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(71) Applicant *(for all designated States except US)*: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BORNSCHEUER, Uwe T.** [DE/DE]; Neuer Brinkhof 25, 17489 Greifswald (DE). **MUSIDLOWSKA-PERSSON, Anna** [PL/SE]; Utsättaregränden 233, SE-22647 Lund (SE). **TRAUTHWEIN, Harald** [DE/DE]; Leonrodstrasse 78, 80636 München (DE).

(74) Common Representative: **DEGUSSA AG**; Intellectual Property Management, PATENTE und MARKEN, Standort Hanau, Postfach 13 45, 63403 Hanau (DE).

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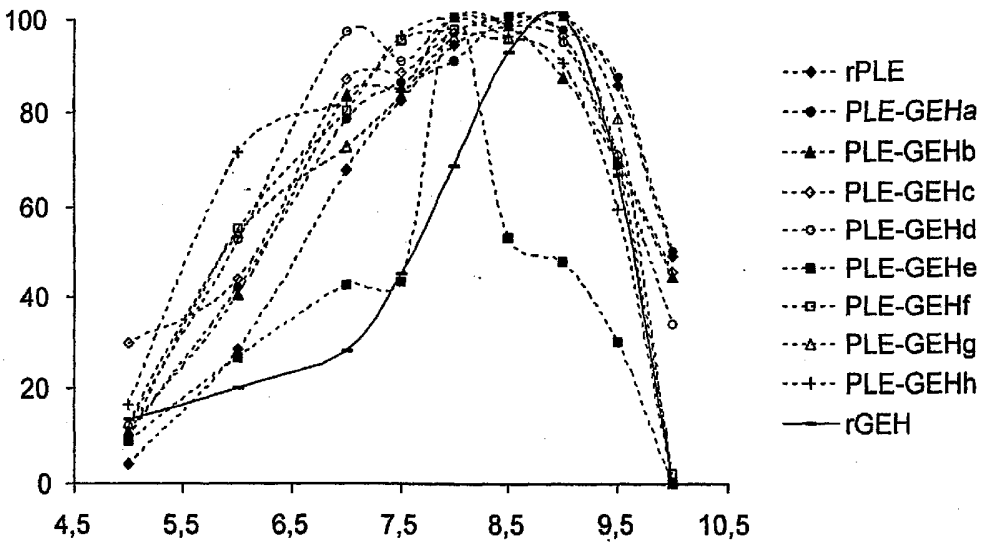
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(54) Title: ARTIFICIAL ESTERASES



(57) Abstract: The invention relates to artificial esterases wherein, on the basis of the amino acid sequence of a pig liver esterase, at least one defined amino acid exchange has been effected, making it possible to modify the enantioselectivity of the enzyme and in some cases to improve the activity and temperature stability.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Artificial esterases

Description:

The invention relates to artificially mutated, enzymatically active, recombinant esterases which are derived from a pig liver esterase sequence and possess a high enzymatic activity.

The importance of esterases as biocatalysts in organic synthesis or as additives in household products, e.g. detergents, has been increasing steadily for many years. This has led research to focus mainly on the preparation of novel esterases that are distinguished on the one hand by their high enantioselectivity in the conversion of their substrates, and on the other hand by a high enzymatic activity.

Despite their great importance and the diversity of their possible applications, only a few recombinant esterases are available. Until recently this also applied particularly to pig liver esterases, which were only obtainable as extracts. These extracts have a variety of disadvantages. Apart from fluctuations in the proportion of esterase between different batches, one particular problem is the presence of other hydrolases in the extract. Furthermore, it has so far proved impossible to separate the extracts into the individual isoenzymes contained therein, so this is another reason why the extracts have fluctuated in their enzymatic activities. In addition, extraction of the enzymes, e.g. from pig liver tissue, carries the risk of viral or toxic contamination of the extract.

To solve these problems, putative pig liver esterase genes were cloned (Matsushima, M., et al., FEBS Lett. 293, 37-41

(1991); Swiss-Prot. Acc. No. Q29550), but the functional expression of an active pig liver esterase enzyme was not successful with the constructs obtained. As an alternative, attempts were also made to obtain other esterases from higher organisms, e.g. a putative glycerol ester hydrolase from the digestive tract of pigs (described in David L. et al., Eur. J. Biochem. 257, 142-148 (1998); Smialowski-Fleter, S. et al., Eur. J. Biochem. 269, 1109-1117 (2002)). However, it has also proved impossible so far to express this recombinant putative esterase gene with enzymatic activity.

The expression of a pure recombinant functional pig liver esterase was only recently successful (WO 02/48322). For the first time, heterologous biotechnological preparation of the recombinant esterase made it possible to obtain pure enzymes uncontaminated by other hydrolases or isoenzymes. The esterases obtained are distinguished by a very good enantioselectivity towards many substrates, which is also in marked contrast to the properties of the conventional pig liver esterase extracts.

However, for many applications, especially technical applications, it would be desirable to have enzymes with modified properties, e.g. a different enantioselectivity or an increased activity.

In an attempt to prepare the above-described putative glycerol ester hydrolase starting from the amino acid sequence of recombinant pig liver esterase, **Seq. ID No. 1** (WO 02/48322), by site directed mutagenesis, a number of artificial mutant enzymes were produced and, surprisingly, the enzymatic activity and temperature stability of some of the artificial enzymes obtained is higher than that of recombinant pig liver esterase or that of the glycerol ester hydrolase produced. Furthermore, the artificial

mutants exhibit a different enantioselectivity from that of rPLE.

The object is thus achieved by recombinant esterases containing an amino acid sequence **Seq. ID No. 1** or a variant thereof with at least 50%, preferably over 70%, particularly preferably over 80% and very particularly preferably over 90% sequence homology, the amino acid sequence in the case of **Seq. ID No. 1** being mutated in at least one of the positional regions from 74 to 79, 90 to 95, 110 to 114, 127 to 141, 193 to 197, 234 to 239, 288 to 296 and 365 to 372. Preferably at least one of positions 76, 77, 92, 93, 112, 129, 133, 134, 138, 139, 195, 236, 237, 290, 294, 367 and 370 is mutated. There is the possibility of exchange of the amino acid given by **Seq. ID No. 1**, in said positions, with another natural or modified amino acid.

In the case of the amino acid sequence **Seq. ID No. 1**, preferred mutants contain the following amino acid exchanges:

- position 76: valine (V) with alanine (A),
- position 77: glutamic acid (E) with glycine (G),
- position 92: threonine (T) with isoleucine (I),
- position 93: leucine (L) with proline (P),
- position 112: lysine (K) with arginine (R),
- position 129: leucine (L) with valine (V),
- position 133: proline (P) with serine (S),
- position 134: methionine (M) with threonine (T),
- position 138: valine (V) with leucine (L),
- position 139: valine (V) with alanine (A),
- position 195: proline (P) with threonine (T),
- position 236: valine (V) with alanine (A),
- position 237: alanine (A) with glycine (G),
- position 290: phenylalanine (F) with leucine (L),
- position 294: glutamine (Q) with proline (P),

- position 367: tyrosine (Y) with phenylalanine (F),
- position 370: alanine (A) with threonine (T).

Preferred mutants possess the indicated amino acid exchanges in positions 92, 93, 112, 138, 139, 236, 237, 367 and 370, which are distinguished by a greater activity increase towards the model substrates tested (cf. **Fig. 3**). It is striking here that the preferred mutations are not located in the direct vicinity of the active centre of the esterase formed by the amino acids in positions 80, 186 and 431, as would have been expected. However, enzymes that are mutated in positions 76, 77, 195, 290 and 294 also exhibit an increased enzymatic activity towards the model substrates. Particularly preferred enzymes contain mutations in positions 76, 77, 112, 236 and 237. Artificial mutant enzymes with a mutation in positions 112, 129, 133 and 134 are distinguished in particular by a high specific activity at elevated temperatures, preferably at above 65°C (**Fig. 2**). However, mutations in positions 195, 236, 237, 290 and 294 also exhibit an increased specific activity at elevated temperatures.

The insertion of mutations in appropriate positions not only increases the activity, but also varies the enantioselectivity of the enzyme. It has also been possible in the case of specific substrates to observe an appreciable increase in enantioselectivity. In particular, the insertion of a mutation in position 77 of rPLE (**Seq. ID No. 1**) has a strong influence on the enantioselectivity (**Fig. 6**). It is furthermore found, e.g. in the hydrolysis of carboxylic acid esters, that the use of rPLE leads preferentially to the formation of (S) enantiomers, while the artificial enzymes are preferable for the synthesis of (R) enantiomers.

The present invention also provides artificial esterases

which, in the case of **Seq. ID No. 1**, have partial sequences shortened by up to 75, preferably up to 50 and particularly preferably up to 25 amino acids at the N-terminal end and/or by up to 100, preferably up to 50, particularly preferably up to 25 and very particularly preferably up to 5 amino acids at the C-terminal end.

Apart from the pure proteins, the artificially mutated esterases claimed also include post-translationally modified enzymes.

Artificially mutated proteins based on the amino acid sequence **Seq. ID No. 1** can be used directly as enzymes or they can multimerize as subunits with identical or different, naturally occurring or artificially mutated subunits. Thus, for example, the proteins claimed can form enzymatically active multimers with the known, naturally occurring subunits of pig liver esterases of the α, β or γ type.

The mutagenized enzymes have other advantages apart from increased enzymatic activity. In particular, the enzymes can be expressed as recombinant proteins and are thus obtainable in pure form, i.e. without interfering and not easily removable contamination due to other hydrolases or isoenzymes. Said expression takes place with maintenance of the enzyme function even with common host cells like *P. pastoris*. It is also easy to produce fusion proteins containing e.g. other functional domains at their C-terminal end, such as a myc-his domain for easier purification. The N terminus can likewise contain fused, functional peptide domains, of which secretory signal domains, e.g. the α-factor signal sequence or the ompA signal sequence, are of particular interest. However, the N terminus of the natural amino acid sequence (Swiss. Prot. Acc. No. Q29550), or a variant thereof with a

homology of over 50%, preferably of over 70% and particularly preferably of over 80%, can also be fused.

The artificial mutant enzymes are prepared via expression of the corresponding mutated nucleic acid sequences. In the case of **Seq. ID No. 29**, the mutations are inserted by site directed mutagenesis, e.g. using the QuikChange Kit from Stratagene, with appropriate primers.

