ala Phe	
195 200 205	
tta tta agt tat ttt aga gca gtg tot tta ttt ggt acg aca aaa gat	732
Leu Leu Ser Tyr Phe Arg Ala Val Ser Leu Phe Gly Thr Thr Lys Asp	
210 215 220	
aaa cct aca gat tct gct tgg tat aag ttt cca gaa atg aaa aca caa	780
Lys Pro Thr Asp Ser Ala Trp Tyr Lys Phe Pro Glu Met Lys Thr Gln	
225 230 235 240	
gat caa tat caa ttt ttt ctt gat gct gga cct tta agt aat ttg aac	828
Asp Gln Tyr Gln Phe Phe Leu Asp Ala Gly Pro Leu Ser Asn Leu Asn	
245 250 255	
aag tat ttc caa tat gac aca cca gac gac aca tct gta tcc aag tct	876
Lys Tyr Phe Gln Tyr Asp Thr Pro Asp Asp Thr Ser Val Ser Lys Ser	
260 265 270	
gat agg ata gat gat gtg ttt tgg aaa gaa att gta gag cat cca aac	924
Asp Arg Ile Asp Asp Val Phe Trp Lys Glu Ile Val Glu His Pro Asn	
275 280 285	
tac gat acg ata tgg aaa tct aaa ggt tta att caa aac cta aaa gat	972
Tyr Asp Thr Ile Trp Lys Ser Lys Gly Leu Ile Gln Asn Leu Lys Asp	
290 295 300	
att aag cca agt gta gcg aca atg att gtg gga ggg tta ttt gat gcc	1020
Ile Lys Pro Ser Val Ala Thr Met Ile Val Gly Gly Leu Phe Asp Ala	
305 310 315 320	
gaa gat tta tat ggg cca ttt gaa act tat aaa acg ata gaa aaa cat	1068
Glu Asp Leu Tyr Gly Pro Phe Glu Thr Tyr Lys Thr Ile Glu Lys His	
325 330 335	
aat cct gat aat tat aat att atg gtt ttt ggg cct tgg gat cat ggt	1116

50/56

Asn Pro Asp Asn Tyr Asn Ile Met Val Phe Gly Pro Trp Asp His Gly	
340 345 350	
ogt tgg gct agg agt gac gtt aaa aat tat gtt gga aat tat ttc ttc	1164
Arg Trp Ala Arg Ser Asp Val Lys Asn Tyr Val Gly Asn Tyr Phe Phe	
355 360 365	
gga gat tct ata tct cta aaa ttt caa cgt gat gtt gaa acg aag ttt	1212
Gly Asp Ser Ile Ser Leu Lys Phe Gln Arg Asp Val Glu Thr Lys Phe	
370 375 380	
ttt aat cat ttt tta aaa gga aaa ggc gac aag aac tca ggg tta cca	1260
Phe Asn His Phe Leu Lys Gly Lys Gly Asp Lys Asn Ser Gly Leu Pro	
385 390 395 400	
gaa gca tat gta ttt gat tot ggt aaa aag gaa tgg agt agc ttt gac	1308
Glu Ala Tyr Val Phe Asp Ser Gly Lys Lys Glu Trp Ser Ser Phe Asp	
405 410 415	
agc tgg cct cca aag caa gca gaa aaa caa gcc atg tat ctt aat gcc	1356
Ser Trp Pro Pro Lys Gln Ala Glu Lys Gln Ala Met Tyr Leu Asn Ala	
420 425 430	
aac caa gag cta tca gat tca aaa aaa gga aat act agt gag aca ttt	1404
Asn Gln Glu Leu Ser Asp Ser Lys Lys Gly Asn Thr Ser Glu Thr Phe	
435 440 445	
gtt agt gat tta aaa cgc cct gta cct tat tcc gaa gat att aaa aca	1452
Val Ser Asp Leu Lys Arg Pro Val Pro Tyr Ser Glu Asp Ile Lys Thr	
450 455 460	
gtt ttc aca cca cga aaa tac atg aca gac gat cag cgt ttt gca gca	1500
Val Phe Thr Pro Arg Lys Tyr Met Thr Asp Asp Gln Arg Phe Ala Ala	
465 470 475 480	
cga cgt cct gat gtt ctt ata ttt gag acc gat att ctt gag gaa gat	1548
Arg Arg Pro Asp Val Leu Ile Phe Glu Thr Asp Ile Leu Glu Glu Asp	
485 490 495	
ata acc tta gct ggt gat att tta ggc cag ctt aat gtg tca act aca	1596

