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GENE CODING FOR ESTERASE AND NOVEL MICROORGANISM CONTAINING SAID GENE

This invention relates to a gene coding for an esterase, a recombinant plasmid containing said gene, a novel microorganism transformed with the recombinant plasmid, and a process for producing an esterase by cultivating the microorganism.

Prior Art

Recently, it has frequently been tried to utilize enzymes such as esterase in hydrolysis reaction. For such a purpose, there are known various esterases, for example, esterases originated from animals (e.g. pig liver, pig pancreas, etc.), and esterases originated from microorganisms, such as *Arthrobacter globiformis*, *Geotrichum candidum*, *Candida cylindracea*, *Pseudomonas fluorescens*, etc.

Further, it is known that an esterase is obtained by utilizing a recombinant DNA technology in microorganism, such as *Rhizopus delemar* (cf. Japanese Patent First Publication (Kokai) No. 87175/1991), *Pseudomonas fragi* (cf. Biochemical and Biophysical Research Communications, Vol. 141, 185-190 (1986)).

However, the known esterases or known methods for producing esterase have various problems. For instance, animal-origin esterases are very expensive. Besides, above-

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mentioned microorganisms-origin esterases of the genus *Arthrobacter*, *Geotrichum*, *Candida*, *Pseudomonas*, or *Rhizopus* had problems such that they were disadvantageous in activity, stability, or specificity, or that the esterase-producing microorganism had no high productivity.

As a result of various investigations, the present inventors found that the desired esterase can be produced favorably by utilizing recombinant DNA technology in a microorganism of the genus *Serratia*. That is, the present inventors have succeeded to obtain a gene coding for an esterase from a microorganism of the genus *Serratia*, and have found that a transformed microorganism having a significantly increased esterase productivity can be obtained by introducing the recombinant plasmid containing the said gene into a host microorganism, and further have found that the desired esterase can be produced by using said transformed microorganism on an industrial scale.

Summary Description of the Invention

An object of the invention is to provide a gene coding for an esterase originated from a microorganism of the genus *Serratia*. Another object of the invention is to provide a mutant strain which has a high productivity of esterase. Another object of the invention is to provide a recombinant plasmid produced by inserting said gene into a vector plasmid. A further object of the invention is to provide a transformed microorganism containing said recombinant plasmid. A still further object of the invention is to provide a method for the

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production of an esterase by cultivating said transformed microorganism and collecting the produced esterase outside and inside the cells of the microorganism.

Brief Description of Drawing

Fig. 1 shows a restriction endonuclease map of the plasmid DNA pLIPE101 isolated from the recombinant cells in Example 2.

Fig. 2 shows restriction endonuclease maps of each of plasmid DNAs, pLIPE111, pLIPE121, pLIPE131, pLIPE141, and pLIPE151, which were produced by deleting the plasmid DNA pLIPE101 using a restriction endonuclease and DNA ligase.

Detailed Description of the Invention

The gene encoding an esterase originated from a microorganism of the genus *Serratia* of this invention is a double stranded DNA of 1839 base pairs having an open reading frame, specifically DNAs having the nucleotide sequences shown in SEQ ID NO:1 and SEQ ID NO:2 hereinafter.

The donor microorganisms for such a gene encoding an esterase include any microorganisms belonging to the genus *Serratia* which has an esterase productivity, for example, *Serratia marcescens* Sr41 (FERM BP-487), *Serratia liquefaciens* ATCC 27592, *Serratia marcescens* ATCC 13880, *Serratia marcescens* ATCC 14764, *Serratia marcescens* ATCC 19180, *Serratia marcescens* ATCC 21074, *Serratia marcescens* ATCC 27117, *Serratia marcescens* ATCC 21212, etc.

The donor microorganism includes also mutant strains derived from wild strains, such as various amino acid-

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requiring strains, various nucleic acid-requiring strains, various vitamin-requiring strains, and the like, for example, *Serratia marcescens* M-1 (FERM BP-4068). Said *Serratia marcescens* M-1 has more than two fold higher esterase productivity than the parent strain *Serratia marcescens* Sr41 and contains an esterase gene coded by the DNA sequence of SEQ ID NO:2. The mutant strain M-1 can be obtained by treating the parent *Serratia marcescens* Sr41 (FERM BP-487) with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) according to a method of Aderburg et al. [Biochemical and Biophysical Research Communications, Vol. 18, 788 (1965)], cultivating the thus treated microorganism on an agar medium containing emulsified triglyceride, and collecting the colony around which the largest clear zone is formed. In the same manner, another mutant strain *Serratia marcescens* G205 having 1.5 fold or higher esterase-productivity can be obtained from the parent strain *Serratia marcescens* Sr41.

The vector plasmid to be inserted with the gene encoding an esterase includes any plasmids which are replicable in transformed cells, but is preferably plasmids which have a copy number of 1 to several thousands and contain a resistant marker against antibiotics such as ampicillin, kanamycin, chloramphenicol, and further contain an appropriate promoter such as *lac*, *tac*, or *trp*. Moreover, the vector plasmids may further contain a plasmid stabilizing gene such as *par* and *par*B.

These vector plasmids include, for example, pLG339

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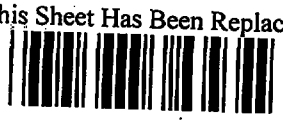
[Gene, Vol. 18, 332 (1982)], pBR322 [Gene, Vol. 2, 95 (1977)], pUC18 [Gene, Vol. 33, 103 (1985)], pUC19 [Gene, Vol. 33, 103 (1985]], pHSG298 [Gene, Vol. 61, 63 (1987)], pHSG299 [Gene, Vol. 61, 63 (1987)], and the like.

The above vector plasmids are commercially available or can be obtained from microbial cells containing these plasmids by a conventional method, for example, by "cleared lysate method" (cf. Yasuyuki Takagi, "Procedure for Experiment in Genetic Engineering", page 125, published by Kodansha, 1980), or by "alkaline lysis method" [cf. Maniatis et al., "Molecular Cloning", page 368, Cold Spring Harbor Laboratory, U.S.A. (1982)].

The host microorganisms (both for the recombination of plasmids and for the expression of the desired esterase), include any microorganisms which can be transformed with the recombinant plasmid and can replicate the plasmid therein, and can express the gene on the plasmid and can produce a functional protein. These host microorganisms are, for example, microorganisms belonging to the genus *Serratia* or the genus *Escherichia*, specifically *Serratia marcescens* Sr41 and various mutant strains derived therefrom, for example, *Serratia marcescens* M-1, *Serratia marcescens* TT392 [cf. Journal of Bacteriology, Vol. 161, 1 (1985)], or *Escherichia coli* K12 DH5 [cf. Maniatis et al., "Molecular Cloning", 2nd volume, A10, Cold Spring Harbor Laboratory, U.S.A. (1989)].

The chromosomal DNA containing the gene encoding an esterase can easily be obtained from microorganisms containing

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said gene by a conventional method, for example, by treating the microbial cells with a lysozyme and further with a surfactant (e.g. sodium lauryl sulfate, sodium N-lauroyl sarcosinate, etc.), extracting the cells thus treated with an organic solvent (e.g. phenol, chloroform, diethyl ether, etc.) to remove proteins, and then precipitating the DNA with ethanol [cf. Journal of Molecular Biology, Vol. 3, 208 (1961), and Biochimica et Biophysica Acta, Vol. 72, 619 (1963)].

The recombinant plasmid comprising the chromosomal DNA containing a gene encoding esterase and the vector plasmid can easily be prepared by a conventional method, for example, by cleaving the chromosomal DNA and the plasmid DNA with an appropriate restriction endonuclease (e.g. *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I, etc.) and then treating the resultant with DNA ligase (e.g. T4 DNA ligase, *E. coli* DNA ligase, etc.), if required, after treating the resultant with a terminal transferase or DNA polymerase [cf. Methods in Enzymology, Vol. 68, 41 (1979), and Yasuyuki Takagi, "Procedure for Experiment in Genetic Engineering", page 135, published by Kodansha, 1980].

Selection of the desired recombinant plasmid containing the esterase gene from a mixture of recombinant plasmids obtained by the above procedure can be done as follows.

Microbial cells being restriction endonuclease deficient and having no esterase activity (e.g. *Escherichia coli* K12 DH5) are transformed with the above mixture of recom-

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binant plasmids, and the thus-transformed cells are spread onto an agar medium containing an emulsified triglyceride, for example, a nutrient agar medium containing tributyrin emulsified with polyoxyethylene cetyl alcohol ether (Brij 58) and also containing a prescribed concentration of an antibiotic. After incubation at 30 to 37°C for 1 to 2 days, the colony of a transformant around which a large clear zone is formed is isolated.

The above introduction of the recombinant plasmid into the host microorganism is carried out by a conventional method, for example, by treating the host cells with an aqueous calcium chloride solution at a low temperature to increase the membrane permeability of the cells and then introducing the recombinant plasmid into the host cells [cf. Journal of Molecular Biology, Vol. 53, 159 (1970)], or by an electroporation method.

Then, the plasmid DNA is extracted from the transformant by "alkaline lysis method" to give a recombinant plasmid containing the gene encoding an esterase.

In order to make the transformation efficient, the recombinant plasmid thus obtained is modified in a microorganism which is restriction endonuclease deficient and is the same species of the host microorganism to be used for expressing the esterase. That is, when the host microorganism to be used is *Serratia marcescens* Sr41, the recombinant plasmid obtained above is introduced into *Serratia marcescens* TT392, which is restriction enzyme deficient strain. The

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recombinant plasmid thus modified is isolated from the microorganism.

The modified recombinant plasmid is then introduced into a host microorganism to obtain the desired transformant suitable for the production of the desired esterase.

The introduction of the recombinant plasmid into the host microorganism can easily be done by the method of Takagi & Kizumi [cf. Journal of Bacteriology, Vol. 161, 1 (1985)]. The host microorganisms to be used for said transformation include the above-mentioned microorganisms but are preferably strains having high esterase productivity. The desired transformant can be obtained by isolating the colony expressing antibitotics resistance.

The transformed microorganisms obtained by the above-mentioned method are, for example, *Serratia marcescens* TA5025 (FERM BP-4067) which is obtained by introducing a recombinant plasmid consisting of pUC19 and the 2.8 kb *Sal*I-*Bam*HI DNA fragment containing an esterase gene into *Serratia marcescens* Sr41; *Serratia marcescens* TBE101 which is obtained by introducing a recombinant plasmid consisting of pBR322 and the 4.0 kb *Sal*I DNA fragment containing an esterase gene into *Serratia marcescens* Sr41. These transformed strains all have the same morphological characteristics as those of the host microorganism *Serratia marcescens* Sr41.

The production of esterase with the transformed microorganism obtained above is carried out by cultivating the microorganism in a medium and collecting the esterase outside

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and inside the cells of the microorganism.

The medium used for the production of esterase includes any conventional medium wherein the microorganism can grow. Suitable medium contains a carbon source such as saccharides (e.g. glucose, sucrose, molasses, etc.), organic acids (e.g. fumaric acid, citric acid, etc.), alcohols (e.g. glycerol, etc.), or amino acids (e.g. alanine, glutamine, asparagine, etc.) and a nitrogen source such as inorganic ammonium salts (e.g. ammonium sulfate, ammonium chloride, etc.), urea, peptone, corn steep liquor, yeast extract, casein hydrolysate, and the like. The carbon source is usually contained in an amount of 1 to 15 % by weight based on the whole weight of the medium, and the nitrogen source is usually contained in an amount of 0.1 to 2.0 % by weight based on the whole weight of the medium. The medium may optionally contain further an appropriate amount of an inorganic salt (e.g. phosphate, magnesium salt, potassium salt, calcium salt, etc.) and/or a metallic ion (e.g. iron, manganese, copper, zinc, etc.). In case of a synthetic medium, it may further contain vitamins or amino acids, and further, inducers for esterase production (e.g. vegetable oils, surfactants, etc.), defoaming agents, antibiotics which are suitable for stabilizing the recombinant plasmid in microorganisms. The medium is preferably adjusted to a pH 5 to 8.

The cultivation of the transformed microorganism is carried out by a conventional method. For example, the microorganism is inoculated into a medium and is cultivated by

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shaking culture, aeration culture, standing culture, and the like. The cultivation conditions may vary depending on the kinds of the medium and cultivation methods, but may be any conditions suitable for growth of the microorganism, usually at the initial pH 5 - 8, at 20 to 40°C, for 1 to 2 days.

