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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present invention relates to lipase variants.

BACKGROUND OF THE INVENTION

[0002] Lipases are useful, e.g., as detergent enzymes to remove lipid or fatty stains from clothes and other textiles, as additives to dough for bread and other baked products. Thus, a lipase derived from *Thermomyces lanuginosus* (synonym *Humicola lanuginosa,* EP 258 068 and EP 305 216) is sold for detergent use under the trade name Lipolase® (product of Novo Nordisk A/S). WO 0060063 (US6939702B1) describes variants of the *T. lanuginosus* lipase with a particularly good first-wash performance in a detergent solution. WO 9704079, WO 9707202 and WO 0032758 also disclose variants of the *T. lanuginosus* lipase.

[0003] In some applications, it is of interest to minimize the formation of odor-generating short-chain fatty acids. Thus, it is known that laundry detergents with lipases may sometimes leave residual odors attached to cloth soiled with milk (EP 430315). WO 02062973 discloses lipase variants where the odor generation has been reduced by attaching a C-terminal extension.

SUMMARY OF THE INVENTION

[0004] The inventors have found that by introducing mutations in certain regions/positions of a lipase it is possible to improve the properties or characteristics of the lipase.

[0005] In one aspect the present invention relates to a variant of a parent lipase, wherein the variant has lipase activity, is at least 80% identical to SEQ ID NO: 2, and comprises the substitutions Q4V+S58A+V60S+S83T+I86V+A150G+E210K+L227G+T231R+N233R+P256K (using SEQ ID NO: 2 for numbering).

[0006] In a further aspect the invention relates to a DNA sequence encoding the lipase variant of the invention, an expression vector harbouring said DNA sequence and a transformed host cell containing the DNA sequence or the expression vector.

[0007] In another aspect, the invention provides a method of producing the lipase variant of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

[0008] Figure 1 shows the alignment of lipases.

SEQUENCE LISTINGS

[0009]

SEQ ID NO: 1 shows the DNA sequence encoding lipase from *Thermomyces lanoginosus.*

SEQ ID NO: 2 shows the amino acid sequence of a lipase from *Thermomyces lanoginosus.*

SEQ ID NO: 3 shows the amino acid sequence of a lipase from *Absidia reflexa.*

SEQ ID NO: 4 shows the amino acid sequence of a lipase from *Absidia corymbifera.*

SEQ ID NO: 5 shows the amino acid sequence of a lipase from *Rhizomucor miehei.*

SEQ ID NO: 6 shows the amino acid sequence of a lipase from *Rhizopus oryzae.*

SEQ ID NO: 7 shows the amino acid sequence of a lipase from *Aspergillus niger.*

SEQ ID NO: 8 shows the amino acid sequence of a lipase from *Aspergillus tubingensis.*

SEQ ID NO: 9 shows the amino acid sequence of a lipase from *Fusarium oxysporrum.*

SEQ ID NO: 10 shows the amino acid sequence of a lipase from *Fusarium heterosporum.*

SEQ ID NO: 11 shows the amino acid sequence of a lipase from *Aspergillus oryzae.*

SEQ ID NO: 12 shows the amino acid sequence of a lipase from *Penicillium camemberti.*

SEQ ID NO: 13 shows the amino acid sequence of a lipase from *Aspergillus foetidus.*

SEQ ID NO: 14 shows the amino acid sequence of a lipase from *Aspergillus niger.*

SEQ ID NO: 15 shows the amino acid sequence of a lipase from *Aspergillus oryzae.*

SEQ ID NO: 16 shows the amino acid sequence of a lipase from *Landerina penisapora.*

DETAILED DESCRIPTION OF THE INVENTION

Parent lipases

[0010] Any suitable parent lipase may be used. In a preferred embodiment, the parent lipase may be a fungal lipase. In another preferred embodiment, the parent lipase may be a lipase with an amino acid sequence having at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% homology as defined in the section "Homology and alignment" to the sequence of the *T. lanuginosus* lipase shown in SEQ ID NO: 2.

[0011] The parent lipase may be a yeast polypeptide such as a *Candida, Kluyveromyces, Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filobasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium, Piromyces*, *Schizophyllum, Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* polypeptide.

[0012] In a preferred aspect, the parent lipase is a Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis, or Saccharomyces oviformis polypeptide having lipase activity.

[0013] In another preferred aspect, the parent lipase is an Aspergillus aculeatus, Aspergillus awamori, Aspergillus fumigatus, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Aspergillus turbigensis, Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides, Fusarium venenatum, Humicola insolens, Thermomyces lanoginosus (synonym: Humicola lanuginose), Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium purpurogenum, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or Trichoderma viride polypeptide.