Thus the present invention also provides nucleic acids that code for the recombinant, artificially mutated pig liver esterases according to the invention, or nucleic acids that are complementary to these coding pig liver esterase nucleic acids, and that hybridize under stringent conditions, where, starting from **Seq. ID No. 29**, which codes for the peptide of **Seq. ID No. 1**, of these are mutated in at least one codon of positions 76, 77, 92, 93, 112, 129, 133, 134, 138, 139, 195, 236, 237, 290, 294, 367 and 370. Thus, for example, mutations can be inserted in positions 227, 230, 275, 278, 335, 385, 397 and 399, 401 and 402, 412, 416 and 417, 583, 707, 710, 870, 881 and 882, 1100 and 1108, preferred base exchanges being apparent from the mutated sequences **Seq. ID No. 30** to **38**.

Stringent hybridization conditions are understood as meaning a hybridization temperature of 60°C and 0.1 x SSC in 0.1% SDS. As also described e.g. in WO 02/48322, the coding DNA sequences can be cloned into conventional vectors and expressed after the transfection of host cells with such vectors in cell culture. An example of a suitable vector is pUC19 or pCYTEX for the transformation of *E. coli*, or pPICZα for the transformation of the yeast *Pichia pastoris*. The species *Aspergillus sp., Schwanniomyces sp., Kluyveromyces sp., Yarrowia sp., Arxula sp., Saccharomyces sp., Hansenula sp.* or *Pichia sp.* include other interesting unicellular organisms which have

proved suitable as hosts for the biotechnological expression of recombinant enzymes. Preferred host organisms which may be mentioned in addition to *P. pastoris* are *Saccharomyces cerevisiae, Aspergillus orycae, Schwanniomyces occidentalis, Kluyveromyces lactis, Yarrowia lipolytica, Arxula adeninivrans, Pichia methanolica, Pichia guilliermondii* or *Hansenula polymorpha*.

In the vectors the coding DNA fragments must be located in the open reading frame belonging to a promoter. Preferred promoters are especially strong promoters, e.g. the lac, lambda, T7 or T4 promoter, the rhamnose-inducible promoter or the alcohol oxidase (AOXI) promoter. The vectors can contain other functional regions. In addition to selection markers and origins of replication, gene regulating elements, e.g. operators, repressors or transcription factors, are of special interest. In particular, it is possible to use a construction of vectors that allows reversible, inducible or repressible expression of the recombinant mutated esterases.

Preferred host cells for transfection with vectors containing the mutated sequences for expression of the esterases are unicellular prokaryotic or eukaryotic organisms such as *Aspergillus sp., S. cerevisiae, Hansenula sp., E. coli* or *P. pastoris*.

Thus the present invention also provides the use of DNA fragments that code for mutant pig liver esterases according to the invention, and optionally for other N- and/or C-terminal domains fused thereto, for cloning into vectors. The invention also provides the use of these vectors for the transformation of cells and the use of such transformed cells or cell cultures for expression of

the recombinant artificial esterases mutated by site directed mutagenesis. The esterases expressed can be isolated e.g. in monomeric form, but it is also possible to prepare enzymatically active, multimeric pig liver esterases and for different units of the multimer to carry different mutations.

Due to the high purity of the recombinant enzymes and the increased specific activity, and in some cases also due to the modified enantioselectivity of the artificially mutated enzymes claimed, they are particularly suitable for organic synthesis. Appropriate substrates for the catalytic conversion are particularly aromatic-aliphatic and aliphatic-aliphatic esters, especially carboxylic acid esters of chiral or prochiral alcohols; the carboxylic acid component preferably contains 2 to 5 carbon atoms and can also be branched. The enzymatic-catalytic resolution of racemic carboxylic acid esters, especially acetates, is characterized by an enhanced R enantioselectivity compared with conversion with rPLE.

The optimal enzymatic activity of recombinant pig liver esterases containing monomeric subunits according to **Seq. ID No. 1** is at a pH of between 5 and 10 and preferably of between 7.5 and 9.5, and at a temperature of between 20°C and 90°C, preferably of between 40°C and 80°C and particularly preferably of between 50°C and 65°C and, for enzymes which in positions 129, 133, 134, 195, 236, 237, 290 or 294, additionally of between 50°C and 80°C.

The recombinant enzymes according to the invention can also be used for the resolution of racemic carboxylic acids or for the conversion of prostereogenic compounds,

especially diols or dicarboxylic acids.

Brief description of the Figures:

Fig. 1 shows the pH dependence of the enzymatic activity of the artificial esterases prepared. Fig. 2 shows the temperature dependence. Fig. 3 shows the measured specific activity of the mutant esterases, compared with commercially available esterase extracts and rPLE, towards methyl butyrate, compared with proline-β-naphthylamide. Fig. 4 and 5 show the enantioselectivity of hydrolysis in respect of selected substrates using the mutated esterases. Fig. 6 shows the *Pichia pastoris* expression vector pPICZαA, which contains the following important sequence elements:

1-940 bp	5′ AOX1 promoter
941-1207 bp	α-factor signal sequence
1208-1274 bp	multiple cloning site
1275-1304 bp	c-myc epitope
1320-1337 bp	his-tag
1341-1682 bp	3′ AOX1 stop site
1683-2094 bp	TEF1, S. cerevisiae transcription elongation factor 1 promoter
2095-2162 bp	EM7, synthetic prokaryotic promoter
2163-2537 bp	Sh ble: Streptoalloteichus hindustanus bleomycin gene (responsible for zeocin resistance)
2538-2855 bp	CYC1 stop site (from the S. cerevisiae CYC1 gene)
2866-3539 bp	pUC ori (origin of replication for E. coli)

Some Examples are given below to illustrate the present invention:

General:

The shuttle vector pPICZαA from Invitrogen was used for cloning in *E. coli* and expression in *P. pastoris* (**Fig. 6**).

The strains used for cloning and expression are listed below:

Species	Strain	Genotype	Supplier
Escherichia coli	DH5α	supE44ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1	Clontech
Escherichia coli	XL10-Gold	Tet^rΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB $lacI^qZ$ ΔM15 Tn10 (Tet^r) Amy Cam^r]	Stratagene
Pichia pastoris	X33	WT	Invitrogen

Tissue preparation, mRNA isolation and cloning of rPLE-cDNA

Cloning of the native pig liver esterase gene in *E. coli* DH5α, containing the sequence **Seq. ID No. 29** coding for rPLE, is carried out as instructed in WO 02/48322 (Examples 1 and 2), where a sequence coding for a myc epitope and a his-tag (**Seq. ID No. 41**) is cloned (pPICZα-mPLE*-tag) directly following the rPLE sequence **Seq. ID No. 29.**

The plasmid DNA was isolated from *E. coli* DH5α using a plasmid preparation method based on the alkaline lysis of cells and the specific binding of DNA to ion exchanger columns (QIAgen).

Small amounts (approx. 1 µg) of the DNA were obtained using the Plasmid Miniprep Kit (QIAgen). After separating the cells from a 3 ml overnight culture by centrifugation (16,000 x g, 1 min, bench centrifuge) and discarding the culture supernatant, isolation of the plasmid continued as instructed by the manufacturer. Larger amounts of plasmid DNA (approx. 10 µg) were prepared using the Plasmid Midiprep Kit (QIAgen). The starting material used in this case was a 50 ml overnight culture, which was centrifuged for 10 min at 4000 x g and 4°C. The plasmid was isolated from the cell pellet according to the manufacturer's instructions.

The resulting vector, pPICZα-mPLE*-tag, is used for site directed mutagenesis.

Example 1: Site directed mutagenesis

Mutagenesis is carried out starting from the pPICZα-mPLE*-tag template. The individual mutations coding for the amino acid exchanges are successively inserted according to **Table 1**, starting in each case from the nucleic acid obtained in the previous mutation step.

Site directed mutagenesis is carried out via a PCR using the QuikChange™ Site Directed Mutagenesis Kit (Stratagene), observing the process conditions indicated by the manufacturer. The plasmid used, pPICZα-mPLE*-tag, is amplified, with the aid of *Pfu* DNA polymerase, with two complementary primers containing the desired mutation, the primers indicated in **Table 2** being used for the site directed mutagenesis. The reaction mixture containing the methylated template DNA is then digested for 1 h at 37°C by the addition of 1 µl of *Dpn*I in order to degrade the DNA. The QIAquik PCR Purification Kit was used as instructed by the manufacturer (QIAgen) to purify the resulting

artificially mutated DNA sequences after the PCR.

17 amino acid codons in **Seq. ID No. 29** were thus modified by the exchange of 21 nucleotides.

The PCR was performed under the following conditions:

Mixture for mutagenesis by the QuikChange method

Component	Amount
10x reaction buffer	5 µl
dsDNA template (pPICZαmPLE*-tag)	10 ng
Primer I	125 ng
Primer II	125 ng
dNTPmix	1 µl
QuikSolution	3 µl
dH_2O	ad 50 µl
PfuTurboDNA polymerase	1 µl

PCR thermocycler programme for mutagenesis using the QuikChange™ method

Step	Temperature [°C]	Time	Number of cycles
Denaturation	95	1:00	1
Denaturation	95	1:00	18
Addition	60	0:50	
Extension	68	10:00	
Extension	68	7:00	1

The mutated plasmids prepared in this way are transformed in *E. coli* for further multiplication. The transformation of *E. coli* (XL10-Gold or DH5α) with the DNA plasmids obtained is effected by the chemical treatment (Mg^{2+}, DMSO) and heat shock of competent cells. For a maximum of 10 transformations, 50 ml of LB medium 1:100 (0.5% (w/v) of yeast extract, 1% (w/v) of tryptone, 1% (w/v) of NaCl, 2% (w/v) of agar for the agar plates) were inoculated with an *E. coli* overnight culture and incubated for 2 - 3 hours at 37°C and 200 rpm until the OD_{600} was 0.4 - 0.7. The

transformation culture was transferred under sterile conditions to a 50 ml Falcon tube and the cells were centrifuged off for 10 min at 1750 x g and 4°C. The cell pellet was resuspended in 2 ml of ice-cold TSS solution (10% (w/v) of PEG 6000, 5% (v/v) of DMSO, 50 mM $MgSO_4$, ad 100 ml of LB medium, autoclaved), distributed into ten 1.5 ml Eppendorf tubes (200 µl each) and then cooled on ice for 5 min. 1-3 µl of the plasmid DNA are then pipetted into each 200 µl aliquot of cell suspension and the transformation mixture is placed on ice. After 20 min the cells are incubated first for 30 - 45 sec at 42°C in a water bath (heat shock) and then, after the addition of 800 µl of LB medium, for 1 h at 37°C. For positive selection of the successfully transformed cells, the cell suspension is streaked on LB_{LS}zeocin-agar plates (1% (w/v) of yeast extract, 1% (w/v) of tryptone, 0.5% (w/v) of NaCl, 2% (w/v) of agar for the agar plates) and incubated overnight at 37°C. The mutated plasmid DNA is isolated and worked up as described above for the rPLE vector. The mutated nucleic acid sequence is then sequenced for control purposes.