The esterase produced outside and inside the cultivated cells is collected by a conventional method. For example, the esterase contained in the medium is collected by means of salting out with an inorganic salt, precipitation with an organic solvent, absorption or desorption with ion exchange resin and various column chromatography, gel filtration, use of protein-precipitating agent, or a combination of these methods. The esterase accumulated within the cells is obtained by firstly disrupting the cells by a physical method such as frictional disrupting device (Dyno Mill) or a chemical means such as treatment with lysozyme, and then collecting the esterase in the cell extract by the above-mentioned method.

The gene encoding the esterase of this invention is not limited to those of the DNA sequences disclosed specifically in the present specification but includes any gene having a DNA sequence obtained by modifications in the sequence such as insertion, deletion, substitution. That is, the esterase gene may artificially be modified directly in a test tube by using a synthetic mutated DNA primer designed on the basis of the DNA sequence of the esterase gene specifically disclosed herein, or by using a chemical mutating agent

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such as formic acid, hydrazine sulfite. Further, a mutant gene may be obtained by treating an esterase producing strain with NTG or UV.

Examples

The present invention is illustrated by the following Examples but should not be construed to be limited thereto.

In the Examples, the esterase activity was measured by a convenient method (using Lipase Kit S, manufactured by Dainippon Pharmaceutical Co., Ltd., Japan). It was also measured by another method comprising subjecting the product to enzymatic reaction in olive oil (as a substrate) at pH 8.0, 37°C for 20 minutes, and then measuring the amount of formed fatty acid. The unit of esterase activity was expressed as μmols of fatty acid formed per minute. Besides, the medium used in Examples has the following formulation, wherein "%" is W/V% unless specified otherwise.

LB medium: 1.0% of Bactotryptone (manufactured by Difco), 0.5% of Bacto Yeast Extract (manufactured by Difco), and 0.5% of sodium chloride.

LBG plate medium: 1.0% of Bactotryptone (manufactured by Difco), 0.5% of Bacto Yeast Extract (manufactured by Difco), 0.5% sodium chloride, and 1.0% of Gellan Gum (manufactured by Wako Pure Chemical Industries, Ltd., Japan).

Tributyrin-containing LBG plate medium: LBG plate medium containing 0.5 v/v% of tributyrin, 0.5% of polyoxyethylene cetyl alcohol ether, and 0.005% of ampicillin.

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Esterase producing medium: 1.0% of dextrin, 2.0% of meast, 0.2% of ammonium sulfate, 0.1% of potassium dihydrogen phosphate, 0.05% of magnesium sulfate heptahydrate, 0.01% of calcium chloride dihydrate, 0.001% of ferrous sulfate hepta hydrate, 0.5% of Tween 80, and 0.1% of colorin.

Example 1

(1) Preparation of chromosomal DNA containing a gene encoding an esterase:

Serratia marcescens Sr41 (FERM BP-487) was subjected to aerobic shaking culture in LB medium (200 ml) at 30°C overnight, and then the cells were collected by centrifugation. The cells were suspended in 0.9% aqueous sodium chloride solution (200 ml) once and then collected by centrifugation in order to wash them. The cells thus washed were suspended in an aqueous solution of 50mM Tris-HCl-50mM disodium ethylenediamine tetraacetate (pH 7.5, 200 ml) containing 200 mg of lysozyme, and the mixture was allowed to stand at room temperature for one hour.

To the mixture was added sodium lauryl sulfate in a concentration of 0.5%, and thereto was further added Protenase K, and the mixture was mildly shaken at 50°C for 3 hours to lyse the cells. The mixture was extracted twice with an equal volume of phenol saturated with an aqueous solution of 10 mM Tris-HCl-1 mM disodium ethylenediamine tetraacetate (pH 8.0) (hereinafter, referred to as "TE"), and further extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform, and then the resultant aqueous phase

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was subjected to precipitation with ethanol. The precipitate was dissolved in TE to prepare a TE solution containing 2.5 mg of the chromosomal DNA containing a gene encoding an esterase.

(2) Preparation of a recombinant plasmid DNA:

The chromosomal DNA prepared in the above (1) (20 μg) was completely digested with restriction endonuclease *Sal*I, which was extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform. To the mixture was added 0.5 volume of 7.5 M ammonium acetate, and the DNA was recovered by centrifugation with two times volume of ethanol.

DNA (0.5 μg) of plasmid vector pUC19, which was completely digested with the same restriction enzyme as above, was dephosphated by treating with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd., Japan, 0.4 unit) at 56°C for one hour, and the resultant was extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform, and thereto was added 0.5 volume of 7.5M ammonium acetate, and then the vector plasmid DNA was recovered by precipitating with two volumes of ethanol. The dephosphated plasmid vector thus obtained was mixed with the chromosomal DNA obtained above (3 μg) and ligated with a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.) at 4°C for 16 hours to give the recombinant plasmid DNA.

(3) Transformation with the recombinant plasmid and preparation of colony bank with *E. coli* host:

The cells of *E. coli* DH5 were treated by the method

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of Hanahan [cf. Journal of Molecular Biology, Vol. 166, 557 (1983)] and thereto was added the reaction mixture containing a plasmid DNA obtained in the above (2), by which the transformation was effected. The cells thus treated were spread onto LBG plate medium containing ampicillin (50 μg/ml), and they were incubated at 37°C overnight to give the transformant (about 20,000 strains) containing recombinant plasmids inserted with fragments of the chromosomal DNA of *Serratia marcescens* Sr41.

(4) Isolation and identification of a transformant strain containing a gene encoding an esterase:

When an esterase producing strain is inoculated to a tributyrin-containing LBG plate medium, the esterase produced in the medium decomposes tributyrin to give fatty acids, by which the triglyceride emulsion around the colony is modified to form a circular clear zone around the colonies. By utilizing this phenomenon, the screening of transformants was effected.

The transformants (about 20,000 strains) obtained in the above (3) were inoculated to a tributyrin-containing LBG plate medium, which was incubated at 37°C overnight, by which one strain forming a clear zone was isolated from the DNA bank of *Sal*I.

The formation of this clear zone will be owing to the activity of esterase, because when *Serratia marcescens* Sr41 was inoculated to a tributyrin-containing LBG plate medium and incubated at 37°C overnight, the formation of a

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clear zone was observed, but when the untransformed *E. coli* DH5 and the *E. Coli* DH5 carrying a plasmid vector pUC19 were inoculated to a tributyrin-containing LBG plate medium and incubated at 37°C overnight, no formation of a clear zone was observed.

Besides, the transformant being capable of formation of a clear zone was cultivated in a LB medium (60 ml) containing ampicillin (200 μg/ml) by aerobic shaking culture at 37°C overnight, and the cells were collected and suspended in a 200 mM Tris-HCl buffer solution (pH 8.0, 20 ml). After disruption of the cells by sonication, the esterase activity of the mixture was measured by the convenient method. As a result, there was observed 1.5 x 10^3 units of esterase activity.

Moreover, the mixture obtained above was subjected to an electrophoresis with SDS polyacrylamide gel. As a result, the product showed a new band, which was not shown in the product from *E. coli* DH5 containing only vector plasmid, at the same position as that in the partially purified standard esterase obtained from the supernatant of the culture of *Serratia marcescens* Sr41.

Example 2

Analysis of plasmid:

A plasmid DNA was prepared from the cells of the transformant obtained in Example 1-(4) by a conventional method [cf. Maniatis et al., "Molecular Cloning", page 368, Cold Spring Harbor Laboratory, U.S.A. (1982)], cleaved with

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various restriction endonucleases and then subjected to an agarose gel electrophoresis. As a result, it was confirmed that this plasmid (hereinafter, referred to as "pLIPE101") contained a *Sal*I DNA fragment of about 4.0 kb.

The restriction endonuclease map of the recombinant DNA fragment of about 4.0 kb contained in pLIPE101 is shown in the accompanying Fig. 1.

The *Sal*I DNA fragment of about 4.0 kb of the plasmid pLIPE101 was cleaved with various restriction endonucleases, and each DNA fragment was subcloned into plasmid vector pUC19, followed by transformation of *E. coli* DH5 therewith. The transformants were cultivated in a tributyrin-containing LBG plate medium in the same manner as described above, and it was determined whether a clear zone was formed or not.

The restriction endonuclease maps of each DNA fragment are shown in the accompanying Fig. 2. As to *E. coli* DH5 transformed with the plasmid containing *Sal*I-*Bam*HI DNA fragment of about 2.8 kb (pLIPE111) and the strain transformed with the plasmid containing *Sal*I-*Bst*PI DNA fragment of about 2.6 kb (pLIPE121), there were observed the formation of a clear zone and esterase activity (measured by the convenient method). However, as to *E. coli* DH5 transformed with the plasmid containing *Sal*I-*Pvu*II DNA fragment of about 1.9 kb (pLIPE131), the plasmid containing *Sal*I-*Bst*PI DNA fragment of about 1.7 kb (pLIPE141), and the plasmid containing *Cla*I-*Sal*I DNA fragment of about 3.6 kb (pLIPE151), there was observed neither the formation of a clear zone nor esterase activity

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(measured by the convenient method).

It was found from the above results that the gene encoding an esterase originated from *Serratia marcescens* Sr41 was present in the *Sal*I-*Bst*PI DNA fragment of about 2.6 kb.

Example 3

Analysis of clone DNA:

[Determination of nucleotide sequence]

The recombinant plasmid pLIPE121 was treated with Kilobase Deletion Kit (manufactured by Takara Shuzo Co., Ltd.) to prepare various deletion plasmids. These plasmids thus obtained were subjected to annealing of a primer, synthesis of complementary chain with Klenow fragment of DNA polymerase [labelled with (α-^{32}P)dCTP (14.8 x 10^6 Bq/pmol, 74 x 10^4 Bq)] according to the dideoxy chain termination method of Sanger et al. [Proc. Natl. Acad. Sci. USA, Vol. 74, 5463 (1977)], and the nucleotide sequences thereof were determined based on the data of an electrophoresis with an 8% urea-modified polyacrylamide gel and autoradiography.

As a result, it has been found that the DNA sequence of the gene encoding an esterase originated from *Serratia marcescens* Sr41 is composed of 1839 base pairs from the initiation codon ATG to the codon GCC.

Besides, the amino acid sequence determined from the nucleotide sequence is shown in SEQ ID NO: 3. The N-terminal Met in this amino acid sequence was not found at the N-terminus of amino acid sequence of the purified enzyme product of the *Serratia*-origin esterase, which will be caused by

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removal of N-terminal methionine from the protein produced in the cells by methionine aminopeptidase present in the cells. Moreover, although *Serratia*-origin esterase is an extracellular enzyme, it has no signal peptide which is usually present at the N-terminus of secretory proteins.

Example 4

Serratia marcescens Sr41 (FERM BP-487) was incubated in a nutrient medium (glucose 0.5%, peptone 1.0%, meat extract 0.3%, yeast extract 1.0%, and sodium chloride 0.5%, pH 7.0, 3 ml) overnight, and the culture thus obtained (0.1 ml) was inoculated in a fresh medium (3 ml) of the same nutrient medium as above, which was incubated at 30°C for 3 hours, and after adding thereto NTG in a concentration of 0.1 mg/ml, was further incubated for additional 30 minutes. The culture broth was centrifuged, and the cells thus obtained were washed three times with a physiological saline solution (by centrifugation method). The cells thus obtained were suspended in a physiological saline solution and spread onto an LBG plate medium containing tributyrin 0.5%, glucose 2% and EDTA 2mM in an amount of 1 - 1000 cells/one plate. After incubating at 30°C for one day, the colony forming a large clear zone was collected to give *Serratia marcescens* M-1 strain (FERM P-12833). The thus-obtained mutant strain (one platinum loop) was inoculated to an esterase-producing medium containing ampicillin (500 μg/ml) and subjected to reciprocating shaking culture (shaking amplitude 7 cm, 120 r.p.m.) at 30°C for 20 hours. The culture broth was centrifuged to give a super-

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natant having an esterase activity of about 80 x 10^3 unit/ml (measured by the convenient method). This strain had about 2.5 times higher esterase productivity than the parent host strain *Serratia marcescens* Sr41.

In the same manner as described in Examples 1 to 3, the chromosomal DNA was isolated from the above *Serratia marcescens* M-1, and the sequence was analyzed. As a result, it had a nucleotide sequence as shown in SEQ ID NO: 2. As is clear therefrom, the sequence was the same as the DNA sequence of that obtained from *Serratia marcescens* Sr41 except that the 9th base was different, that is, it was composed of 1839 base pairs from the initiation codon ATG to the codon GCC. The amino acid sequence determined therefrom was the same as that deduced from SEQ ID NO: 1.

