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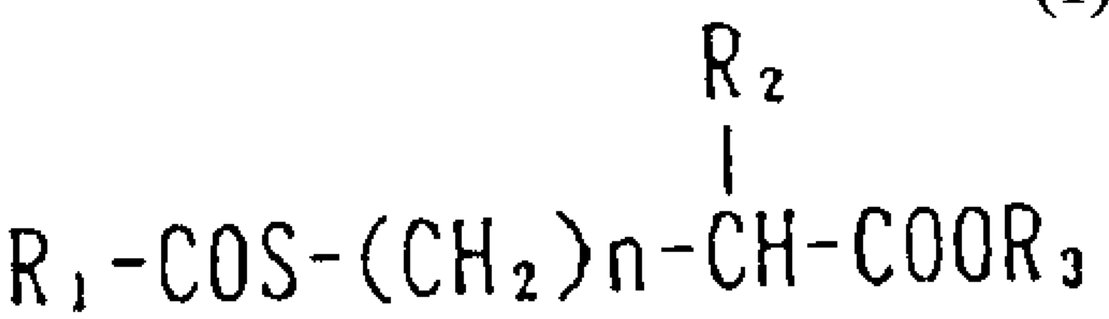
(54) Titre : GENES DE TYPE ESTERASE, ESTERASE, PLASMIDES RECOMBINANTS ET TRANSFORMANTS CONTENANT LES PLASMIDES RECOMBINANTS ET METHODES POUR LA PRODUCTION D'ACIDES CARBOXYLIQUES OPTIQUEMENT ACTIFS ET DE LEURS ESTERS ENANTIOMERIQUES A L'AIDE DE CES TRANSFORMANTS

(54) Title: ESTERASE GENES, ESTERASE, RECOMBINANT PLASMIDS AND TRANSFORMANTS CONTAINING THE RECOMBINANT PLASMID AND METHODS OF PRODUCING OPTICALLY ACTIVE CARBOXYLIC ACIDS AND THEIR ENANTIOMERIC ESTERS USING SAID TRANSFORMANTS

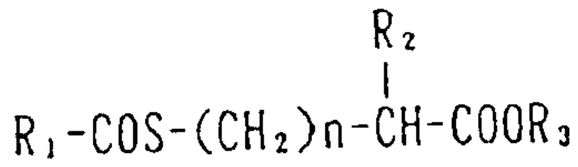
(I)



(57) Abrégé/Abstract:
The present invention relates to a DNA fragment containing a nucleotide sequence that encodes an amino acid sequence of esterase, said esterase asymmetrically hydrolyzing carboxylic acid esters represented by the formula (I); (see formula I) (wherein R_1 is alkyl, aralkyl or aryl, R_2 and R_3 are alkyl, and n is 1 or 2) an esterase encoded by the DNA fragment, a recombinant plasmid containing the DNA fragment, a microorganism transformed with the recombinant plasmid and methods of producing optically active carboxylic acids and their enantiomeric esters.

ABSTRACT

The present invention relates to a DNA fragment containing a nucleotide sequence that encodes an amino acid sequence of esterase, said esterase asymmetrically hydrolyzing carboxylic acid esters represented by the formula (I);



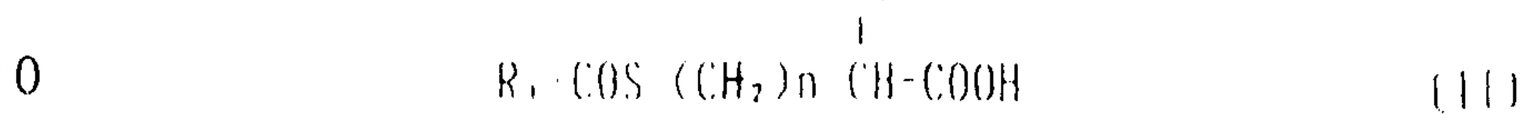
(wherein R_1 is alkyl, aralkyl or aryl, R_2 and R_3 are alkyl, and n is 1 or 2) an esterase encoded by the DNA fragment, a recombinant plasmid containing the DNA fragment, a microorganism transformed with the recombinant plasmid and methods of producing optically active carboxylic acids and their enantiomeric esters.

ESTERASE GENES, ESTERASE, RECOMBINANT PLASMIDS AND TRANSFORMANTS CONTAINING THE RECOMBINANT PLASMID AND METHODS OF PRODUCING OPTICALLY ACTIVE CARBOXYLIC ACIDS AND THEIR ENANTIOMERIC ESTERS USING SAID TRANSFORMANTS

Background of the Invention

The present invention relates to a DNA fragment containing a nucleotide sequence encoding esterase which is highly heat stable and asymmetrically hydrolyzes carboxylic acid esters, an esterase encoded by the DNA fragment, a recombinant plasmid containing the DNA fragment, a microorganism transformed with the recombinant plasmid and methods of producing optically active carboxylic acids and their enantiomeric esters.

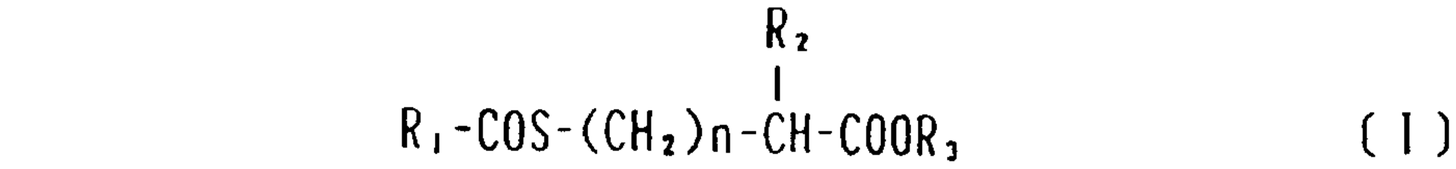
An optically active carboxylic acid and its enantiomeric ester, which are represented by the general formula (II);



(II)

(wherein R_1 is alkyl, aralkyl or aryl, R_2 is alkyl, and n is 1 or 2), are useful as a raw material of various physiologically active substances.

The present inventors disclosed methods of asymmetrically hydrolyzing a racemic mixture of carboxylic acids esters to produce an optically active carboxylic acid and its enantiomeric ester using an enzyme or a microorganism, the carboxylic acid esters being represented by the general formula (I);



(I)

(wherein R_1, R_2 and n are the same meaning as described above and R_3 is alkyl)

[see Japanese Patent Publication KOKAI No. 12992/1985, No. 12993/1985].

The present inventors isolated *Pseudomonas putida* (FERM BP-3846) from a soil and found that it produces esterase having a strong, asymmetrical hydrolysis activity for a racemic mixture of carboxylic acid esters, and disclosed the bacterium in Japanese Patent Publication KOKAI No. 222798/1989).

To obtain a microorganism having a stronger enzymatic activity, the recombinant DNA technology is often utilized these days. There has been a method known in the art in which a transformed microorganism is used to increase the production of esterase involved in hydrolysis reaction: a DNA fragment encoding an esterase gene derived from *Pseudomonas fluorescens* IF03018 is prepared; a host microorganism is transformed with a plasmid having the DNA fragment; esterase is produced from the transformed microorganism (Japanese Patent Publication KOKAI No. 67190/1989).

Typically, bacterial cells are directly utilized as "enzyme" rather than utilizing an enzyme purified by a complicated manipulation such as isolation and purification when carboxylic acid

esters of the formula (I) are required to be asymmetrically hydrolyzed. In such a method, an efficient reaction relies on several factors such as a strong enzyme activity of bacterial cells, the property of an enzyme that serves to produce carboxylic acids with high optical purity and a strong stability in various conditions such as temperature, pH and the like.

Heat labile enzymes are susceptible to thermal inactivation as reaction time progresses so that it is not easy to increase a reaction rate by raising reaction temperature or to reuse recovered enzyme. Bacterial cells that produce enzyme, which has an excellent heat stability and a strong enzymatic activity, are necessary for efficient reaction.

The recombinant DNA technology is an effective method of increasing the enzymatic activity of enzyme produced by bacteria as described in Japanese Patent Publication KOKAI No. 67190/1989. The properties of enzyme essentially depends on a DNA sequence that encodes an enzyme. To obtain a highly stable enzyme that satisfies the above requirement, one needs to find a DNA fragment encoding the enzyme.

The Japanese Patent Publication KOKAI No. 67190/1989 discloses a microorganism transformed with a recombinant plasmid containing a DNA fragment. The DNA fragment comprises the sequence encoding an esterase gene derived from *Pseudomonas fluorescens* IF03018. Esterase produced by the microorganism is less heat stable and is inactivated in several hours, for example, at 45^{o}C or more.

It is a primary object of the present invention to prepare a DNA fragment encoding a highly heat stable esterase for the asymmetrically hydrolysis of carboxylic acid esters of the formula (I) and to efficiently carry out the asymmetrical hydrolysis using a transformant that contains the DNA fragment and efficiently produces the enzyme.

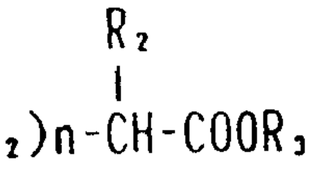
The present inventors have investigated an efficient method of asymmetrically hydrolyzing carboxylic acid esters of the formula (I) using enzyme. We have successfully found that a microorganism transformed with a recombinant plasmid containing an esterase gene derived from *Pseudomonas putida* (FERM BP-3846), which is isolated from a soil, is useful for obtaining a high yield of reaction products and for carrying out an efficient reaction in a short period of time. Because the microorganism produces enzyme having a strong enzymatic activity and the enzyme is not inactivated with time even at a reaction temperature of $50^{o}C$ or more.

In addition, we have isolated a highly heat stable, novel esterase substantially in a pure form from the transformed microorganism and successfully characterized the physical and chemical properties of the enzyme.