Part of the mutated plasmid DNA isolated is used to produce the next mutant nucleic acid sequence.

Nine mutated nucleic acid sequences were produced in this way. The coding DNA sequences of the artificial esterases are shown in **Seq. ID No. 2** to **10**. The mutations are listed in **Table 1** on the basis of the amino acid sequences.

Table 1: Mutations in the amino acid sequences of the rPLE variants

Designation	Mutations*)
PLE-GEHa	V76A, E77G (**Seq. ID No. 2**)
PLE-GEHb	V76A, E77G, T92I, L93P (**Seq. ID No. 3**)
PLE-GEHc	V76A, E77G, T92I, L93P, P195T (**Seq. ID No. 4**)
PLE-GEHd	V76A, E77G, T92I, L93P, P195T, V236A, A237G (**Seq. ID No. 5**)
PLE-GEHe	V76A, E77G, T92I, L93P, P195T, V236A, A237G, F290L, Q294P (**Seq. ID No. 6**)
PLE-GEHf	V76A, E77G, T92I, L93P, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T (**Seq. ID No. 7**)
PLE-GEHg	V76A, E77G, T92I, L93P, L129V, P133S, M134T, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T (**Seq. ID No. 8**)
PLE-GEHh	V76A, E77G, T92I, L93P, L129V, P133S, M134T, V138L, V139A, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T (**Seq. ID No. 9**)
rGEH	V76A, E77G, T92I, L93P, K112R L129V, P133S, M134T, V138L, V139A, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T **(Seq. ID No. 10)**

*) The information in the Table represents the position and the amino acid exchange performed in the case of Seq. ID No. 1; for example, "V76A" denotes the exchange of valine (V) in position 76 with alanine (A).

Table 2: Sequences of the primers used for the site directed mutagenesis

Designation	Primer
PLE-GEHa	
(Seq. ID No. 11)	5′ gccaggaccc agtag**c**ggg**g**g cagatgacct cagatc
(Seq. ID No. 12)	5′ gatctgaggt catctg**c**ccc **g**ctactgggt cctggc
PLE-GEHb	
(Seq. ID No. 13)	5′ cggaaaggag aggctca**t**tc **c**ggagttttc tgaagac
(Seq. ID No. 14)	5′ gtcttcagaa aactcc**g**ga**a** tgagcctctc ctttccg
PLE-GEHc	
(Seq. ID No. 15)	5′ ccaactttgg aggcgac**a**ca ggctctgtga cc
(Seq. ID No. 16)	5′ ggtcacagag cctg**t**gtcgc ctccaaagtt gg
PLE-GEHd	
(Seq. ID No. 17)	5′ ggcgtggccc tcactg**c**tg**g** cctggtcagg aagg
(Seq. ID No. 18)	5′ ccttcctgac cagg**c**ca**g**ca gtgagggcca cgcc
PLE-GEHe	
(Seq. ID No. 19)	5′ gaaattttta actcttgatt t**g**catggaga c**ccc**agagag agccatccc
(Seq. ID No. 20)	5′ gggatggctc tctct**gg**ggt ctccatg**c**aa atcaagagtt aaaaatttc
PLE-GEHf	
(Seq. ID No. 21)	5′ ctcctgtgga agtc**c**ttccc catc**a**ctaac atccctgagg
	5′ cctcagggat gttag**t**gatg gggaa**g**gact tccacaggag
(Seq. ID No. 22)	
PLE-GEHg	
(Seq. ID No. 23)	5′ ggaggcctgg t**g**gtgggcgg ggca**t**c**cacc** tatgatgggg tggtg
(Seq. ID No. 24)	5′ caccacccca tcata**ggtg**g **a**tgccccgcc caccaccagg cctcc
PLE-GEHh	
(Seq. ID No. 25)	5′ catccaccta tgatggg**c**tg g**cc**cttgctg cgcatg
(Seq. ID No. 26)	5′ catgcgcagc aag**gg**cca**g**c ccatcatagg tggatg
rGEH	
(Seq. ID No. 27)	GEH-F9: 5′ gctgacctga caa**g**gagggg cagactg
(Seq. ID No. 28)	GEH-R9: 5′ cagtctgccc ctc**c**ttgtca ggtcagc

The nucleotides used to insert the particular mutation are printed in bold.

Example 2: Preparation of the modified enzymes

To prepare larger amounts of linearized plasmid for the transformation of *P. pastoris*, the plasmids obtained in Example 1 are subjected to a preparative restriction digestion with *Pme*I. This is done by adding the following buffer solution to 6 µg of plasmid DNA:

dH_2O ad 65 µl

Buffer 4 (Promega)	6.5 µl
BSA solution	0.65 µl
*Pme*I	5 µl

The plasmid DNA is digested overnight at 25°C.

The digested plasmid DNA is purified using the QIAquik PCR Purification Kit as instructed by the manufacturer (QIAgen).

For transformation in *P. pastoris*, 50 ml of YPD medium (yeast extract-peptone-dextrose medium: 1% (w/v) of yeast extract, 2% (w/v) of peptone, with 10% (v/v) of 10 x D solution added after autoclaving, 2% (w/v) of agar for the agar plates) are cultivated with *P. pastoris* X33, with shaking at 30°C, until the OD_{600} is 1.0. The resulting cell suspension is centrifuged for 10 min at 1500 x g and room temperature, the supernatant is discarded and the cells are washed once with 25 ml of sterile water. The cells are centrifuged off again and taken up in 1 ml of 100 mM LiCl and the cell suspension is then transferred to a microreaction vessel. The cells are sedimented again (max. speed in the bench centrifuge, 15 s), the supernatant is pipetted off and discarded and the cell pellet is resuspended in 400 µl of 100 mM LiCl. 50 µl portions of the resuspended cells are distributed into 1.5 ml microreaction vessels for further direct processing. Shortly before transformation, the cells are centrifuged off again and the LiCl solution above the cells is removed. For transformation, solutions are pipetted onto the cells in the following order: 240 µl of 50% (w/v) PEG, 36 µl of 1 M LiCl, 25 µl of 2 mg/ml fragmented herring sperm DNA and 5-10 µg of plasmid DNA in 50 µl of sterile water. The cells are completely dissolved by vortexing for 1 min and are subsequently incubated with the plasmid DNA for 30 min at 30°C without

shaking and then for 20-25 min at 42°C (heat shock). The cells are centrifuged off (3500-6000 x g), the supernatant is discarded and the pellet is resuspended in 1 ml of YPD and shaken at 30°C. After 1 h and 4 h, 25, 50 and 100 µl portions are plated on YPD agar plates containing 100 µg/ml of zeocin. The agar plates are incubated at 30°C until visible colonies appear (2-3 days).

For the identification of enzymatically active *P. pastoris* clones, individual transformed cells are transferred to YPD-zeocin-agar plates. After 24 hours' incubation at 30°C, 100 µl of methanol are added to the cover of the Petri dish every 24 h in order to induce esterase production. After 2-3 days the agar plates are covered with a layer of 10 ml of soft agar (0.5% of agar in water) mixed with 100 µl of α-naphthyl acetate (40 mg/ml in DMF) and 100 µl of Fast Red TR (100 mg/ml in DMSO). α-Naphthol is produced during hydrolysis and forms a coloured complex with Fast Red. Positive clones are stained brown.

After the identification of enzymatically active clones, 10 ml of BMGY medium (2% (w/v) of peptone, 1% (w/v) of yeast extract, 1% (v/v) of glycerol, with 10% (v/v) of phosphate buffer (1 M, pH 6.0) and 0.2% (v/v) of 500 x B added after autoclaving) are inoculated with the clones in a 50 ml tube. Expression takes place on a small scale at 30°C and 200 rpm. When the OD_{600} is in the range 2-6 (after approx. 16 h), the cells are centrifuged off (2000 x g, 5 min, RT) and resuspended in 2 ml of BMMY medium (2% (w/v) of peptone, 1% (w/v) of yeast extract, with 10% (v/v) of phosphate buffer (1 M, pH 6.0), 0.2% (v/v) of 500 x B and 0.5% (v/v) of methanol added after autoclaving). 20 ml of the BMMY medium were inoculated with this suspension in 100 ml flasks (until the OD was approx. 1). The cultures were incubated at 30°C and 200 rpm and methanol (0.5% of the culture volume) was added every 24 h. Samples are

taken after 0.5, 24 and 48 h and the supernatant containing the expressed enzyme is concentrated by ultrafiltration. This is done using a Centrikons-Plus 30K with an Ultracel-PL membrane, which affords a 25- to 100-fold concentration of the esterase solution. The activity of the concentrated supernatant is determined by means of a pNPA assay. After concentration by ultrafiltration, the success of the enzyme expression is checked by native PAGE.

For the expression of larger amounts of enzyme, 100 ml of BMGY medium are inoculated in 500 ml flasks in an overnight culture (3 ml of YPD medium) and incubated at 30°C and 200 rpm. When the OD_{600} is in the range 2-6 (after approx. 16 h), the cells are centrifuged off (2000 x g, 5 min, RT) and resuspended in 10 ml of BMMY medium. 200 to 400 ml of the BMMY medium are inoculated with this suspension in 1 l or 2 l flasks (until the OD is approx. 1). The cultures are incubated at 30°C and 200 rpm and methanol (0.5% of the culture volume) is added every 24 h. Cultivation is carried out for 72 hours, after which the cells are centrifuged off and the supernatant is processed further. The activity of the expressed enzymes is determined by means of a pNPA assay (cf. Ex. 3.4 for instructions).