[0014] In another preferred aspect, the parent lipase is a Thermomyces lipase.

[0015] In a more preferred aspect, the parent lipase is a Thermomyces lanuginosus lipase. In an even more preferred embodiment the parent lipase is the lipase of SEQ ID NO: 2.

Variant lipases

[0016] The lipase variants of the present disclosure comprise, as compared to the parent lipase, at least three substitutions selected from the group consisting of:

1. a) at least two substitution in Region I, and
2. b) at least one substitution in Region II, and

3. c) at least one substitution in Region III, and
4. d) at least one substitution in Region IV;'

and wherein the variant has lipase activity.

[0017] Accordingly, the invention provides a variant of a parent lipase, wherein the variant has lipase activity, is at least 80% identical to SEQ ID NO:2, and comprises the substitutions Q4V+S58A+V60S+S83T+I86V+A150G+E210K+L227G+T231R+N233R+P256K (using SEQ ID NO: 2 for numbering).

[0018] In a preferred embodiment the variant lipase is a variant of a Thermomyces lipase, more preferably, a *T. lanuginosus* lipase, and even more preferably, the *T. lanuginosus* lipase shown in SEQ ID NO: 2. In a preferred embodiment. the variant lipase has at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2.

[0019] The variant lipase may be a variant of a parent lipase encoded by a gene derived/obtained from one of the following parent organisms: Candida, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces, or Yarrowia, Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Filobasidium, Fusarium, Humicola, Magnaporthe, Mucor, Myceliophthora, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Schizophyllum, Talaromyces, Thermoascus, Thielavia, Tolypocladium, or Trichoderma. In a preferred embodiment, the variant lipase further has at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to a parent lipase encoded by a gene derived/obtained from one of the following parent organisms: Candida, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces, or Yarrowia, Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Filobasidium, Fusarium, Humicola, Magnaporthe, Mucor, Myceliophthora, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Schizophyllum, Talaromyces, Thermoascus, Thielavia, Tolypocladium, or Trichoderma.

[0020] In a preferred aspect, the variant lipase is a variant Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis, or Saccharomyces oviformis. In a preferred embodiment, the variant lipase further has at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to a parent lipase encoded by a gene derived/obtained from Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis, or Saccharomyces oviformis.

[0021] The variant lipase may be a variant of a parent lipase encoded by a gene derived/obtained from one of the following parent organisms: Aspergillus aculeatus, Aspergillus awamori, Aspergillus fumigatus, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Aspergillus turbigensis, Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum,

Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides, Fusarium venenatum, Humicola insolens, Thermomyces lanoginosus (synonym: Humicola lanuginose), Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium purpurogenum, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or Trichoderma viride. In a preferred embodiment, the variant lipase has further at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to a parent lipase encoded by a gene derived/obtained from one of the following parent organisms: Aspergillus aculeatus, Aspergillus awamori, Aspergillus fumigatus, Aspergillus foetidus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Aspergillus turbigensis, Fusarium bactridioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichioides, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides, Fusarium venenatum, Humicola insolens, Thermomyces lanoginosus (synonym: Humicola lanuginose), Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium purpurogenum, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or Trichoderma viride.

[0022] In another preferred embodiment, the variant is a variant of a Thermomyces lipase.

[0023] In a more preferred embodiment, the parent lipase is a Thermomyces lanuginosus lipase. In an even more preferred embodiment the parent lipase is the lipase of SEQ ID NO: 2.

Identification of regions and substitutions

[0024] The positions referred to in Region I through Region IV below are the positions of the amino acid residues in SEQ ID NO: 2. To find the corresponding (or homologous) positions in a different lipase, the procedure described in "Homology and alignment" is used.

Substitutions in Region I

[0025] Region I consists of amino acid residues surrounding the N-terminal residue E1. In this region, it is preferred to substitute an amino acid of the parent lipase with a more positive amino acid. The lipase variant may comprise at least two substitutions in Region I, such as three, four, five or six substitutions in Region I, and include Q4V, L227G, T231R and N233R

[0026] Amino acid residues corresponding to the following positions are comprised by Region I: 1, 2 to 11 and 223-239. The following positions are of particular interest: 1, 4, 8, 11, 223, 227, 229, 231, 233, 234, 236.

[0027] In particular the following additional substitutions have been identified: X1N/*.

[0028] In a preferred embodiment the variant lipase has at least 85%, 90%, 95%, 96%, 97%, 98%, 99% identity to SEQ ID NO:2

[0029] In a most preferred embodiment the variant lipase is a variant of the lipase having the amino acid sequence of SEQ ID NO: 2.