Summary of the Invention

The present invention provides a DNA fragment containing a nucleotide sequence that encodes the amino acid sequence of esterase as described in SEQ ID No. 2,

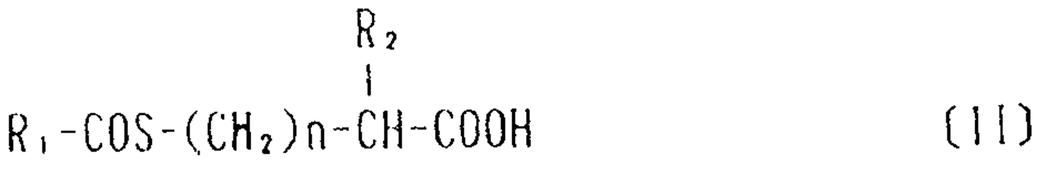
the esterase asymmetrically hydrolyzing carboxylic acid esters represented by the formula (I);



(I)

(wherein R_1 is alkyl, aralkyl or aryl, R_2 and R_3 are alkyl, and n is 1 or 2)

to give an optically active carboxylic acid represented by the formula (II);



(II)

(wherein R_1, R_2, and n are the same meaning as described above).

In addition, the present invention provides an esterase having an amino acid sequence as described in SEQ ID No.2, an esterase encoded by a DNA fragment as described in SEQ ID No. 3, a recombinant plasmid containing the DNA fragment and a microorganism transformed with the recombinant plasmid.

Furthermore, the present invention provides a method of producing optically active carboxylic acids represented by the formula (II) and their enantiomeric esters, by reacting a racemic mixture of carboxylic acid esters represented by the formula (I) with the culture or bacterial cells of the transformant or bacterial-cell-treated materials.

The method of the present invention efficiently produce optically active carboxylic acids represented by the formula (II), and their enantiomeric esters from a racemic mixture of carboxylic acid esters represented by the formula (I).

The esterase of the present invention has the following physical and chemical properties.

(1) Optimum pH: 7.0 (see Fig. 4)

(2) Stability of esterase in varied pH: 6.0-8.0 (see Fig. 5)

(3) Optimum temperature: 60-70 ^{o}C (see Fig. 6)

(4) Heat stability: up to 70 ^{o}C (see Fig. 7)

(5) Molecular weight: about 30,000 (by SDS-PAGE)

(6) Isoelectric point: pH 3.90±0.1(by isoelectric focusing)

(7) Amino acid sequence: sequence described in SEQ ID No. 2

(8) Specificity: mainly hydrolyze carboxylic acid esters having 8 or less carbon atoms as well as aromatic alcohols such as acetyl esters

Description of the Figures

Fig. 1 shows the cloning vector pUC19.

Fig. 2 shows a restriction map of recombinant plasmid pPE101 comprising the DNA fragment that contains the esterase structural gene of the present invention.

Fig. 3 shows deleted DNA fragments and their positions relative to the EcoRI fragment in pPE101, a recombinant plasmid constructed by incorporating the DNA fragment into the pUC19, and esterase activity of microorganisms transformed with the recombinant plasmids.

Fig. 4 shows an optimum pH of the esterase of the present invention.

Fig. 5. shows stability of esterase in varied pH.

Fig. 6 shows an optimum temperature of the esterase of the present invention.

Fig. 7 shows heat stability of the esterase of the present invention.

In the Figures, the symbols are defined as follows:

Amp	=	ampicillin resistance coding region
lacZ	=	ß-galactosidase structural gene
lac i	=	repressor structural gene
①	=	region derived from pUC19
②	=	region derived from the chromosomal DNA of *Pseudomonas putida*
B	=	BglII
C	=	ClaI
EI	=	EcoRI
EV	=	EcoRV
P	=	PvuII
S	=	SalI
Sm	=	SmaI
Ps	=	PstI

Detailed Description of the Invention

In the general formula (I) and (II),

preferable alkyl represented by R_1 is (C_1-C_6) alkyl, for example, methyl and ethyl,

aralkyl is, for example, benzyl,

aryl is, for example, phenyl, and

preferable alkyl represented by R_2 or R_3 is (C_1-C_6) alkyl, for example, methyl and ethyl.

Carboxylic acid esters represented by the formula (I), include methyl ß-acetylthio-α-methylpropionate, methyl S-acetyl-ß-mercaptoisobutyrate, methyl S-acetyl-γ-mercapto-α-methyl-n-butyrate,

methyl S-benzoyl-ß-mercaptoisobutyrate and methyl S-phenylacetyl-ß-mercaptoisobutyrate.

The DNA fragment of the present invention includes, for example, a DNA fragment comprising the whole or part of a nucleotide sequence described in SEQ ID No. 1 and a DNA fragment comprising the whole or part of a nucleotide sequence described in SEQ ID No. 3.

In addition, the DNA fragment of the present invention may be obtained by cloning a desirable esterase gene derived from the chromosome of a microorganism or by synthesizing a desirable sequence by a DNA synthesizer.

The nucleotide sequence may be a single-stranded or a double-stranded DNA. Either of them can be used in the present invention.

Microorganisms suitable as a chromosome donor in cloning are, for example, *Pseudomonas putida* (FERM BP-3846).

The following will illustrate cloning of a DNA fragment containing a whole nucleotide sequence described in SEQ ID No. 1.

Chromosomal DNA is obtained from *Pseudomonas putida* (FERM BP-3846) by the Marmur et al method [J.Marmur et al., J.Mol.Biol., 3:208, 1961] and is then partially digested with EcoRI. After digestion, restriction fragments are obtained.

The plasmid pUC19 was digested with EcoRI, which cleaves the EcoRI site of a multiple cloning site in the lacZ gene(one of the genes in lac operon of *E. coli*) to give a linear DNA.

The EcoRI digested chromosomal DNA and pUC19 are ligated using T4 DNA ligase.

The reaction product is used to transform $CaCl_2$-treated *E. coli* JM105 cells (one of the strains of *E. coli* K-12). The transformation

mixture is spread on an LB agar plate containing ampicillin, IPTG (isopropyl ß-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside), and incubated.

Transformants containing the DNA-fragment-free pUC19 at its EcoRI site become ampicillin resistance and grow to form colonies. When induced by IPTG, these transformants also express ß-galactosidase that cut X-Gal to make blue colonies. Conversely, transformants containing the DNA-fragment-containing pUC19 at its EcoRI site become ampicillin resistance and are unable to express ß-galactosidase. These transformants make white colonies.

After incubation on a plate, positive transformants are selected by picking white colonies. The positive transformants are then screened for esterase activities.

Screening may be carried out as described below.

The colonies on the plate are transferred to a filter paper soaked in a solution of 10 mM Tris-HCl/pH7.5, 0.01% bromocresolpurple and 100 ppm of methyl DL-ß-acetylthio-α-methylpropionate. The filter is incubated at room temperature for several hours. Transformants having an esterase activity produce carboxylic acid and change the color of bromocresolpurple (pH indicator) from purple to yellow around the colony because of a decrease of pH. Transformants having an esterase gene can be, therefore, observed with the naked eye.

The position of an esterase gene in the recombinant plasmid is determined by the following method.

Plasmids are isolated from the transformant having an esterase activity. EcoRI DNA fragments are then obtained by digesting the plasmid DNA with EcoRI. The EcoRI digest is further digested with various restriction enzymes. The restriction fragments are inserted into the plasmid pUC19 to give recombinant plasmids. *E. coli* JM105 is

transformed with the recombinant plasmids. The transformants are tested for an esterase activity. Among the positive transformants, a transformant comprising a minimum length DNA fragment for an esterase gene expression is selected. The nucleotide sequence of the DNA fragment is determined by various methods and SEQ ID No. 1 is obtained.

Cloning vectors include the pBR and pUC series that are a high-copy-number plasmid stably maintained in *E. coli*, pHY300PLK used for *Bacillus subtilis* and vectors derived from RSF1010 that have a wide host range.

Host microorganisms used for transformation include *E. coli*, yeast, *Bacillus subtilis* and *Actinomycetes*.

Microorganisms transformed with a recombinant plasmid containing a esterase gene encoding a heat stable esterase produce enzyme that has similar enzymatic properties to those of a parental strain, *Pseudomonas putida* (FERM BP-3846). In addition, the transformant has by far higher enzymatic activity than the DNA donor bacterium because the transformant contains a high-copy-number plasmid. The transformant is grown in a culture medium and used in a form such as culture, bacterial cells or bacterial-cell-treated materials.

The transformed microorganism is typically grown in a liquid culture medium or in a solid culture medium. For example, an LB culture medium can be used. Culture is carried out at 10-50^{O}C, pH2-11. Aeration agitation culture is also utilized to facilitate growth.

Hydrolysis may be carried out by adding carboxylic acid esters of the formula (I) to culture at the beginning of culture or in the middle of culture or after completion of culture.

Alternatively, grown bacterial cells are harvested by centrifugation and may be added to a solution containing carboxylic acid

esters of the formula (I). When bacterial cells are used, bacterial cells are dried by, for example, lyophilization or spray-drying, or are treated by organic solvents such as acetone or toluene, or are disrupted to give broken cells, or are extracted to give bacterial-cell-treated materials. Solvents used for reaction include deionized water or buffer. The preferable concentration of carboxylic acid esters of formula (I) in a solvent or in a culture medium may be 0.01-50 wt%. Carboxylic acid esters of formula (I) suspended in water may be added to carry out reaction. Solvents, methanol or acetone, may be added to help dissolve esters. The pH of a reaction mixture is 2-11, preferably 5-8. As reaction proceeds, the pH of a reaction mixture decreases due to the increase of the reaction product, optically active carboxylic acids of the formula (II). The pH of a reaction mixture should be adjusted with an appropriate neutralizing agent. An enzyme produced by the transformant of the present invention is highly heat stable so that reaction temperature may be in the range of 5-80 ^{o}C. If the property of the enzyme produced by the transformant of the present invention is attempted to be optimized, 45 ^{o}C or more is preferable for reaction. At that temperature range, the enzyme can maintain its activity during reaction.

Isolation and purification of a reaction product from a reaction mixture or a culture may be carried out by the method known in the art, for example, extraction, recrystalization and column chromatography.