51/56

Ile Thr Leu Ala Gly Asp Ile Leu Ala Gln Leu Asn Val Ser Thr Thr	
500 505 510	
ggg aca gat gca gat tgg att gtc aaa ata gta gat gtt cat cca gca	1644
Gly Thr Asp Ala Asp Trp Ile Val Lys Ile Val Asp Val His Pro Ala	
515 520 525	
gat gct gag gag caa aaa gaa ggt atg caa gac cat tta tca atg agt	1692
Asp Ala Glu Glu Gln Lys Glu Gly Met Gln Asp His Leu Ser Met Ser	
530 535 540	
aat tat cat ttg atg gtg agg agt gaa gtg atg cgc ggt cgt ttt aga	1740
Asn Tyr His Leu Met Val Arg Ser Glu Val Met Arg Gly Arg Phe Arg	
545 550 555 560	
aat agt ttt gaa aac cca gag cca ttt gtg cca aac caa cca aca gat	1788
Asn Ser Phe Glu Asn Pro Glu Pro Phe Val Pro Asn Gln Pro Thr Asp	
565 570 575	
gtc aat atc aag tta caa gat gta cat cat aca ttt aaa aaa ggt cac	1836
Val Asn Ile Lys Leu Gln Asp Val His His Thr Phe Lys Lys Gly His	
580 585 590	
aaa tta caa gtg caa gtt cag agt acg tgg ttt cca ctt att gat ttg	1884
Lys Leu Gln Val Gln Val Gln Ser Thr Trp Phe Pro Leu Ile Asp Leu	
595 600 605	
aac cag caa aca ttt gtg cct aat att tat aaa gca aaa gaa ago gat	1932
Asn Pro Gln Thr Phe Val Pro Asn Ile Tyr Lys Ala Lys Glu Ser Asp	
610 615 620	
ttt aaa acc caa aca cat tcg gtt ttt aac gat tct aaa att gag ttt	1980
Phe Lys Thr Gln Thr His Ser Val Phe Asn Asp Ser Lys Ile Glu Phe	
625 630 635 640	
acg gtt ttg aaa taagagtaga tgactaaatt tgccaaggta gatttagtct tttt	2036
Thr Val Leu Lys	