Example 5

Preparation of a strain having high esterase productivity:

The plasmid pLIPE111 was introduced into a restriction endonuclease-deficient strain, *Serratia marcescens* TT392, to give a transformed strain. The pLIPE111 plasmid DNA modified with *Serratia marcescens* was extracted from the cells of the transformed strain by alkaline lysis method. Then, *Serratia marcescens* Sr41 cells were transformed with the plasmid DNA obtained above by electroporation method to give a transformant *Serratia marcescens* TA5025 (FERM P-12607). The thus-obtained transformant (one platinum loop) was inoculated to an esterase-producing medium containing

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ampicillin (500 μg/ml) and subjected to reciprocating shaking culture (shaking amplitude 7 cm, 120 r.p.m.) at 30°C for 20 hours. The culture broth was centrifuged to give a supernatant having an esterase activity of about 1.9×10^5 unit/ml (measured by the convenient method). This strain had about 6 times higher esterase productivity than the host strain *Serratia marcescens* Sr41.

Example 6

Production of esterase:

An esterase-producing medium (20 liters) containing ampicillin (500 μg/ml) was charged into a 30 liter-volume jar fermenter and was sterilized. Previously, the above *Serratia marcescens* TA5025 was incubated in the same medium by reciprocating shaking culture at 30°C for 20 hours in the same manner as above. The pre-culture broth thus obtained (200 ml) was inoculated to the above medium and incubated under aeration at 30°C with stirring at 200 r.p.m., 0.5 vvm for 18 hours. The culture broth was centrifuged to remove the cells, and the supernatant (18 liters) was concentrated with a ultrafilter (AIL-1010, manufactured by Asahi Chemical Industry Co., Ltd.) to give a crude enzyme solution (2000 ml) which had an esterase activity of 1.7×10^6 unit/ml (measured by the convenient method).

Example 7

Production of esterase:

An esterase-producing medium (20 liters) was charged

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into a 30 liter-volume jar fermenter and was sterilized. Previously, *Serratia marcescens* M-1 was incubated in the same medium by reciprocating shaking culture at 30°C for 20 hours in the same manner as above. The pre-culture broth thus obtained (200 ml) was inoculated to the above medium and incubated under aeration at 30°C with stirring at 200 r.p.m., 0.5 vvm for 18 hours. The culture broth was centrifuged to remove the cells, and the supernatant (18 liters) was concentrated with a ultrafilter (AIL-1010, manufactured by Asahi Chemical Industry Co., Ltd.) to give a crude enzyme solution (2000 ml) which had an esterase activity of 7.9×10^5 unit/ml (measured by the convenient method).

Effects of the Invention

The mutant strain and the transformant containing a recombinant plasmid inserted with a gene encoding an esterase of the present invention have excellent esterase productivity, and the cultivation of the transformant can give the desired esterase in high purity on a large scale.

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SEQ ID NO:1:
LENGTH: 1839 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
STRAIN: Serratia marcescens Sr41

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ATG GGC ATC TTT AGC TAT AAG GAT CTG GAC GAA AAC GCG TCA AAG GCG    48
CTG TTT TCC GAC GCC TTG GCC ATC TCT ACC TAC GCT TAC CAC AAT ATC    96
GAT AAC GGC TTC GAT GAA GGC TAT CAC CAG ACC GGT TTC GGC CTC GGT   144
CTG CCG CTG ACG CTG ATC ACG GCG CTC ATC GGC AGC ACC CAG TCG CAG   192
GGC GGC CTG CCC GGT CTT CCC TGG AAC CCC GAC TCC GAA CAG GCC GCG   240
CAG GAC GCG GTG AAC AAT GCC GGC TGG TCA GTG ATC GAC GCC GCG CAA   288
TTG GGA TAC GCC GGC AAA ACC GAT GCG CGC GGC ACC TAC TAC GGC GAA   336
ACC GCC GGT TAC ACC ACC GCT CAG GCC GAG GTG CTG GGC AAA TAT GAC   384
AGC GAA GGC AAT CTC ACC GCC ATT GGC ATC TCA TTT CGC GGT ACC AGC   432
GGC CCG CGC GAG TCG CTG ATC GGC GAT ACC ATC GGC GAT GTG ATT AAC   480
GAT CTG TTG GCC GGG TTC GGG CCG AAA GCT ATG CGA CGC TAT ACG CTG   528
AAG GCC TTC GGC AAT TTG CTG GGG GAC GTG GCG AAA TTC GCG CAG GCC   576
CAC GGG CTG AGC GGC GAA GAC GTG GTG ATC AGC GGC CAC AGC CTC GGC   624
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TAC GAA GCC GGC GGC AAG GTG ATC AAC ATC GGC TAT GAG AAC GAT CCG   768
GTG TTT CGC GCG CTC GAC GGC ACC TCG CTG ACC CTG CCG TCA TTG GGC   816
GTT CAC GAT GCG CCG CAT ACC TCC GCC ACC AAC AAT ATC GTC AAC TTC   864
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CTC AAC ATT CCG ACC TGG CTA TCC CAC CTG CCG TTC TTC TAT CAG GAT   960
GGT CTG ATG CGG GTG CTG AAC TCC GAG TTT TAT TCG CTG ACC GAC AAA  1008
GAC TCG ACC ATC ATC GTC TCC AAC CTG TCG AAC GTC ACG CGC GGC AGT  1056
ACC TGG GTG GAA GAT CTG AAC CGC AAC GCG GAA ACG CAC AGC GGG CCG  1104
ACG TTT ATC ATC GGC AGC GAC GGC AAT GAT TTG ATC AAG GGC GGC AAA  1152
GGC AAC GAC TAT CTC GAG GGC CGC GAC GGT GAC GAT ATC TTC CGC GAC  1200
GCC GGC GGC TAT AAC CTG ATC GCC GGC GGC AAA GGC CAC AAT ATC TTC  1248
GAT ACC CAG CAG GCG TTG AAA AAC ACC GAG GTC GCC TAC GAC GGC AAT  1296
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GAG GTG GAT CAT CAG GTG ACC GCC GCC GGA TTG AAA TCG GAT TCG GGC  1440
CTC AAA GCC TAT GCG GCC GCC ACC ACC GGC GGC GAC GGC GAT GAC GTC  1488
CTG CAG GCT CGC AGC CAC GAC GCC TGG CTG TTC GGC AAC GCC GGC AAC  1536
GAC ACG CTT ATC GGC CAT GCC GGC GGC AAC CTG ACC TTC GTC GGC GGC  1584
AGC GGC GAT GAC ATC CTG AAG GGG GTC GGC AAC GGC AAT ACC TTC CTG  1632
TTC AGC GGC GAT TTC GGC CGC GAC CAG CTG TAT GGC TTC AAC GCC ACC  1680
GAC AAA CTG GTA TTT ATC GGC ACC GAG GGC GCC AGC GGG AAT ATC CGC  1728
GAC TAT GCC ACG CAG CAA AAC GAC GAT CTG GTG CTG GCC TTC GGC CAC  1776
AGC CAG GTC ACG CTG ATC GGC GTC TCG CTC GAT CAT TTC AAC CCC GAT  1824
CAG GTG GTG TTG GCC                                             1839
```

SEQ ID NO:2:
LENGTH: 1839 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
STRAIN: Serratia marcescens M-1

```
ATG GGC ATT TTT AGC TAT AAG GAT CTG GAC GAA AAC GCG TCA AAG GCG    48
CTG TTT TCC GAC GCC TTG GCC ATC TCT ACC TAC GCT TAC CAC AAT ATC    96
GAT AAC GGC TTC GAT GAA GGC TAT CAC CAG ACC GGT TTC GGC CTC GGT   144
CTG CCG CTG ACG CTG ATC ACG GCG CTC ATC GGC AGC ACC CAG TCG CAG   192
GGC GGC CTG CCC GGT CTT CCC TGG AAC CCC GAC TCC GAA CAG GCC GCG   240
CAG GAC GCG GTG AAC AAT GCC GGC TGG TCA GTG ATC GAC GCC GCG CAA   288
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TAC GAA GCC GGC GGC AAG GTG ATC AAC ATC GGC TAT GAG AAC GAT CCG   768
GTG TTT CGC GCG CTC GAC GGC ACC TCG CTG ACC CTG CCG TCA TTG GGC   816
GTT CAC GAT GCG CCG CAT ACC TCC GCC ACC AAC AAT ATC GTC AAC TTC   864
AAC GAC CAC TAC GCG TCG GAC GCC TGG AAT CTG TTG CCG TTC TCC ATT   912
CTC AAC ATT CCG ACC TGG CTA TCC CAC CTG CCG TTC TTC TAT CAG GAT   960
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ACG TTT ATC ATC GGC AGC GAC GGC AAT GAT TTG ATC AAG GGC GGC AAA  1152
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GCC GGC GGC TAT AAC CTG ATC GCC GGC GGC AAA GGC CAC AAT ATC TTC  1248
GAT ACC CAG CAG GCG TTG AAA AAC ACC GAG GTC GCC TAC GAC GGC AAT  1296
ACG CTT TAC CTG CGC GAC GCC AAA GGC GGC ATT ACG CTG GCG GAC GAC  1344
ATC AGC ACC CTG CGC AGC AAA GAA ACC TCC TGG CTG ATT TTC AGC AAA  1392
GAG GTG GAT CAT CAG GTG ACC GCC GCC GGA TTG AAA TCG GAT TCG GGC  1440
CTC AAA GCC TAT GCG GCC GCC ACC ACC GGC GGC GAC GGC GAT GAC GTC  1488
CTG CAG GCT CGC AGC CAC GAC GCC TGG CTG TTC GGC AAC GCC GGC AAC  1536
GAC ACG CTT ATC GGC CAT GCC GGC GGC AAC CTG ACC TTC GTC GGC GGC  1584
AGC GGC GAT GAC ATC CTG AAG GGG GTC GGC AAC GGC AAT ACC TTC CTG  1632
TTC AGC GGC GAT TTC GGC CGC GAC CAG CTG TAT GGC TTC AAC GCC ACC  1680
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AGC CAG GTC ACG CTG ATC GGC GTC TCG CTC GAT CAT TTC AAC CCC GAT  1824
CAG GTG GTG TTG GCC                                             1839
```

SEQ ID NO:3:

LENGTH: 613 amino acids

TYPE: amino acid

TOPOLOGY:linear

MOLECULE TYPE:peptide

```
Met Gly Ile Phe Ser Tyr Lys Asp Leu Asp Glu Asn Ala Ser Lys Ala
 1               5                  10                  15
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Asp Asn Gly Phe Asp Glu Gly Tyr His Gln Thr Gly Phe Gly Leu Gly
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Leu Pro Leu Thr Leu Ile Thr Ala Leu Ile Gly Ser Thr Gln Ser Gln
50 55 60

Gly Gly Leu Pro Gly Leu Pro Trp Asn Pro Asp Ser Glu Gln Ala Ala
65 70 75 80

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

Gly Pro Arg Glu Ser Leu Ile Gly Asp Thr Ile Gly Asp Val Ile Asn
145 150 155 160

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Lys Ala Phe Gly Asn Leu Leu Gly Asp Val Ala Lys Phe Ala Gln Ala
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His Gly Leu Ser Gly Glu Asp Val Val Ile Ser Gly His Ser Leu Gly
195 200 205

Gly Leu Ala Val Asn Ser Met Ala Ala Gln Ser Asp Ala Thr Trp Gly
210 215 220

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290 295 300

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355 360 365

Thr Phe Ile Ile Gly Ser Asp Gly Asn Asp Leu Ile Lys Gly Gly Lys
370 375 380

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385 390 395 400

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405 410 415

Asp Thr Gln Gln Ala Leu Lys Asn Thr Glu Val Ala Tyr Asp Gly Asn
420 425 430

Thr Leu Tyr Leu Arg Asp Ala Lys Gly Gly Ile Thr Leu Ala Asp Asp
435 440 445

Ile Ser Thr Leu Arg Ser Lys Glu Thr Ser Trp Leu Ile Phe Ser Lys
450 455 460

Glu Val Asp His Gln Val Thr Ala Ala Gly Leu Lys Ser Asp Ser Gly
465 470 475 480

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

Leu Gln Ala Arg Ser His Asp Ala Trp Leu Phe Gly Asn Ala Gly Asn
500 505 510

Asp Thr Leu Ile Gly His Ala Gly Gly Asn Leu Thr Phe Val Gly Gly
515 520 525

Ser Gly Asp Asp Ile Leu Lys Gly Val Gly Asn Gly Asn Thr Phe Leu
530 535 540

Phe Ser Gly Asp Phe Gly Arg Asp Gln Leu Tyr Gly Phe Asn Ala Thr
545 550 555 560

Asp Lys Leu Val Phe Ile Gly Thr Glu Gly Ala Ser Gly Asn Ile Arg
565 570 575

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580 585 590

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Ser Gln Val Thr Leu Ile Gly Val Ser Leu Asp His Phe Asn Pro Asp
595 600 605

Gln Val Val Leu Ala
610

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What is claimed is:

1. A gene encoding an esterase originated from a microorganism of the genus *Serratia*.

2. The gene as claimed in claim 1 which has a DNA sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2.

3. A recombinant plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*.

4. A transformant which contains a recombinant plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*.

5. The transformant as claimed in claim 4, wherein the host microorganism is a microorganism of the genus *Serratia* or a microorganism of the genus *Escherichia*.