A novel esterase, one of the present invention, may be obtained from the culture of the transformant of the present invention by the method known in the art: Enzyme endogenouly produced in the transformant is extracted by any method in the art to give bacterial-cell free extract. The extract is concentrated using ammonium sulfate, loaded

on an ion-exchange chromatography column, gel-filtrated or is repetitiously subjected to the procedure described above to give pure enzyme.

Example

Example 1

1-(1) Preparation of Chromosomal DNA

Chromosomal DNA was isolated from *Pseudomonas putida* (FERM BP-3846) by the Marmur et al method [J.Marmur et al., J.Mol.Biol., 3:208, 1961] as described below:

Pseudomonas putida (FERM BP-3846) was inoculated into 100 ml of an LB culture medium (1% polypeptone, 0.5 % yeast extract, 0.5 % NaCl) and incubated at 37°C overnight. After the incubation, bacterial cells were harvested by centrifugation and about 3 g of bacterial cells (wet weight) was then suspended in 24 ml of TEG buffer [25 mM Tris-HCl, 10 mM EDTA (ethylenediaminetetraacetic acid), 50 mM glucose, pH8.0]. 1 ml of lysozyme (suspended in TEG buffer, concentration: 10 mg/ml) was added to the suspension. The suspension was stirred at 32 °C for 30 minutes. After the stirring, 2 ml of a 12.5 % SDS solution was added to the bacterial lysate. The mixture was stirred. 6.75 ml of a 5 M sodium perchlorate solution was added to the lysate mixture. The lysate mixture was stirred. 33.8 ml of a chloroform/ isoamylalcohol [24:1(v/v)] mixture was added to the lysate mixture. The mixture was gently stirred for 30 minutes.

After the stirring, the mixture was centrifuged. The supernatant was saved. Two volume of ice cold ethanol was gently added to the supernatant. DNA was removed from ethanol by winding it with a glass stick. The DNA was dissolved in 9 ml of 0.1 X SSC (15 mM NaCl, 1.5

mM sodium citrate). 1 ml of 10 X SSC (1.5 M NaCl, 150 mM sodium citrate) was added to the DNA solution. RNaseA was added to the DNA solution to a final concentration of 50 μg/ml. The DNA mixture was gently stirred for 30 minutes. After the stirring, 10.2 ml of a chloroform/ isoamylalcohol [24:1(v/v)] mixture was added to the DNA mixture. The mixture was gently stirred for 30 minutes.

After the stirring, the mixture was centrifuged. The supernatant was saved. 1 ml of an acetic acid/EDTA (3M sodium acetate, 0.01 M EDTA, pH 7.0) mixture was added to the supernatant. Isopropylalcohol (total volume: 5.4 ml) was dropwise added to the mixture while stirring the mixture with a glass stick. DNA was removed by winding it with a glass stick. The DNA was dissolved in 3 ml of 1 X SSC (0.15 M NaCl, 15 mM sodium citrate) to give 2 μg/μl of DNA.

1-(2) Preparation of Recombinant Plasmids

EcoRI was added to 5 μl of chromosomal DNA (corresponds to 10 μg of DNA) obtained in 1-(1) to a final concentration of 2 units/μg DNA. The reaction mixture was incubated at 37 ^{o}C for 30 minutes according to the method known in the art. After the incubation, EcoRI partially digested DNA was obtained. In the meantime, 1 μg of plasmid pUC19 DNA was digested with EcoRI according to the method known in the art to give a linear plasmid DNA.

The digested chromosome DNA fragments and pUC19 fragments were combined. T4 DNA ligase was added to the DNA mixture and incubated to give a ligation product.

1-(3) Isolation of Recombinant Plasmids

E. coli JM105 (TAKARA SYUZO Co., LTD) was grown in an LB culture medium at 37 ^{o}C for 2-3 hours. After the incubation, bacterial cells were harvested by centrifugation. The bacterial cells were suspended in an ice cold 50 mM $CaCl_2$ solution. The suspension was then centrifuged. The cell pellet was resuspended in a fresh 50 mM $CaCl_2$ solution. The suspension was placed on ice for 30 minutes.

After 30 minute incubation, about 100-200 µl of the suspension was transferred to an Eppendorf tube. The ligation product (about 2 µg of DNA) obtained in 1-(2) was added to the tube. The tube was further incubated on ice for 30 minutes. The bacterial cells were then heat-shocked at 42 ^{o}C for 2 minutes. 1 ml of an LB culture medium was added to the tube, which was incubated at 37 ^{o}C for 60 minutes.

After the incubation, the transformation product, about 100 µl each, was spread on an LB agar culture medium plate containing100 µg/ml of ampicillin, 0.5 mM IPTG, 0.2 % X-Gal. The plate was incubated at 37 ^{o}C overnight.

Of colonies appeared on the plate, white colonies were tested for esterase activity. Positive transformants that contain recombinant plasmids comprising an esterase gene were selected as described below.

Measurement of Esterase Activity

The white colonies on the plate were transferred to a filter paper soaked in a solution of 10 mM Tris-HCl/pH7.5, 0.01% bromocresolpurple and 100 ppm of methyl DL-ß-acetylthio-α-methylpropionate. The filter was incubated at room temperature for several hours. Transformants having a esterase activity produced carboxylic acid, which changed the color of bromocresolpurple (pH indicator) from purple to yellow around the colony because of a decrease

of pH. Positive transformants that contain recombinant plasmids comprising an esterase gene were thus obtained.

One of the positive transformants was grown and the plasmid DNA was prepared according to the method described by Birnboim et al., in Nucleic acid Res., 7: 1513-1523, 1979. The restriction map of the plasmid was constructed and is shown in Fig. 2. The recombinant plasmid shown in Fig. 2 was designated as pPE101.

1-(4) Esterase Gene Region in pPE101

pPE101 was digested with EcoRI according to the method known in the art. The restriction fragments were electrophoresed and a DNA fragment having EcoRI ends (large EcoRI digest) was obtained. The large EcoRI digest was further digested with various restriction enzymes to give smaller DNA fragments. Several restriction maps are shown in Fig. 3.

The smaller DNA fragments were then inserted into a multiple cloning site within the lac Z region of pUC19. The resulting recombinant plasmids were used to transform *E. coli* JM105. Transformants were tested for esterase activity.

Preparation of plasmid DNA, transformation and measurement of esterase activity were carried out according to the methods described in 1-(2) and (3). When inserting the smaller fragments into the pUC19, a linker was synthesized and used if it was necessary.

The esterase activity of the transformant is also shown in Fig. 3. According to the esterase activity, about 1.3kb ClaI/SmaI DNA fragment and about 1.2kb ClaI/PstI DNA fragment were found to contain an esterase gene.

E. coli JM105 transformed with the plasmid pPE116 was designated as *E. coli* JM105 (pPE116). *E. coli* JM105 (pPE116) was deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, under the name of MR-2101, and assigned the accession number FERM BP-3838.

E. coli C600 transformed with the plasmid pPE117 was designated as *E. coli* C600 (pPE117). *E. coli* C600 (pPE117) was deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, under the name of MR-2103, and assigned the accession number FERM BP-3835.

1-(5) Nucleotide Sequence of Esterase Structural Gene

The whole nucleotide sequence of the ClaI/SmaI DNA fragment obtained in 1-(4) was determined by the dideoxy chain termination method (F. Sanger, Science., 214: 1205, 1981) using a phage vector. The nucleotide sequence was then analyzed and the positions of an SD sequence and a DNA sequence essential for an enzyme activity were located. Based on the analysis, an open reading frame and a corresponding amino acid sequence were determined. The results are shown in SEQ ID No. 4.

1-(6) Nucleotide Sequences of Esterase Gene and Deduced Amino Acid Sequences

The whole nucleotide sequence of the DNA fragment in pPE117 was determined by the dideoxy chain termination method (F. Sanger, Science., 214: 1205, 1981) using M13 phage vector. The nucleotide sequence of about 1.2kb DNA fragment derived from the chromosomal DNA of *Pseudomonas putida* (FERM BP-3846) is the one described in

SEQ ID No. 3. The study of the nucleotide sequence described in SEQ ID No. 3 revealed the following: only an open reading frame encompassing the region essential for enzyme activity is present in the nucleotide sequence described in SEQ ID No. 1; an SD sequence situated several bases upstream of the translation initiation codon as well as the open reading frame is present in the nucleotide sequence described in SEQ ID No. 3. The amino acid sequence deduced from the nucleotide sequence described in SEQ ID No. 1 is shown in SEQ ID No. 2.

1-(7) Asymmetrical Hydrolysis of Methyl DL-ß-acethylthio-α-methylpropionate

E. coli JM105(pPE116) was cultured with shaking in 500 ml of an LB culture medium containing 50 µg/µl of ampicillin at 37 ^{o}C overnight. After incubation, bacterial cells were harvested by centrifugation. The whole bacterial cell was suspended in 200 ml of a 5 % methyl DL-ß-acetylthio-α-methylpropionate solution. The reaction was carried out at 45^{o}C for three hours while adjusting the pH with 0.1 N NaOH to pH 7. After the reaction, the bacterial cells were removed by centrifugation. Unreacted methyl ß-acetylthio-α-methylpropionate in the supernatant was removed using ethyl acetate. The pH of the aqueous layer of the resultant extract was adjusted with diluted sulfuric acid to 2.0 or less. The mixture was then extracted with ethyl acetate to give a D-ß-acetylthio-α-methylpropionate extract. Anhydrous sodium sulfate was added to the extract. After dehydration, the solvent was vapored out to give oily substance. Part of the substance was removed and diluted with water. The resulting diluted sample was subjected to a quantitative analysis of ß-acetylthio-α-methylpropionate by HPLC. Additionally, part of the substance was dissolved in chloroform. The optical rotation of the

resulting sample was polarimetrically analyzed (PM-101, Union Giken). As a result, 1.5 g of a desired product was obtained. Specific rotation was calculated by the following equation:

$$[\alpha]_D^{25} = -58.1 \ (C=2.00 \ CHCl_3)$$

The specific rotation was -58.3, which was the same value as that of a sample obtained from *Pseudomonas putida* (FERM BP-3846).

1-(8) Measurement of Esterase Activity

E. coli C600 (pPE117) cells were tested for asymmetric hydrolysis of methyl DL-ß-acetylthio-α-methylpropionate according to the similar method described in 1-(7).

The enzymatic activity of *Pseudomonas putida* (FERM BP-3846), *E. coli* JM105(pPE116) and *E. coli* C600 (pPE117) during the first one hour was measured and compared. The results are shown in Table 1.

Table 1

Bacterial strain	Relative specific activity*
Pseudomonas putida (FERM BP-3846)	1
E. coli JM105 (pPE116)[FERM BP-3838]	2
E. coli C600 (pPE117)[FERM BP-3835]	200

Pseudomonas putida (FERM BP-3846) is assumed to be 1

As is shown in Table 1, the specific activity of *E. coli* C600 (pPE117) [FERM BP-3835] is about 200 times as much as that of *Pseudomonas putida* (FERM BP-3846).

Example 2

Heat Stability Test of Esterase

Pseudomonas fluorescens IF03018, *Pseudomonas putida* 2068 (FERM BP-3846) and *E. coli* JM105(pPE116) [FERM BP-3838] were separately grown in the following condition.

(a) *Pseudomonas fluorescens* IF03018:

Culture medium; LB culture medium (500 ml)

Growth temperature; 30 ^{o}C

Incubation period; overnight

(b) *Pseudomonas putida* 2068 (FERM BP-3846):

Culture medium; LB culture medium (500 ml)

Growth temperature; 30 ^{o}C

Incubation period; overnight

(c) *E. coli* JM105(pPE116) [FERM BP-3838]

Culture medium; LB culture medium containing 50 μg/ml of ampicillin (500 ml)

Growth temperature; 30 ^{o}C

Incubation period; overnight

Bacterial cells thus obtained were tested for heat stability of esterase by the method described below.

Bacterial cells were harvested. 1.0 g of the cells (wet weight) was suspended in 100 ml of 0.05 M phosphate buffer/pH 7.0 containing 5.0 g of methyl DL-ß-acethylthio-α-methylpropionate. The suspension was incubated at 30 ^{o}C for enzyme reaction. As the reaction proceeded, 0.1 N NaOH was dropwise added to the suspension to maintain the pH at 7.0. The total volume (Q_1) of NaOH used to maintain the pH during first one hour was measured.

1.0 g of the cells (wet weight) was suspended in 100 ml of 0.05 M phosphate buffer/pH 7.0. The suspension was incubated for three hours at a temperature selected from the range of 20 - 80 ^{o}C. After incubation, 5.0 g of methyl DL-ß-acethylthio-α-methylpropionate was added to the suspension. The suspension was incubated at 30 ^{o}C for enzyme reaction. As the reaction proceeded, 0.1 N NaOH was added dropwise to the suspension to maintain the pH at 7.0. The total volume (Q_2) of NaOH used to maintain the pH during first one hour was measured.

Heat unaffected esterase activity is calculated by the following equation;

$$\text{Heat unaffected esterase activity} = (Q_2 \div Q_1) \times 100$$

The results are shown in Table 2.

Table 2

Incubation temperature (^{o}C)	Esterase activity		
	Bacterial strain		
	a	b	c
30	100	100	100
40	85	100	100
50	70	100	100
60	30	100	100
70	5	65	67
80	0	0	0

As is evident from Table 2, the heat stability of the esterase produced by *Pseudomonas putida* 2068 (FERM BP-3846) and *E. coli* JM105(pPE116) is higher than that of esterase produced by *Pseudomonas fluorescens* IF03018.

Example 3

3-(1) Purification of Novel Esterase

E. coli C600 (pPE117)[FERM BP-3835] was grown in a culture medium (1 % polypeptone, 0.5 % yeast extract, 0.5 % NaCl, 2 % glucose, pH 7.0) in aeration-agitation fermenter at 37 ^{o}C for 14 hours.

Bacterial cells were harvested by centrifugation and 15 g of bacterial cells was obtained. The bacterial cells were suspended in an equal amount of M/20 phosphate buffer. The bacterial cells were sonicated and then centrifuged. The supernatant was saved and concentrated using ammonium sulfate. The concentrate was then put on a DEAE Sephadex* D-50 (Pharmacia) column equilibrated with M/20 phosphate buffer. A linear gradient solution, 0 - 0.5M NaCl, was added to the column to elute esterase. Esterase active fractions were collected and concentrated. The concentrated sample was put on a Sephadex G-100 (Pharmacia) column equilibrated with M/20 phosphate buffer. Esterase was eluted with M/20 phosphate buffer. Esterase active fractions were pooled and concentrated. 500 mg of purified esterase was obtained.

The molecular weight of the esterase was determined by SDS-PAGE and found that it was about 30,000. The isoelectric point of the esterase was determined by the isoelectric focusing (Fast System, Pharmacia) and found that it was pH 3.90±0.1.

pH for optimum esterase activity and stability of the esterase in different pHs are shown in Figs. 4 and 5.

Temperature for optimum esterase activity and heat stability of the esterase are shown in Figs. 6 and 7.

3-(2) Analysis of N-terminal Amino Acid Sequence of Novel Esterase

N-terminal amino acid sequence of the novel esterase was analyzed by amino acid sequence autoanalyzer (SHIMADZU CORPORATION) and found as follows:

*Trade-mark

NH_2 terminus

1 - 2 - 3 - 4 - 5 - 6 - 7

Ser Tyr Val Thr Thr Lys Asp

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(ii) TITLE OF INVENTION:

(iii) NUMBER OF SEQUENCES:

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE

(B) STREET:

(C) CITY:

(D) STATE:

(E) COUNTRY:

(F) ZIP:

(v) COMPUTER READABLE FORM;

(A) MEDIUM TYPE:

(B) COMPUTER:

(C) OPERATING SYSTEM:

(D) SOFTWARE:

(vii) ATTORNEY/AGENT INFORMATION:

(A) NAME:

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE:

(B) TELEFAX:

(2) INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 831

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Pseudomonas putida

(B) STRAIN: MR-2068 (FERM BP-3846)

(ix) FEATURES: 1 - 831 P CDS

(xi) SEQUENCE DESCRIPTION:

ATG AGC TAT GTA ACC ACG AAG GAC GGC GTA CAG ATC TTC TAC AAG 45

GAC TGG GGC CCG CGC GAT GCG CCG GTC ATC CAC TTC CAC CAC GGC 90

TGG CCG CTC AGT GCC GAC GAC TGG GAC GCG CAG ATG CTG TTC TTC 135

CTC GCC CAC GGT TAC CGC GTG GTC GCC CAC GAC CGC CGC GGC CAT 180

GGC CGC TCC AGC CAG GTA TGG GAC GGC CAC GAC ATG GAC CAC TAC 225

GCC GAC GAC GTA GCC GCA GTG GTG GCC CAC CTG GGC ATT CAG GGC 270

GCC GTG CAT GTC GGC CAC TCG ACC GGT GGC GGT GAG GTG GTG CGC 315

TAC ATG GCC CGA CAC CCT GCA GAC AAG GTG GCC AAG GCC GTG CTG 360

ATC GCC GCC GTA CCG CCG TTG ATG GTG CAG ACT CCC GAT AAT CCC 405

GGT GGC CTG CCC AAA TCC GTT TTC GAC GGC TTC CAG GCC CAG GTC 450

GCC AGC AAC CGC GCG CAG TTC TAC CGG GAT GTG CCG GCA GGG CCG 495

TTC TAC GGC TAC AAC CGC CCC GGT GTC GAC GCC AGC GAA GGC ATC 540

ATC GGC AAC TGG TGG CGC CAG GGC ATG ATC GGT AGC GCC AAG GCC 585

CAT TAC GAT GGC ATC GTG GCG TTT TCC CAG ACC GAC TTC ACC GAA 630

GAC CTG AAG GGC ATT ACC CAG CCG GTG CTG GTG ATG CAT GGC GAC 675

GAC GAC CAG ATC GTG CCG TAT GAG AAC TCC GGG CTG CTG TCG GCC 720

AAG CTG CTG CCC AAT GGC ACA CTG AAG ACC TAC CAG GGC TAC CCG 765

CAT GGC ATG CCG ACC ACC CAT GCC GAT GTG ATC AAT GCG GAT TTG 810

CTG GCG TTT ATC CGT AGC TGA 831

(3) INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 276

(B) TYPE: Amino acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY:

(ii) MOLECULE TYPE: Peptide

(vi) ORIGINAL SOURCE

(A) ORGANISM: Pseudomonas putida

(B) STRAIN: MR-2068 (FERM BP-3846)

(ix) FEATURES:

(xi) SEQUENCE DESCRIPTION:

Met Ser Tyr Val Thr Thr Lys Asp Gly Val Gln Ile Phe Tyr Lys
1 5 10 15

Asp Trp Gly Pro Arg Asp Ala Pro Val Ile His Phe His His Gly
20 25 30

Trp Pro Leu Ser Ala Asp Asp Trp Asp Ala Gln Met Leu Phe Phe
35 40 45

Leu Ala His Gly Tyr Arg Val Val Ala His Asp Arg Arg Gly His
50 55 60

Gly Arg Ser Ser Gln Val Trp Asp Gly His Asp Met Asp His Tyr
65 70 75

Ala Asp Asp Val Ala Ala Val Val Ala His Leu Gly Ile Gln Gly
80 85 90
Ala Val His Val Gly His Ser Thr Gly Gly Gly Glu Val Val Arg
95 100 105
Tyr Met Ala Arg His Pro Ala Asp Lys Val Ala Lys Ala Val Leu
110 115 120
Ile Ala Ala Val Pro Pro Leu Met Val Gln Thr Pro Asp Asn Pro
125 130 135
Gly Gly Leu Pro Lys Ser Val Phe Asp Gly Phe Gln Ala Gln Val
140 145 150
Ala Ser Asn Arg Ala Gln Phe Tyr Arg Asp Val Pro Ala Gly Pro
155 160 165
Phe Tyr Gly Tyr Asn Arg Pro Gly Val Asp Ala Ser Glu Gly Ile
170 175 180
Ile Gly Asn Trp Trp Arg Gln Gly Met Ile Gly Ser Ala Lys Ala
185 190 195
His Tyr Asp Gly Ile Val Ala Phe Ser Gln Thr Asp Phe Thr Glu
200 205 210
Asp Leu Lys Gly Ile Thr Gln Pro Val Leu Val Met His Gly Asp
215 220 225
Asp Asp Gln Ile Val Pro Tyr Glu Asn Ser Gly Leu Leu Ser Ala
230 235 240
Lys Leu Leu Pro Asn Gly Thr Leu Lys Thr Tyr Gln Gly Tyr Pro
245 250 255
His Gly Met Pro Thr Thr His Ala Asp Val Ile Asn Ala Asp Leu
260 265 270
Leu Ala Phe Ile Arg Ser
275