52/56

<210> 27

<211> 644

<212> PRT

<213> *Psychocloerpens burtonensis*

<400> 27

Met Lys Thr Leu Phe Lys Leu Leu Leu Phe Val Phe Val Leu Thr
1 5 10 15

Ser Cys Asn Lys Ala Asn Lys Asp Ala Thr Glu Ile Val Lys Thr Glu
20 25 30

Val Glu Asp Thr Tyr Val Lys Asp Asn Tyr Asn Lys Gln Glu Val Thr
35 40 45

Ile Glu Met Arg Asp Gly Ile Lys Leu His Thr Thr Ile Tyr Ser Pro
50 55 60

Lys Asp Glu Ser Gln Thr Tyr Pro Ile Leu Met Met Arg Thr Pro Tyr
65 70 75 80

Ser Ser Gln Pro Tyr Gly Asp Asn Glu Phe Lys Thr Lys Ile Gly Pro
85 90 95

Asn Val His Leu Met Lys Glu Gly Asn Ile Val Val Tyr Gln Asp Val
100 105 110

Arg Gly Arg Trp Met Ser Glu Gly Val Tyr Asp Asn Met Arg Ala Tyr
115 120 125

Ile Pro Asn Lys Thr Glu Asp Ser Gln Ile Asp Glu Ala Ser Asp Thr

53/56

130

135

140

Tyr Asp Thr Ile Asp Trp Leu Val Asn Asn Val Glu Asn Asn Asn Gly
145 150 155 160

Asn Val Gly Thr Trp Gly Ile Ser Tyr Pro Gly Phe Tyr Ala Thr Tyr
165 170 175

Ser Thr Ile Asp Ala His Pro Ala Leu Lys Ala Ala Ser Pro Gln Ala
180 185 190

Cys Ile Gly Asp Phe Phe Phe Asp Asp Phe His His Asn Gly Ala Phe
195 200 205

Leu Leu Ser Tyr Phe Arg Ala Val Ser Leu Phe Gly Thr Thr Lys Asp
210 215 220

Lys Pro Thr Asp Ser Ala Trp Tyr Lys Phe Pro Glu Met Lys Thr Gln
225 230 235 240

Asp Gln Tyr Gln Phe Phe Leu Asp Ala Gly Pro Leu Ser Asn Leu Asn
245 250 255

Lys Tyr Phe Gln Tyr Asp Thr Pro Asp Asp Thr Ser Val Ser Lys Ser
260 265 270

Asp Arg Ile Asp Asp Val Phe Trp Lys Glu Ile Val Glu His Pro Asn
275 280 285

Tyr Asp Thr Ile Trp Lys Ser Lys Gly Leu Ile Gln Asn Leu Lys Asp

54/56

290

295

300

Ile Lys Pro Ser Val Ala Thr Met Ile Val Gly Gly Leu Phe Asp Ala
305 310 315 320

Glu Asp Leu Tyr Gly Pro Phe Glu Thr Tyr Lys Thr Ile Glu Lys His
325 330 335

Asn Pro Asp Asn Tyr Asn Ile Met Val Phe Gly Pro Trp Asp His Gly
340 345 350

Arg Trp Ala Arg Ser Asp Val Lys Asn Tyr Val Gly Asn Tyr Phe Phe
355 360 365

Gly Asp Ser Ile Ser Leu Lys Phe Gln Arg Asp Val Glu Thr Lys Phe
370 375 380

Phe Asn His Phe Leu Lys Gly Lys Gly Asp Lys Asn Ser Gly Leu Pro
385 390 395 400

Glu Ala Tyr Val Phe Asp Ser Gly Lys Lys Glu Trp Ser Ser Phe Asp
405 410 415

Ser Trp Pro Pro Lys Gln Ala Glu Lys Gln Ala Met Tyr Leu Asn Ala
420 425 430

Asn Gln Glu Leu Ser Asp Ser Lys Lys Gly Asn Thr Ser Glu Thr Phe
435 440 445

Val Ser Asp Leu Lys Arg Pro Val Pro Tyr Ser Glu Asp Ile Lys Thr

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Val Phe Thr Pro Arg Lys Tyr Met Thr Asp Asp Gln Arg Phe Ala Ala
465 470 475 480

Arg Arg Pro Asp Val Leu Ile Phe Glu Thr Asp Ile Leu Glu Glu Asp
485 490 495

Ile Thr Leu Ala Gly Asp Ile Leu Ala Gln Leu Asn Val Ser Thr Thr
500 505 510

Gly Thr Asp Ala Asp Trp Ile Val Lys Ile Val Asp Val His Pro Ala
515 520 525

Asp Ala Glu Glu Gln Lys Glu Gly Met Gln Asp His Leu Ser Met Ser
530 535 540

Asn Tyr His Leu Met Val Arg Ser Glu Val Met Arg Gly Arg Phe Arg
545 550 555 560

Asn Ser Phe Glu Asn Pro Glu Pro Phe Val Pro Asn Gln Pro Thr Asp
565 570 575

Val Asn Ile Lys Leu Gln Asp Val His His Thr Phe Lys Lys Gly His
580 585 590

Lys Leu Gln Val Gln Val Gln Ser Thr Trp Phe Pro Leu Ile Asp Leu
595 600 605

Asn Pro Gln Thr Phe Val Pro Asn Ile Tyr Lys Ala Lys Glu Ser Asp

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610

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620

Phe Lys Thr Gln Thr His Ser Val Phe Asn Asp Ser Lys Ile Glu Phe
625 630 635 640

Thr Val Leu Lys

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP 2004/000620**A. CLASSIFICATION OF SUBJECT MATTER**Int.Cl⁷ **C12P 21/02, C12N 15/57 // C12N 9/48, C07K 5/075**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl⁷ **C12P 21/02, C12N 15/57, C12N 9/48-9/64, C07K 5/075, A23L 1/236**Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Japanese Utility Model Gazette 1922-1996, Japanese Publication of Unexamined Utility Model Applications 1971-2004, Japanese Registered Utility Model Gazette 1994-2004, Japanese Gazette Containing the Utility Model 1996-2004

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI/BIOSIS (DIALOG), PUBMED, JSTPlus, SwissProt/PIR/Geneseq**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/58478 A1 (HOLLAND SWEETENER COMPANY V.O.F.) 2000.10.05 & CA 2365594 A & AU 3843900 A & EP 1096011 A1 & EP 1165799 A1 & US 2002/0064834 A1 & CN 1353757 A & JP 2002-539835 A & BR 10398 A	1-17
A	EP 269390 A2 (GENENCOR INC) 1988.06.01 & JP 01-160495 A & EP 269390 B1 & DE 3787155 B & JP 07-51075 B1 & CA 1339674 B	1-17
A	JP 04-311389 A (MERCIAN CORP.) 1992.11.04 (Family:none)	1-17

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26.03.2004

Date of mailing of the international search report

13.4.2004

Name and mailing address of the ISA/JP

Japan Patent Office**3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan**

Authorized officer

HAJIME KAMIJOTelephone No. **+81-3-3581-1101 Ext. 3448****4B****9453**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP 2004/000620

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing
- b. format of material
- ☐ in written format
- ☒ in computer readable form
- c. time of filing/furnishing
- ☐ contained in the international application as filed
- ☒ filed together with the international application in computer readable form
- ☐ furnished subsequently to this Authority for the purposes of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: