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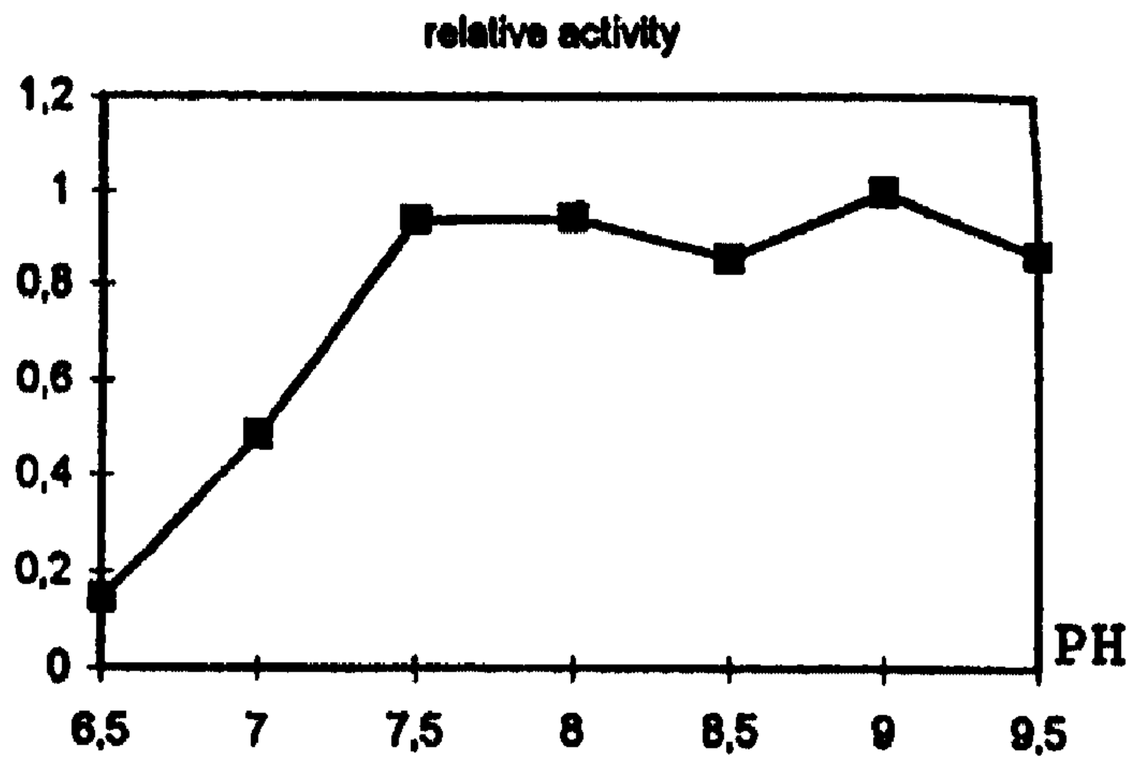
(54) **EPOXYDE HYDROLASE**
(54) **EPOXIDE HYDROLASE**



(57) La présente invention concerne une séquence de nucléotides isolés et purifiés d'origine microbienne codant une époxyde hydrolase, le vecteur comprenant ladite séquence de nucléotides, la cellule hôte recombinante transformée par la séquence de nucléotides et la séquence d'acide aminé d'époxyde hydrolase codée par la séquence de nucléotides et/ou exprimée par la cellule hôte recombinante.

(57) The present invention is related to an isolated and purified nucleotide sequence from microbial origin, encoding an epoxide hydrolase, the vector comprising said nucleotide sequence, the recombinant host cell transformed by said nucleotide sequence and the epoxide hydrolase amino acid sequence encoded by said nucleotide sequence and/or expressed by said recombinant host cell.

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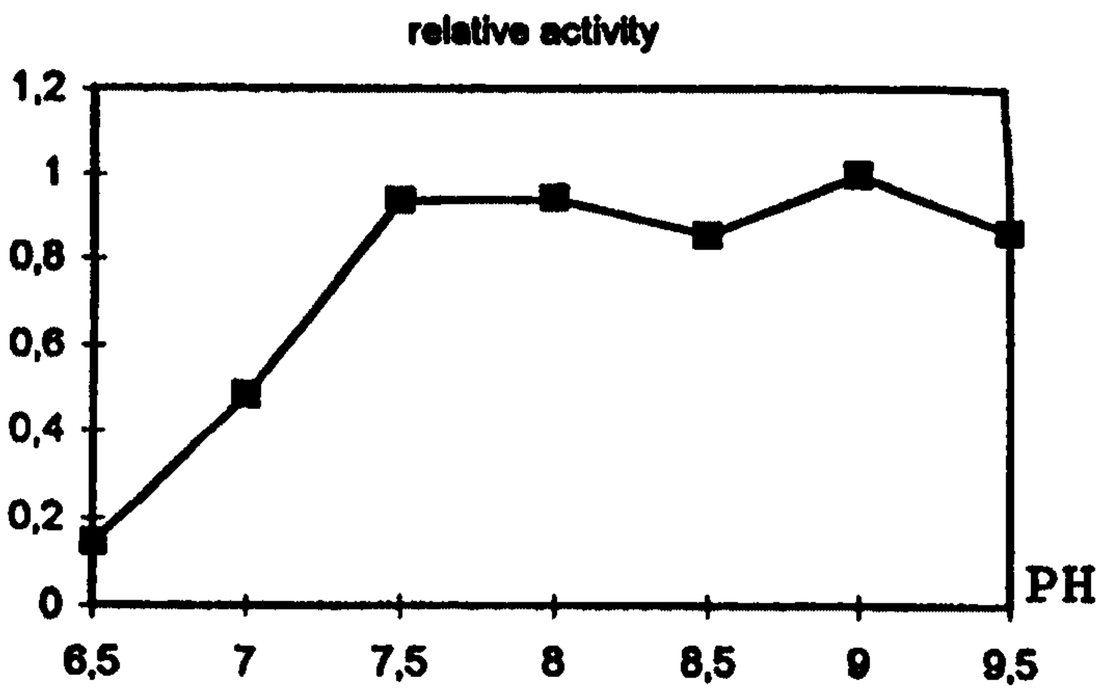
(57) Abstract

The present invention is related to an isolated and purified nucleotide sequence from microbial origin, encoding an epoxide hydrolase, the vector comprising said nucleotide sequence, the recombinant host cell transformed by said nucleotide sequence and the epoxide hydrolase amino acid sequence encoded by said nucleotide sequence and/or expressed by said recombinant host cell.



EPOXIDE HYDROLASE

Field of the invention

The present invention relates to epoxide hydrolase nucleotidic and amino acid sequences and to their use in the enantiomeric hydrolysis of epoxides.

Background of the present invention

Epoxides

Epoxides are used as chiral building blocks in the organic synthesis of fine chemicals, especially enantiomerically pure compounds. They are reactive molecules as their ring may be easily open to give a broad range of products. For this reason, they occupy a place of importance among the precursors of pharmaceuticals and speciality chemicals. Some chemical methods exist for preparing them from optically active precursors, but no efficient asymmetric syntheses involving asymmetrisation or resolution methods are known with the exception of the Sharpless-epoxidation method, which is limited to allylic alcohols (Katsuki et al., *J. Am. Chem. Soc.* **102**, p. 5974 (1980)). The use of biological reactions to perform epoxides synthesis has also been investigated (see for example the review of de Bont J.A.M., *Tetrahedron: Asymmetry* 4, p. 1331 (1993)).

Other epoxides, like halogenated aliphatic epoxides, are potential pollutants that are released into the environment from various industrial sources or that may be formed during the transformation of other synthetic chemicals. For example, epichlorohydrin (3-chloro-1,2-epoxypropane) is a widely used industrial chemical that is well recognised as mutagenic and carcinogenic.

Epoxide hydrolases

Epoxide hydrolases (EC3.3.2.3.) are hydrolytic enzymes which catalyse the opening of an epoxide ring converting their substrate to the corresponding diol. One of their most interesting property is that they are generally highly regio- and enantioselective, allowing the preparation of pure enantiomers.

Epoxide hydrolases have been studied in a variety of organisms. The best studied are those from mammals. They are found mainly in liver, testis, kidney, ovary and lung. They have been intensively characterised because of their involvement in the metabolism of xenobiotics (detoxification of cytotoxic, mutagenic and carcinogenic intermediates) (Seidegard et al., *Biochemica et Biophysica Acta* **695**, p. 251 (1983)).

Epoxide hydrolases have been also described in other higher eukaryotes like plants and insects.

Due to their low availability, enzymes from such sources are not of practical value for large-scale processes. The microbial world represents a suitable alternative due to the possibility of cultivating micro-organisms on a large-scale. The use of whole cells to perform the biotransformation of epoxides has been investigated. Microbial epoxide hydrolases have been

already described in a variety of micro-organisms. Some examples of such descriptions are summarised hereafter :

- *Aspergillus niger* LPC521 and *Beauvaria sulfurescens* ATCC7159 possess enantiocomplementary epoxide hydrolases that hydrolyse the two racemic forms of styrene epoxide (Pedragosa-Moreau et al., *J. Org. Chem.* **58**, p. 5533 (1993)).
- *Diplodia gossipina* ATCC16391 catalyses the kinetic resolution of racemic indene oxide into 1(S), 2(R) indene oxide (Zhang et al., *J. Ferment. Bioeng.* **80**, p. 244 (1995)).
- Epichlorhydrin (3-chloro-1,2-epoxypropane) is transformed in (R)-3-chloro-2-propanol by a epoxide hydrolase characterised in *Corynebacterium sp* strain N-1074 (Nakamura et al., *J. Bact.* **174**, p. 7613 (1992)). A similar enzyme has been purified from *Pseudomonas sp.* strain AD1 (Jacobs et al., *Eur. J. Biochem.* **202**, p. 1217 (1991)).
- An epoxide hydrolase that catalyses the asymmetric hydrolysis of various racemic epoxides in chiral epoxides and diols has been isolated from *Rhodococcus sp.* NCIMB 11216 (Mischitz et al., *Biotechnol. Lett.* **17**, p. 893 (1995)).
- A strain of *Flavobacterium sp.* is able to convert trans-1-epoxysuccinic acid in mesotartaric acid (Martin et al., *Biochem. J.* **70**, p. 405 (1955)).
- The epoxide hydrolase of *Nocardia tartaricans* catalyses the hydrolysis of cis-epoxysuccinate to give L(+)tartaric acid (Patentschrift DE 2605921). The same reaction could be performed by some other micro-organisms like Achromobacter, *Alcaligenes* (Patent US-3957579), *Acinetobacter tartarogenes*, *Agrobacterium aureum*,

Agrobacterium viscosum, *Rhizobium validum*, *Pseudomonas sp.* (Offenlegungsschrift DT 2619311).

A characteristic common to all these examples is the use of whole cells or whole crude extracts of the cells to perform the reaction. The enzyme can be liberated either by breaking the cells by physical disruption or by permeabilising the cell wall and/or the cell membrane by the use of detergents.

Tartaric acid

Tartaric acid is used by the food industry for various applications (additive in soft drinks, food preservative, raw material for the synthesis of emulsifiers,...). It is possible to synthesise chemically the tartaric acid starting from maleic acid but this process gives a racemic product composed of L(+)-tartaric acid and D(+)-tartaric acid. In food, only the L(+) form of tartaric acid is authorised as the D(+) is considered as harmful for human health.

L(+)-tartaric acid is produced naturally as a by-product during wine fermentation, but the supply of this compound is variable from year to year as it is very dependant of the climate.

The enzymatic hydrolysis of cis-epoxysuccinate by a cis-epoxysuccinate hydrolase allows the obtention of the only L(+) form of tartaric acid. This biotransformation would thus represent a valuable alternative for the production of L(+) tartaric acid.

State of the art

Rink et al. (*J. of Biological Chemistry* 272 (23) (June 6, 1997)) describe the primary structure and catalytic mechanisms of the epoxide hydrolase from the strain *Agrobacterium radiobacter* AD1.

Murdiyatmo et al. (*Biochemical Journal* Vol. 284, pp. 87-93 (May 1992)) describe the molecular biology of the 2-haloacid-halidohydrolase-IVa from *Pseudomonas cepacia* MBA4.

Mischitz et al. (*Biotechnology Letters* No. 17(9), pp. 893-898 (1995)) describe the isolation of a highly enantioselective epoxide hydrolase from a strain of *Rhodococcus sp.* having a molecular weight of 33-35000 kD and obtained from gel filtration chromatography SDS page. Said document states that the optimum temperature of the epoxide hydrolase is 30 ^{o}C. However, said document never describes the amino acid sequence of said enzyme and the possible nucleotide sequence encoding said enzyme.

Yamagishi & Cho (*Annals of New York Academy of Sciences* Vol. 799, pp. 784-785 (1996)) describe the enzymatic preparation of tartaric acid from cis-epoxysuccinic acid by the strain *Pseudomonas putida* MCI3037.

A similar preparation of tartaric acid is described in the Japanese patent application JP-08245497 by treating cis-epoxytartaric acid with a culture of a *Pseudomonas* micro-organism.

Summary of the invention

The present invention is related to a isolated and purified nucleotide sequence from *Rhodococcus*

rhodochrous, preferably a strain having the deposit umber LMGP-18079, encoding an epoxide hydrolase.

According to the invention, said nucleotide sequence (genomic DNA, cDNA, RNA) presents more than 50%, preferably more than 70%, more preferably more than 90% homology with the sequence SEQ ID NO 5 described hereafter.

According to a preferred embodiment of the present invention, said isolated and purified nucleotide sequence corresponds to the nucleotide sequence SEQ ID NO 5 or a portion thereof encoding a peptide having an epoxide hydrolase activity.

It is meant by "a portion of the nucleotide sequence SEQ ID NO 5", a fragment of said sequence SEQ ID NO 5 having more than 100 nucleotides of said nucleotide sequence and encoding a protein characterised by an epoxide hydrolase enzymatic activity similar to the epoxide hydrolase activity of the complete amino-acid sequence SEQ ID NO 6.

Preferably, said portion has an epoxide hydrolase enzymatic activity of more than 80% of the epoxide hydrolase enzymatic activity of the amino acid sequence SEQ ID NO 6, preferably has an epoxide hydrolase enzymatic activity corresponding to the one of the amino acid sequence corresponding to SEQ ID NO 6.

Another aspect of the present invention is related to a recombinant nucleotide sequence comprising, operably linked to the nucleotide sequence according to the invention and above-describes, one or more adjacent regulatory sequence(s), preferably originating from homologous micro-organisms.

However, said adjacent regulatory sequences can also be originating from heterologous micro-organisms.

These adjacent regulatory sequences are specific sequences such as promoters, secretion and termination signal sequences.

Another aspect of the present invention is related to the vector comprising the nucleotide sequence(s) according to the invention, possibly operably linked to one or more adjacent regulatory sequence(s) originating from homologous or from heterologous micro-organisms.

It is meant by "a vector", any biochemical construct which can be used for the introduction of a nucleotide sequence (by transduction or transfection) into a cell. Advantageously, the vector according to the invention is selected from the group consisting of plasmids, viruses, phagemides, liposomes, cationic vesicles or a mixture thereof. Said vector may comprise already one or more of the above-described adjacent regulatory sequence(s).

Preferably, said vector is a plasmid having the deposit number LMBP-3666.

The present invention is also related to the host cell, preferably a recombinant host cell, transformed by the nucleotide sequence or the vector according to the invention above-described.

It is meant by "a host cell transformed by the nucleotide sequence or the vector according to the invention", a cell having incorporated said nucleotide sequence or said vector and which does not comprise naturally said nucleotide sequence or said vector.

Preferably, said host cell is also capable of expressing said nucleotide sequence or said vector and allows advantageously the production of an amino acid sequence encoded by said nucleotide sequence or by said

vector. The isolated and purified nucleotide sequence according to the invention can be either integrated into the genome of the selected host cell or present on a episomal vector in said host cell.