6. A transformant *Serratia marcescens* TA5025 (FERM BP-4067).

7. A method for the production of an esterase, which comprises cultivating a microorganism transformed with a recombinant plasmid (said plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*), and collecting the produced esterase outside and inside the cells.

8. A microorganism of the genus *Serratia* which contains a gene having a DNA sequence shown in SEQ ID NO: 2.

9. A method for the production of an esterase, which comprises cultivating the microorganism as set forth in

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claim 8 in a medium, and collecting the esterase produced outside and inside the cells.

10. A mutant strain *Serratia marcescens* M-1 (FERM BP-4068).

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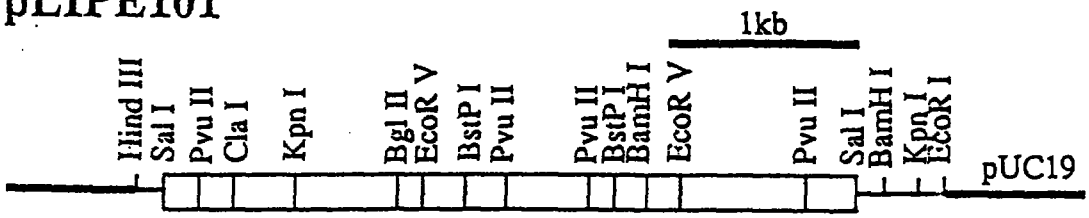
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Abstract of the Disclosure:

A gene encoding an esterase originated from a microorganism of the genus *Serratia*, a mutant strain having high esterase productivity containing said gene, a recombinant plasmid comprising said gene inserted into a vector plasmid, a novel microorganism transformed with said recombinant plasmid, and a method for the production of an esterase which comprises cultivating the mutant strain or the novel microorganism as set forth above in a medium and collecting the produced esterase outside and inside the cells. Said mutant strain having high esterase productivity and new transformed microorganism have excellent esterase productivity and can produce the desired esterase in high purity on a large scale.

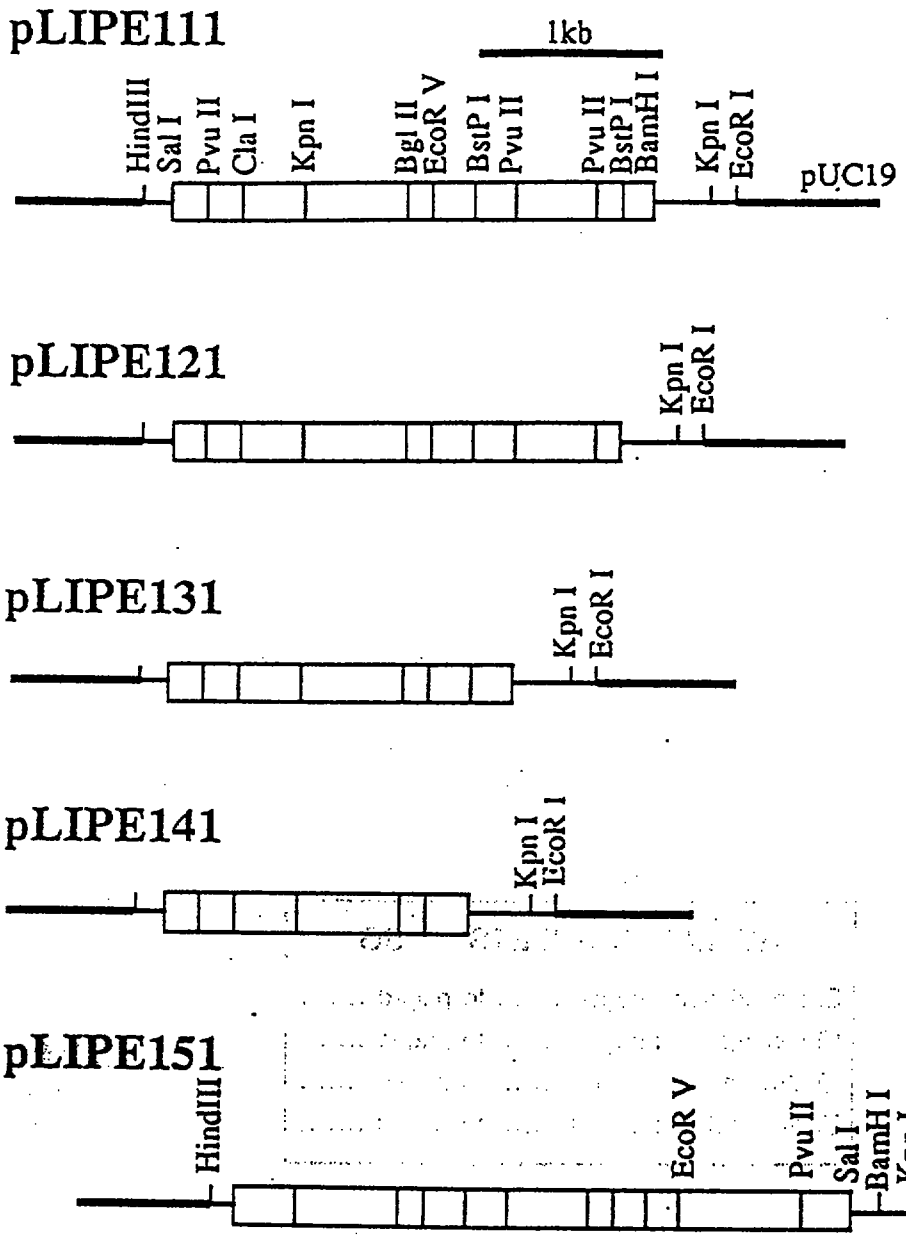
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Fig. 1



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Fig. 2



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☒ Description : paged 1 to paged 27
☒ Claim(s) : paged 28 to paged 29
☐ Drawing(s) : paged 1 to paged 2
☒ Abstract : paged 30 to paged 1
DATE OF RECEIPT 25/2/97

GENE CODING FOR ESTERASE AND NOVEL MICROORGANISM CONTAINING SAID GENE

This invention relates to a gene coding for an esterase, a recombinant plasmid containing said gene, a novel microorganism transformed with the recombinant plasmid, and a process for producing an esterase by cultivating the microorganism.

Prior Art

Recently, it has frequently been tried to utilize enzymes such as esterase in hydrolysis reaction. For such a purpose, there are known various esterases, for example, esterases originated from animals (e.g. pig liver, pig pancreas, etc.), and esterases originated from microorganisms, such as *Arthrobacter globiformis*, *Geotrichum candidum*, *Candida cylindracea*, *Pseudomonas fluorescens*, etc.

Further, it is known that an esterase is obtained by utilizing a recombinant DNA technology in microorganism, such as *Rhizopus delemar* (cf. Japanese Patent First Publication (Kokai) No. 87175/1991), *Pseudomonas fragi* (cf. Biochemical and Biophysical Research Communications, Vol. 141, 185-190 (1986)).

However, the known esterases or known methods for producing esterase have various problems. For instance, animal-origin esterases are very expensive. Besides, above-

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mentioned microorganisms-origin esterases of the genus *Arthrobacter*, *Geotrichum*, *Candida*, *Pseudomonas*, or *Rhizopus* had problems such that they were disadvantageous in activity, stability, or specificity, or that the esterase-producing microorganism had no high productivity.

As a result of various investigations, the present inventors found that the desired esterase can be produced favorably by utilizing recombinant DNA technology in a microorganism of the genus *Serratia*. That is, the present inventors have succeeded to obtain a gene coding for an esterase from a microorganism of the genus *Serratia*, and have found that a transformed microorganism having a significantly increased esterase productivity can be obtained by introducing the recombinant plasmid containing the said gene into a host microorganism, and further have found that the desired esterase can be produced by using said transformed microorganism on an industrial scale.

Summary Description of the Invention

An object of the invention is to provide a gene coding for an esterase originated from a microorganism of the genus *Serratia*. Another object of the invention is to provide a mutant strain which has a high productivity of esterase. Another object of the invention is to provide a recombinant plasmid produced by inserting said gene into a vector plasmid. A further object of the invention is to provide a transformed microorganism containing said recombinant plasmid. A still further object of the invention is to provide a method for the

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production of an esterase by cultivating said transformed microorganism and collecting the produced esterase outside and inside the cells of the microorganism.

Brief Description of Drawing

Fig. 1 shows a restriction endonuclease map of the plasmid DNA pLIPE101 isolated from the recombinant cells in Example 2.

Fig. 2 shows restriction endonuclease maps of each of plasmid DNAs, pLIPE111, pLIPE121, pLIPE131, pLIPE141, and pLIPE151, which were produced by deleting the plasmid DNA pLIPE101 using a restriction endonuclease and DNA ligase.

Detailed Description of the Invention

The gene encoding an esterase originated from a microorganism of the genus *Serratia* of this invention is a double stranded DNA of 1839 base pairs having an open reading frame, specifically DNAs having the nucleotide sequences shown in SEQ ID NO:1 and SEQ ID NO:2 hereinafter.

The donor microorganisms for such a gene encoding an esterase include any microorganisms belonging to the genus *Serratia* which has an esterase productivity, for example, *Serratia marcescens* Sr41 (FERM BP-487), *Serratia liquefaciens* ATCC 27592, *Serratia marcescens* ATCC 13880, *Serratia marcescens* ATCC 14764, *Serratia marcescens* ATCC 19180, *Serratia marcescens* ATCC 21074, *Serratia marcescens* ATCC 27117, *Serratia marcescens* ATCC 21212, etc.

The donor microorganism includes also mutant strains derived from wild strains, such as various amino acid-

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requiring strains, various nucleic acid-requiring strains, various vitamin-requiring strains, and the like, for example, *Serratia marcescens* M-1 (FERM BP-4068). Said *Serratia marcescens* M-1 has more than two fold higher esterase productivity than the parent strain *Serratia marcescens* Sr41 and contains an esterase gene coded by the DNA sequence of SEQ ID NO:2. The mutant strain M-1 can be obtained by treating the parent *Serratia marcescens* Sr41 (FERM BP-487) with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) according to a method of Aderburg et al. [Biochemical and Biophysical Research Communications, Vol. 18, 788 (1965)], cultivating the thus treated microorganism on an agar medium containing emulsified triglyceride, and collecting the colony around which the largest clear zone is formed. In the same manner, another mutant strain *Serratia marcescens* G205 having 1.5 fold or higher esterase-productivity can be obtained from the parent strain *Serratia marcescens* Sr41.

The vector plasmid to be inserted with the gene encoding an esterase includes any plasmids which are replicable in transformed cells, but is preferably plasmids which have a copy number of 1 to several thousands and contain a resistant marker against antibiotics such as ampicillin, kanamycin, chloramphenicol, and further contain an appropriate promoter such as *lac*, *tac*, or *trp*. Moreover, the vector plasmids may further contain a plasmid stabilizing gene such as *par* and *par*B.

These vector plasmids include, for example, pLG339

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[Gene, Vol. 18, 332 (1982)], pBR322 [Gene, Vol. 2, 95 (1977)], pUC18 [Gene, Vol. 33, 103 (1985)], pUC19 [Gene, Vol. 33, 103 (1985], pHSG298 [Gene, Vol. 61, 63 (1987)], pHSG299 [Gene, Vol. 61, 63 (1987)], and the like.

The above vector plasmids are commercially available or can be obtained from microbial cells containing these plasmids by a conventional method, for example, by "cleared lysate method" (cf. Yasuyuki Takagi, "Procedure for Experiment in Genetic Engineering", page 125, published by Kodansha, 1980), or by "alkaline lysis method" [cf. Maniatis et al., "Molecular Cloning", page 368, Cold Spring Harbor Laboratory, U.S.A. (1982)].