Substitutions in Region II

[0030] Region II consists of amino acid residues in contact with substrate on one side of the acyl chain and one side of the alcohol part. In this region it is preferred to substitute an amino acid of the parent lipase with a more positive amino acid or with a less hydrophobic amino acid.

[0031] The lipase variant may comprise at least one substitution in Region II, such as two, three, four, five or six substitutions in Region II, and include E210K and P256K

[0032] Amino acid residues corresponding to the following positions are comprised by Region II: 202 to 211 and 249 to 269. The following positions are of particular interest : 202, 210, 211, 253, 254, 255, 256.

[0033] In particular the following substitutions have been identified: X202G, X255Y/V/A and and X259G/M/Q/V.

[0034] In a preferred embodiment the variant lipase has at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2

[0035] In a most preferred embodiment the variant lipase is a variant of the lipase having the amino acid sequence of SEQ ID NO: 2.

Substitutions in Region III

[0036] Region III consists of amino acid residues that form a flexible structure and thus allowing the substrate to get into the active site. In this region it is preferred to substitute an amino acid of the parent lipase with a more positive amino acid or a less hydrophobic amino acid.

[0037] The lipase variant may comprise at least one substitution in Region III, such as two, three, four, five or six substitutions in Region III, and include S83Tand 186V.

[0038] Amino acid residues corresponding to the following positions are comprised by Region

III: 82 to 102. The following positions are of particular interest: 83, 86, 87, 90, 91, 95, 96, 99.

[0039] In particular the following additional substitutions have been identified: X90A/R.

[0040] In a preferred embodiment the variant lipase has at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2

[0041] In a most preferred embodiment the variant lipase is a variant of the lipase having the amino acid sequence of SEQ ID NO: 2.

Substitutions in Region IV

[0042] Region IV consists of amino acid residues that bind electrostatically to a surface. In this region it is preferred to substitute an amino acid of the parent lipase with a more positive amino acid.

[0043] The lipase variant may comprise at least one substitution in Region IV, such as two, three, four, five or six substitutions in Region IV, and include S58A and V60S.

[0044] Amino acid residues corresponding to the following positions are comprised by Region IV: 27 and 54 to 62. The following positions are of particular interest: 27, 56, 57, 58, 60.

[0045] In particular the following substitutions have been identified: X27R.

[0046] In a preferred embodiment the variant lipase has at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 2

[0047] In a most preferred embodiment the variant lipase is a variant of the lipase having the amino acid sequence of SEQ ID NO: 2.

Amino acids at other positions

[0048] The parent lipase may optionally comprise additional alterations, e.g, substitution of other amino acids, particularly less than 10, less than 9, less than 8, less than 7, less than 6, less than 5 alterations as compared to a parent lipase. Examples are substitutions corresponding to one or more of the positions 24, 37, 38, 46, 74, 81, 83, 115, 127, 131, 137, 143, 147, 150, 199, 200, 203, 206, 211, 263, 264, 265, 267 and 269 of the parent lipase. In a particular embodiment there is a substitution in at least one of the positions corresponding to position 81, 147, 150, 227 and 249. In a preferred embodiment the at least one substitution is selected from the group consisting of X38R, X81Q/E, X143S/C/N/D/A, X147M/Y, X150G/K, X227G and X249R/I/L.

[0049] The variant may comprise substitutions outside the defined Region I to IV; the number of substitutions outside the defined Region I to IV is preferably less than six, such as five, four, three, two or one substitution.

[0050] Further substitutions may, e.g., be made according to principles known in the art, e.g. substitutions described in WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and WO 97/07202.

Lipase variants

[0051] Variant lipases include parent lipases identical to SEQ ID NO:2 having the substitutions listed in the below table (using SEQ ID NO:2 for numbering).
(Q4V, L227G, T231 Rand N233R) (E210K and P256K) (S83Tand 186V) (S58A and V60S).

Region I	Region II	Region III	Region IV	Outside regions
Q4V + L227G + T231 R + N233R	E210K + P256K	S83T + I86V	S58A + V60S	A150G

Nomenclature for amino acid modifications

[0052] In describing lipase variants according to the invention, the following nomenclature is used for ease of reference:

Original amino acid(s):position(s):substituted amino acid(s)

[0053] According to this nomenclature, for instance the substitution of glutamic acid for glycine in position 195 is shown as G195E. A deletion of glycine in the same position is shown as G195*, and insertion of an additional amino acid residue such as lysine is shown as G195GK.