The results of the pNPA assay are shown in **Table 3.**

Table 3: Volumetric and specific activity in the supernatant after cultivation and concentration

	Volume	Activity in supernatant		Activity in concentrate		Specific activity
	[ml]	[U/ml]	[U]	[U/ml]	[U]	[U/mg]
PLE-GEH a	250	0.53	132.5	22.4	67	62.7
PLE-GEH b	250	0.65	162.5	17.5	79	58.9
PLE-GEH c	250	0.32	80.0	9.1	23	22.8
PLE-GEH d	250	0.28	70.0	16.2	62	376.7
PLE-GEH e	250	0.22	55.0	10.0	40	34.5
PLE-GEH f	250	0.50	125.0	10.3	46	20.1
PLE-GEH g	250	0.16	40.0	2.2	20	16.2
PLE-GEH h	400	0.23	91.6	9.2	46	3.6
rGEH	200	0.15	30.0	1.44	10	24.7

Example 3: Further characterization of the enzymes

3.1. Determination of the molecular weight

The molecular weight of the enzymes was determined with a Ferguson Plot (native gel) and a gradient gel (PhastSystem):

With the Ferguson Plot, enzyme trimers with a molecular weight of approx. 180 kDa were found preferentially. With the gradient gel (PhastSystem), in which the semilogarithmic plot of molecular weight against migration distance of the protein shows a linear dependence, a molecular weight of approx. 180 kDa is again found.

3.2. Determination of the isoelectric point

The isoelectric focusing of the enzymes was carried out with a PhastSystem. This was done using two different gels, one with a pH range of 3 - 9 and the other with a pH range of 4 - 6.5. In addition, for the purposes of comparison, all the isoelectric points were also calculated theoretically with a computer program (DNAsis). The results are listed in **Table 4.**

Table 4: Results of the pI determination

Enzyme	IEF 4-6.5 pH range		IEF 3-9 pH range		Determined with DNAsis
rPLE	5.28	4.71	5.34	4.67	5.62
PLE-GEH a	5.46	4.57	5.58	4.51	5.69
PLE-GEH b	5.44	4.59	5.58	4.59	5.69
PLE-GEH c	5.46	4.69	5.54	4.59	5.69
PLE-GEH d	5.44	4.55	5.56	4.49	5.69
PLE-GEH e	5.46	4.62	5.56	4.42	5.69
PLE-GEH f	5.44	4.56	5.52	4.26	5.69
PLE-GEH g	5.44	4.60	5.52	4.26	5.69
PLE-GEH h	5.50	4.55	5.60	4.34	5.69
rGEH	5.50	4.55	5.60	4.34	5.69

3.3. Determination of the protein content

The protein content was determined by SDS-PAGE. Different concentrations of BSA were used for the standard curve. The intensity of the protein bands was measured with the computer program NIH-Imager after the gel had been scanned, and this was used to calculate the protein content (**Table 5**). Conventional methods (Bradford, BCA test) have proved unsuitable for determining the protein content in the supernatant of a *Pichia* culture because interfering peptide-containing constituents are present in the medium.

Table 5: Protein contents of the recombinant enzymes

Enzyme	µg/ml
rPLE	67
PLE-GEHa	357
PLE-GEHb	297
PLE-GEHc	400
PLE-GEHd	43
PLE-GEHe	290
PLE-GEHf	513
PLE-GEHg	136
PLE-GEHh	2570
rGEH	58

3.4. Kinetic data

The kinetic data were determined by means of a pNPA assay (concentration range 0.1-5 mM). K_m and V_{max} were calculated from a Lineweaver-Burk plot.

Table 6: Kinetic data of the recombinant enzymes

Enzyme	V_{max} [µmol/min]	K_m [mM]	V_{max}/K_m [µmol/min/mM]
rPLE	120	1.10	110
PLE-GEHa	145	0.74	196
PLE-GEHb	125	0.56	222
PLE-GEHc	37	0.60	63
PLE-GEHd	588	0.65	909
PLE-GEHe	40	0.40	100
PLE-GEHf	34	0.50	67
PLE-GEHg	30	0.50	60
PLE-GEHh	6	0.48	12
rGEH	47	1.1	43

Determination of the esterase activities by means of a pNPA assay

The hydrolysis of p-nitrophenyl acetate (pNPA) is used for the photometric determination of esterase activity. The product formed is p-nitrophenol, which is quantified at a wavelength of 410 nm. The molar extinction coefficient (ε) is pH-dependent and the extinction coefficients determined for different pH values are indicated below.

Extinction coefficients for p-nitrophenol as a function of pH

pH	ε [M^{-1} cm^{-1}]
5.0	$1.37 \cdot 10^3$
6.0	$3.21 \cdot 10^3$
7.0	$11.68 \cdot 10^3$
7.5	$15.60 \cdot 10^3$
8.0	$17.10 \cdot 10^3$
8.5	$18.00 \cdot 10^3$
9.0	$18.10 \cdot 10^3$
9.5	$18.11 \cdot 10^3$
10.0	$18.16 \cdot 10^3$

The reaction rate is measured over one minute at one-second intervals in a 1 ml cuvette at room temperature. The assay solution used consists of 800 µl of sodium phosphate buffer (50 mM, pH 7.5), 100 µl of enzyme solution and 100 µl of substrate solution (pNPA in DMSO, 10 mM for standard measurements or from 0.1 to 100 mM for determinations of enzyme kinetics). The autohydrolysis of pNPA in buffer is measured in each case as a blank. 1 U of esterase activity corresponds to the amount of enzyme that liberates 1 µl of p-nitrophenol in one minute.

Example 4: pH profiles of the enzymatic activity

The pH profiles were determined by means of a pNPA assay (performed as in Ex. 3). The activity was measured in phosphate buffer at the appropriate pH values. The profiles are very similar, with the exception of the mutant GEH-PLEe. The highest activity was observed for all the enzymes in the pH range from 8 to 9 (**Table 7, Fig. 1**).

Table 7: Volumetric and specific activity of the recombinant enzymes at optimum pH

Enzyme	pH	Spec. activity [U/mg]
rPLE	9	95.5
PLE-GEHa	8.5	84.6
PLE-GEHb	8	90.2
PLE-GEHc	8.5	32.3
PLE-GEHd	8	516.3
PLE-GEHe	8	134.1
PLE-GEHf	8.5	23.6
PLE-GEHg	9	19.9
PLE-GEHh	8	3.9
rGEH	8	26.0

The pH profiles of the recombinant mutant enzymes are represented graphically in **Fig. 1.** The highest activity was taken as 100% for each enzyme and compared with the other activities.

Example 5: Temperature profiles

The temperature profiles were determined by the pH stat method (carried out as in Ex. 6) at different temperatures. Tributyrin was used as the substrate. 0.5 U (pNPA) of enzyme was used in each case for 30 ml of substrate-containing emulsion. The butyric acid liberated was titrated with 0.01 M NaOH, the pH being kept constant at 7.5 (cf. **Table 8, Fig. 2** for results). As the number of mutations increases, so too does the temperature stability of the enzymes: at 70°C rPLE is no longer active and rGEH is at its optimum.

Table 8: Specific activity of the recombinant enzymes at optimum temperature

Enzyme	Temperature	Spec. activity [U/mg]
rPLE	60	67.3
PLE-GEHa	60	139.8
PLE-GEHb	60	117.6
PLE-GEHc	60	39.3
PLE-GEHd	60	1165.1
PLE-GEHe	60	110.6
PLE-GEHf	60	87.6
PLE-GEHg	70	291.7
PLE-GEHh	70	52.3
rGEH	70	451.0

The temperature profiles of the recombinant mutant enzymes are shown in **Fig. 2.** The highest activity was taken as 100% for each enzyme and compared with the other activities.

Example 6: Hydrolysis of methyl butyrate and proline-β-naphthylamide

The hydrolysis of methyl butyrate was carried out by the pH stat method, a known amount of enzyme being added to a substrate solution (30 ml) consisting of 2% w/v of gum arabic and 5% v/v of substrate in water, emulsified with a homogenizer. The acid liberated in the reaction is back-titrated with sodium hydroxide solution in order to keep the pH constant. This is done by titrating the liberated butyric acid with 0.01 M NaOH, the pH being kept constant at 7.5 (at 37°C).

The amount of sodium hydroxide solution consumed is used to calculate the amount of liberated acid that correlates with the activity of the enzyme, 1 U corresponding to the amount of enzyme that liberates 1 µmol of acid per minute. 1 U (from pNPA assay) of enzyme is used in each case for 30 ml of substrate-containing emulsion.

The results of the hydrolysis are shown in **Table 9.**

The hydrolysis of proline-β-naphthylamide is followed photometrically by means of a Fast Garnet assay using 0.5 U (pNPA) of enzyme in each case. β-Naphthylamine liberated in the reaction forms a pink complex with Fast Garnet, which can be detected at 520 nm ($\varepsilon = 24.03 \cdot 10^3$ M^{-1} cm^{-1}).

The reaction mixture (500 µl), consisting of Tris/HCl (0.1 M, pH 8.0), 50 µl of substrate solution (0.2 mM in DMSO) and esterase solution (0.4 - 0.5 U (from pNPA assay)), is reacted in a thermomixer at 37°C. After 30 min, Fast Garnet solution (1.5 ml, 15% w/v, first dissolved with ethanol and then topped up with 4% Brij solution) is added and the absorption is measured immediately.

1 U of amidase activity corresponds to the amount of enzyme that liberates 1 µmol of β-naphthylamine in one minute.

The results of the hydrolysis are likewise shown in **Table 9.**

Table 9: Results of the hydrolysis of methyl butyrate and proline-β-naphthylamide (as reference substance) with all the recombinant enzymes

Enzyme	MB [U/mg]	PβNA [U/mg]	MB/PβNA
Fluka PLE	122.9	0.23	534
Chirazyme E2	34.4	0.29	118
rPLE	2.92	0.075	39
PLE-GEHa	6.88	0.043	162
PLE-GEHb	10.10	0.035	290
PLE-GEHc	4.09	0.024	173
PLE-GEHd	88.15	0.409	216
PLE-GEHe	7.14	0.043	167
PLE-GEHf	5.31	0.023	231
PLE-GEHg	0.82	0.032	25
PLE-GEHh	0.74	0.006	135
rGEH	5.0	0.055	91

The results of the reactions are also given in **Fig. 3**, which shows the specific activity of commercially available esterases (PLE Fluka and PLE Chirazyme E2) and recombinant esterases towards methyl butyrate/proline-β-naphthylamide. It is seen that the activity of mutants a to f and h is greater than that of rPLE.