Advantageously, the recombinant host cell according to the invention is selected from the group consisting of the microbial world, preferably bacteria or fungi, including yeast.

Preferably, said recombinant host cell is modified to obtain an expression of the epoxide hydrolase enzyme at high level.

Preferably, said expression at high level is obtained by the use of adjacent regulatory sequences being capable of directing the overexpression of the nucleotide sequence according to the invention in the recombinant host cell.

Another aspect of the present invention is related to the isolated and purified (from possible contaminants) epoxide hydrolase amino acid sequence encoded by the isolated and purified nucleotide sequence and/or expressed by the recombinant host cell according to the invention.

The isolated and purified epoxide hydrolase amino acid sequence according to the invention is also characterised by an advantageous pH activity profile having a high enzymatic activity (more than 80% of the optimum enzyme activity) between 7.0 and 10, preferably between 7.5 and 9.5 (see Fig. 5).

Advantageously, said isolated and purified epoxide hydrolase has a molecular weight comprised between 26 and 30 kD, preferably a molecular weight about 28 kD (theoretical molecular weight 28,136 kD).

Said epoxide hydrolase amino acid sequence or peptide is extra-cellular or intra-cellular expressed and/or secreted by the recombinant host cell according to the invention.

According to a preferred embodiment of the present invention, the isolated and purified epoxide hydrolase amino acid sequence presents more than 50%, preferably more than 70%, more preferably more than 90% homology with the amino acid sequence SEQ ID NO 6.

According to another preferred embodiment of the present invention, the isolated and purified epoxide hydrolase amino acid sequence has the amino acid sequence of SEQ ID NO 6 or a smaller portion of said amino acid sequence (of more than 50 amino-acids, preferably more than 100 amino-acids), which has at least more than 80% of the epoxide hydrolase activity of the complete amino acid sequence SEQ ID NO 6, preferably more than 95% of the epoxide hydrolase activity of the complete amino acid sequence SEQ ID NO 6. In other words, the isolated and purified epoxide hydrolase amino acid sequence according to the invention can be deleted partially while maintaining its enzymatic activity, which can be measured by methods well known by the person skilled in the art. Said isolated and purified epoxide hydrolase or its portion has a molecular weight lower than 30 kD, preferably about 28 kD or lower.

An epoxide hydrolase of interest is identified via an enzymatic assay not critical for the present invention, such as the hydrolysis of cis-epoxysuccinate in L(+)tartaric acid. To perform this assay the micro-organism is cultivated in an appropriate medium to produce the enzyme of interest (e.g. by induction with

cis-epoxysuccinate). The whole cells or the culture medium are separately tested for the enzymatic activity.

Once an epoxide hydrolase has been identified, the DNA sequence encoding such epoxide hydrolase may be obtained from the micro-organisms which naturally produce it or from the recombinant cells according to the invention by culturing said micro-organisms or said cells in the appropriate medium to induce and produce the enzyme (epoxide hydrolase) of interest, isolating the desired epoxide hydrolase using known methods such as column chromatography, and determining the "active portion" of the amino acid sequence of the purified enzyme.

The DNA sequence encoding the epoxide hydrolase is obtained from a gene library of the micro-organism from which the epoxide hydrolase has been purified.

According to the present invention an oligonucleotide probe was derived from a portion of the amino acid sequence determined above. This oligonucleotide probe was used to screen a partial gene library of the micro-organism from which the epoxide hydrolase of interest has been purified. A 8 kb BamHI-EcoRI genomic DNA fragment containing the epoxide hydrolase-encoding sequence has been obtained as an insert in the plasmid vector pBlueScript SK(+). The resulting plasmid was designated pREHBE.

The size of the insert of the plasmid pREHBE has been reduced to 1.4 kb by digestion with the restriction enzymes SphI and XhoI and insertion in appropriate restriction sites of the plasmid vector pBluescript SK(+). The resulting plasmid was designated pREHXS and has been deposited (in E. coli DH10B) at the LMBP (BCCM/LMBP Plasmid collection, Laboratorium voor

Moleculaire Biologie, Universiteit Gent, K.L. Ledenganckstraat, 35, B-9000 Gent) under the accession number LMBP-3666. The entire sequence of the insert was determined by manual sequencing using techniques well known by the person skilled in the art.

The expression constructs as described above allowed their expression in a *Escherichia coli* host. According to the present invention, said *Escherichia coli* host containing the plasmid pREHXS is cultivated in an appropriate medium (e.g. LB medium) and containing the appropriate antibiotic to allow the continuous presence of the plasmid in host cells, then the cells are collected and the epoxide hydrolase activity of the enzyme is determined. It is in the scope of the present invention not to limit the expression of the epoxide hydrolase gene to *Escherichia coli*. According to the present invention, the DNA fragment encoding the epoxide hydrolase could be advantageously expressed in bacteria or fungi, including yeast. To permit the expression of the gene of interest in such hosts, the DNA sequence encoding the epoxide hydrolase could eventually be operably linked to DNA sequences that will permit the (over)expression in the chosen host (e.g. promoters, terminators, UAS, ... sequences).

The DNA sequence encoding the epoxide hydrolase is preferably modified to allow the secretion (extra cellular expression) of the epoxide hydrolase in the host cell culture medium. Such modification is usually done by adding a DNA sequence encoding a leader sequence that is recognised by the secretion machinery of the chosen host and allows the recovery of the epoxide hydrolase in its culture medium.

The present invention also provides the conditions (culture medium, temperature, ...) for the cultivation of the host selected for the expression of the epoxide hydrolase.

A last aspect of the present invention is related to the use of the recombinant host cell according to the invention or the isolated and purified epoxide hydrolase amino acid sequence according to the invention for the hydrolysis of an epoxide, preferably the cis-epoxysuccinate.

The new enzyme according to the invention can be advantageously used to hydrolyse epoxide rings found in epoxide substrates. Known examples of epoxides are styrene epoxides, octene epoxides, naphthalene epoxides, phenantrene epoxides, benzene oxide, estroxide, androstene oxide, epichlorhydrin, and cis-epoxysuccinate. Preferably, the epoxide hydrolase according to the invention can be used for the hydrolysis of the cis-epoxysuccinate but does not allow an hydrolysis of the epoxide substrate epichlorhydrin.

The enzyme according to the invention can be used under several forms : the cells can be used directly after cultivation with or without permeabilisation, and the enzyme could be used as a cell extract (i.e. portions of the host cell which has been submitted to one or more centrifugation and extraction step(s)) or as a purified protein. Any of the form above-described can be used in combination with another enzyme under any of the above-described forms. Furthermore, the enzyme can be recovered from the culture medium of a host cell expressing and secreting the epoxide hydrolase outside the cells. In this case, the enzymatic preparation can be used either as

a crude preparation or as a partially or totally purified preparation in combination or not with one or several other enzyme(s).

These whole cells, cell extracts, cell-free extracts or purified epoxide hydrolase can be fixed (immobilised by any conventional means on a solid support such as a chromatography column) to allow a continuous hydrolysis of the epoxide substrate or to allow a recycling of the enzymatic preparation (whole cells, cell extracts, cell-free extracts, totally or partially purified epoxide hydrolase, etc.).

The invention will be described in further details in the following examples by reference to the enclosed drawings, which are not in any way intended to limit the scope of the invention as claimed.

Brief description of the drawings

Figure 1 shows the content of fractions eluted from the MonoQ column and the activity associated with these fractions.

Figure 2 shows the result of a typical Southern blotting experiment using a oligonucleotide based on the amino acid sequence of the cis-epoxysuccinate hydrolase to probe total genomic DNA of *Rhodococcus rhodochrous* LMGP-18079.

Figure 3 shows the nucleotide sequence of the *Rhodococcus rhodochrous* LMGP-18079 cis-epoxysuccinate hydrolase gene and its flanking regions.

Figure 4 shows the amino acid sequence deduced from the sequence of the *Rhodococcus rhodochrous* LMGP-18079 cis-epoxysuccinate hydrolase gene. The

amino acid sequences obtained by chemical sequencing of the enzyme are underlined.