(4) INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1120

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Pseudomonas putida

(B) STRAIN: MR-2068 (FERM BP-3846)

(ix) FEATURES: 93 - 923 P CDS

(xi) SEQUENCE DESCRIPTION:

CTGCAGTGCA GTCACCACAA ATTCCGGCGC CAAGCAAAAT TCCTCCTATT CTCAATAGCT 60

CACTTCGCTT CCTGCACACA GGAGACCCGA CC ATG AGC TAT GTA ACC ACG AAG 113

GAC GGC GTA CAG ATC TTC TAC AAG GAC TGG GGC CCG CGC GAT GCG CCG 161

GTC ATC CAC TTC CAC CAC GGC TGG CCG CTC AGT GCC GAC GAC TGG GAC 209

GCG CAG ATG CTG TTC TTC CTC GCC CAC GGT TAC CGC GTG GTC GCC CAC 257

GAC CGC CGC GGC CAT GGC CGC TCC AGC CAG GTA TGG GAC GGC CAC GAC 305

ATG GAC CAC TAC GCC GAC GAC GTA GCC GCA GTG GTG GCC CAC CTG GGC 353

ATT CAG GGC GCC GTG CAT GTC GGC CAC TCG ACC GGT GGC GGT GAG GTG 401

GTG CGC TAC ATG GCC CGA CAC CCT GCA GAC AAG GTG GCC AAG GCC GTG 449

CTG ATC GCC GCC GTA CCG CCG TTG ATG GTG CAG ACT CCC GAT AAT CCC 497

GGT GGC CTG CCC AAA TCC GTT TTC GAC GGC TTC CAG GCC CAG GTC GCC 545

AGC AAC CGC GCG CAG TTC TAC CGG GAT GTG CCG GCA GGG CCG TTC TAC 593

GGC TAC AAC CGC CCC GGT GTC GAC GCC AGC GAA GGC ATC ATC GGC AAC 641

TGG TGG CGC CAG GGC ATG ATC GGT AGC GCC AAG GCC CAT TAC GAT GGC 689

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ATC GTG GCG TTT TCC CAG ACC GAC TTC ACC GAA GAC CTG AAG GGC ATT     737
ACC CAG CCG GTG CTG GTG ATG CAT GGC GAC GAC GAC CAG ATC GTG CCG     785
TAT GAG AAC TCC GGG CTG CTG TCG GCC AAG CTG CTG CCC AAT GGC ACA     833
CTG AAG ACC TAC CAG GGC TAC CCG CAT GGC ATG CCG ACC ACC CAT GCC     881
GAT GTG ATC AAT GCG GAT TTG CTG GCG TTT ATC CGT AGC TGATGTGATC      930
GCCTGCACCG GCCTCTTCGC GGGCACTGGC AACACACCTC CCCCAGGATT ACCATGTCAC  990
GCTTCTAGTG CGGCCCTTTG CCGCCCCTTG CCTCCCTGCC TGCCAAAACC CCATGCCCTT 1050
CGAACTCACC GTAGAACCCC TCACCCTGCT GATCCTGGCC CTGGTCGCCT TCGTCGCCGG 1110
TTTCATCGAT                                                       1120
```

(4) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1329

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE

(A) ORGANISM: Pseudomonas putida

(B) STRAIN: MR-2068 (FERM BP-3846)

(ix) FEATURES:

(A) NAME/KEY: mat peptide

(B)LOCATION: 302.. 1129

(C) IDENTIFICATION METHOD: dideoxy chain termination method (F. Sanger)

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION:

```
CCCGGGCCGT GAGCGATGCC ATCCTCGGTG ACGACGACCT GCTGGCGCTA TATCAAGGCA 60

TCGACAACGG CCGCTTCCCC GGTGGCGACC TGCTGGCCGC ACCGCTGGAA GCCGCCGCCA 120

AGGCCTGGTA CCGGATGCGC GACCGCGCCT GATCGCCTGG CACCGCTCCT ACACGGCGCC 180

GGGCAGGCCG GAAGCATGGT GCAAGCCCAC TGCAGTGCAG TCACCACAAA TTCCGGCGCC 240

AAGCAAAATT CCTCCTATTC TCAATAGCTC ACTTCGCTTC CTGCACACAG GAGACCCGAC 300

C ATG AGC TAT GTA ACC ACG AAG GAC GGC GTA CAG ATC TTC TAC AAG GAC 349
  Met Ser Tyr Val Thr Thr Lys Asp Gly Val Gln Ile Phe Tyr Lys Asp
  1               5                  10                  15

TGG GGC CCG CGC GAT GCG CCG GTC ATC CAC TTC CAC CAC GGC TGG CCG   397
Trp Gly Pro Arg Asp Ala Pro Val Ile His Phe His His Gly Trp Pro
              20                  25                  30

CTC AGT GCC GAC GAC TGG GAC GCG CAG ATG CTG TTC TTC CTC GCC CAC   445
Leu Ser Ala Asp Asp Trp Asp Ala Gln Met Leu Phe Phe Leu Ala His
          35                  40                  45

GGT TAC CGC GTG GTC GCC CAC GAC CGC CGC GGC CAT GGC CGC TCC AGC   493
Gly Tyr Arg Val Val Ala His Asp Arg Arg Gly His Gly Arg Ser Ser
      50                  55                  60

CAG GTA TGG GAC GGC CAC GAC ATG GAC CAC TAC GCC GAC GAC GTA GCC   541
Gln Val Trp Asp Gly His Asp Met Asp His Tyr Ala Asp Asp Val Ala
  65                  70                  75                  80

GCA GTG GTG GCC CAC CTG GGC ATT CAG GGC GCC GTG CAT GTC GGC CAC   589
Ala Val Val Ala His Leu Gly Ile Gln Gly Ala Val His Val Gly His
                  85                  90                  95

TCG ACC GGT GGC GGT GAG GTG GTG CGC TAC ATG GCC CGA CAC CCT GCA   637
Ser Thr Gly Gly Gly Glu Val Val Arg Tyr Met Ala Arg His Pro Ala
             100                 105                 110
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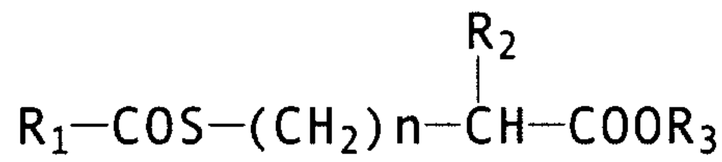
GAC AAG GTG GCC AAG GCC GTG CTG ATC GCC GCC GTA CCG CCG TTG ATG    685
Asp Lys Val Ala Lys Ala Val Leu Ile Ala Ala Val Pro Pro Leu Met
        115                 120                 125
GTG CAG ACT CCC GAT AAT CCC GGT GGC CTG CCC AAA TCC GTT TTC GAC    733
Val Gln Thr Pro Asp Asn Pro Gly Gly Leu Pro Lys Ser Val Phe Asp
    130                 135                 140
GGC TTC CAG GCC CAG GTC GCC AGC AAC CGC GCG CAG TTC TAC CGG GAT    781
Gly Phe Gln Ala Gln Val Ala Ser Asn Arg Ala Gln Phe Tyr Arg Asp
145                 150                 155                 160
GTG CCG GCA GGG CCG TTC TAC GGC TAC AAC CGC CCC GGT GTC GAC GCC    829
Val Pro Ala Gly Pro Phe Tyr Gly Tyr Asn Arg Pro Gly Val Asp Ala
                165                 170                 175
AGC GAA GGC ATC ATC GGC AAC TGG TGG CGC CAG GGC ATG ATC GGT AGC    877
Ser Glu Gly Ile Ile Gly Asn Trp Trp Arg Gln Gly Met Ile Gly Ser
            180                 185                 190
GCC AAG GCC CAT TAC GAT GGC ATC GTG GCG TTT TCC CAG ACC GAC TTC    925
Ala Lys Ala His Tyr Asp Gly Ile Val Ala Phe Ser Gln Thr Asp Phe
        195                 200                 205
ACC GAA GAC CTG AAG GGC ATT ACC CAG CCG GTG CTG GTG ATG CAT GGC    973
Thr Glu Asp Leu Lys Gly Ile Thr Gln Pro Val Leu Val Met His Gly
    210                 215                 220
GAC GAC GAC CAG ATC GTG CCG TAT GAG AAC TCC GGG CTG CTG TCG GCC   1021
Asp Asp Asp Gln Ile Val Pro Tyr Glu Asn Ser Gly Leu Leu Ser Ala
225                 230                 235                 240
AAG CTG CTG CCC AAT GGC ACA CTG AAG ACC TAC CAG GGC TAC CCG CAT   1069
Lys Leu Leu Pro Asn Gly Thr Leu Lys Thr Tyr Gln Gly Tyr Pro His
                245                 250                 255
```