The host microorganisms (both for the recombination of plasmids and for the expression of the desired esterase), include any microorganisms which can be transformed with the recombinant plasmid and can replicate the plasmid therein, and can express the gene on the plasmid and can produce a functional protein. These host microorganisms are, for example, microorganisms belonging to the genus *Serratia* or the genus *Escherichia*, specifically *Serratia marcescens* Sr41 and various mutant strains derived therefrom, for example, *Serratia marcescens* M-1, *Serratia marcescens* TT392 [cf. Journal of Bacteriology, Vol. 161, 1 (1985)], or *Escherichia coli* K12 DH5 [cf. Maniatis et al., "Molecular Cloning", 2nd volume, A10, Cold Spring Harbor Laboratory, U.S.A. (1989)].

The chromosomal DNA containing the gene encoding an esterase can easily be obtained from microorganisms containing

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said gene by a conventional method, for example, by treating the microbial cells with a lysozyme and further with a surfactant (e.g. sodium lauryl sulfate, sodium N-lauroyl sarcosinate, etc.), extracting the cells thus treated with an organic solvent (e.g. phenol, chloroform, diethyl ether, etc.) to remove proteins, and then precipitating the DNA with ethanol [cf. Journal of Molecular Biology, Vol. 3, 208 (1961), and Biochimica et Biophysica Acta, Vol. 72, 619 (1963)].

The recombinant plasmid comprising the chromosomal DNA containing a gene encoding esterase and the vector plasmid can easily be prepared by a conventional method, for example, by cleaving the chromosomal DNA and the plasmid DNA with an appropriate restriction endonuclease (e.g. *Eco*RI, *Bam*HI, *Hind*III, *Sal*I, etc.) and then treating the resultant with DNA ligase (e.g. T4 DNA ligase, *E. coli* DNA ligase, etc.), if required, after treating the resultant with a terminal transferase or DNA polymerase [cf. Methods in Enzymology, Vol. 68, 41 (1979), and Yasuyuki Takagi, "Procedure for Experiment in Genetic Engineering", page 135, published by Kodansha, 1980].

Selection of the desired recombinant plasmid containing the esterase gene from a mixture of recombinant plasmids obtained by the above procedure can be done as follows.

Microbial cells being restriction endonuclease deficient and having no esterase activity (e.g. *Escherichia coli* K12 DH5) are transformed with the above mixture of recom-

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binant plasmids, and the thus-transformed cells are spread onto an agar medium containing an emulsified triglyceride, for example, a nutrient agar medium containing tributyrin emulsified with polyoxyethylene cetyl alcohol ether (Brij 58) and also containing a prescribed concentration of an antibiotic. After incubation at 30 to 37°C for 1 to 2 days, the colony of a transformant around which a large clear zone is formed is isolated.

The above introduction of the recombinant plasmid into the host microorganism is carried out by a conventional method, for example, by treating the host cells with an aqueous calcium chloride solution at a low temperature to increase the membrane permeability of the cells and then introducing the recombinant plasmid into the host cells [cf. Journal of Molecular Biology, Vol. 53, 159 (1970)], or by an electroporation method.

Then, the plasmid DNA is extracted from the transformant by "alkaline lysis method" to give a recombinant plasmid containing the gene encoding an esterase.

In order to make the transformation efficient, the recombinant plasmid thus obtained is modified in a microorganism which is restriction endonuclease deficient and is the same species of the host microorganism to be used for expressing the esterase. That is, when the host microorganism to be used is *Serratia marcescens* Sr41, the recombinant plasmid obtained above is introduced into *Serratia marcescens* TT392, which is restriction enzyme deficient strain. The

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recombinant plasmid thus modified is isolated from the microorganism.

The modified recombinant plasmid is then introduced into a host microorganism to obtain the desired transformant suitable for the production of the desired esterase.

The introduction of the recombinant plasmid into the host microorganism can easily be done by the method of Takagi & Kizumi [cf. Journal of Bacteriology, Vol. 161, 1 (1985)]. The host microorganisms to be used for said transformation include the above-mentioned microorganisms but are preferably strains having high esterase productivity. The desired transformant can be obtained by isolating the colony expressing antibitotics resistance.

The transformed microorganisms obtained by the above-mentioned method are, for example, *Serratia marcescens* TA5025 (FERM BP-4067) which is obtained by introducing a recombinant plasmid consisting of pUC19 and the 2.8 kb *Sal*I-*Bam*HI DNA fragment containing an esterase gene into *Serratia marcescens* Sr41; *Serratia marcescens* TBE101 which is obtained by introducing a recombinant plasmid consisting of pBR322 and the 4.0 kb *Sal*I DNA fragment containing an esterase gene into *Serratia marcescens* Sr41. These transformed strains all have the same morphological characteristics as those of the host microorganism *Serratia marcescens* Sr41.

The production of esterase with the transformed microorganism obtained above is carried out by cultivating the microorganism in a medium and collecting the esterase outside

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and inside the cells of the microorganism.

The medium used for the production of esterase includes any conventional medium wherein the microorganism can grow. Suitable medium contains a carbon source such as saccharides (e.g. glucose, sucrose, molasses, etc.), organic acids (e.g. fumaric acid, citric acid, etc.), alcohols (e.g. glycerol, etc.), or amino acids (e.g. alanine, glutamine, asparagine, etc.) and a nitrogen source such as inorganic ammonium salts (e.g. ammonium sulfate, ammonium chloride, etc.), urea, peptone, corn steep liquor, yeast extract, casein hydrolysate, and the like. The carbon source is usually contained in an amount of 1 to 15 % by weight based on the whole weight of the medium, and the nitrogen source is usually contained in an amount of 0.1 to 2.0 % by weight based on the whole weight of the medium. The medium may optionally contain further an appropriate amount of an inorganic salt (e.g. phosphate, magnesium salt, potassium salt, calcium salt, etc.) and/or a metallic ion (e.g. iron, manganese, copper, zinc, etc.). In case of a synthetic medium, it may further contain vitamins or amino acids, and further, inducers for esterase production (e.g. vegetable oils, surfactants, etc.), defoaming agents, antibiotics which are suitable for stabilizing the recombinant plasmid in microorganisms. The medium is preferably adjusted to a pH 5 to 8.

The cultivation of the transformed microorganism is carried out by a conventional method. For example, the microorganism is inoculated into a medium and is cultivated by

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shaking culture, aeration culture, standing culture, and the like. The cultivation conditions may vary depending on the kinds of the medium and cultivation methods, but may be any conditions suitable for growth of the microorganism, usually at the initial pH 5 - 8, at 20 to 40°C, for 1 to 2 days.

The esterase produced outside and inside the cultivated cells is collected by a conventional method. For example, the esterase contained in the medium is collected by means of salting out with an inorganic salt, precipitation with an organic solvent, absorption or desorption with ion exchange resin and various column chromatography, gel filtration, use of protein-precipitating agent, or a combination of these methods. The esterase accumulated within the cells is obtained by firstly disrupting the cells by a physical method such as frictional disrupting device (Dyno Mill) or a chemical means such as treatment with lysozyme, and then collecting the esterase in the cell extract by the above-mentioned method.

The gene encoding the esterase of this invention is not limited to those of the DNA sequences disclosed specifically in the present specification but includes any gene having a DNA sequence obtained by modifications in the sequence such as insertion, deletion, substitution. That is, the esterase gene may artificially be modified directly in a test tube by using a synthetic mutated DNA primer designed on the basis of the DNA sequence of the esterase gene specifically disclosed herein, or by using a chemical mutating agent

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such as formic acid, hydrazine sulfite. Further, a mutant gene may be obtained by treating an esterase producing strain with NTG or UV.

Examples

The present invention is illustrated by the following Examples but should not be construed to be limited thereto.

In the Examples, the esterase activity was measured by a convenient method (using Lipase Kit S, manufactured by Dainippon Pharmaceutical Co., Ltd., Japan). It was also measured by another method comprising subjecting the product to enzymatic reaction in olive oil (as a substrate) at pH 8.0, 37°C for 20 minutes, and then measuring the amount of formed fatty acid. The unit of esterase activity was expressed as μmols of fatty acid formed per minute. Besides, the medium used in Examples has the following formulation, wherein "%" is W/V% unless specified otherwise.

LB medium: 1.0% of Bactotryptone (manufactured by Difco), 0.5% of Bacto Yeast Extract (manufactured by Difco), and 0.5% of sodium chloride.

LBG plate medium: 1.0% of Bactotryptone (manufactured by Difco), 0.5% of Bacto Yeast Extract (manufactured by Difco), 0.5% sodium chloride, and 1.0% of Gellan Gum (manufactured by Wako Pure Chemical Industries, Ltd., Japan).

Tributyrin-containing LBG plate medium: LBG plate medium containing 0.5 v/v% of tributyrin, 0.5% of polyoxyethylene cetyl alcohol ether, and 0.005% of ampicillin.

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Esterase producing medium: 1.0% of dextrin, 2.0% of meast, 0.2% of ammonium sulfate, 0.1% of potassium dihydrogen phosphate, 0.05% of magnesium sulfate heptahydrate, 0.01% of calcium chloride dihydrate, 0.001% of ferrous sulfate hepta hydrate, 0.5% of Tween 80, and 0.1% of colorin.

Example 1

(1) Preparation of chromosomal DNA containing a gene encoding an esterase:

Serratia marcescens Sr41 (FERM BP-487) was subjected to aerobic shaking culture in LB medium (200 ml) at 30°C overnight, and then the cells were collected by centrifugation. The cells were suspended in 0.9% aqueous sodium chloride solution (200 ml) once and then collected by centrifugation in order to wash them. The cells thus washed were suspended in an aqueous solution of 50mM Tris-HCl-50mM disodium ethylenediamine tetraacetate (pH 7.5, 200 ml) containing 200 mg of lysozyme, and the mixture was allowed to stand at room temperature for one hour.

To the mixture was added sodium lauryl sulfate in a concentration of 0.5%, and thereto was further added Protenase K, and the mixture was mildly shaken at 50°C for 3 hours to lyse the cells. The mixture was extracted twice with an equal volume of phenol saturated with an aqueous solution of 10 mM Tris-HCl-1 mM disodium ethylenediamine tetraacetate (pH 8.0) (hereinafter, referred to as "TE"), and further extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform, and then the resultant aqueous phase

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was subjected to precipitation with ethanol. The precipitate was dissolved in TE to prepare a TE solution containing 2.5 mg of the chromosomal DNA containing a gene encoding an esterase.

(2) Preparation of a recombinant plasmid DNA:

The chromosomal DNA prepared in the above (1) (20 μg) was completely digested with restriction endonuclease *SalI*, which was extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform. To the mixture was added 0.5 volume of 7.5 M ammonium acetate, and the DNA was recovered by centrifugation with two times volume of ethanol.

DNA (0.5 μg) of plasmid vector pUC19, which was completely digested with the same restriction enzyme as above, was dephosphated by treating with alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd., Japan, 0.4 unit) at 56°C for one hour, and the resultant was extracted twice with a mixture of equal volume of TE-saturated phenol and chloroform, and thereto was added 0.5 volume of 7.5M ammonium acetate, and then the vector plasmid DNA was recovered by precipitating with two volumes of ethanol. The dephosphated plasmid vector thus obtained was mixed with the chromosomal DNA obtained above (3 μg) and ligated with a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.) at 4°C for 16 hours to give the recombinant plasmid DNA.

(3) Transformation with the recombinant plasmid and preparation of colony bank with *E. coli* host:

The cells of *E. coli* DH5 were treated by the method

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of Hanahan [cf. Journal of Molecular Biology, Vol. 166, 557 (1983)] and thereto was added the reaction mixture containing a plasmid DNA obtained in the above (2), by which the transformation was effected. The cells thus treated were spread onto LBG plate medium containing ampicillin (50 μg/ml), and they were incubated at 37°C overnight to give the transformant (about 20,000 strains) containing recombinant plasmids inserted with fragments of the chromosomal DNA of *Serratia marcescens* Sr41.

(4) Isolation and identification of a transformant strain containing a gene encoding an esterase:

When an esterase producing strain is inoculated to a tributyrin-containing LBG plate medium, the esterase produced in the medium decomposes tributyrin to give fatty acids, by which the triglyceride emulsion around the colony is modified to form a circular clear zone around the colonies. By utilizing this phenomenon, the screening of transformants was effected.

The transformants (about 20,000 strains) obtained in the above (3) were inoculated to a tributyrin-containing LBG plate medium, which was incubated at 37°C overnight, by which one strain forming a clear zone was isolated from the DNA bank of *SalI*.