Example 7: Study of the stereoselectivities of the enzymatic hydrolysis

The hydrolysis is carried out in 1.5 ml reaction vessels in a thermomixer (Eppendorf) at 37°C. 0.5 U of esterase (based on the pNPA test) is used in each case for 1 ml of substrate solution (10 mM in sodium phosphate buffer pH 7.5, 50 mM). Samples are taken from the reaction mixture at the times indicated in the Tables. The reaction is stopped by extracting the mixture with methylene chloride and drying the organic phase over anhydrous sodium sulfate.

The enantiomeric purity and the conversion are determined by gas chromatography. The results are shown in **Tables 10** to **15** and in **Fig. 4**.

Table 10: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-1-ethyl acetate (all the enzymes exhibit an (R) preference)

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$]	[%ee$_P$]	[%]	
rPLE	2.5 h	72	60	55	8
PLE-GEH a	1.5 h	96	87	53	54
PLE-GEH b	1.5 h	88	85	51	35
PLE-GEH c	3 h	91	83	52	34
PLE-GEH d	1.5 h	90	76	54	23
PLE-GEH e	1.5 h	89	79	53	24
PLE-GEH f	1 h	97	70	58	23
PLE-GEH g	0.5 h	81	60	58	10
PLE-GEH h	0.5 h	72	64	53	10
rGEH	0.75 h	85	65	57	12

Table 11: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-1-propyl acetate (all the enzymes exhibit an (R) preference)

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$]	[%ee$_P$]	[%]	
rPLE	6 h	35	33	51	3
PLE-GEH a	2 h	53	57	48	6
PLE-GEH b	2 h	46	58	44	6
PLE-GEH c	4 h	56	54	51	6
PLE-GEH d	4 h	55	50	53	5
PLE-GEH e	3 h	51	55	48	6
PLE-GEH f	2 h	50	54	48	5
PLE-GEH g	15 min	23	30	44	2
PLE-GEH h	15 min	31	31	49	3
rGEH	30 min	32	21	60	2

Table 12: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-3-butyl acetate

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$]	[%ee$_P$]	[%]	
rPLE	5 h	38 (R)	34 (S)	53	3
PLE-GEH a	4 h	37 (R)	32 (S)	54	3
PLE-GEH b	4 h	32 (R)	36 (S)	47	3
PLE-GEH c	6 h	30 (R)	38 (S)	45	3
PLE-GEH d	7 h	49 (R)	63 (S)	44	7
PLE-GEH e	7 h	35 (R)	57 (S)	39	5
PLE-GEH f	6 h	47 (R)	54 (S)	47	5
PLE-GEH g	2 h	60 (S)	58 (R)	51	7
PLE-GEH h	2 h	58 (S)	56 (R)	51	6
rGEH	1.5 h	12 (S)	9 (R)	58	1.3

Table 13: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-2-propyl acetate (all the enzymes exhibit an (S) preference)

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$] [c]	[%ee$_P$] [c]	[%]	
rPLE	2 h	80	61	57	10
PLE-GEH a	2 h	79	66	54	12
PLE-GEH b	2 h	62	66	49	9
PLE-GEH c	2 h	33	32	51	3
PLE-GEH d	2 h	71	73	49	13
PLE-GEH e	2 h	63	54	54	6
PLE-GEH f	2 h	71	64	53	9
PLE-GEH g	2 h	-	-	50	1
PLE-GEH h	1 h	6.8	6.0	53	1.2
rGEH	3 h	9.5	9.5	50	1.3

Table 14: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-2-pentyl acetate

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$]	[%ee$_P$]	[%]	
rPLE	2 h	89 (R)	75 (S)	54	20
PLE-GEH a	2 h	64 (R)	55 (S)	54	6
PLE-GEH b	2 h	71 (R)	50 (S)	59	6
PLE-GEH c	2 h	48 (R)	60 (S)	45	6
PLE-GEH d	2 h	58 (R)	61 (S)	49	7
PLE-GEH e	2 h	59 (R)	67 (S)	47	9
PLE-GEH f	0.5 h	63 (R)	61(S)	51	8
PLE-GEH g	0.5 h	5.4 (S)	6.2 (R)	47	1.2
PLE-GEH h	0.5 h	5.4 (S)	5.2 (R)	51	1.2
rGEH	1.5 h	3.1 (S)	6.3 (R)	33	1.2

Table 15: Enantioselectivity of different recombinant esterases in the hydrolysis of 1-phenyl-2-butyl acetate (all the enzymes exhibit an (S) preference)

Enzyme	Time	Enantiomeric excess		Conversion	E
		[%ee$_S$]	[%ee$_P$]	[%]	
rPLE	2 h	60	93	39	53
PLE-GEH a	2 h	89	71	56	17
PLE-GEH b	2 h	80	76	51	18
PLE-GEH c	2 h	75	67	53	11
PLE-GEH d	2 h	89	77	54	23
PLE-GEH e	2.5 h	77	77	50	18
PLE-GEH f	2 h	82	75	52	17
PLE-GEH g	1 h	8	9	47	1.3
PLE-GEH h	45 min	9	9	51	1.3
rGEH	4 h	57	51	53	5

Fig. 4 is a graphical representation of the results obtained. Above the X-axis: (S) preference, below: (R) preference. Values above the X-axis indicate an S preference of the particular enzyme and values below the X-axis indicate an R preference. The R preference of the esterase was increased, or the S-preference reduced, by the inserted mutations. The mutated enzymes tested are thus particularly suitable for the preparation of R enantiomers.

Example 8: Further study of the enantioselectivity

To delimit more precisely the mutations which have a particularly strong influence on the enantioselectivity of the hydrolysis, two other artificial mutant enzymes, **Seq. ID No. 39** (PLE-GEHa1) and **40** (PLE-GEHa2), were prepared as described in Examples 1 and 2. 1-Phenylethyl acetate (grey) and 1-phenyl-1-ethyl acetate (black) were used as the model substrates and converted enzymatically as described in Example 7. The E value is calculated for a conversion of approx. 50%. The results are illustrated in **Fig. 5**, which shows that the amino acid exchange in position 77, in particular, is responsible for the increase in enantioselectivity in respect of the R enantiomers.

Claims:

1. Artificial esterases containing an amino acid sequence **Seq. ID No. 1**, or a variant thereof with a sequence homology of at least 50%, characterized in that, in the case of **Seq. ID No. 1**, the amino acid sequence has at least one mutation in the positional regions from 74 to 79, 90 to 95, 110 to 114, 127 to 141, 193 to 197, 234 to 239, 288 to 296 and 365 to 372, it being possible for the enzyme to be shortened by up to 75 amino acids at the N-terminal end and/or by up to 100 amino acids at the C-terminal end.

2. Artificial esterases according to Claim 1, characterized in that, in the case of the amino acid sequence **Seq. ID No. 1**, at least one of the following amino acid exchanges is present:

- position 76: valine (V) with alanine (A),
- position 77: glutamic acid (E) with glycine (G),
- position 92: threonine (T) with isoleucine (I),
- position 93: leucine (L) with proline (P),
- position 112: lysine (K) with arginine (R),
- position 129: leucine (L) with valine (V),
- position 133: proline (P) with serine (S),
- position 134: methionine (M) with threonine (T),
- position 138: valine (V) with leucine (L),
- position 139: valine (V) with alanine (A),
- position 195: proline (P) with threonine (T),
- position 236: valine (V) with alanine (A),
- position 237: alanine (A) with glycine (G),
- position 290: phenylalanine (F) with leucine (L),
- position 294: glutamine (Q) with proline (P),
- position 367: tyrosine (Y) with phenylalanine (F),
- position 370: alanine (A) with threonine (T).

3. Artificial esterases with an increased specific activity, according to Claim 1 or 2, characterized in that they are mutated in at least one of positions 76, 77, 92, 93, 112, 138, 139, 195, 290, 294, 236, 237, 367 and 370.

4. Artificial esterases with a modified enantioselectivity compared with rPLE, according to one of the preceding claims, characterized in that they are mutated in position 77.

5. Artificial esterases with an increased temperature stability, according to one of the preceding claims, characterized in that they are mutated in at least one of positions 112, 129, 133, 134, 195, 236, 237, 290 and 294.

6. Artificial esterases according to Claim 1 or 2, characterized in that they contain an amino acid sequence according to **Seq. ID No. 2, 3, 4, 5, 6, 7, 8, 9** or **10.**

7. Multimeric esterases containing at least one artificial esterase according to one of the preceding claims as a subunit.

8. Recombinant nucleic acids coding for artificial esterases according to Claim 1 or 2, where, in the case of **Seq. ID No. 29,** at least one of the codons 76, 77, 92, 93, 112, 129, 133, 134, 138, 139, 195, 236, 237, 290, 294, 367 and 370 is mutated, or a nucleic acid hybridizing with its complementary sequence under stringent conditions, in which at least one of said codons is mutated.

9. Recombinant nucleic acids coding for artificial esterases according to Claim 7, characterized in that the nucleic acids contain a sequence according to **Seq. ID No. 30, 31, 32, 33, 34, 35, 36, 37** or **38.**

10. Recombinant vectors containing a nucleic acid sequence according to Claim 8 or 9.

11. Expression systems comprising a host cell transfected with a recombinant vector according to Claim 10.

12. Use of recombinant nucleic acids according to Claim 8 or 9 or vectors according to Claim 10 or an expression system according to Claim 11 for the expression of artificial esterases according to one of Claims 1 to 6 or multimeric esterases according to Claim 7.

13. Use of artificial esterases according to one of Claims 1 to 6 or multimeric esterases according to Claim 7 for the biocatalytic conversion of carboxylic acid derivatives.