Figure 5 represents the relative enzymatic activity of the enzyme according to the invention.

Examples

Example 1 : Partial purification of an epoxide hydrolase

Strain

Rhodococcus rhodochrous LMGP-18079 is routinely maintained on agar slants containing 0.4% glucose, 0.4% yeast extract, 1% malt extract and 2% agar.

Determination of the cis-epoxysuccinate-hydrolase activity

The enzymatic reaction is performed at 37 °C in a final volume of 9.3 ml that contains the following components : 0.7 ml of 0.1N Tris.HCl buffer pH 8.0, 2.6 ml of a 1.14% Triton X-100 solution, 5.0 ml of a 30% sodium cis-epoxysuccinate solution and 1.0 ml of the cell suspension. The reaction is stopped by diluting the mixture between 100 and 500 times with H_2O acidified to pH 2.2 with H_3PO_4.

The amount of L-tartaric acid formed during the reaction is determined by HPLC at room temperature on a Vydac C18 column (cat no. 201HS3410) with a flow rate between 400 and 600 µl/min and a sample volume of 20 µl. The solvent is the same as the one used to dilute the samples. The detection of cis-epoxysuccinate and tartaric acid occurs at 210 nm.

Activity is expressed as the amount of L(+)-tartaric acid formed in a day by 1 gram of dried cells.

For rapid qualitative analysis of soluble fractions containing the enzyme, the enzymatic reaction is performed at 37 °C in a final volume of 1 ml in 10 mM Tris-HCl pH 8.0 with 0.5 ml sodium cis-epoxysuccinate (30%) as substrate.

Cultivation of the strain and induction of the cis-epoxysuccinate-hydrolase activity

The standard medium has the following composition (in g/l) : propylene glycol 10, Yeast extract 2, KH_2PO_4 2, $Na_2HPO_4.2H_2O$ 2.2, $(NH_4)_2SO_4$ 3, $CaCl_2.2H_2O$ 0.03, $MnSO_4.H_2O$ 0.003, $MgSO_4.7H_2O$ 0.1, $FeSO_4.7H_2O$ 0.01. The pH is adjusted to 7.0 and the culture is incubated at 30 °C with agitation. The cis-epoxysuccinate hydrolase activity is induced by adding after 24 hours 10 g/l of cis-epoxysuccinate. Cells are collected by centrifugation and stored at -20 °C until use.

Partial purification of the cis-epoxysuccinate hydrolase

1. Obtention of the cell-free extract

About 26 g of frozen cells are suspended in 35 ml of potassium phosphate buffer 50 mM pH 7.2 containing 5 mM DTT, 0.5 mM PMSF, 5 mM EDTA and 2 µg/ml pepstatine.

The cells are disrupted with glass beads using a Braun MSK homogeniser cooled by liquid CO_2.

The homogenate is first centrifuged at 7000 rpm in the SS34 rotor of the Sorvall RC5B centrifuge. The resulting pellet is re-extracted with 50 ml of cold buffer and centrifuged again. The two combined supernatants are then centrifuged for 20 min at 16000 rpm in the same rotor at 4 °C. A final centrifugation step of 60 min at 38000 rpm

in the rotor A641 of the Beckman L8-70 centrifuge gives the cell-free extract.

The extract (138 ml) is dialysed overnight at 4 °C against 2 l of potassium buffer 50 mM pH 7.2 containing 1M $(NH_4)_2SO_4$, 2.5 mM DTT and 2.5 mM EDTA.

2. Chromatography on Phenyl-Sepharose.

After dialysis the extract is loaded on a 30 ml Phenyl Sepharose (Pharmacia Biotech) column equilibrated with the dialysis buffer. After washing with the same buffer, the bound proteins are eluted at 3 ml/ min with 240 ml of a 1M-0M $(NH_4)_2SO_4$ linear gradient in the same buffer. Fractions of 3 ml are collected and assayed for cis-epoxysuccinate-hydrolase activity.

Active fractions are pooled and dialysed against 50 mM potassium phosphate buffer pH 7.2 containing 2.5 mM EDTA and 2.5 mM DTT.

3. Chromatography on Q-Sepharose

The dialysate of step 2 is applied on a 10 ml Q-Sepharose (Pharmacia Biotech) column equilibrated with the dialysing buffer. After washing with the same buffer, the bound protein are eluted from the column at 1 ml/min with a 80 ml linear gradient of NaCl (0-0.5M) in the same buffer. Fractions of 1 ml are collected and assayed for cis-epoxysuccinate-hydrolase activity.

4. Chromatography on MonoQ

About 1/10 of the pooled active fractions of step 3 is diluted 2.7-fold with 50 mM potassium phosphate buffer pH 7.2 containing 2.5 mM EDTA and 2.5 mM DTT and loaded on a MonoQ HR 5/5 column (Pharmacia Biotech). The

proteins are eluted from the column with a linear gradient of NaCl (0-0.5M) in the same buffer at 0.25 ml/min. Fractions of 0.25 ml are collected and assayed for cis-epoxysuccinate-hydrolase activity.

5. Electrophoresis on SDS-PAGE

25 µl of the fractions collected from the MonoQ column are applied on a 12.5% acrylamide SDS gel (Mini-PROTEAN II Electrophoresis system - Bio-Rad). Proteins on the gel are visualised by staining with Coomassie Blue.

Example 2 : Determination of the amino acid sequence of the cis-epoxysuccinate hydrolase

General procedures were followed to perform the N-terminal sequencing of the protein after electrophoresis on a SDS-polyacrylamide gel and electroblotting on a PVDF Immobilon-P membrane (Millipore). An automatic 477A Protein Sequencer coupled to a HPLC 120A Analyser (Applied Biosystem) was used.

For the determination of the sequence of internal fragments, the protein was first digested on the membrane with trypsine. The resulting peptides were separated by reverse phase chromatography on HPLC, and subjected to N-terminal sequencing as above.

The following sequences have been obtained:

N-terminal :	MQLNNANDNTQF	SEQ ID NO 1
1st internal peptide :	SWPDVPSGLEQLR	SEQ ID NO 2
2nd internal peptide :	RPLEYGPTGR	SEQ ID NO 3

Example 3 : Identification of the cis-epoxysuccinate-hydrolase gene

1. Design of the oligonucleotide probe

Based on the N-terminal sequence of the protein (SEQ ID NO 1) a oligonucleotide labelled with digoxigenine has been synthesised. This oligonucleotide has the following sequence :

5' AAYAAYGCNAAYGAYAAYAC 3' SEQ ID NO 4

Y represents either C or T, N represents any of the four bases.

2. Hybridisation of the oligonucleotide with total Rhodococcus rhodochrous DNA

2.1. Isolation of genomic DNA

Rhodococcus rhodochrous LMGP-18079 strain is cultivated overnight in 200 ml of LBroth à 37 °C. After collection by centrifugation, cells are washed for 30 minutes at 80 °C with TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0), centrifuged again, resuspended in 15 ml of TSE buffer (Tris-HCl 50 mM, Sucrose 200 mM, EDTA 1 mM, pH 8.0) with 120 mg of lysozyme and incubated for 2 hours at 37 °C before addition of 1.5 ml of 250 mM EDTA, pH 8.0. The mixture is further incubated for 60 minutes. After addition of 1 ml of STE (SDS 20%, Tris-HCl 50 mM, EDTA 20 mM, pH 8.0), the mixture is incubated for 30 minutes at 55 °C.

DNA is separated from the contaminating material by successive extractions with phenol and phenol-chloroform and by precipitation with ethanol.

2.2. Southern blotting

About 6 µg of *Rhodococcus rhodochrous* DNA are digested with various restriction enzymes (EcoRI, BamHI, SalI, PstI) or by a combination of these restriction enzymes (EcoRI + BamHI, EcoRI + PstI, EcoRI + SalI, BamHI + SalI, BamHI + PstI, SalI + PstI). The samples are loaded on a 20x20 cm 1% agarose gel and run overnight at 40 volts in buffer TAE (Tris-Acetate 40 mM, EDTA 1 mM, pH 8.0).