The formation of this clear zone will be owing to the activity of esterase, because when *Serratia marcescens* Sr41 was inoculated to a tributyrin-containing LBG plate medium and incubated at 37°C overnight, the formation of a

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clear zone was observed, but when the untransformed *E. coli* DH5 and the *E. Coli* DH5 carrying a plasmid vector pUC19 were inoculated to a tributyrin-containing LBG plate medium and incubated at 37°C overnight, no formation of a clear zone was observed.

Besides, the transformant being capable of formation of a clear zone was cultivated in a LB medium (60 ml) containing ampicillin (200 μg/ml) by aerobic shaking culture at 37°C overnight, and the cells were collected and suspended in a 200 mM Tris-HCl buffer solution (pH 8.0, 20 ml). After disruption of the cells by sonication, the esterase activity of the mixture was measured by the convenient method. As a result, there was observed 1.5×10^3 units of esterase activity.

Moreover, the mixture obtained above was subjected to an electrophoresis with SDS polyacrylamide gel. As a result, the product showed a new band, which was not shown in the product from *E. coli* DH5 containing only vector plasmid, at the same position as that in the partially purified standard esterase obtained from the supernatant of the culture of *Serratia marcescens* Sr41.

Example 2

Analysis of plasmid:

A plasmid DNA was prepared from the cells of the transformant obtained in Example 1-(4) by a conventional method [cf. Maniatis et al., "Molecular Cloning", page 368, Cold Spring Harbor Laboratory, U.S.A. (1982)], cleaved with

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various restriction endonucleases and then subjected to an agarose gel electrophoresis. As a result, it was confirmed that this plasmid (hereinafter, referred to as "pLIPE101") contained a *Sal*I DNA fragment of about 4.0 kb.

The restriction endonuclease map of the recombinant DNA fragment of about 4.0 kb contained in pLIPE101 is shown in the accompanying Fig. 1.

The *Sal*I DNA fragment of about 4.0 kb of the plasmid pLIPE101 was cleaved with various restriction endonucleases, and each DNA fragment was subcloned into plasmid vector pUC19, followed by transformation of *E. coli* DH5 therewith. The transformants were cultivated in a tributyrin-containing LBG plate medium in the same manner as described above, and it was determined whether a clear zone was formed or not.

The restriction endonuclease maps of each DNA fragment are shown in the accompanying Fig. 2. As to *E. coli* DH5 transformed with the plasmid containing *Sal*I-*Bam*HI DNA fragment of about 2.8 kb (pLIPE111) and the strain transformed with the plasmid containing *Sal*I-*Bst*PI DNA fragment of about 2.6 kb (pLIPE121), there were observed the formation of a clear zone and esterase activity (measured by the convenient method). However, as to *E. coli* DH5 transformed with the plasmid containing *Sal*I-*Pvu*II DNA fragment of about 1.9 kb (pLIPE131), the plasmid containing *Sal*I-*Bst*PI DNA fragment of about 1.7 kb (pLIPE141), and the plasmid containing *Cla*I-*Sal*I DNA fragment of about 3.6 kb (pLIPE151), there was observed neither the formation of a clear zone nor esterase activity

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(measured by the convenient method).

It was found from the above results that the gene encoding an esterase originated from *Serratia marcescens* Sr41 was present in the *SalI-BstPI* DNA fragment of about 2.6 kb.

Example 3

Analysis of clone DNA:

[Determination of nucleotide sequence]

The recombinant plasmid pLIPE121 was treated with Kilobase Deletion Kit (manufactured by Takara Shuzo Co., Ltd.) to prepare various deletion plasmids. These plasmids thus obtained were subjected to annealing of a primer, synthesis of complementary chain with Klenow fragment of DNA polymerase [labelled with (α-^{32}P)dCTP (14.8 x 10^6 Bq/pmol, 74 x 10^4 Bq)] according to the dideoxy chain termination method of Sanger et al. [Proc. Natl. Acad. Sci. USA, Vol. 74, 5463 (1977)], and the nucleotide sequences thereof were determined based on the data of an electrophoresis with an 8% urea-modified polyacrylamide gel and autoradiography.

As a result, it has been found that the DNA sequence of the gene encoding an esterase originated from *Serratia marcescens* Sr41 is composed of 1839 base pairs from the initiation codon ATG to the codon GCC.

Besides, the amino acid sequence determined from the nucleotide sequence is shown in SEQ ID NO: 3. The N-terminal Met in this amino acid sequence was not found at the N-terminus of amino acid sequence of the purified enzyme product of the *Serratia*-origin esterase, which will be caused by

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removal of N-terminal methionine from the protein produced in the cells by methionine aminopeptidase present in the cells. Moreover, although *Serratia*-origin esterase is an extracellular enzyme, it has no signal peptide which is usually present at the N-terminus of secretory proteins.

Example 4

Serratia marcescens Sr41 (FERM BP-487) was incubated in a nutrient medium (glucose 0.5%, peptone 1.0%, meat extract 0.3%, yeast extract 1.0%, and sodium chloride 0.5%, pH 7.0, 3 ml) overnight, and the culture thus obtained (0.1 ml) was inoculated in a fresh medium (3 ml) of the same nutrient medium as above, which was incubated at 30°C for 3 hours, and after adding thereto NTG in a concentration of 0.1 mg/ml, was further incubated for additional 30 minutes. The culture broth was centrifuged, and the cells thus obtained were washed three times with a physiological saline solution (by centrifugation method). The cells thus obtained were suspended in a physiological saline solution and spread onto an LBG plate medium containing tributyrin 0.5%, glucose 2% and EDTA 2mM in an amount of 1 - 1000 cells/one plate. After incubating at 30°C for one day, the colony forming a large clear zone was collected to give *Serratia marcescens* M-1 strain (FERM P-12833). The thus-obtained mutant strain (one platinum loop) was inoculated to an esterase-producing medium containing ampicillin (500 μg/ml) and subjected to reciprocating shaking culture (shaking amplitude 7 cm, 120 r.p.m.) at 30°C for 20 hours. The culture broth was centrifuged to give a super-

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natant having an esterase activity of about 80 x 10^3 unit/ml (measured by the convenient method). This strain had about 2.5 times higher esterase productivity than the parent host strain *Serratia marcescens* Sr41.

In the same manner as described in Examples 1 to 3, the chromosomal DNA was isolated from the above *Serratia marcescens* M-1, and the sequence was analyzed. As a result, it had a nucleotide sequence as shown in SEQ ID NO: 2. As is clear therefrom, the sequence was the same as the DNA sequence of that obtained from *Serratia marcescens* Sr41 except that the 9th base was different, that is, it was composed of 1839 base pairs from the initiation codon ATG to the codon GCC. The amino acid sequence determined therefrom was the same as that deduced from SEQ ID NO: 1.

Example 5

Preparation of a strain having high esterase productivity:

The plasmid pLIPE111 was introduced into a restriction endonuclease-deficient strain, *Serratia marcescens* TT392, to give a transformed strain. The pLIPE111 plasmid DNA modified with *Serratia marcescens* was extracted from the cells of the transformed strain by alkaline lysis method. Then, *Serratia marcescens* Sr41 cells were transformed with the plasmid DNA obtained above by electroporation method to give a transformant *Serratia marcescens* TA5025 (FERM P-12607). The thus-obtained transformant (one platinum loop) was inoculated to an esterase-producing medium containing

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ampicillin (500 µg/ml) and subjected to reciprocating shaking culture (shaking amplitude 7 cm, 120 r.p.m.) at 30°C for 20 hours. The culture broth was centrifuged to give a supernatant having an esterase activity of about 1.9×10^5 unit/ml (measured by the convenient method). This strain had about 6 times higher esterase productivity than the host strain *Serratia marcescens* Sr41.

Example 6

Production of esterase:

An esterase-producing medium (20 liters) containing ampicillin (500 µg/ml) was charged into a 30 liter-volume jar fermenter and was sterilized. Previously, the above *Serratia marcescens* TA5025 was incubated in the same medium by reciprocating shaking culture at 30°C for 20 hours in the same manner as above. The pre-culture broth thus obtained (200 ml) was inoculated to the above medium and incubated under aeration at 30°C with stirring at 200 r.p.m., 0.5 vvm for 18 hours. The culture broth was centrifuged to remove the cells, and the supernatant (18 liters) was concentrated with a ultrafilter (AIL-1010, manufactured by Asahi Chemical Industry Co., Ltd.) to give a crude enzyme solution (2000 ml) which had an esterase activity of 1.7×10^6 unit/ml (measured by the convenient method).

Example 7

Production of esterase:

An esterase-producing medium (20 liters) was charged

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into a 30 liter-volume jar fermenter and was sterilized. Previously, *Serratia marcescens* M-1 was incubated in the same medium by reciprocating shaking culture at 30°C for 20 hours in the same manner as above. The pre-culture broth thus obtained (200 ml) was inoculated to the above medium and incubated under aeration at 30°C with stirring at 200 r.p.m., 0.5 vvm for 18 hours. The culture broth was centrifuged to remove the cells, and the supernatant (18 liters) was concentrated with a ultrafilter (AIL-1010, manufactured by Asahi Chemical Industry Co., Ltd.) to give a crude enzyme solution (2000 ml) which had an esterase activity of 7.9×10^5 unit/ml (measured by the convenient method).

Effects of the Invention

The mutant strain and the transformant containing a recombinant plasmid inserted with a gene encoding an esterase of the present invention have excellent esterase productivity, and the cultivation of the transformant can give the desired esterase in high purity on a large scale.

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SEQ ID NO:1:
LENGTH: 1839 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
STRAIN: Serratia marcescens Sr41

```
ATG GGC ATC TTT AGC TAT AAG GAT CTG GAC GAA AAC GCG TCA AAG GCG    48
CTG TTT TCC GAC GCC TTG GCC ATC TCT ACC TAC GCT TAC CAC AAT ATC    96
GAT AAC GGC TTC GAT GAA GGC TAT CAC CAG ACC GGT TTC GGC CTC GGT   144
CTG CCG CTG ACG CTG ATC ACG GCG CTC ATC GGC AGC ACC CAG TCG CAG   192
GGC GGC CTG CCC GGT CTT CCC TGG AAC CCC GAC TCC GAA CAG GCC GCG   240
CAG GAC GCG GTG AAC AAT GCC GGC TGG TCA GTG ATC GAC GCC GCG CAA   288
TTG GGA TAC GCC GGC AAA ACC GAT GCG CGC GGC ACC TAC TAC GGC GAA   336
ACC GCC GGT TAC ACC ACC GCT CAG GCC GAG GTG CTG GGC AAA TAT GAC   384
AGC GAA GGC AAT CTC ACC GCC ATT GGC ATC TCA TTT CGC GGT ACC AGC   432
GGC CCG CGC GAG TCG CTG ATC GGC GAT ACC ATC GGC GAT GTG ATT AAC   480
GAT CTG TTG GCC GGG TTC GGG CCG AAA GCT ATG CGA CGC TAT ACG CTG   528
AAG GCC TTC GGC AAT TTG CTG GGG GAC GTG GCG AAA TTC GCG CAG GCC   576
CAC GGG CTG AGC GGC GAA GAC GTG GTG ATC AGC GGC CAC AGC CTC GGC   624
GGG CTG GCG GTC AAC AGC ATG GCG GCG CAG AGC GAC GCA ACC TGG GGC   672
GGC TTC TAC GCG CAG TCC AAC TAT GTC GCC TTC GCC TCG CCG ACC CAG   720
TAC GAA GCC GGC GGC AAG GTG ATC AAC ATC GGC TAT GAG AAC GAT CCG   768
GTG TTT CGC GCG CTC GAC GGC ACC TCG CTG ACC CTG CCG TCA TTG GGC   816
GTT CAC GAT GCG CCG CAT ACC TCC GCC ACC AAC AAT ATC GTC AAC TTC   864
AAC GAC CAC TAC GCG TCG GAC GCC TGG AAT CTG TTG CCG TTC TCC ATT   912
CTC AAC ATT CCG ACC TGG CTA TCC CAC CTG CCG TTC TTC TAT CAG GAT   960
GGT CTG ATG CGG GTG CTG AAC TCC GAG TTT TAT TCG CTG ACC GAC AAA  1008
GAC TCG ACC ATC ATC GTC TCC AAC CTG TCG AAC GTC ACG CGC GGC AGT  1056
ACC TGG GTG GAA GAT CTG AAC CGC AAC GCG GAA ACG CAC AGC GGG CCG  1104
ACG TTT ATC ATC GGC AGC GAC GGC AAT GAT TTG ATC AAG GGC GGC AAA  1152
GGC AAC GAC TAT CTC GAG GGC CGC GAC GGT GAC GAT ATC TTC CGC GAC  1200
GCC GGC GGC TAT AAC CTG ATC GCC GGC GGC AAA GGC CAC AAT ATC TTC  1248
GAT ACC CAG CAG GCG TTG AAA AAC ACC GAG GTC GCC TAC GAC GGC AAT  1296
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ACG CTT TAC CTG CGC GAC GCC AAA GGC GGC ATT ACG CTG GCG GAC GAC  1344
ATC AGC ACC CTG CGC AGC AAA GAA ACC TCC TGG CTG ATT TTC AGC AAA  1392
GAG GTG GAT CAT CAG GTG ACC GCC GCC GGA TTG AAA TCG GAT TCG GGC  1440
CTC AAA GCC TAT GCG GCC GCC ACC ACC GGC GGC GAC GGC GAT GAC GTC  1488
CTG CAG GCT CGC AGC CAC GAC GCC TGG CTG TTC GGC AAC GCC GGC AAC  1536
GAC ACG CTT ATC GGC CAT GCC GGC GGC AAC CTG ACC TTC GTC GGC GGC  1584
AGC GGC GAT GAC ATC CTG AAG GGG GTC GGC AAC GGC AAT ACC TTC CTG  1632
TTC AGC GGC GAT TTC GGC CGC GAC CAG CTG TAT GGC TTC AAC GCC ACC  1680
GAC AAA CTG GTA TTT ATC GGC ACC GAG GGC GCC AGC GGG AAT ATC CGC  1728
GAC TAT GCC ACG CAG CAA AAC GAC GAT CTG GTG CTG GCC TTC GGC CAC  1776
AGC CAG GTC ACG CTG ATC GGC GTC TCG CTC GAT CAT TTC AAC CCC GAT  1824
CAG GTG GTG TTG GCC                                             1839
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SEQ ID NO:2:
LENGTH: 1839 base pairs.
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: Genomic DNA
STRAIN: Serratia marcescens M-1