14. Use of artificial esterases or multimeric esterases according to Claim 13 for the hydrolysis or synthesis of carboxylic acid esters.

15. Use of artificial esterases or multimeric esterases according to Claim 13 for the enantioselective conversion of carboxylic acid derivatives.

16. Use of artificial esterases according to one of Claims 1 to 6 or multimeric esterases according to Claim 7 as additives in detergents or household cleaners.

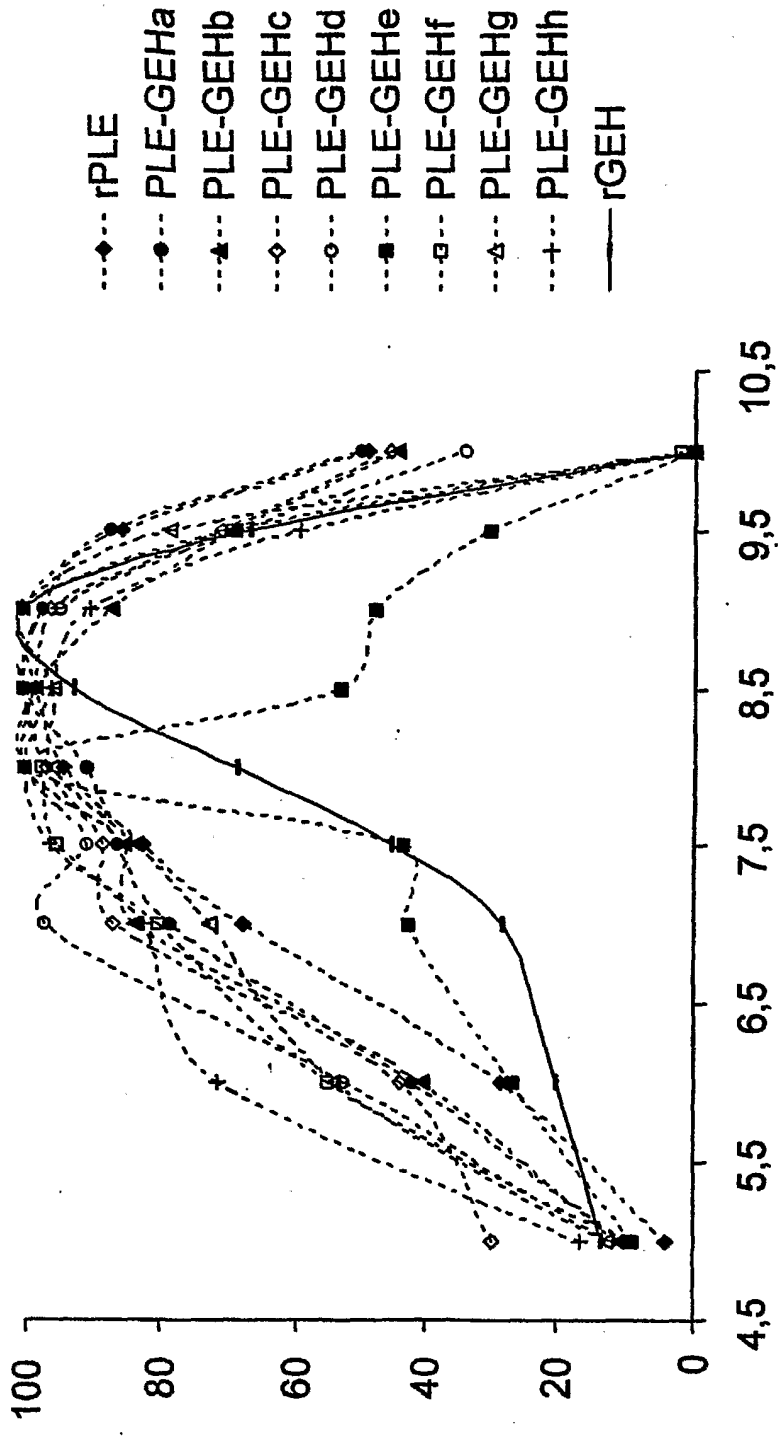

rPLE
PLE-GEHa
PLE-GEHb
PLE-GEHc
PLE-GEHd
PLE-GEHe
PLE-GEHf
PLE-GEHg
PLE-GEHh
rGEH
100
80
60
40
20
0
4,5
5,5
6,5
7,5
8,5
9,5
10,5

Fig. 1

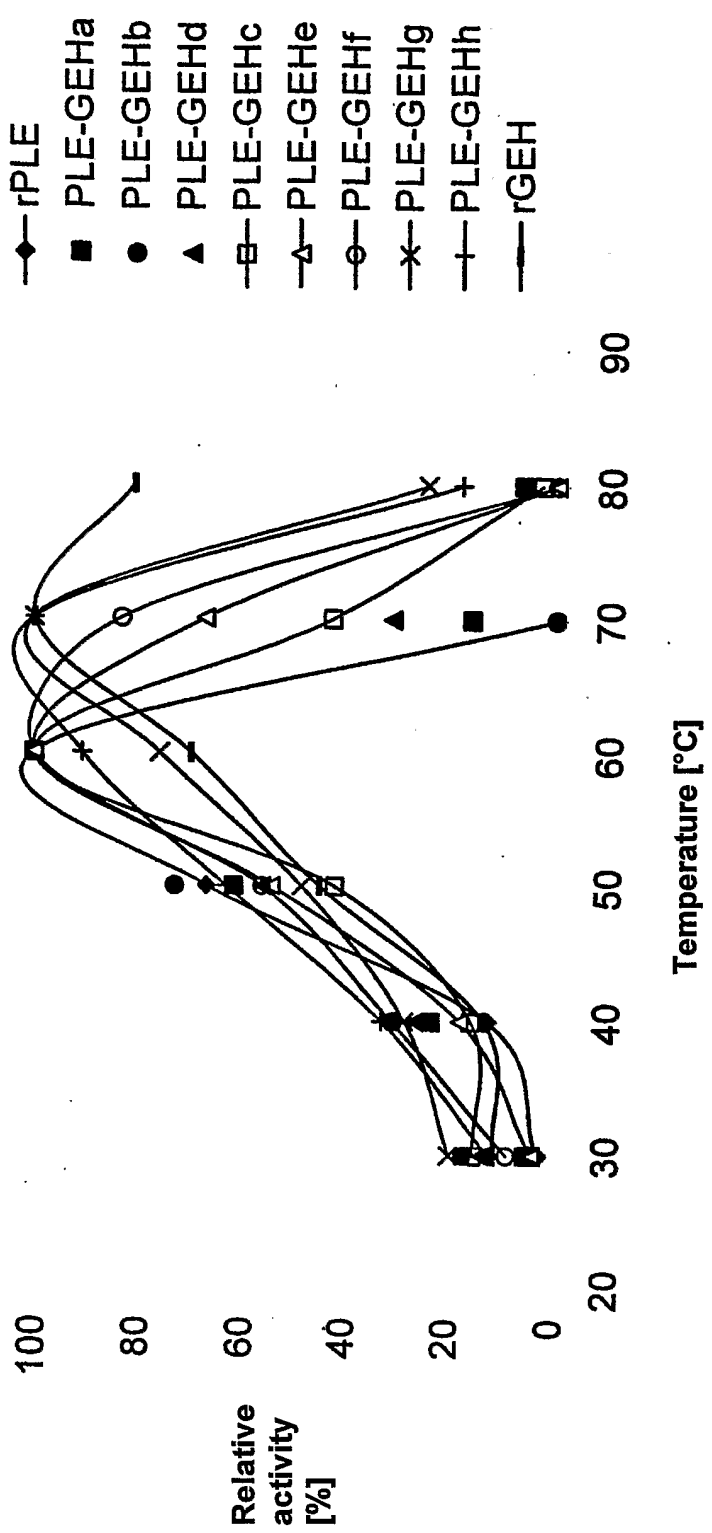

rPLE
PLE-GEHa
PLE-GEHb
PLE-GEHd
PLE-GEHc
PLE-GEHe
PLE-GEHf
PLE-GEHg
PLE-GEHh
rGEH
Relative activity [%]
100
80
60
40
20
0
20
30
40
50
60
70
80
90
Temperature [°C]