After migration the DNA the gel is treated and transferred on a Hybond N+ membrane (Amersham) as recommended by the manufacturer. Hybridisation buffers and detection conditions were those recommended in the digoxigenine detection kit (Boehringer Mannheim). Prehybridisation was performed overnight at 42 °C. Hybridisation occurred at 42 °C for 6 hours. The washing conditions were the following : 2 x 5 minutes at room temperature with 2xSSC, 0.1% SDS, 2 x 15 min at 42 °C with 0.5xSSC 0.1% SDS and 2 x 15 min at 42 °C with 0.1xSSC 0.1 % SDS.

The result of a typical Southern blot experiment is shown on the figure 2.

Example 4 : Cloning of the cis-epoxysuccinate hydrolase gene

1. Construction of a partial gene library

About 15 µg of total chromosomal DNA of *Rhodococcus rhodochrous* are digested with the restriction enzymes BamHI and SalI. The plasmid pBluescript SK(+) (Stratagene) is digested by the same enzymes. The digested DNA is separated on a 1% agarose in TAE buffer (cf. above). Fragments of agarose corresponding to the appropriate

length as determined by analysing the results of the Southern blot (1600-2200 bp) are cut off the gel. The DNA of the agarose blocks is extracted with the aid of a QIAEX II Gel extraction kit (QIAGEN). A ligation is performed between the plasmid vector and the *Rhodococcus* DNA fragments and the ligation mixture is used to transform *E. coli* DH10B by electroporation using the standard protocol (Bio-Rad Gene Pulser II).

2. Cloning of the cis-epoxysuccinate hydrolase gene

2.1. Cloning of an incomplete cis-epoxysuccinate hydrolase gene

Transformants from step 1 are pooled in groups of ten clones. Plasmid DNA is extracted from these groups. The purified DNA is digested with the restriction enzymes BamHI and SalI and subjected to electrophoresis in agarose gel in order to perform a Southern blotting as described in example 2.

The individual clones from the positive groups are analysed again following the same protocol.

One positive clone (pREHBS) is retained for further analysis. It contains an insert with a size higher than expected (about 6.8 kb) that results from a partial digestion of the chromosomal DNA of the *Rhodococcus rhodochrous* strain.

The size of the insert is reduced, by digestion with restriction enzymes and ligation, to a length of about 1800 bp that corresponds to the expected BamHI-SalI fragment. DNA sequencing of the ends of this insert demonstrates the presence of the sequence corresponding to the oligonucleotide used to screen the

library. However, it appears that the 3′ end of the gene is missing.

2.2. Obtaining the complete gene

A partial gene library is constructed following the protocol described in 1. but using the restriction enzymes BamHI and EcoRI. This library is screened with the same oligonucleotide. A positive clone (pREHBE) is subjected to DNA sequencing on the BamHI side to confirm the overlap with the BamHI-SalI fragment.

The size of the insert of pREHBE is about 8 kb. Restriction analysis of the insert of pREHBE shows that the gene is located on a 1.4 kb SphI-XhoI fragment. To clone this fragment, the plasmid pREHBE is digested first with SphI, treated with the Klenow fragment of DNA polymerase and digested with XhoI. The fragment of interest is purified after agarose gel electrophoresis and inserted by ligation in pBluescriptSK(+) that has been digested by BamHI, treated with the Klenow fragment of DNA polymerase and digested with XhoI. The plasmid obtained is termed pREHXS and has been deposited (in *E. coli* DH10B) under the accession number LMBP-3666.

Example 5 : Characterisation of the cis-epoxysuccinate gene

The DNA sequence of the insert of pREHXS is determined manually using the dideoxynucleotide chain-termination procedure (Sanger, F., Nickelen, S., Coulson, A.R., Proc. Nat. Acad. Sci. USA, 74, p. 5463 (1977)). Specific oligonucleotides are used as primers in the sequencing reactions. Computer analysis is done with the PC/GENE program.

The complete nucleotide sequence is determined and is given in figure 3 (SEQ ID NO 5).

The sequence obtained comprises 1366 bp, 67 bp in the 5′ non-coding region and 543 bp in the 3′ non-coding region. The polypeptide derived from the coding sequence is 253 amino acids long and has a predicted molecular weight of 28 kDa. The amino acid sequence of the cis-epoxysuccinate hydrolase is given in figure 4 (SEQ ID NO 6) The fragments of the amino acid sequence determined in example 2 are present in this sequence. SEQ ID NO 1 corresponds to the amino acids 1 to 12, SEQ ID NO 2 corresponds to the amino acids 112 to 124 and SEQ ID NO 3 corresponds to amino acids 209 to 218.

Example 6 : Expression of the cis-epoxysuccinate-hydrolase gene in *Escherichia coli*

The *Escherichia coli* strain DH10B containing the plasmid pREHXS is cultivated overnight in 2 x LB medium (Yeast Extract 1%, Bacto-tryptone 2%, NaCl 2%) supplemented with 100µg/ml of ampicillin. The same strain with the pBluescriptSK(+) plasmid without insert is used as a control.

The activity of the cis-epoxysuccinate hydrolase is determined in the cells collected from the two cultures using the protocol described in example 1. No activity could be detected in the control. About 150 units is present in the strain expressing the cis-epoxysuccinate gene.

Example 7 : Production of L-tartaric acid

1. Enzyme production

The *E. coli* strain containing the plasmid pREHXS is grown for 8 hours in 100 ml at 35 °C with agitation in LB medium supplemented with 100µg/ml ampicillin. The culture is transferred in 1 litre of the same medium and cultivated again for 8 hours. This culture is used to inoculate a 15 l Braun Biostat E fermentor. After 24 hours, the culture is stopped and the cells are collected by centrifugation for 10 min. at 10000g and 4 °C. Cells are washed once with 10 mM potassium phosphate buffer pH 7.0 and centrifuged again. Finally the cells are suspended in one volume of the buffer and stored at -20 °C until use or dried by lyophilisation. The cells can also be dried by spray drying. The cells are preferably stored in closed bags or containers to protect them from humidity. Storage at lower temperature and storage under vacuum can extend the shelf life of the dried cells. In a sealed polyethylene bag the dried cells remain at 90% of their original activity after 3 months storage at ambient temperature (25 °C).

This enzymatic activity is advantageously maintained constant at a pH higher than 7, preferably at a pH comprised between 7.0 and 10, more preferably at a pH comprised between 7.5 and 9.5.

2. Bioconversion of cis-epoxysuccinate

The bioconversion is performed in a 15 l Braun Biostat E fermentor. 10 l of a 20% cis-epoxysuccinate are mixed with 50 g of dried cells and 625 ml of a 2% (w/v) Triton X-100 solution. The pH is maintained constant at 8.0

with NaOH or HCl. The mixture is incubated under agitation (200 rpm) at 37 °C for 40 hours. The disappearance of the cis-epoxysuccinate and the synthesis of L-tartaric acid is followed by HPLC (see example 1).

A deposit has been made according to the Budapest Treaty for the micro-organisms *Rhodococcus rhodochrous* under the deposit number LMGP-18079 at the BCCM/LMG Plasmid collection, Laboratorium voor Moleculaire Biologie, Universiteit Gent, K.L. Ledenganckstraat, 35, B-9000 Gent and for *E. coli* containing the plasmid comprising the sequence according to the invention under the deposit number LMBP-3666 at the BCCM/LMBP Plasmid collection, Laboratorium voor Moleculaire Biologie, Universiteit Gent, K.L. Ledenganckstraat, 35, B-9000 Gent .