```
ATG GGC ATT TTT AGC TAT AAG GAT CTG GAC GAA AAC GCG TCA AAG GCG   48
CTG TTT TCC GAC GCC TTG GCC ATC TCT ACC TAC GCT TAC CAC AAT ATC   96
GAT AAC GGC TTC GAT GAA GGC TAT CAC CAG ACC GGT TTC GGC CTC GGT  144
CTG CCG CTG ACG CTG ATC ACG GCG CTC ATC GGC AGC ACC CAG TCG CAG  192
GGC GGC CTG CCC GGT CTT CCC TGG AAC CCC GAC TCC GAA CAG GCC GCG  240
CAG GAC GCG GTG AAC AAT GCC GGC TGG TCA GTG ATC GAC GCC GCG CAA  288
TTG GGA TAC GCC GGC AAA ACC GAT GCG CGC GGC ACC TAC TAC GGC GAA  336
ACC GCC GGT TAC ACC ACC GCT CAG GCC GAG GTG CTG GGC AAA TAT GAC  384
AGC GAA GGC AAT CTC ACC GCC ATT GGC ATC TCA TTT CGC GGT ACC AGC  432
GGC CCG CGC GAG TCG CTG ATC GGC GAT ACC ATC GGC GAT GTG ATT AAC  480
GAT CTG TTG GCC GGG TTC GGG CCG AAA GCT ATG CGA CGC TAT ACG CTG  528
AAG GCC TTC GGC AAT TTG CTG GGG GAC GTG GCG AAA TTC GCG CAG GCC  576
CAC GGG CTG AGC GGC GAA GAC GTG GTG ATC AGC GGC CAC AGC CTC GGC  624
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GGG CTG GCG GTC AAC AGC ATG GCG GCG CAG AGC GAC GCA ACC TGG GGC   672
GGC TTC TAC GCG CAG TCC AAC TAT GTC GCC TTC GCC TCG CCG ACC CAG   720
TAC GAA GCC GGC GGC AAG GTG ATC AAC ATC GGC TAT GAG AAC GAT CCG   768
GTG TTT CGC GCG CTC GAC GGC ACC TCG CTG ACC CTG CCG TCA TTG GGC   816
GTT CAC GAT GCG CCG CAT ACC TCC GCC ACC AAC AAT ATC GTC AAC TTC   864
AAC GAC CAC TAC GCG TCG GAC GCC TGG AAT CTG TTG CCG TTC TCC ATT   912
CTC AAC ATT CCG ACC TGG CTA TCC CAC CTG CCG TTC TTC TAT CAG GAT   960
GGT CTG ATG CGG GTG CTG AAC TCC GAG TTT TAT TCG CTG ACC GAC AAA  1008
GAC TCG ACC ATC ATC GTC TCC AAC CTG TCG AAC GTC ACG CGC GGC AGT  1056
ACC TGG GTG GAA GAT CTG AAC CGC AAC GCG GAA ACG CAC AGC GGG CCG  1104
ACG TTT ATC ATC GGC AGC GAC GGC AAT GAT TTG ATC AAG GGC GGC AAA  1152
GGC AAC GAC TAT CTC GAG GGC CGC GAC GGT GAC GAT ATC TTC CGC GAC  1200
GCC GGC GGC TAT AAC CTG ATC GCC GGC GGC AAA GGC CAC AAT ATC TTC  1248
GAT ACC CAG CAG GCG TTG AAA AAC ACC GAG GTC GCC TAC GAC GGC AAT  1296
ACG CTT TAC CTG CGC GAC GCC AAA GGC GGC ATT ACG CTG GCG GAC GAC  1344
ATC AGC ACC CTG CGC AGC AAA GAA ACC TCC TGG CTG ATT TTC AGC AAA  1392
GAG GTG GAT CAT CAG GTG ACC GCC GCC GGA TTG AAA TCG GAT TCG GGC  1440
CTC AAA GCC TAT GCG GCC GCC ACC ACC GGC GGC GAC GGC GAT GAC GTC  1488
CTG CAG GCT CGC AGC CAC GAC GCC TGG CTG TTC GGC AAC GCC GGC AAC  1536
GAC ACG CTT ATC GGC CAT GCC GGC GGC AAC CTG ACC TTC GTC GGC GGC  1584
AGC GGC GAT GAC ATC CTG AAG GGG GTC GGC AAC GGC AAT ACC TTC CTG  1632
TTC AGC GGC GAT TTC GGC CGC GAC CAG CTG TAT GGC TTC AAC GCC ACC  1680
GAC AAA CTG GTA TTT ATC GGC ACC GAG GGC GCC AGC GGG AAT ATC CGC  1728
GAC TAT GCC ACG CAG CAA AAC GAC GAT CTG GTG CTG GCC TTC GGC CAC  1776
AGC CAG GTC ACG CTG ATC GGC GTC TCG CTC GAT CAT TTC AAC CCC GAT  1824
CAG GTG GTG TTG GCC                                             1839
```

SEQ ID NO:3:

LENGTH: 613 amino acids

TYPE: amino acid

TOPOLOGY:linear

MOLECULE TYPE:peptide

```
Met Gly Ile Phe Ser Tyr Lys Asp Leu Asp Glu Asn Ala Ser Lys Ala
 1               5                  10                  15
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Leu Phe Ser Asp Ala Leu Ala Ile Ser Thr Tyr Ala Tyr His Asn Ile
20 25 30

Asp Asn Gly Phe Asp Glu Gly Tyr His Gln Thr Gly Phe Gly Leu Gly
35 40 45

Leu Pro Leu Thr Leu Ile Thr Ala Leu Ile Gly Ser Thr Gln Ser Gln
50 55 60

Gly Gly Leu Pro Gly Leu Pro Trp Asn Pro Asp Ser Glu Gln Ala Ala
65 70 75 80

Gln Asp Ala Val Asn Asn Ala Gly Trp Ser Val Ile Asp Ala Ala Gln
85 90 95

Leu Gly Tyr Ala Gly Lys Thr Asp Ala Arg Gly Thr Tyr Tyr Gly Glu
100 105 110

Thr Ala Gly Tyr Thr Thr Ala Gln Ala Glu Val Leu Gly Lys Tyr Asp
115 120 125

Ser Glu Gly Asn Leu Thr Ala Ile Gly Ile Ser Phe Arg Gly Thr Ser
130 135 140

Gly Pro Arg Glu Ser Leu Ile Gly Asp Thr Ile Gly Asp Val Ile Asn
145 150 155 160

Asp Leu Leu Ala Gly Phe Gly Pro Lys Ala Met Arg Arg Tyr Thr Leu
165 170 175

Lys Ala Phe Gly Asn Leu Leu Gly Asp Val Ala Lys Phe Ala Gln Ala
180 185 190

His Gly Leu Ser Gly Glu Asp Val Val Ile Ser Gly His Ser Leu Gly
195 200 205

Gly Leu Ala Val Asn Ser Met Ala Ala Gln Ser Asp Ala Thr Trp Gly
210 215 220

Gly Phe Tyr Ala Gln Ser Asn Tyr Val Ala Phe Ala Ser Pro Thr Gln
225 230 235 240

Tyr Glu Ala Gly Gly Lys Val Ile Asn Ile Gly Tyr Glu Asn Asp Pro
245 250 255

Val Phe Arg Ala Leu Asp Gly Thr Ser Leu Thr Leu Pro Ser Leu Gly
260 265 270

Val His Asp Ala Pro His Thr Ser Ala Thr Asn Asn Ile Val Asn Phe
275 280 285

Asn Asp His Tyr Ala Ser Asp Ala Trp Asn Leu Leu Pro Phe Ser Ile
290 295 300

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Leu Asn Ile Pro Thr Trp Leu Ser His Leu Pro Phe Phe Tyr Gln Asp
305 310 315 320

Gly Leu Met Arg Val Leu Asn Ser Glu Phe Tyr Ser Leu Thr Asp Lys
325 330 335

Asp Ser Thr Ile Ile Val Ser Asn Leu Ser Asn Val Thr Arg Gly Ser
340 345 350

Thr Trp Val Glu Asp Leu Asn Arg Asn Ala Glu Thr His Ser Gly Pro
355 360 365

Thr Phe Ile Ile Gly Ser Asp Gly Asn Asp Leu Ile Lys Gly Gly Lys
370 375 380

Gly Asn Asp Tyr Leu Glu Gly Arg Asp Gly Asp Asp Ile Phe Arg Asp
385 390 395 400

Ala Gly Gly Tyr Asn Leu Ile Ala Gly Gly Lys Gly His Asn Ile Phe
405 410 415

Asp Thr Gln Gln Ala Leu Lys Asn Thr Glu Val Ala Tyr Asp Gly Asn
420 425 430

Thr Leu Tyr Leu Arg Asp Ala Lys Gly Gly Ile Thr Leu Ala Asp Asp
435 440 445

Ile Ser Thr Leu Arg Ser Lys Glu Thr Ser Trp Leu Ile Phe Ser Lys
450 455 460

Glu Val Asp His Gln Val Thr Ala Ala Gly Leu Lys Ser Asp Ser Gly
465 470 475 480

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

Leu Gln Ala Arg Ser His Asp Ala Trp Leu Phe Gly Asn Ala Gly Asn
500 505 510

Asp Thr Leu Ile Gly His Ala Gly Gly Asn Leu Thr Phe Val Gly Gly
515 520 525

Ser Gly Asp Asp Ile Leu Lys Gly Val Gly Asn Gly Asn Thr Phe Leu
530 535 540

Phe Ser Gly Asp Phe Gly Arg Asp Gln Leu Tyr Gly Phe Asn Ala Thr
545 550 555 560

Asp Lys Leu Val Phe Ile Gly Thr Glu Gly Ala Ser Gly Asn Ile Arg
565 570 575

Asp Tyr Ala Thr Gln Gln Asn Asp Asp Leu Val Leu Ala Phe Gly His
580 585 590

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Ser Gln Val Thr Leu Ile Gly Val Ser Leu Asp His Phe Asn Pro Asp
595 600 605

Gln Val Val Leu Ala
610

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What is claimed is:

1. A gene encoding an esterase originated from a microorganism of the genus *Serratia*.

2. The gene as claimed in claim 1 which has a DNA sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2.

3. A recombinant plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*.

4. A transformant which contains a recombinant plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*.

5. The transformant as claimed in claim 4, wherein the host microorganism is a microorganism of the genus *Serratia* or a microorganism of the genus *Escherichia*.