```
GGC ATG CCG ACC ACC GAT GCC GAT GTG ATC AAT GCG GAT TTG CTG GCG  1117
Gly Met Pro Thr Thr His Ala Asp Val Ile Asn Ala Asp Leu Leu Ala
            260                 265                 270
TTT ATC CGT AGC TGA TGTGATCGCC TGCACCGGCC TCTTCGCGGG CACTGGCAAC  1172
Phe Ile Arg Ser
        275 276
ACACCTCCC CCAGGATTAC CATGTCACGC TTCTAGTGCG GCCCTTTGCC GCCCCTTGC  1230
CTCCCTGCC TGCCAAAACC CCATGCCCTT CGAACTCACC GTAGAACCCC TCACCCTGC  1298
TGATCCTGG CCCTGGTCGC CTTCGTCGCC GGTTTCATCG AT                    1329
```

CLAIMS:

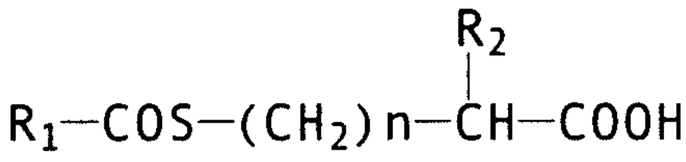
1. A DNA fragment containing a nucleotide sequence that encodes the amino acid sequence of esterase as described in SEQ ID No. 2, the esterase asymmetrically hydrolyzing carboxylic acid esters represented by the formula (I):



(I)

(wherein R_1 is alkyl, aralkyl or aryl; R_2 and R_3 are each alkyl; and n is 1 or 2)

to give an optically active carboxylic acid represented by the formula (II):



(II)

(wherein R_1, R_2, and n have the same meanings as described above).

2. An esterase comprising the amino acid sequence as described in SEQ ID No. 2.

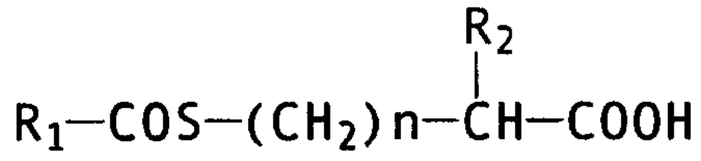
3. The DNA fragment of claim 1, comprising the whole nucleotide sequence as described in SEQ ID No. 1.

4. The DNA fragment of claim 1, comprising the whole nucleotide sequence as described in SEQ ID No. 3.

5. A recombinant plasmid comprising the DNA fragment of claim 1, 3 or 4.