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: PURATOS N.V.
(B) STREET: Industrialaan 25
(C) CITY: Groot-Bijgaarden
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-1702

(ii) TITLE OF INVENTION: Epoxide hydrolase

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Gln Leu Asn Asn Ala Asn Asp Asn Thr Gln Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Trp Pro Asp Val Pro Ser Gly Leu Glu Gln Leu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Pro Leu Glu Tyr Gly Pro Thr Gly Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAYAAYGCNA AYGAYAAYAC 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCTTATCCT GGTAGCGCCC GTAATTTCGT GGGCTATCTT CGTTCTTCCG AGTGGATTGT 60

GAGCACAATG CAACTGAACA ATGCGAACGA CAACACGCAG TTCCGGGCCC TGCTTTTCGA 120

CGTGCAGGGG ACTCTGACAG ATTTCCGTTC CACACTCATC GAGCACGGCT TATCGATTCT 180

CGGAGACAGA GTGGATCGAG AACTCTGGGA GGAATTGGTC GACCAATGGC GCGGCTGCTA 240

TCGAGACGAG CTCGATTCCT TGGTCAAACA GGAGAAATGG CGCTCGGTCC GCGCCGTGTA 300

CCGAGATTCT CTTATCAATC TTCTCGCAAA ATTCTCTGAC AGTTTCTGCG CCACCTCGGC 360

CGAAGTGGAA TTGCTGACCG ATGGTTGGGA ACGTCTTCGG TCGTGGCCGG ACGTCCCCTC 420

TGGATTGGAA CAGCTGCGGT CTAAGTACCT CGTCGCGGCA CTGACGAATG CGGACTTTTC 480

CCATCGTC AACGTCGGGC GTAGCGCCAA ACTGCAATGG GACGCTGTTC TTTCAGCTCA 540

ACTCTTTGGA GCCTACAAGC CCCACCGGTC AACATATGAG GGAGCCGCGA CACTCCTGGG 600

TATCGCTCCG TCAGAGATCC TCATGGTCGC CTCCCATGCA TACGATCTCG AAGCGGCGCG 660

GGAAGTGGGA GCCGGCACAG CGTACGTCAG ACGGCCACTG GAATACGGAC CGACGGGGCG 720

AACCGAGGAC GTTCCCGATG GACGTTTCGA TTTCTTGGTC GACAGCATCA GTGAACTGGC 780

TGATCAGCTG GGCTGCCCAC GACTCGGTGG AACTGCCGGT ATCGATTGAC ATCGACCGGC 840

GGTTCCACGC GTCCCTTGTT TTCGGTGCCT GCATTCCCCG TGACGGCAAT TCAGTCACGG 900

GGAATGCAGG CTCTGCTCTG TGCCCAGGAT TTCAGGCGCT TCCCAGGCAA CTGGAGTTTT 960

TGCAGCGTAG AGGCGCGCTT CGTTCATGAG TTCCTGTGCG GGGGGTGTGA GATGACTCCG 1020

CCTACAGACA GCGGCGATGT GAAGGTAGGA CTCCGGGACG AATGCGGTGC ACGGTGGCGC 1080

CGACCTGCCG TGCCGATGGC GCCCAGGAGG ACGGCATGAC AGCCTGTCCG AATCCGCTGA 1140

GCACCATCGG CAGGATCGAG GTCCGGTGGG CAACTTCGGC GACGATGGTG GCTTCGACAC 1200

CGCTGGCGAG AACCTCGTCG ACCAGCGTTC GCATCAATGA TCCTCGCTGG GAGACAATCA 1260

ATCGGAAGCC GGACAGTTCC GACCGGTAAA TTTCCGGACG GTCCGGCGTC TCTGACCCGG 1320

GCCCGGACAT GAGGATCAGC GGTTGACTTT CCAGTGCCAC AACCTC 1366

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 253 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Gln Leu Asn Asn Ala Asn Asp Asn Thr Gln Phe Arg Ala Leu Leu
1 5 10 15

Phe Asp Val Gln Gly Thr Leu Thr Asp Phe Arg Ser Thr Leu Ile Glu
20 25 30

His Gly Leu Ser Ile Leu Gly Asp Arg Val Asp Arg Glu Leu Trp Glu
35 40 45

Glu Leu Val Asp Gln Trp Arg Gly Cys Tyr Arg Asp Glu Leu Asp Ser
50 55 60

Leu Val Lys Gln Glu Lys Trp Arg Ser Val Arg Ala Val Tyr Arg Asp
65 70 75 80

Ser Leu Ile Asn Leu Leu Ala Lys Phe Ser Asp Ser Phe Cys Ala Thr
85 90 95

Ser Ala Glu Val Glu Leu Leu Thr Asp Gly Trp Glu Arg Leu Arg Ser
100 105 110

Trp Pro Asp Val Pro Ser Gly Leu Glu Gln Leu Arg Ser Lys Tyr Leu
115 120 125

Val Ala Ala Leu Thr Asn Ala Asp Phe Ser Ala Ile Val Asn Val Gly
130 135 140

Arg Ser Ala Lys Leu Gln Trp Asp Ala Val Leu Ser Ala Gln Leu Phe
145 150 155 160

Gly Ala Tyr Lys Pro His Arg Ser Thr Tyr Glu Gly Ala Ala Thr Leu
165 170 175

Leu Gly Ile Ala Pro Ser Glu Ile Leu Met Val Ala Ser His Ala Tyr
180 185 190

Asp Leu Glu Ala Ala Arg Glu Val Gly Ala Gly Thr Ala Tyr Val Arg
195 200 205

Arg Pro Leu Glu Tyr Gly Pro Thr Gly Arg Thr Glu Asp Val Pro Asp
210 215 220

Gly Arg Phe Asp Phe Leu Val Asp Ser Ile Ser Glu Leu Ala Asp Gln
225 230 235 240

Leu Gly Cys Pro Arg Leu Gly Gly Thr Ala Gly Ile Asp
245 250

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

.CTGCAGAG GAGGAGCACA ATGCAACTG 29

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGAATTCCT GGGCACAGAG C 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGGATGCATA GGAGGTAACA TATGTTTAAG 30

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGGAATTCAA TGGAAGGTGC GTTATAACG 29

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AACTGCAGCA ATGCAACTGA ACAATGCG 28

CLAIMS

1. An isolated and purified epoxide hydrolase comprising an amino acid sequence having more than 70% homology with the amino acid sequence SEQ ID NO 6 and in which 80% of its optimal enzymatic activity is obtained at a pH comprised between 7.5 and 9.5.

2. An isolated and purified epoxide hydrolase according to claim 1, comprising an amino acid sequence having more than 90% homology with the amino acid sequence SEQ ID NO 6 and in which 80% of its optimal enzymatic activity is obtained at a pH comprised between 7.5 and 9.5.

3. An isolated and purified epoxide hydrolase amino acid sequence having the amino acid sequence of SEQ ID NO 6 or a portion thereof having an epoxide hydrolase enzymatic activity.

4. The isolated and purified epoxide hydrolase amino acid sequence according to any one of the preceding claims, having a molecular weight lower than 30 kD.

5. The isolated and purified epoxide hydrolase according to any one of the preceding claims, characterised in that it is intracellularly expressed by a recombinant host cell.

6. The isolated and purified epoxide hydrolase according to any one of the claims 1 to 4, characterised in that it is extracellularly expressed by a recombinant host cell.

7. A nucleotide sequence encoding the isolated and purified epoxide hydrolase amino acid sequence according to any one of the preceding claims.

8. The nucleotide sequence according to claim 7, having more than 90% homology with the nucleotide sequence SEQ ID NO 5.

9. An isolated and purified nucleotide sequence, corresponding to the nucleotide sequence SEQ ID NO 5 or a portion thereof encoding the epoxide hydrolase amino acid sequence according to any one of the claims 1 to 6.

10. The nucleotide sequence according to any one of the claims 7 to 9, isolated and purified from *Rhodococcus* species.

11. The isolated and purified nucleotide sequence according to claim 10, isolated from *Rhodococcus rhodocrous*, preferably from *Rhodococcus rhodocrous* having the deposit number LMGP-18079.

12. A recombinant nucleotide sequence comprising, operably linked to the nucleotide sequence according to any one of the claims 4 to 10, one or more adjacent regulatory sequence(s).

13. The recombinant nucleotide sequence according to claim 12, comprising one or more adjacent regulatory sequence(s) originating from homologous micro-organisms.