[0054] Where a specific lipase contains a "deletion" in comparison with other lipases and an insertion is made in such a position this is indicated as *36D for insertion of an aspartic acid in position 36.

[0055] Multiple mutations are separated by pluses, i.e.: R170Y+G195E, representing mutations in positions 170 and 195 substituting tyrosine and glutamic acid for arginine and glycine, respectively.

[0056] X231 indicates the amino acid in a parent polypeptide corresponding to position 231,

when applying the described alignment procedure. X231R indicates that the amino acid is replaced with R. For SEQ ID NO: 2 X is T, and X231R thus indicates a substitution of T in position 231 with R. Where the amino acid in a position (e.g. 231) may be substituted by another amino acid selected from a group of amino acids, e.g. the group consisting of R and P and Y, this will be indicated by X231R/P/Y.

[0057] In all cases, the accepted IUPAC single letter or triple letter amino acid abbreviation is employed.

Amino acid grouping

[0058] In this specification, amino acids are classified as negatively charged, positively charged or electrically neutral according to their electric charge at pH 10. Thus, negative amino acids are E, D, C (cysteine) and Y, particularly E and D. Positive amino acids are R, K and H, particularly R and K. Neutral amino acids are G, A, V, L, I, P, F, W, S, T, M, N, Q and C when forming part of a disulfide bridge. A substitution with another amino acid in the same group (negative, positive or neutral) is termed a conservative substitution.

[0059] The neutral amino acids may be divided into hydrophobic or non-polar (G, A, V, L, I, P, F, W and C as part of a disulfide bridge) and hydrophilic or polar (S, T, M, N, Q).

Amino acid identity

[0060] The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity".

[0061] For purposes of the present invention, the alignment of two amino acid sequences is determined by using the Needle program from the EMBOSS package (http://emboss.org) version 2.8.0. The Needle program implements the global alignment algorithm described in Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453. The substitution matrix used is BLOSUM62, gap opening penalty is 10, and gap extension penalty is 0.5.

[0062] The degree of identity between an amino acid sequence of the present invention ("invention sequence"; e.g. amino acids 1 to 269 of SEQ ID NO: 2) and a different amino acid sequence ("foreign sequence") is calculated as the number of exact matches in an alignment of the two sequences, divided by the length of the "invention sequence" or the length of the "foreign sequence", whichever is the shortest. The result is expressed in percent identity.

[0063] An exact match occurs when the "invention sequence" and the "foreign sequence" have identical amino acid residues in the same positions of the overlap. The length of a sequence is the number of amino acid residues in the sequence (e.g. the length of SEQ ID NO:

2 is 269).

[0064] The above procedure may be used for calculation of identity as well as homology and for alignment. In the context of the present invention homology and alignment has been calculated as described below.

Homology and alignment

[0065] For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

[0066] In the present invention, corresponding (or homologous) positions in the lipase sequences of *Absidia reflexa, Absidia corymbefera, Rhizmucor miehei, Rhizopus delemar, Aspergillus niger, Aspergillus tubigensis, Fusarium oxysporum, Fusarium heterosporum, Aspergillus oryzea, Penicilium camembertii, Aspergillus foetidus, Aspergillus niger, Thermomyces lanoginosus (synonym: Humicola lanuginose) and Landerina penisapora* are defined by the alignment shown in Figure 1.

[0067] To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Figure 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Hybridization

[0068] The present invention also relates to isolated polypeptides having lipase activity which are encoded by polynucleotides which hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with (i) nucleotides 178 to 660 of SEQ ID NO: 1, (ii) the cDNA sequence contained in nucleotides 178 to 660 of SEQ ID NO: 1,

(iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York). A subsequence of SEQ ID NO: 1 contains at least 100 contiguous nucleotides or preferably at least 200 contiguous nucleotides. Moreover, the subsequence may encode a polypeptide fragment which has lipase activity.

[0069] For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 ug/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

[0070] For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at least at 45 °C (very low stringency), more preferably at least at 50 °C (low stringency), more preferably at least at 55 °C (medium stringency), more preferably at least at 60 °C (medium-high stringency), even more preferably at least at 65 °C (high stringency), and most preferably at least at 70 °C (very high stringency).

DNA sequence, Expression vector, Host cell, Production of lipase

[0071] The invention provides a DNA sequence encoding the lipase of the invention, an expression vector harboring the DNA sequence, and a transformed host cell containing the DNA sequence or the expression vector. These may be obtained by methods known in the art.