6. A transformant obtainable by transforming a host microorganism with the recombinant plasmid of claim 5.

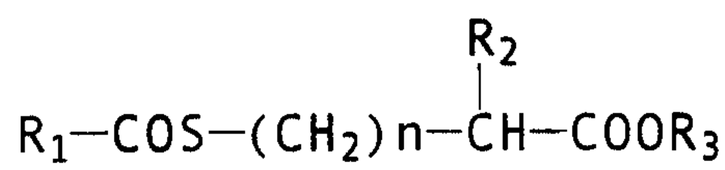
7. A method of producing an optically active carboxylic acid represented by the formula (II):



(II)

(wherein R_1 is alkyl, aralkyl or aryl; R_2 is alkyl; and n is 1 or 2) and its enantiomeric ester, which method comprises:

reacting a racemic mixture of a carboxylic acid ester represented by the formula (I):



(I)

(wherein R_3 is alkyl; and R_1, R_2, and n have the same meanings as described above) with a culture or bacterial cell of the transformant of claim 6 or a material obtained by drying, treating with an organic solvent, disrupting or extracting the bacterial cell of the transformant of claim 6.

8. The method as described in claim 7, in which the transformant is *E. coli*.

9. The method as described in claim 7 or 8, in which the reaction is carried out at a temperature of 45°C or more but less than 80°C.

10. The method as described in claim 9, wherein the reaction is carried out at a temperature of from 45°C to 70°C at a pH of 6 to 9.

11. The method as described in any one of claims 7 to 10, wherein R_1 is $(C_1\text{-}C_6)$alkyl, benzyl or phenyl; and R_2 and R_3 are each $(C_1\text{-}C_6)$alkyl.

12. The method as described in any one of claims 7 to 10, wherein the carboxylic acid ester represented by the