14. A vector comprising the nucleotide sequence according to any one of the claims 7 to 13.

15. The vector according to claim 14, being a plasmid incorporated in *E. coli* and having the deposit number LMBP-3666.

16. A recombinant host cell transformed by the nucleotide sequence according to any one of the claims 7 to 13 or the vector according to claim 14 or 15.

17. The recombinant host cell according to claim 16, which is selected from the group consisting of bacteria or fungi, including yeast.

18. A solid support immobilising an element selected from the group consisting of the cell according to claim 16 or 17, a cell extract of the cell according to claim 16 or 17 and/or the isolated and purified epoxide hydrolase according to any one of the claims 1 to 6.

19. Use of the recombinant host cell according to claim 16 or 17 or of the epoxide hydrolase according to any one of the claims 1 to 6 or of the solid support according to claim 18 for the hydrolysis of an epoxide.

20. Use according to claim 19, for the hydrolysis of the cis-epoxysuccinate.

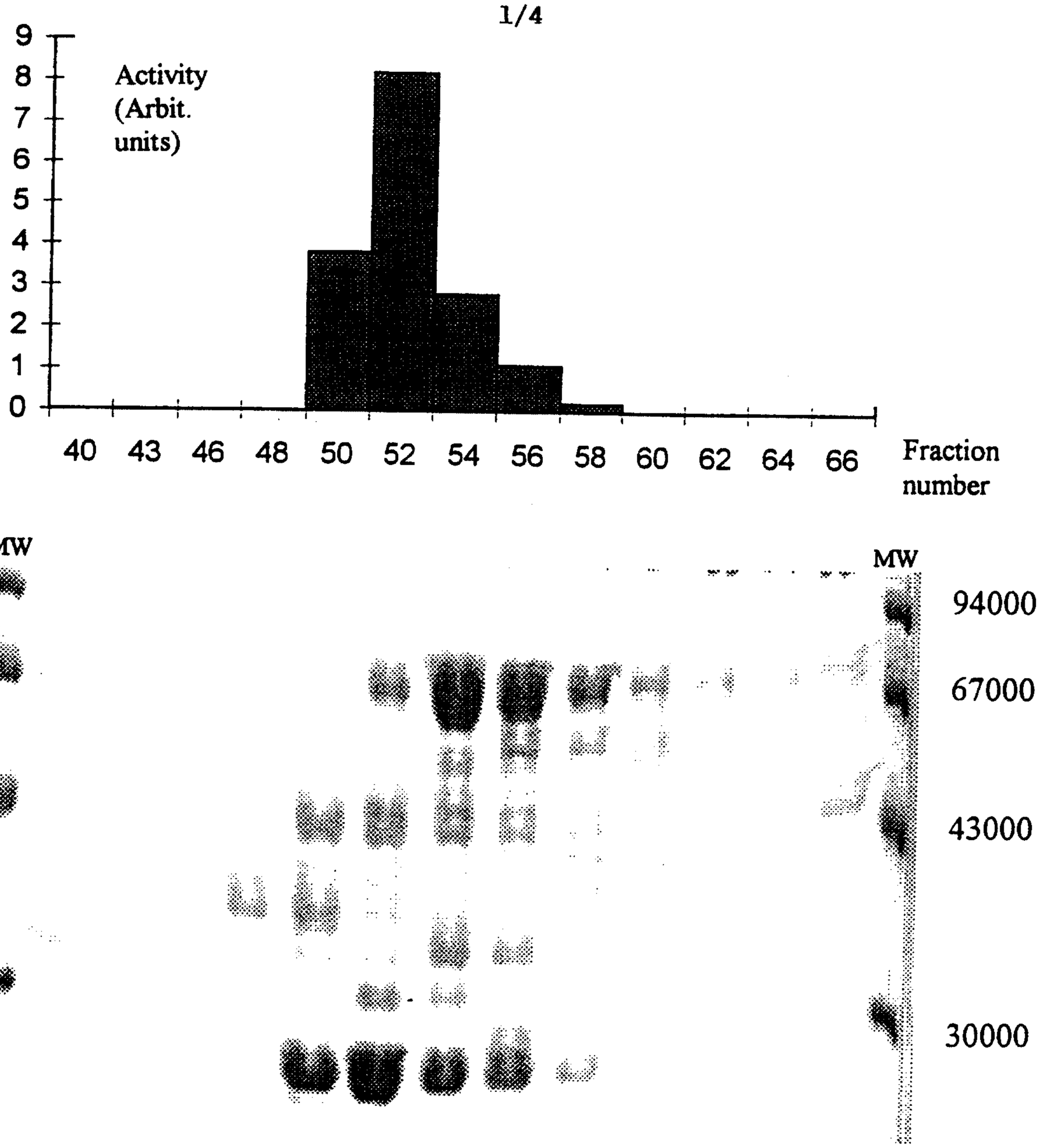

9
8
7
6
5
4
3
2
1
0
Activity (Arbit. units)
40 43 46 48 50 52 54 56 58 60 62 64 66
Fraction number
MW
MW
94000
67000
43000
30000