[0072] The invention also provides a method of producing the lipase by culturing the transformed host cell under conditions conducive for the production of the lipase and recovering the lipase from the resulting broth. The method may be practiced according to principles known in the art.

Lipase activity

Lipase activity on tributyrin at neutral pH (LU)

[0073] A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 or 9 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU) equals the amount of enzyme capable of releasing 1 micro mol butyric acid/min at pH 7.

Benefit Risk

[0074] The Benefit Risk factor describing the performance compared to the reduced risk for odor smell is defined as: BR = RP_{avg} / R, as described below.

Uses

[0075] Enzymes of the present invention may find industrial use, e.g. be included in detergent compositions for removing of fatty matter.

[0076] Items disclosed in the application.

Item 1. A variant of a parent lipase, wherein the variant comprises at least three substitutions selected from the group consisting of (using SEQ ID NO: 2 for numbering):

1. a) at least two substitutions in Region I, and
2. b) at least one substitution in Region II, and
3. c) at least one substitution in Region III, and
4. d) at least one substitution in Region IV;

and wherein the variant has lipase activity.

Item 2. A lipase variant according to item 1, wherein the at least two substitutions in Region I of the parent lipase comprises substitutions of the amino acids at the positions corresponding to the positions 231 and 233 (using SEQ ID NO:2 for numbering).

Item 3. A lipase variant according to item 2, wherein the at least two substitutions in Region I of the parent lipase comprises substitutions of an R of the amino acids at the positions corresponding to the positions 231 and 233 (using SEQ ID NO: 2 for numbering).

Item 4. A lipase variant according to item 1, wherein the variant lipase is at least 80%. 85%, 90%, 95%, 96%, 97%, 98%, 99% identical to SEQ ID NO: 2.

Item 5. A lipase variant according to item 1, wherein the parent lipase is the lipase having the amino acid sequence of SEQ ID NO: 2.

Item 6. A lipase variant according to item 2, wherein the lipase comprises a further substitution in the position corresponding to position 4 and/or position 227 of SEQ ID NO: 2.

Item 7. A lipase variant according to item 6, wherein the lipase has a substitution corresponding to X4V and X227G (using SEQ ID NO: 2 for numbering).

Item 8. A lipase variant according to item 1, wherein the at least one substitution in Region II of the parent lipase comprises substitutions selected from the group consisting of substitutions in

positions corresponding to the positions 202, 210, 211, 255 and 256 (using SEQ ID NO: 2 for numbering).

Item 9. A lipase variant according to item 8, wherein the at least one substitution in the parent lipase is selected from the group consisting of X202G, X210K, X211 L, X255Y/V and X256K (using SEQ ID NO: 2 for numbering).

Item 10. A lipase variant according to item 1, wherein the at least one substitution in Region III of the parent lipase comprises substitutions selected from the group consisting of substitutions in positions corresponding to the positions 83, 86 and 90 (using SEQ ID NO: 2 for numbering).

Item 11. A lipase variant according to item 10, wherein the at least one substitution in the parent lipase is selected from the group consisting of X83T, X86V and X90A/R (using SEQ ID NO: 2 for numbering).

Item 12. A lipase variant according to item 1, wherein the at least one substitution in Region IV of the parent lipase comprises substitutions selected from the group consisting of substitutions in positions corresponding to the positions 27, 58 and 60 (using SEQ ID NO: 2 for numbering).

Item 13. A lipase variant according to item 1, wherein the at least one substitution in Region IV of the parent lipase comprises substitutions selected from the group consisting of X27R, X58N/A/G/P/T and X60S/V/G/N/R/K/A/L (using SEQ ID NO: 2 for numbering).

Item 14. A lipase variant according to item 1, wherein the lipase variant further comprises at least one substitution in the parent lipase is selected from the group consisting of substitutions in positions corresponding to position 81, 147, 150, 227 and 249 (using SEQ ID NO: 2 for numbering).

Item 15. A lipase variant according to item 1, wherein the lipase variant further comprises at least one substitution selected from the group consisting of X81Q/E, X147M/Y, X150G and X249R/I/L (using SEQ ID NO:2 for numbering).

Item 16. A lipase variant of item 1, wherein the lipase comprises a substitution selected from the following group of substitutions:

1. a) T231 R + N233R + I255Y
2. b) I202G + T231R + N233R
3. c) I86V + L227G + T231 R + N233R + P256K
4. d) Q4V + S58N + V60S + T231 R + N233R
5. e) S58N + V60S + I90R + T231 R + N233R
6. f) I90A + T231 R + N233R + I255V
7. g) S58N + V60S + I86V + A150G + L227G + T231R + N233R + P256K
8. h) S58N + V60S + L147M + F211L + T231R + N233R
9. i) Q4V + S58A + V60S + S83T + I86V + A150G + E210K + L227G + T231R + N233R + P256K
10. j) S58N + V60S + I86V + A150G + L227G + T231R + N233R + P256K

Item 17. A DNA sequence encoding the lipase variant of items 1-16.