Fig. 2

Fig. 3

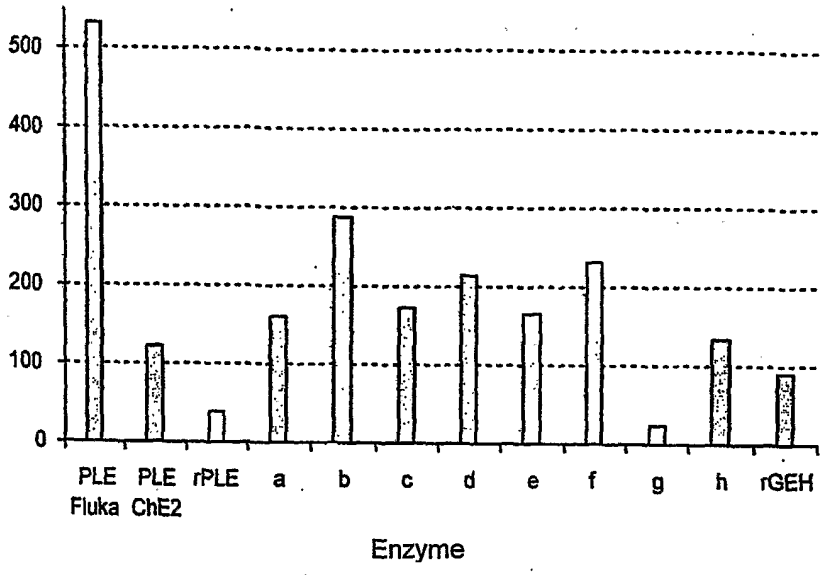

500
400
300
200
100
0
PLE Fluka
PLE ChE2
rPLE
a
b
c
d
e
f
g
h
rGEH
Enzyme

Fig. 4

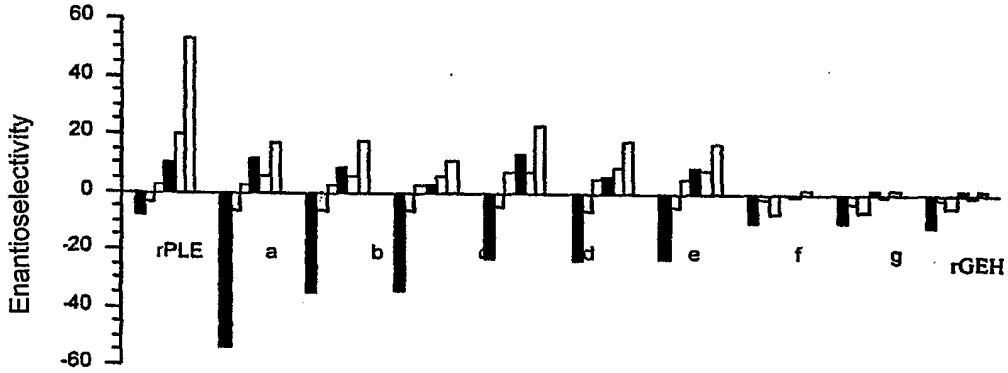

60
40
20
0
-20
-40
-60
Enantioselectivity
rPLE
a
b
d
e
f
g
rGEH

Fig. 5

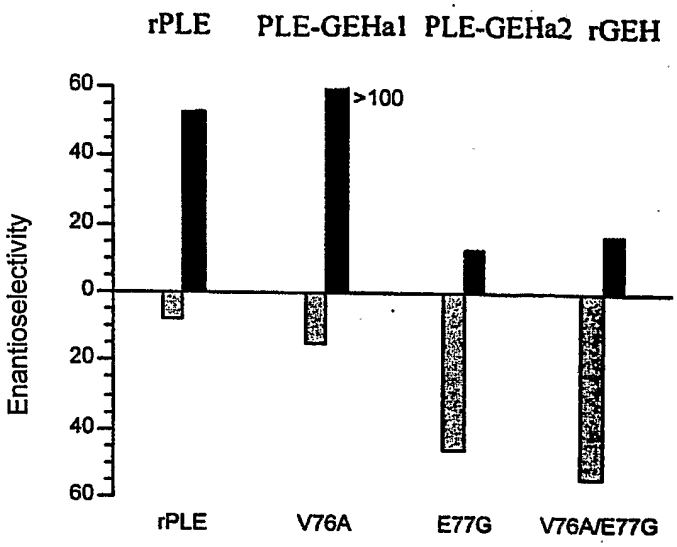
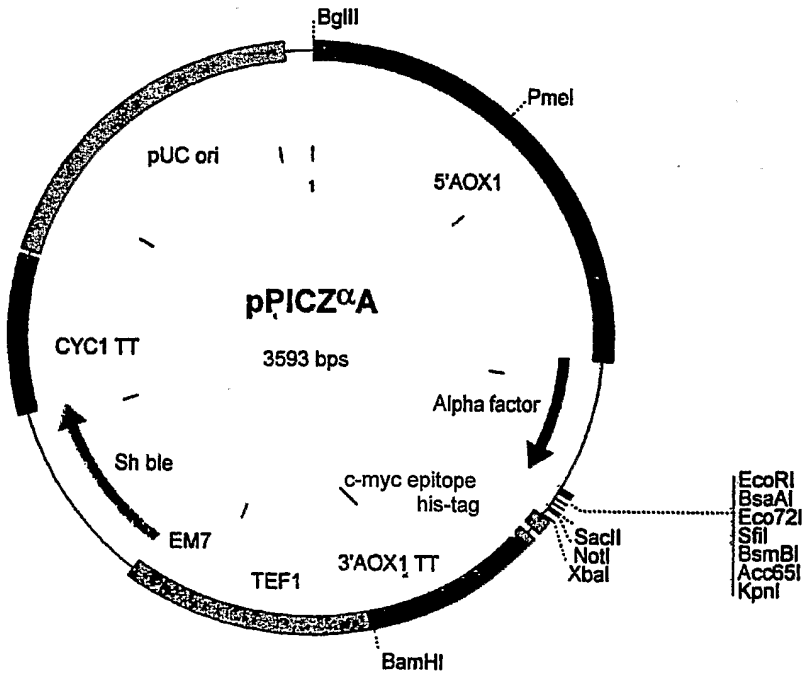

rPLE
PLE-GEHa1
PLE-GEHa2
rGEH
Enantioselectivity
60
40
20
0
20
40
60
>100
rPLE
V76A
E77G
V76A/E77G

Fig. 6


BglII
PmeI
pUC ori
5'AOX1
pPICZαA
3593 bps
CYC1 TT
Alpha factor
Sh ble
c-myc epitope
his-tag
EM7
TEF1
3'AOX1 TT
SacII
NotI
XbaI
EcoRI
BsaAI
Eco72I
SfiI
BsmBI
Acc65I
KpnI
BamHI

SEQUENCE LISTING

<110> Degussa AG

<120> Artificial esterases with high enzymatic activity

<130> 202dg06.de

<140>
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<160> 41

<170> PatentIn Ver. 2.1

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<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: rPLE

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
             20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
         35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
     50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Val Glu Gln Met Thr
 65                  70                  75                  80

Ser Asp Leu Phe Thr Asn Gly Lys Glu Arg Leu Thr Leu Glu Phe Ser
                 85                  90                  95

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190
Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205
```

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
260 265 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
275 280 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
290 295 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305 310 315 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
355 360 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys

530 535 540

<210> 2
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH a

<400> 2
Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
20 25 30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
35 40 45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
50 55 60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
65 70 75 80

Ser Asp Leu Phe Thr Asn Gly Lys Glu Arg Leu Thr Leu Glu Phe Ser
85 90 95

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
100 105 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
115 120 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
130 135 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145 150 155 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
165 170 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
180 185 190

Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
195 200 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

```
Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
        355                 360                 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
    450                 455                 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465                 470                 475                 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
                485                 490                 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
            500                 505                 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
        515                 520                 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
    530                 535                 540
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<210> 3
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH b

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
            20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
        35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
    50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
65                  70                  75                  80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270
Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
```

325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
355 360 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
530 535 540

<210> 4
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH c
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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15
Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
20 25 30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
35 40 45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
50 55 60

```
Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
 65                  70                  75                  80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
        355                 360                 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400
```

```
Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
    450                 455                 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465                 470                 475                 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
                485                 490                 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
            500                 505                 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
        515                 520                 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
    530                 535                 540
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<210> 5
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH d

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
             20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
         35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
     50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
 65                  70                  75                  80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
```

```
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
        355                 360                 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445
```

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
530 535 540

<210> 6
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH e

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
20 25 30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
35 40 45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
50 55 60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
65 70 75 80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
100 105 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
115 120 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
130 135 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145 150 155 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
165 170 175

```
Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Leu His Gly Asp Pro Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
        355                 360                 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
    450                 455                 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465                 470                 475                 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
                485                 490                 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
```

```
            500                 505                 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
        515                 520                 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
    530                 535                 540


<210> 7
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial
      esterase rPLE-rGEH f

<400> 7
Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
             20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
         35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
     50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
 65                  70                  75                  80

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


Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220
```

```
Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Leu His Gly Asp Pro Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Phe Pro
        355                 360                 365

Ile Thr Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
    450                 455                 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465                 470                 475                 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
                485                 490                 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
            500                 505                 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
        515                 520                 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
    530                 535                 540
```

<210> 8

<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH g

<400> 8

Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
20 25 30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
35 40 45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
50 55 60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
65 70 75 80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
100 105 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
115 120 125

Val Gly Gly Ala Ser Thr Tyr Asp Gly Val Val Leu Ala Ala His Glu
130 135 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145 150 155 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
165 170 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
180 185 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
195 200 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
260 265 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu

275 280 285

Asp Leu His Gly Asp Pro Arg Glu Ser His Pro Phe Leu Pro Thr Val
290 295 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305 310 315 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Phe Pro
355 360 365

Ile Thr Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
530 535 540

<210> 9
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: rPLE-rGEH h

<400> 9
Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15

```
Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
            20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
        35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
    50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
65                  70                  75                  80

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Lys
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Val Gly Gly Ala Ser Thr Tyr Asp Gly Leu Ala Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175
Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
    210                 215                 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225                 230                 235                 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
                245                 250                 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
            260                 265                 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
        275                 280                 285

Asp Leu His Gly Asp Pro Arg Glu Ser His Pro Phe Leu Pro Thr Val
    290                 295                 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305                 310                 315                 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
                325                 330                 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
            340                 345                 350
```

```
Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Phe Pro
        355                 360                 365

Ile Thr Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
    370                 375                 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385                 390                 395                 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
                405                 410                 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
            420                 425                 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
        435                 440                 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
    450                 455                 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465                 470                 475                 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
                485                 490                 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
            500                 505                 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
        515                 520                 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
    530                 535                 540
```

<210> 10
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: rGEH

<400> 10

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
             20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
         35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
     50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Gly Gln Met Thr
 65                  70                  75                  80
```

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

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Arg
100 105 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
115 120 125

Val Gly Gly Ala Ser Thr Tyr Asp Gly Leu Ala Leu Ala Ala His Glu
130 135 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145 150 155 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
165 170 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
180 185 190

Gly Asp Thr Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
195 200 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Ala Gly Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
260 265 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
275 280 285

Asp Leu His Gly Asp Pro Arg Glu Ser His Pro Phe Leu Pro Thr Val
290 295 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305 310 315 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Phe Pro
355 360 365

Ile Thr Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
530 535 540

<210> 11
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 11
gccaggaccc agtagcgggg cagatgacct cagatc 36

<210> 12
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 12
gatctgaggt catctgcccc gctactgggt cctggc 36

<210> 13
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 13
cggaaaggag aggctcattc cggagttttc tgaagac 37

<210> 14
<211> 37

<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 14
gtcttcagaa aactccggaa tgagcctctc ctttccg 37

<210> 15
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 15
ccaactttgg aggcgacaca ggctctgtga cc 32

<210> 16
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 16
ggtcacagag cctgtgtcgc ctccaaagtt gg 32

<210> 17
<211> 34
<212> DNA
<213> Artificial sequence
<220>
<223> Description of the artificial sequence: Primer

<400> 17
ggcgtggccc tcactgctgg cctggtcagg aagg 34

<210> 18
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 18
ccttcctgac caggccagca gtgagggcca cgcc 34

<210> 19
<211> 49
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 19
gaaattttta actcttgatt tgcatggaga ccccagagag agccatccc 49