Fig 1

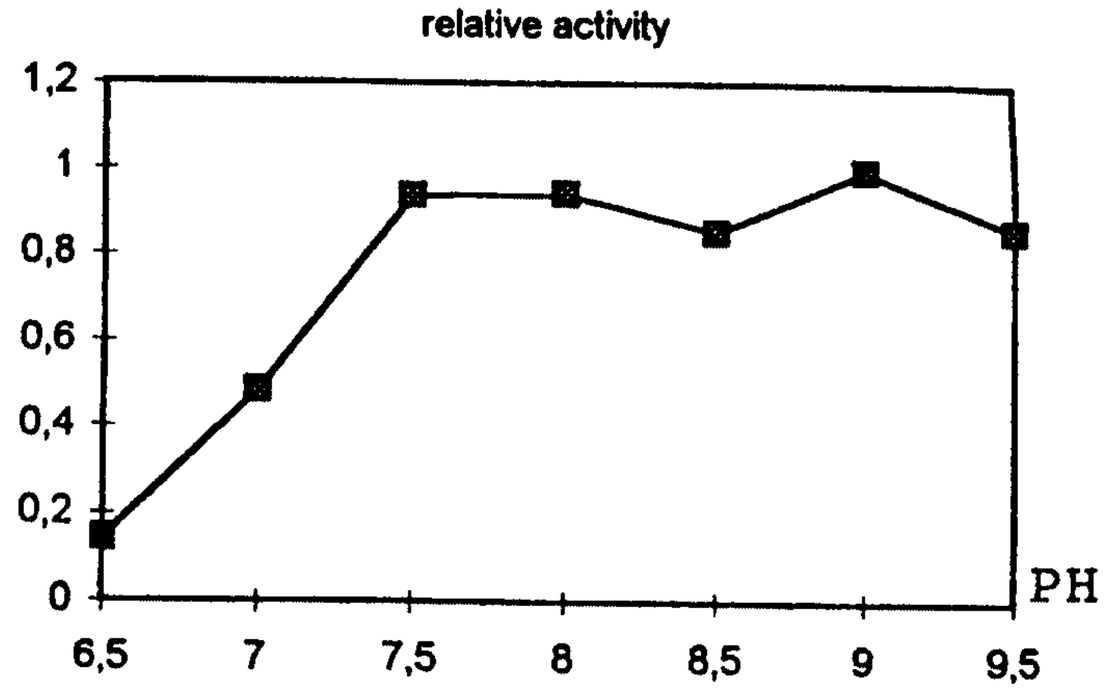

relative activity
1,2
1
0,8
0,6
0,4
0,2
0
6,5 7 7,5 8 8,5 9 9,5
PH

FIG.5

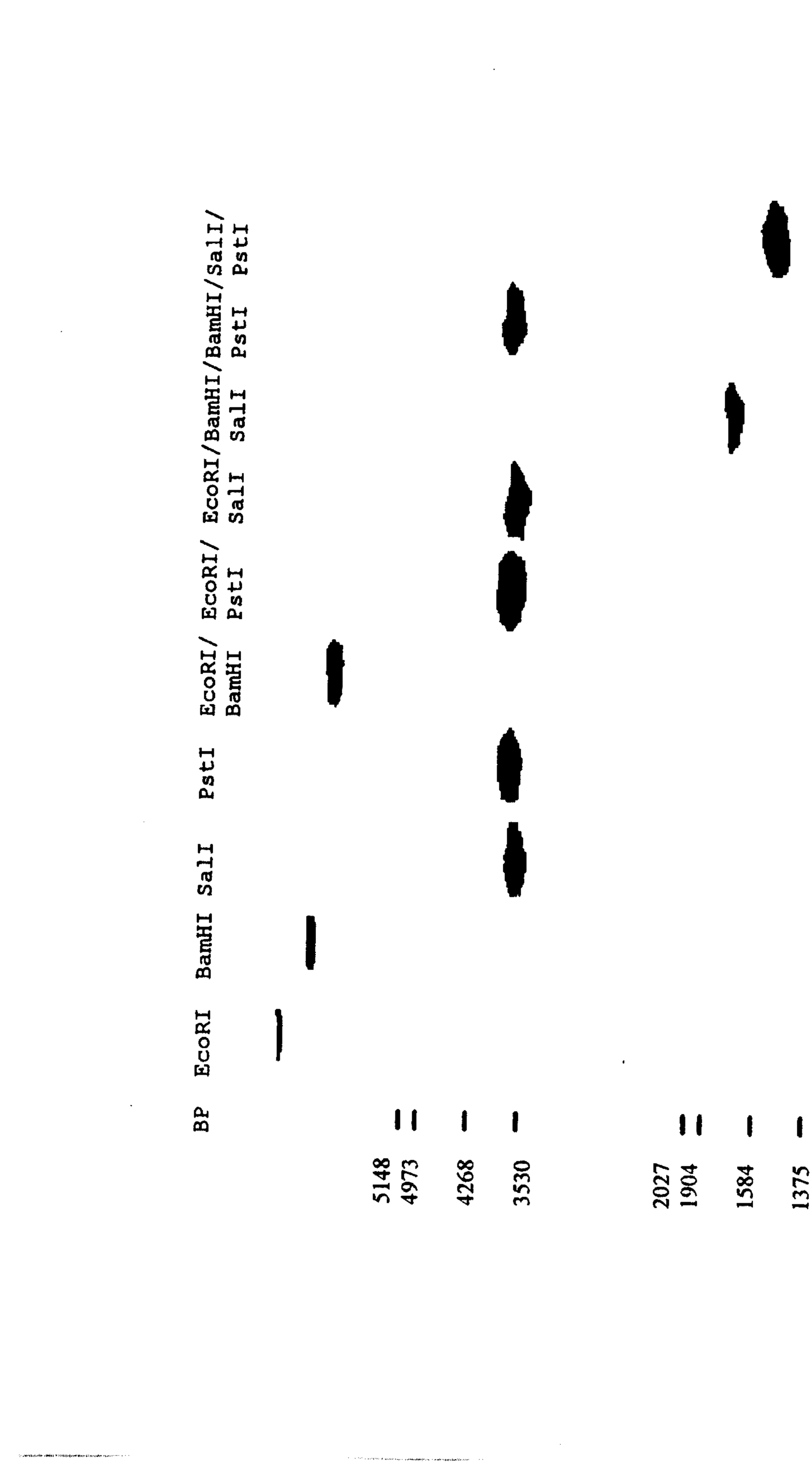

BP
EcoRI
BamHI
SalI
PstI
EcoRI/ BamHI
EcoRI/ PstI
BamHI/ SalI
EcoRI/ SalI
EcoRI/ PstI
BamHI/ PstI
SalI/ PstI
5148
4973
4268
3530
2027
1904
1584
1375

Fig 2

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
   1 GTCTTATCCT GGTAGCGCCC GTAATTTCGT GGGCTATCTT CGTTCTTCCG AGTGGATTGT
  61 GAGCACAATG CAACTGAACA ATGCGAACGA CAACACGCAG TTCCGGGCCC TGCTTTTCGA
 121 CGTGCAGGGG ACTCTGACAG ATTTCCGTTC CACACTCATC GAGCACGGCT TATCGATTCT
 181 CGGAGACAGA GTGGATCGAG AACTCTGGGA GGAATTGGTC GACCAATGGC GCGGCTGCTA
 241 TCGAGACGAG CTCGATTCCT TGGTCAAACA GGAGAAATGG CGCTCGGTCC GCGCCGTGTA
 301 CCGAGATTCT CTTATCAATC TTCTCGCAAA ATTCTCTGAC AGTTTCTGCG CCACCTCGGC
 361 CGAAGTGGAA TTGCTGACCG ATGGTTGGGA ACGTCTTCGG TCGTGGCCGG ACGTCCCCTC
 421 TGGATTGGAA CAGCTGCGGT CTAAGTACCT CGTCGCGGCA CTGACGAATG CGGACTTTTC
 481 TGCCATCGTC AACGTCGGGC GTAGCGCCAA ACTGCAATGG GACGCTGTTC TTTCAGCTCA
 541 ACTCTTTGGA GCCTACAAGC CCCACCGGTC AACATATGAG GGAGCCGCGA CACTCCTGGG
 601 TATCGCTCCG TCAGAGATCC TCATGGTCGC CTCCCATGCA TACGATCTCG AAGCGGCGCG
 661 GGAAGTGGGA GCCGGCACAG CGTACGTCAG ACGGCCACTG GAATACGGAC CGACGGGGCG
 721 AACCGAGGAC GTTCCCGATG GACGTTTCGA TTTCTTGGTC GACAGCATCA GTGAACTGGC
 781 TGATCAGCTG GGCTGCCCAC GACTCGGTGG AACTGCCGGT ATCGATTGAC ATCGACCGGC
 841 GGTTCCACGC GTCCCTTGTT TTCGGTGCCT GCATTCCCCG TGACGGCAAT TCAGTCACGG
 901 GGAATGCAGG CTCTGCTCTG TGCCCAGGAT TTCAGGCGCT TCCCAGGCAA CTGGAGTTTT
 961 TGCAGCGTAG AGGCGCGCTT CGTTCATGAG TTCCTGTGCG GGGGGTGTGA GATGACTCCG
1021 CCTACAGACA GCGGCGATGT GAAGGTAGGA CTCCGGGACG AATGCGGTGC ACGGTGGCGC
1081 CGACCTGCCG TGCCGATGGC GCCCAGGAGG ACGGCATGAC AGCCTGTCCG AATCCGCTGA
1141 GCACCATCGG CAGGATCGAG GTCCGGTGGG CAACTTCGGC GACGATGGTG GCTTCGACAC
1201 CGCTGGCGAG AACCTCGTCG ACCAGCGTTC GCATCAATGA TCCTCGCTGG GAGACAATCA
1261 ATCGGAAGCC GGACAGTTCC GACCGGTAAA TTTCCGGACG GTCCGGCGTC TCTGACCCGG
1321 GCCCGGACAT GAGGATCAGC GGTTGACTTT CCAGTGCCAC AACCTC
```

Fig 3

```
                    5         10        15        20        25        30
                    |         |         |         |         |         |
  1 M Q L N N A N D N T Q F R A L L F D V Q G T L T D F R S T L
 31 I E H G L S I L G D R V D R E L W E E L V D Q W R G C Y R D
 61 E L D S L V K Q E K W R S V R A V Y R D S L I N L L A K F S
 91 D S F C A T S A E V E L L T D G W E R L R S W P D V P S G L
121 E Q L R S K Y L V A A L T N A D F S A I V N V G R S A K L Q
151 W D A V L S A Q L F G A Y K P H R S T Y E G A A T L L G I A
181 P S E I L M V A S H A Y D L E A A R E V G A G T A Y V R R P
211 L E Y G P T G R T E D V P D G R F D F L V D S I S E L A D Q
241 L G C P R L G G T A G I D
```

Fig 4

CA 02306979