Item 18. An expression vector harbouring the DNA sequence of item 17.

Item 19. A transformed host cell containing the DNA sequence of item 17.

Item 20. A method of producing a lipase variant, which method comprises culturing the transformed host cell of item 19 under conditions conducive for the production of the lipase variant and recovering the lipase variant from the resulting broth.

Item 21. A variant of SEQ ID NO: 2 comprising at least one of the mutations Q4V, S58N/A/G/P/T, I90R or Q249I/L.

EXAMPLES

[0077] Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Media and Solutions

Product	Tradename
LAS :	Surfac PS
Zeolite A	Wessalith P

Other ingredients used are standard laboratory reagents.

Materials

Product Supplier

[0078] EMPA221 EMPA St. Gallen, Lerchfeldstrasse 5, CH-9014 St. Gallen, Switzerland

Example 1

Production of enzyme

[0079] A plasmid containing the gene encoding the lipase is constructed and transformed into a suitable host cell using standard methods of the art.

[0080] Fermentation is carried out as a fed-batch fermentation using a constant medium temperature of 34 °C and a start volume of 1.2 liter. The initial pH of the medium is set to 6.5. Once the pH has increased to 7.0 this value is maintained through addition of 10% H3PO4. The level of dissolved oxygen in the medium is controlled by varying the agitation rate and using a fixed aeration rate of 1.0 liter air per liter medium per minute. The feed addition rate is maintained at a constant level during the entire fed-batch phase.
The batch medium contains maltose syrup as carbon source, urea and yeast extract as nitrogen source and a mixture of trace metals and salts. The feed added continuously during the fed-batch phase contains maltose syrup as carbon source whereas yeast extract and urea is added in order to assure a sufficient supply of nitrogen.

[0081] Purification of the lipase may be done by use of standard methods known in the art, e.g. by filtering the fermentation supernatant and subsequent hydrophobic chromatography and ion exchange chromatography, e.g. as described in EP 0 851 913 EP, Example 3.

Example 2

AMSA - Automated Mechanical Stress Assay - for calculation of Relative Performance (RP)

[0082] The enzyme variants of the present application are tested using the Automatic Mechanical Stress Assay (AMSA). With the AMSA test the wash performance of a large quantity of small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid firmly squeezing the textile swatch to be washed against all the slot openings. During the washing time, the plate, test solutions, textile and lid are vigorously shaken to bring the test solution in contact with the textile and apply mechanical stress. For further description see WO 02/42740 especially the paragraph "Special method embodiments" at page 23-24. The containers, which contain the detergent test solution, consist of cylindrical holes (6 mm diameter, 10 mm depth) in a metal plate. The stained fabric (test material) lies on the top of the metal plate and is used as a lid and seal on the containers. Another metal plate lies on the top of the stained fabric to avoid any spillage from each container. The two metal plate together with the stained fabric are vibrated up and down at a frequency of 30 Hz with an amplitude of 2 mm.

[0083] The assay is conducted under the experimental conditions specified below:
Table 3

Test solution	0.5 g/l LAS
	0.52 g/l Na2CO3
	1.07 g/l Zeolite A
	0.52 g/l Na3Citrat

Test solution volume	160 micro l
pH	As is (≈9.9)
Wash time	20 minutes
Temperature	30 °C
Water hardness	15 °dH
	Ratio of Ca^2+/Mg^2+/$NaHCO_3$: 4:1:7.5
Enzyme concentration in test solution	0.125, 0.25, 0.50, 1.0 mg ep / l
Drying	Performance: After washing the textile pieces is immediately flushed in tap water and air-dried at 85C in 5 min
	Odor: After washing the textile pieces is immediately flushed in tap water and dried at room temperature (20 °C) for 2 hours
Test material	Cream turmeric swatch as described below (EM-PA221 used as cotton textile)

[0084] Cream-turmeric swatches were prepared by mixing 5 g of turmeric (Santa Maria, Denmark) with 100 g cream (38% fat, Aria, Denmark) at 50 °C, the mixture was left at this temperature for about 20 minutes and filtered (50 °C) to remove any undissolved particles. The mixture is cooled to 20 °C) woven cotton swatches, EMPA221, were immersed in the cream-turmeric mixture and afterwards allowed to dry at room temperature over night and frozen until use. The preparation of cream-turmeric swatches is disclosed in the patent application WO 2006/125437.