formula (I) is a member selected from the group consisting of methyl β-acetylthio-α-methylpropionate, methyl S-acetyl-β-mercaptoisobutyrate, methyl S-acetyl-γ-mercapto-α-methyl-n-butyrate, methyl S-benzoyl-β-mercaptoisobutyrate and methyl S-phenylacetyl-β-mercaptoisobutyrate.

13. The recombinant plasmid pPE116.

14. The recombinant plasmid pPE117.

15. A transformant obtained by transforming a host microorganism selected from the group consisting of *E. coli*, yeast, *Bacillus subtilis* and *Actinomycetes* with a recombinant plasmid which is derived from the plasmid pUC19 and contains the DNA fragment of claim 1, 3 or 4.

16. A transformant of claim 15, wherein the DNA fragment is contained at an EcoRI site of a multiple cloning site in the lacZ gene of the plasmid pUC19.

17. A transformant of claim 15 or 16, wherein the host microorganism is *E. coli*.

18. The transformant *E. coli* JM105 (pPE116).

19. The transformant *E. coli* C600 (pPE117).

SMART & BIGGAR
OTTAWA, CANADA

PATENT AGENTS

FIG.1

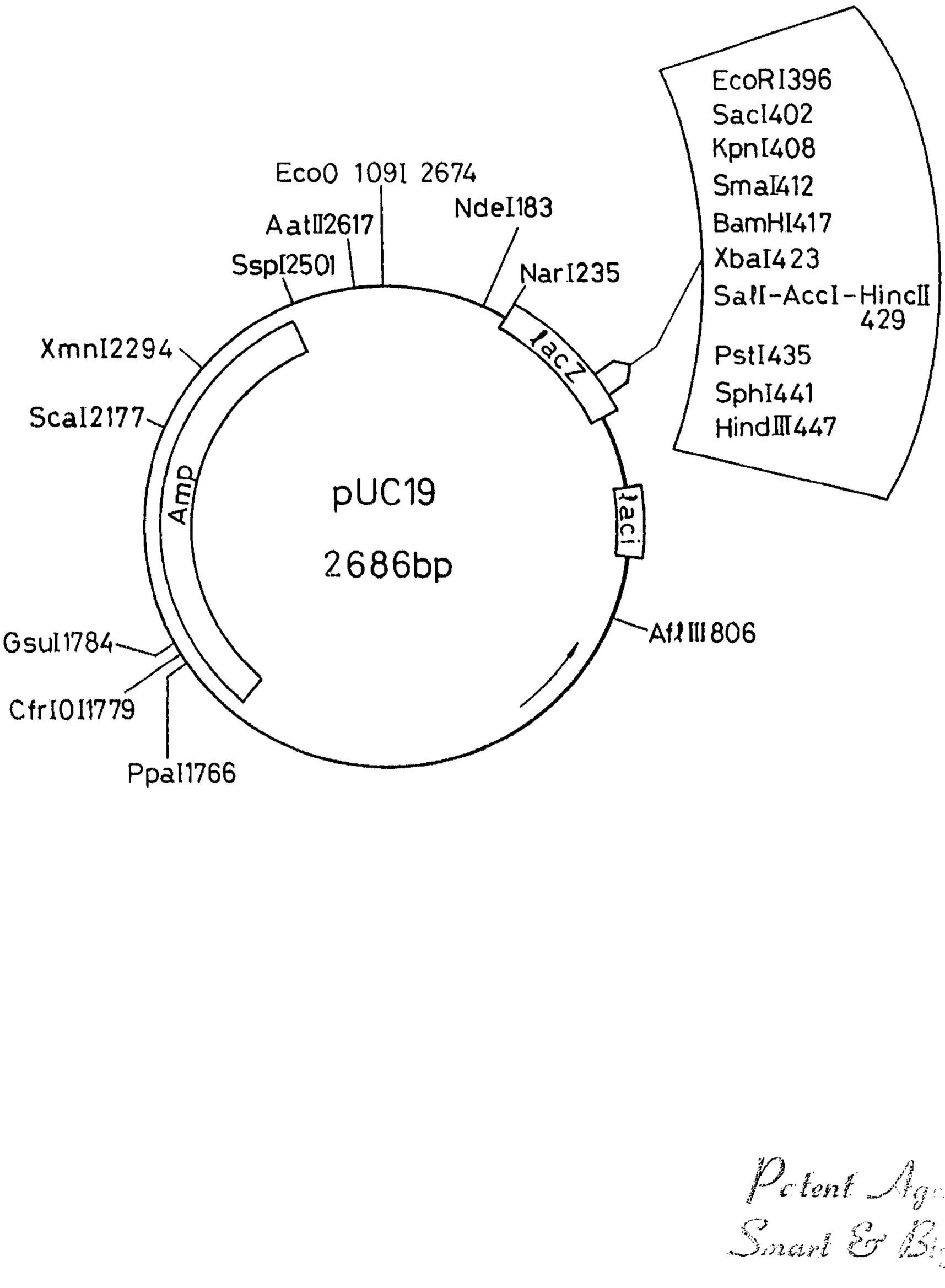


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FIG.2

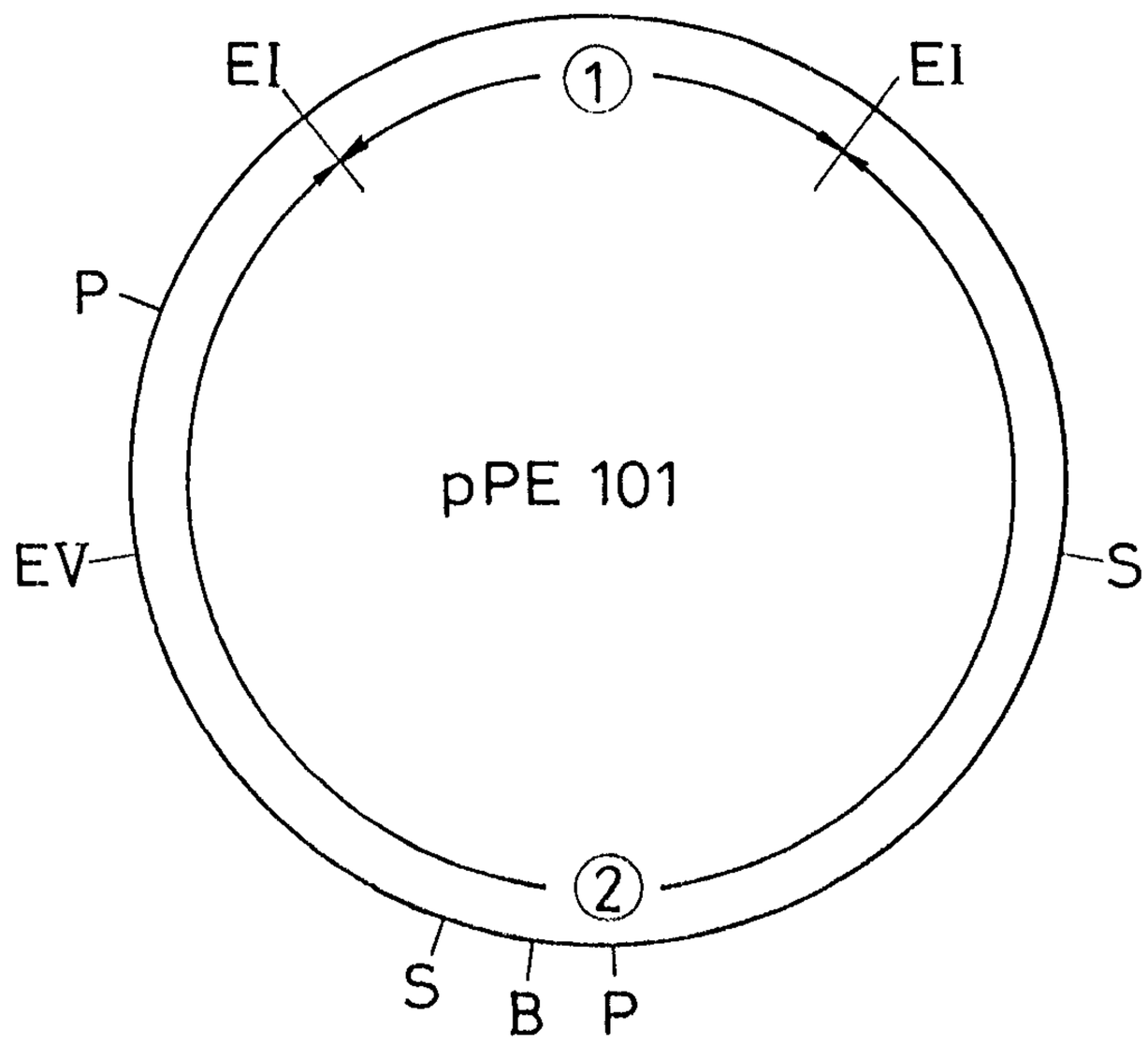

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FIG.3

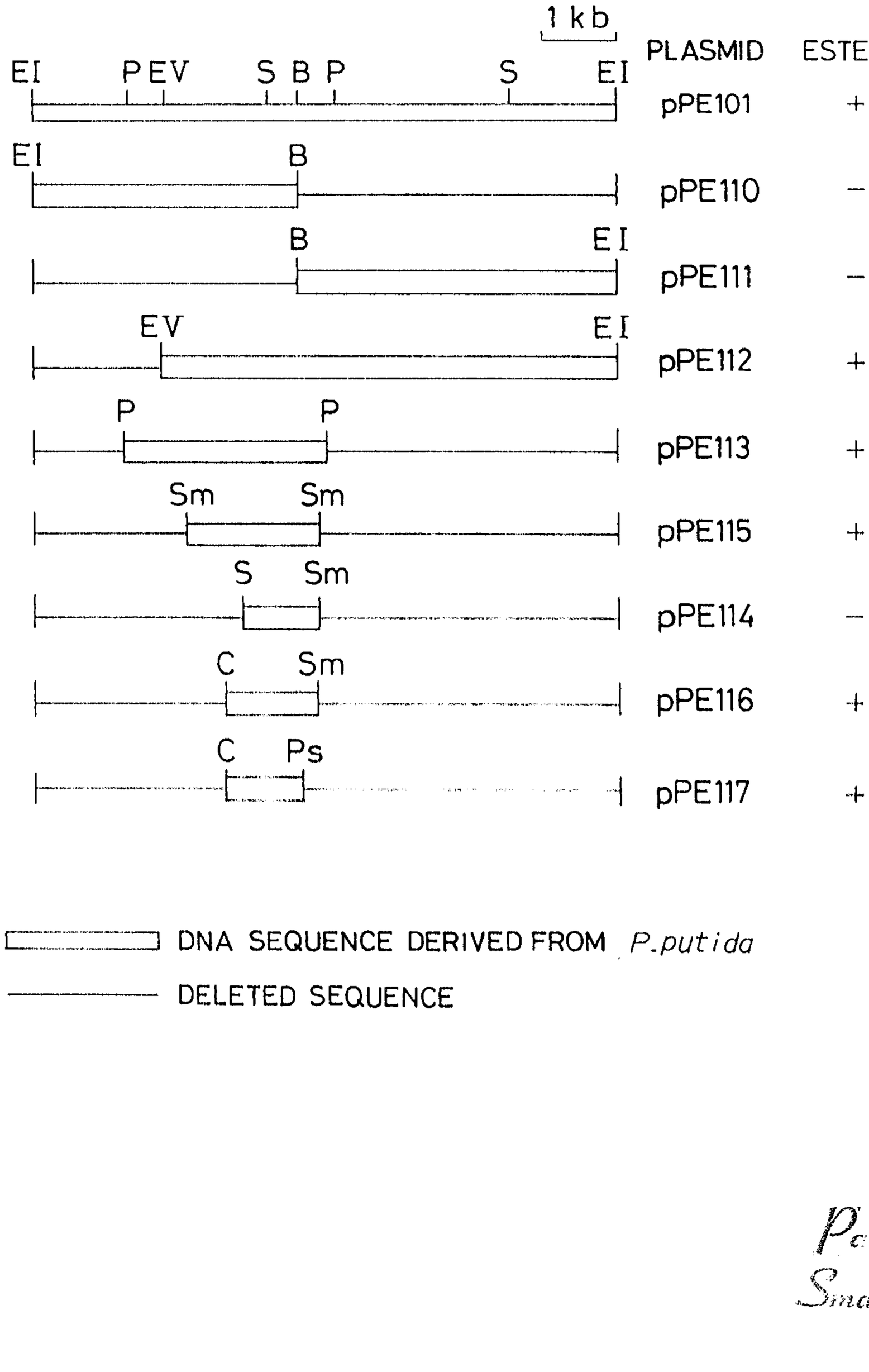

FIG.4

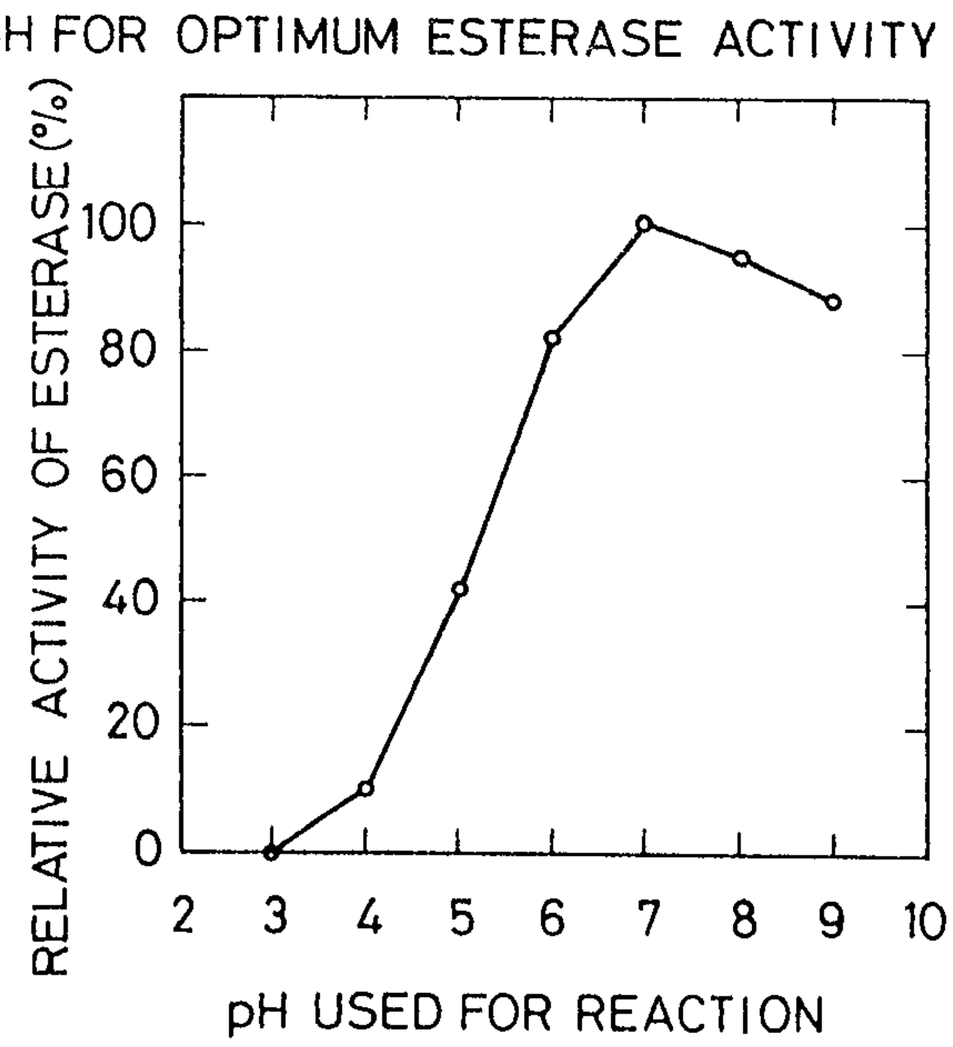

FIG.5

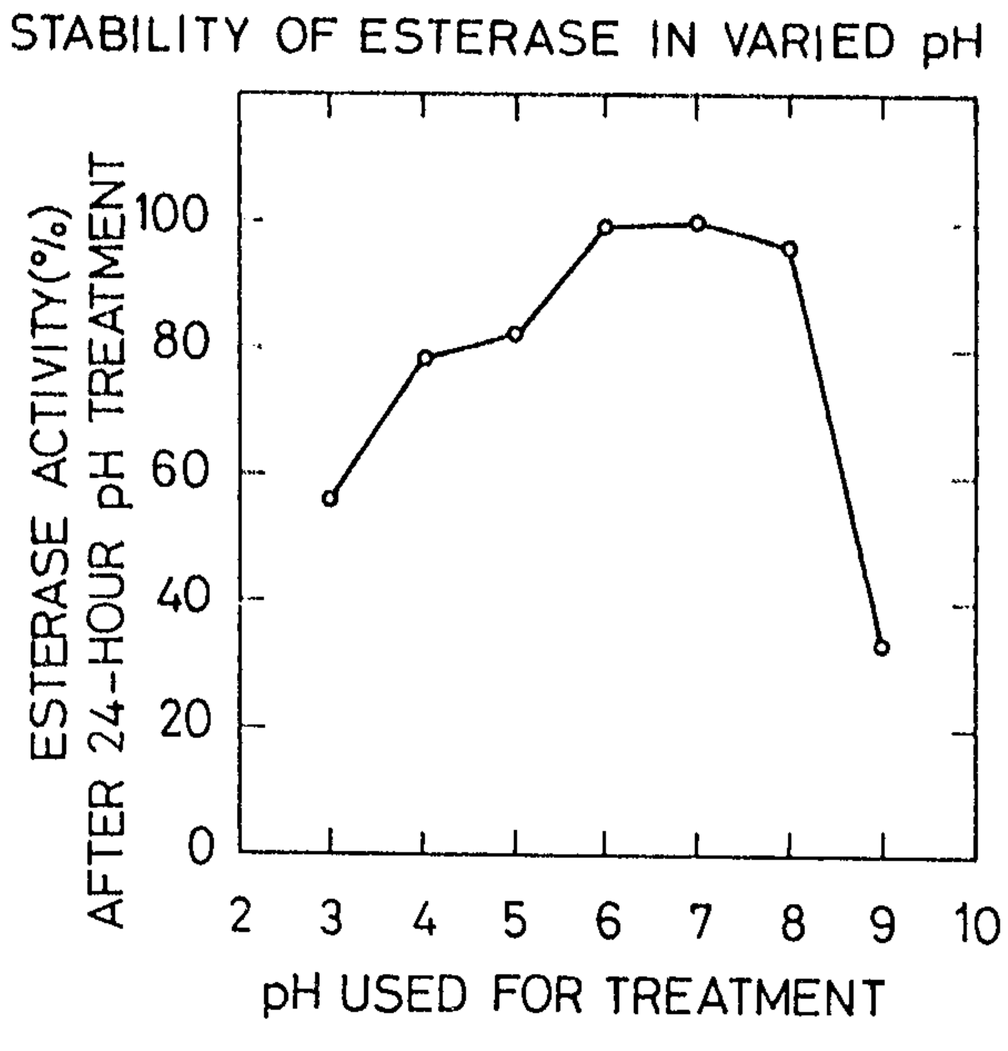

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FIG.6

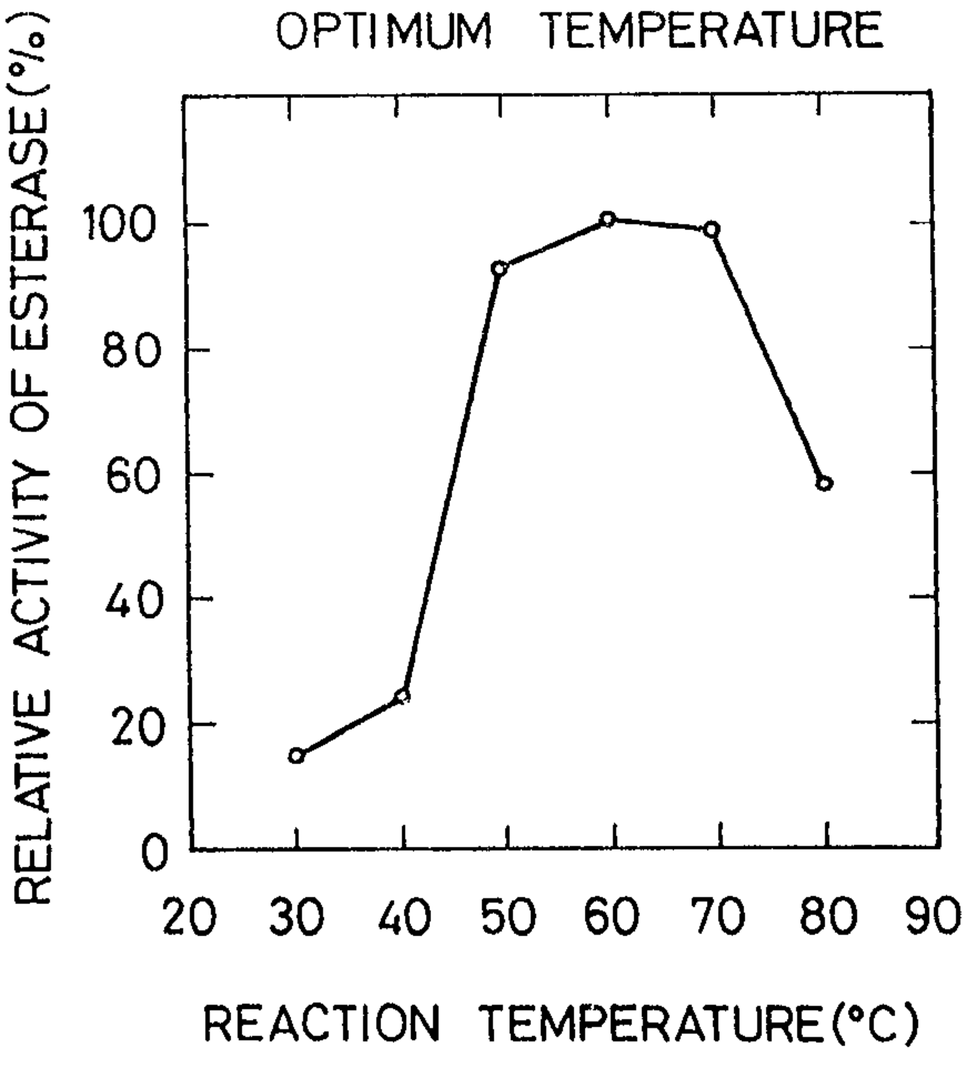
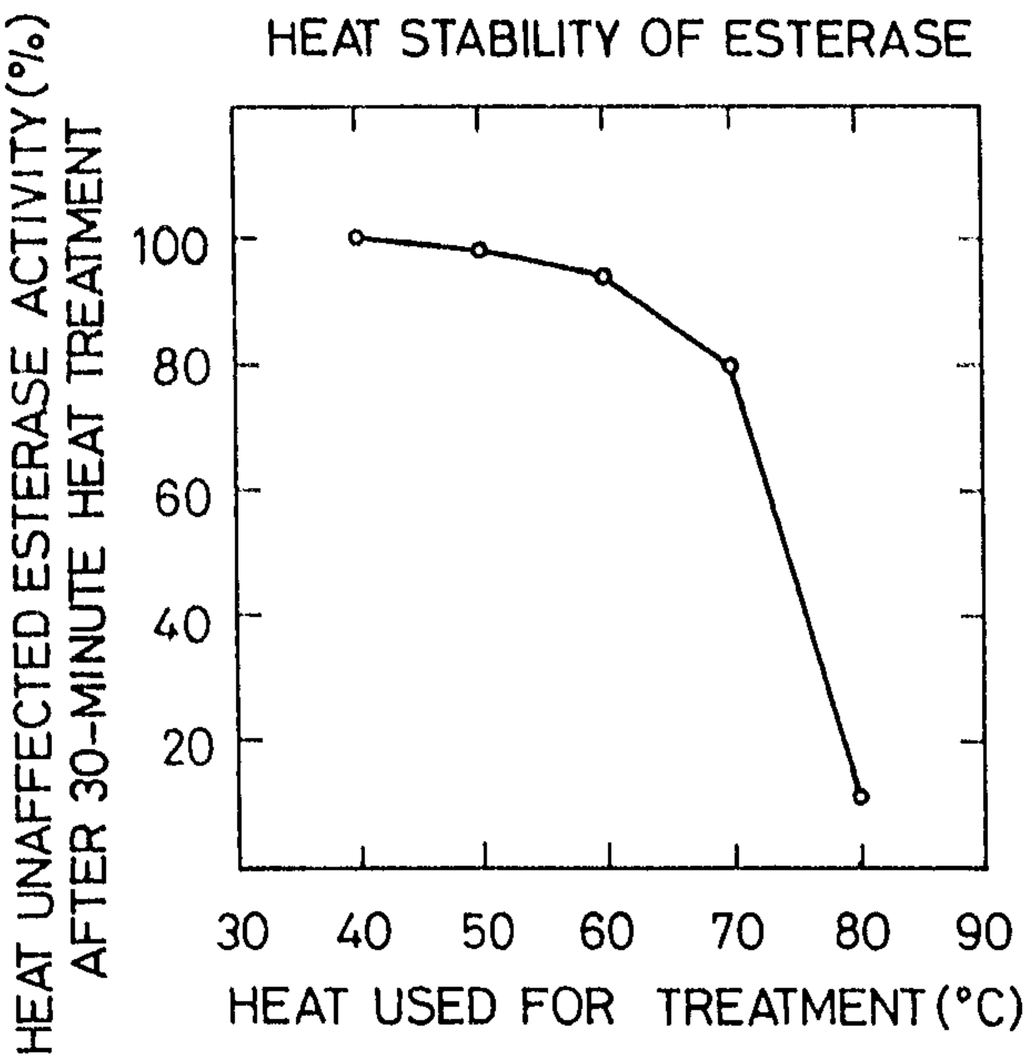


FIG.7



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