<210> 20
<211> 49
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 20
gggatggctc tctctggggt ctccatgcaa atcaagagtt aaaaatttc 49

<210> 21
<211> 40
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 21
ctcctgtgga agtccttccc catcactaac atccctgagg 40

<210> 22
<211> 40
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 22
cctcagggat gttagtgatg gggaaggact tccacaggag 40

<210> 23
<211> 45
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 23
ggaggcctgg tggtgggcgg ggcatccacc tatgatgggg tggtg 45

<210> 24
<211> 45
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 24
caccacccca tcataggtgg atgccccgcc caccaccagg cctcc 45

<210> 25
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 25
catccaccta tgatgggctg gcccttgctg cgcatg 36

<210> 26
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 26
catgcgcagc aagggccagc ccatcatagg tggatg 36

<210> 27
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer
<400> 27
gctgacctga caaggagggg cagactg 27

<210> 28
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Primer

<400> 28
cagtctgccc ctccttgtca ggtcagc 27

<210> 29
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: rPLE sequence

<400> 29
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60

gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagtgga gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcactctgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggatgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acccaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgttgc cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg cctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttt catggagacc aaagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 30
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH a

<400> 30
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcactctgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acccaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgttgc cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg cctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttt catggagacc aaagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380

ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 31
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH b

<400> 31
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acccaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgttgc cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg cctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttt catggagacc aaagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 32
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH c

<400> 32
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300

tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgttgc cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg cctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttt catggagacc aaagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
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ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
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ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
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tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 33
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH d
<400> 33
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
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ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttt catggagacc aaagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 34
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH e

<400> 34
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gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttg catggagacc ccagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtccta ccccatcgct aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 35
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH f

<400> 35
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gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtgttgggc ggggcaccaa tgtatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600

tttggagagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttg catggagacc ccagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtcctt ccccatcact aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 36
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH g

<400> 36
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtggtgggc ggggcatcca cctatgatgg ggtggtgctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggggagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttg catggagacc ccagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtcctt ccccatcact aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 37
<211> 1632

<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Sequence rPLE-rGEH h
<400> 37
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gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagagaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtggtgggc ggggcatcca cctatgatgg gctggccctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggggagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttg catggagacc ccagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960
aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtcctt ccccatcact aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
gcaggagccc ccacctacat gtatgagttt cagtatcgcc caagcttctc atcggacaag 1320
aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag 1632

<210> 38
<211> 1632
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: rGEH

<400> 38
gggcagccag cctcgccgcc tgttgtggac actgcccagg gccgagtcct ggggaagtac 60
gtcagcttag aaggcctggc acagccggtg gccgtcttcc tgggagtccc ttttgccaag 120
ccccctctcg gatccttgag gtttgctccg ccgcagcctg cagaaccatg gagcttcgtg 180
aagaacacca cctcctaccc tcccatgtgc tgccaggacc cagtagcggg gcagatgacc 240
tcagatctat ttaccaacgg aaaggagagg ctcattccgg agttttctga agactgtctc 300
tacctaaata tttacacccc tgctgacctg acagggaggg gcagactgcc ggtgatggtg 360
tggatccacg gaggaggcct ggtggtgggc ggggcatcca cctatgatgg gctggccctt 420
gctgcgcatg aaaacgtggt ggtggtggcc atccagtacc gcctgggcat ctggggattc 480
ttcagcacag gggacgaaca cagccggggc aactggggtc acttggacca ggtggccgca 540
ctgcactggg tccaggagaa catcgccaac tttggaggcg acacaggctc tgtgaccatc 600
tttggggagt cagcaggagg ggaaagtgtc tctgttctgg tgttgtctcc cttggccaag 660
aacctcttcc accgggccat ctctgagagt ggcgtggccc tcactgctgg cctggtcagg 720
aaggacatga aggctgcagc taagcaaatt gctgtccttg ctgggtgtaa aaccaccacc 780
tcggctgtct ttgttcactg tctgcgccag aagtcggagg acgagctctt ggacttaacg 840
ctgaagatga aatttttaac tcttgatttg catggagacc ccagagagag ccatcccttc 900
ctgcccactg tggtggatgg agtgctgctg cccaagatgc ctgaagagat tctggctgag 960

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aaggatttca acactgtccc ctacatcgtg ggaatcaaca agcaagagtt tggctggctt 1020
ctgccaacga tgatgggctt ccccctctct gaaggcaagc tggaccagaa gacggccacg 1080
tcactcctgt ggaagtcctt ccccatcact aacatccctg aggaactgac tccagtggcc 1140
actgacaagt atttgggggg gacagacgac cccgtcaaaa agaaagacct gttcctggac 1200
ttgatggggg atgtggtgtt tggtgtccca tctgtgacgg tggcccgtca acacagagat 1260
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aaacccaaga cggtgatcgg ggaccacggg gatgagatct tctccgtctt tggttttcca 1380
ctgttaaaag gcgatgcccc agaagaggag gtcagtctca gcaagacggt gatgaaattc 1440
tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg 1500
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg 1560
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca 1620
cccaagataa ag                                                     1632
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<210> 39
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH a1

<400> 39

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Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
  1               5                  10                  15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
             20                  25                  30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
         35                  40                  45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
     50                  55                  60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Ala Glu Gln Met Thr
 65                  70                  75                  80

Ser Asp Leu Phe Thr Asn Gly Lys Glu Arg Leu Thr Leu Glu Phe Ser
                 85                  90                  95

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Arg
            100                 105                 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
        115                 120                 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
    130                 135                 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145                 150                 155                 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
                165                 170                 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
            180                 185                 190

Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
        195                 200                 205
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Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
260 265 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
275 280 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
290 295 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305 310 315 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
355 360 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
515 520 525

Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
530 535 540

<210> 40
<211> 544
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence: Artificial esterase rPLE-rGEH a2

<400> 40
Gly Gln Pro Ala Ser Pro Pro Val Val Asp Thr Ala Gln Gly Arg Val
1 5 10 15

Leu Gly Lys Tyr Val Ser Leu Glu Gly Leu Ala Gln Pro Val Ala Val
20 25 30

Phe Leu Gly Val Pro Phe Ala Lys Pro Pro Leu Gly Ser Leu Arg Phe
35 40 45

Ala Pro Pro Gln Pro Ala Glu Pro Trp Ser Phe Val Lys Asn Thr Thr
50 55 60

Ser Tyr Pro Pro Met Cys Cys Gln Asp Pro Val Val Gly Gln Met Thr
65 70 75 80

Ser Asp Leu Phe Thr Asn Gly Lys Glu Arg Leu Thr Leu Glu Phe Ser
85 90 95

Glu Asp Cys Leu Tyr Leu Asn Ile Tyr Thr Pro Ala Asp Leu Thr Arg
100 105 110

Arg Gly Arg Leu Pro Val Met Val Trp Ile His Gly Gly Gly Leu Val
115 120 125

Leu Gly Gly Ala Pro Met Tyr Asp Gly Val Val Leu Ala Ala His Glu
130 135 140

Asn Val Val Val Val Ala Ile Gln Tyr Arg Leu Gly Ile Trp Gly Phe
145 150 155 160

Phe Ser Thr Gly Asp Glu His Ser Arg Gly Asn Trp Gly His Leu Asp
165 170 175

Gln Val Ala Ala Leu His Trp Val Gln Glu Asn Ile Ala Asn Phe Gly
180 185 190

Gly Asp Pro Gly Ser Val Thr Ile Phe Gly Glu Ser Ala Gly Gly Glu
195 200 205

Ser Val Ser Val Leu Val Leu Ser Pro Leu Ala Lys Asn Leu Phe His
210 215 220

Arg Ala Ile Ser Glu Ser Gly Val Ala Leu Thr Val Ala Leu Val Arg
225 230 235 240

Lys Asp Met Lys Ala Ala Ala Lys Gln Ile Ala Val Leu Ala Gly Cys
245 250 255

Lys Thr Thr Thr Ser Ala Val Phe Val His Cys Leu Arg Gln Lys Ser
260 265 270

Glu Asp Glu Leu Leu Asp Leu Thr Leu Lys Met Lys Phe Leu Thr Leu
275 280 285

Asp Phe His Gly Asp Gln Arg Glu Ser His Pro Phe Leu Pro Thr Val
290 295 300

Val Asp Gly Val Leu Leu Pro Lys Met Pro Glu Glu Ile Leu Ala Glu
305 310 315 320

Lys Asp Phe Asn Thr Val Pro Tyr Ile Val Gly Ile Asn Lys Gln Glu
325 330 335

Phe Gly Trp Leu Leu Pro Thr Met Met Gly Phe Pro Leu Ser Glu Gly
340 345 350

Lys Leu Asp Gln Lys Thr Ala Thr Ser Leu Leu Trp Lys Ser Tyr Pro
355 360 365

Ile Ala Asn Ile Pro Glu Glu Leu Thr Pro Val Ala Thr Asp Lys Tyr
370 375 380

Leu Gly Gly Thr Asp Asp Pro Val Lys Lys Lys Asp Leu Phe Leu Asp
385 390 395 400

Leu Met Gly Asp Val Val Phe Gly Val Pro Ser Val Thr Val Ala Arg
405 410 415

Gln His Arg Asp Ala Gly Ala Pro Thr Tyr Met Tyr Glu Phe Gln Tyr
420 425 430

Arg Pro Ser Phe Ser Ser Asp Lys Lys Pro Lys Thr Val Ile Gly Asp
435 440 445

His Gly Asp Glu Ile Phe Ser Val Phe Gly Phe Pro Leu Leu Lys Gly
450 455 460

Asp Ala Pro Glu Glu Glu Val Ser Leu Ser Lys Thr Val Met Lys Phe
465 470 475 480

Trp Ala Asn Phe Ala Arg Ser Gly Asn Pro Asn Gly Glu Gly Leu Pro
485 490 495

His Trp Pro Met Tyr Asp Gln Glu Glu Gly Tyr Leu Gln Ile Gly Val
500 505 510

Asn Thr Gln Ala Ala Lys Arg Leu Lys Gly Glu Glu Val Ala Phe Trp
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Asn Asp Leu Leu Ser Lys Glu Ala Ala Lys Lys Pro Pro Lys Ile Lys
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sequence domain

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catcatcatt ga 72