[0085] The performance of the enzyme variant is measured as the brightness of the color of the textile samples washed with that specific enzyme variant. Brightness can also be expressed as the intensity of the light reflected from the textile sample when luminated with white light. When the textile is stained the intensity of the reflected light is lower, than that of a clean textile. Therefore the intensity of the reflected light can be used to measure wash performance of an enzyme variant.

[0086] Color measurements are made with a professional flatbed scanner (PFU DL2400pro), which is used to capture an image of the washed textile samples. The scans are made with a resolution of 200 dpi and with an output color depth of 24 bits. In order to get accurate results, the scanner is frequently calibrated with a Kodak reflective IT8 target.

[0087] To extract a value for the light intensity from the scanned images, a special designed software application is used (Novozymes Color Vector Analyzer). The program retrieves the 24 bit pixel values from the image and converts them into values for red, green and blue (RGB).

The intensity value (Int) is calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

$$Int = \sqrt{r^2 + g^2 + b^2}$$

[0088] The wash performance (P) of the variants is calculated in accordance with the below formula:

P = Int(v) – Int(r)

where

Int(v) is the light intensity value of textile surface washed with enzyme, and

Int(r) is the light intensity value of textile surface washed without enzyme.

A relative performance score is given as the result of the AMSA wash in accordance with the definition:

Relative Performance scores (RP) are summing up the performances (P) of the tested enzyme variants against the reference enzyme:

RP = P(test enzyme) / P(reference enzyme).

RPavg indicates the average relative performance compared to the reference enzyme at all four enzyme concentrations (0.125, 0.25, 0.5, 1.0 mg ep/l)

RPavg = avg(RP(0.125), RP(0.25) RP(0.5), RP(1.0))

[0089] A variant is considered to exhibit improved wash performance, if it performs better than the reference.

In the context of the present invention the reference enzyme is the lipase of SEQ ID NO: 2 with the substitutions T231 R + N233R.

Example 3

GC - Gas Chromatograph - for calculation of risk factor

[0090] The butyric acid release from the lipase washed swatches were measured by Solid Phase Micro Extraction Gas Chromatography (SPME-GC) using the following method. Four textile pieces (5 mm in diameter), washed in the specified solution in Table 3 containing 1 mg/l lipase, were transferred to a Gas Chromatograph (GC) vial. The samples were analysed on a Varian 3800 GC equipped with a Stabilwax- DA w/Integra-Guard column (30m, 0.32 mm ID and 0.25 micro-m df) and a Carboxen PDMS SPME fibre (75 micro-m). Each sample was preincubated for 10 min at 40°C followed by 20 min sampling with the SPME fibre in the head-space over the textile pieces. The sample was subsequently injected onto the column (injector temperature=250°C). Column flow = 2 ml Helium/min. Column oven temperature gradient: 0

min = 40°C, 2 min = 40°C, 22 min = 240°C, 32 min = 240°C. The butyric acid was detected by FID detection and the amount of butyric acid was calculated based on a butyric acid standard curve.

[0091] The Risk Performance Odour, R, of a lipase variant is the ratio between the amount of released butyric acid from the lipase variant washed swatch and the amount of released butyric acid from a swatch washed with the lipase of SEQ ID NO: 2 with the substitutions T231R + N233R (reference enzyme), after both values have been corrected for the amount of released butyric acid from a non-lipase washed swatch. The risk (R) of the variants is calculated in accordance with the below formula:

Odour =measured in micro g butyric acid developed at 1 mg enzyme protein / I corrected for blank

$$Alpha_{test\ enzyme} = Odour_{test\ enzyme} - Blank$$

$$Alpha_{reference\ enzyme} = Odour_{reference\ enzyme} - Blank$$

$$R = Alpha_{test\ enzyme} / Alpha_{reference\ enzyme}$$

A variant is considered to exhibit reduced odor compared to the reference if the R factor is lower than 1.

Example 4

Activity (LU) relative to absorbance at 280nm

[0092] The activity of a lipase relative to the absorbance at 280 nm is determined by the following assay:

LU/A280:

[0093] The activity of the lipase is determined as described above in the section Lipase activity. The absorbance of the lipase at 280 nm is measured (A280) and the ratio LU/A280 is calculated. The relative LU/A280 is calculated as the LU/A280 of the variant divided by the LU/A280 of a reference enzyme. In the context of the present invention the reference enzyme is the lipase of SEQ ID NO:2 with the substitutions T231 R + N233R.