6. A transformant *Serratia marcescens* TA5025 (FERM BP-4067).

7. A method for the production of an esterase, which comprises cultivating a microorganism transformed with a recombinant plasmid (said plasmid comprising a vector plasmid containing a gene encoding an esterase originated from a microorganism of the genus *Serratia*), and collecting the produced esterase outside and inside the cells.

8. A microorganism of the genus *Serratia* which contains a gene having a DNA sequence shown in SEQ ID NO: 2.

9. A method for the production of an esterase, which comprises cultivating the microorganism as set forth in

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claim 8 in a medium, and collecting the esterase produced outside and inside the cells.

10. A mutant strain *Serratia marcescens* M-1 (FERM BP-4068).

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GENE CODING FOR ESTERASE AND NOVEL MICROORGANISM CONTAINING SAID GENE

Abstract of the Disclosure:

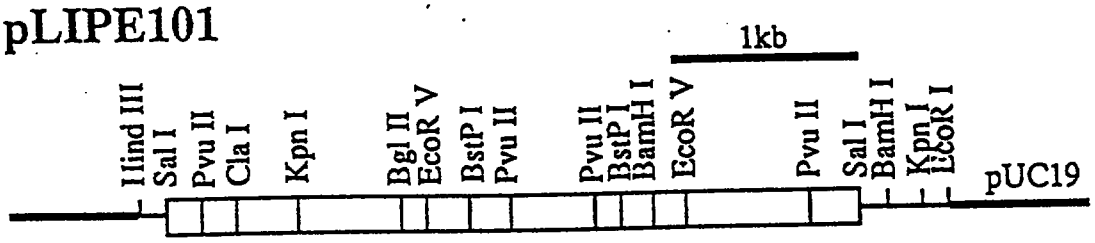
A gene encoding an esterase originated from a microorganism of the genus *Serratia*, a mutant strain having high esterase productivity containing said gene, a recombinant plasmid comprising said gene inserted into a vector plasmid, a novel microorganism transformed with said recombinant plasmid, and a method for the production of an esterase which comprises cultivating the mutant strain or the novel microorganism as set forth above in a medium and collecting the produced esterase outside and inside the cells. Said mutant strain having high esterase productivity and new transformed microorganism have excellent esterase productivity and can produce the desired esterase in high purity on a large scale.

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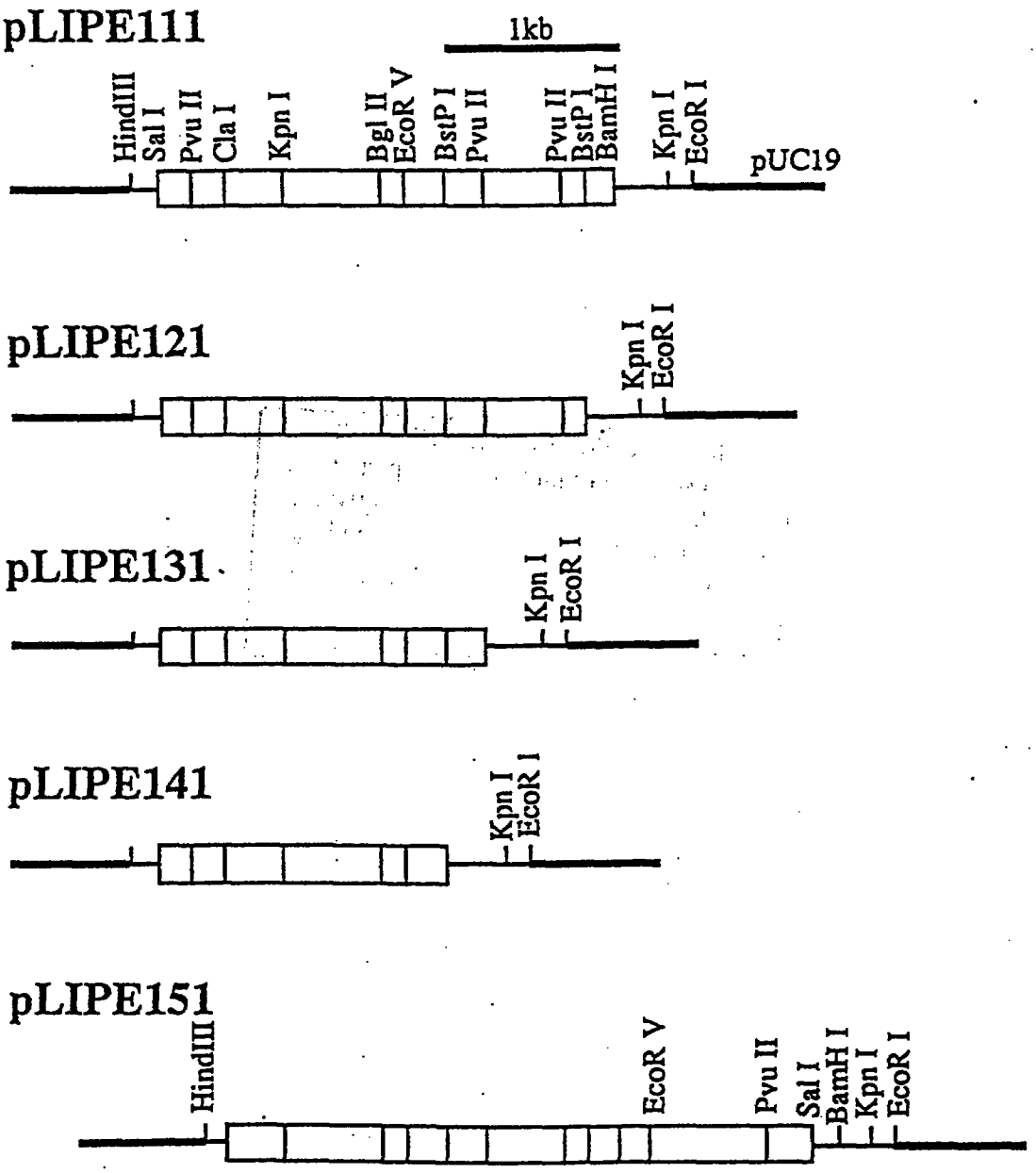
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Fig. 1



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Fig. 2



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The claims defining the invention are as follows:

1. A transformant *Serratia marcescens* which contains a recombinant plasmid including a vector plasmid containing a gene encoding an esterase, wherein said gene is a gene having the DNA sequence as shown in SEQ ID NO: 1 obtained from *Serratia marcescens* Sr41 (FERM BP-487) or a gene having the DNA sequence as shown in SEQ ID NO: 2 obtained from *Serratia marcescens* M-1 (FERM BP-4068).

2. A transformant *Serratia marcescens* TA5025 (FERM BP-4067).

3. A method for the production of an esterase, which includes cultivating a microorganism *Serratia marcescens* transformed with a recombinant plasmid including a vector plasmid containing a gene encoding an esterase (said gene includes the DNA sequence as shown in SEQ ID NO: 1 obtained from *Serratia marcescens* Sr41 (FERM BP-487) or the DNA sequence as shown in SEQ ID NO: 2 obtained from *Serratia marcescens* M-1 (FERM BP-4068)) and collecting the produced esterase outside and inside the cells.

4. A method for the production of an esterase, which includes cultivating the microorganism as set forth in claim 3 in a medium, and collecting the esterase produced outside and inside the cells.

5. A mutant strain *Serratia marcescens* M-1 (FERM BP-4068).

6. A transformant according to claim 1 substantially as hereinbefore described with reference to any one of the examples, sequence identifications or accompanying drawings.

7. A method according to claim 3 or 4 substantially as hereinbefore described with reference to any one of the examples, identifications or accompanying drawings.

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removal of N-terminal methionine from the protein produced in the cells by methionine aminopeptidase present in the cells. Moreover, although *Serratia*-origin esterase is an extracellular enzyme, it has no signal peptide which is usually present at the N-terminus of secretory proteins.

Example 4

Serratia marcescens Sr41 (FERM BP-487) was incubated in a nutrient medium (glucose 0.5%, peptone 1.0%, meat extract 0.3%, yeast extract 1.0%, and sodium chloride 0.5%, pH 7.0, 3 ml) overnight, and the culture thus obtained (0.1 ml) was inoculated in a fresh medium (3 ml) of the same nutrient medium as above, which was incubated at 30°C for 3 hours, and after adding thereto NTG in a concentration of 0.1 mg/ml, was further incubated for additional 30 minutes. The culture broth was centrifuged, and the cells thus obtained were washed three times with a physiological saline solution (by centrifugation method). The cells thus obtained were suspended in a physiological saline solution and spread onto an LBG plate medium containing tributyrin 0.5%, glucose 2% and EDTA 2mM in an amount of 1 - 1000 cells/one plate. After incubating at 30°C for one day, the colony forming a large clear zone was collected to give *Serratia marcescens* M-1 strain (FERM BP-4068). The thus-obtained mutant strain (one platinum loop) was inoculated to an esterase-producing medium containing ampicillin (500 μg/ml) and subjected to reciprocating shaking culture (shaking amplitude 7 cm, 120 r.p.m.) at 30°C for 20 hours. The culture broth was centrifuged to give a super-

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natant having an esterase activity of about 80 x 10^3 unit/ml (measured by the convenient method). This strain had about 2.5 times higher esterase productivity than the parent host strain *Serratia marcescens* Sr41.

In the same manner as described in Examples 1 to 3, the chromosomal DNA was isolated from the above *Serratia marcescens* M-1, and the sequence was analyzed. As a result, it had a nucleotide sequence as shown in SEQ ID NO: 2. As is clear therefrom, the sequence was the same as the DNA sequence of that obtained from *Serratia marcescens* Sr41 except that the 9th base was different, that is, it was composed of 1839 base pairs from the initiation codon ATG to the codon GCC. The amino acid sequence determined therefrom was the same as that deduced from SEQ ID NO: 1.

Example 5

Preparation of a strain having high esterase productivity:

The plasmid pLIPE111 was introduced into a restriction endonuclease-deficient strain, *Serratia marcescens* TT392, to give a transformed strain. The pLIPE111 plasmid DNA modified with *Serratia marcescens* was extracted from the cells of the transformed strain by alkaline lysis method. Then, *Serratia marcescens* Sr41 cells were transformed with the plasmid DNA obtained above by electroporation method to give a transformant *Serratia marcescens* TA5025 (FERM BP-4067). The thus-obtained transformant (one platinum loop) was inoculated to an esterase-producing medium containing

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The claims defining the invention are as follows:

1. A transformant *Serratia marcescens* which contains a recombinant plasmid including a vector plasmid containing a gene encoding an esterase, wherein said gene is a gene having the DNA sequence as shown in SEQ ID NO: 1 obtained from *Serratia marcescens* Sr41 (FERM BP-487) or a gene having the DNA sequence as shown in SEQ ID NO: 2 obtained from *Serratia marcescens* M-1 (FERM BP-4068).

2. A transformant *Serratia marcescens* TA5025 (FERM BP-4067).

3. A method for the production of an esterase, which includes cultivating a microorganism *Serratia marcescens* transformed with a recombinant plasmid including a vector plasmid containing a gene encoding an esterase (said gene includes the DNA sequence as shown in SEQ ID NO: 1 obtained from *Serratia marcescens* Sr41 (FERM BP-487) or the DNA sequence as shown in SEQ ID NO: 2 obtained from *Serratia marcescens* M-1 (FERM BP-4068)) and collecting the produced esterase outside and inside the cells.

4. A method for the production of an esterase, which includes cultivating the microorganism of claim 5 in a medium, and collecting the esterase produced outside and inside the cells.

5. A mutant strain *Serratia marcescens* M-1 (FERM BP-4068).

6. A transformant according to claim 1 substantially as hereinbefore described with reference to any one of the examples, sequence identifications or accompanying drawings.

7. A method according to claim 3 or 4 substantially as hereinbefore described with reference to any one of the examples, identifications or accompanying drawings.

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GENE CODING FOR ESTERASE AND NOVEL MICROORGANISM CONTAINING SAID GENE

Abstract of the Disclosure:

A gene encoding an esterase originated from a microorganism of the genus *Serratia*, a mutant strain having high esterase productivity containing said gene, a recombinant plasmid comprising said gene inserted into a vector plasmid, a novel microorganism transformed with said recombinant plasmid, and a method for the production of an esterase which comprises cultivating the mutant strain or the novel microorganism as set forth above in a medium and collecting the produced esterase outside and inside the cells. Said mutant strain having high esterase productivity and new transformed microorganism have excellent esterase productivity and can produce the desired esterase in high purity on a large scale.