Example 5

BR - Benefit Risk

[0094] The Benefit Risk factor describing the performance compared to the reduced risk for odour smell is thus defined as:

$BR = RP_{avg} / R$

A variant is considered to exhibit improved wash performance and reduced odor, if the BR factor is higher than 1.

[0095] Applying the above methods the following results were obtained:

Table 4

Variant	Mutations in SEQ ID NO: 2	Average RP (RP_{avg})	BR	LU/A280
1	I202G +T231 R +N233R	0.84	1.41	not determined
2	I86V +L227G +T231R +N233R +P256K	1.08	1.52	1700
3	Q4V +S58N +V60S +T231R +N233R	0.87	1.73	1950
4	S58N +V60S +I90R +T231R +N233R	1.06	1.27	2250
5	I255Y +T231R +N233R	1.19	1.17	3600
6	I90A +T231R +N233R +I255V	1.13	1.14	2700
Reference	T231R +N233R	1.00	1.00	3650
7	G91A +E99K +T231R +N233R +Q249R +270H +271T +272P +273S +274S +275G +276R +277G +278G +279H +280R	0.43	not determined	850
8	G91A +E99K +T231 R +N233R + 249R +270H +271T +272P +273S +274S +275G +276R +277G +278G	0.13	not determined	500

The reference lipase and variants 7 and 8 in Table 4 are described in WO 2000/060063.

Example 6

BR - Benefit Risk

[0096] The Benefit Risk was measured for the variants listed in Table 5. The Benefit Risk factor was measured in the same way as described in Example 5 and it was found to be above 1 for all the listed variants.

Table 5

Variant	Mutations in SEQ ID NO: 2
Reference	T231R + N233R
9	L97V+ T231R+N233R
10	A150G+T231R+N233R
11	190R+T231R+N233R
12	I202V+T231R+N233R
13	L227G+ T231R+ N233R+ P256K
14	I90A+ T231 R+ N233R
15	T231R+N233R+ I255P
16	I90V+I255V+T231R+N233R
17	F211L+ L227G+ T231R+ N233R+ I255L+ P256K
18	S58N+ V60S+ T231 R+ N233R+ Q249L
19	S58N+ V60S+ T231 R+ N233R+ Q249I
20	A150G+ L227G+ T231R+ N233R+ P256K
21	K46L+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254I
22	Q4L+ E43T+ K46I+ S58N+ V60S+ T231 R+ N233R+ Q249L+ D254I
23	Q4L+ S58N+ V60S+ T231 R+ N233R+ Q249L+ D254I
24	K46I+ S58N+ V60S+ T231 R+ N233R+ Q249L+ D254L
25	K46L+ S58N+ V60S+ K223I+ T231 R+ N233R+ D254I
26	E43T+ K46I+ S58N+ V60S+ T231 R+ N233R+ Q249L+ D254I
27	S58N+ V60S+ I86V+ A150G+ L227G+ T231 R+ N233R+ P256K
28	K24R+ K46R+ K74R+ I86V+ K98R+ K127R+ D137K+ A150G+ K223R+ T231 R+ N233R
29	S58A+V60A+ I86V+T231R+N233R
30	K24R+ K46R+ S58N+ V60S+ K74R+ I86V+ K98R+ K127R+ D137K+ K223R+ T231 R+ N233R
31	S58A+ V60A+ I86V+ A150G+ T231 R+ N233R
32	S58N+ V60V+ D62G+ T231 R+ N233R
33	Q4V+ S58N+ V60S+ I86V+ T231 R+ N233R+ Q249L
34	Q4V+ S58N+ V60S+ I86V+ A150G+ T231R+ N233R+ I255V
35	Q4V+ S58N+ V60S+ I90A+ A150G+ T231R+ N233R+ I255V
36	Y53A+ S58N+ V60S+ T231 R+ N233R+ P256L
37	I202L+ T231 R+ N233R+ I255A
38	S58A+ V60S+ I86V+ A150G+ L227G+ T231 R+ N233R+ P256K
39	D27R+ S58N+ V60S+ I86V+ A150G+ L227G+ T231 R+ N233R+ P256K

Variant	Mutations in SEQ ID NO: 2
Reference	T231R + N233R
40	V60K+ I86V+ A150G+ L227G+ T231 R+ N233R+ P256K
41	Q4V+ S58A+ V60S+ S83T+ I86V+ A150G+ E210K+ L227G+ T231R+ N233R+ P256K
42	Q4V+ V60K+ S83T+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
43	D27R+ V60K+ I86V+ A150G+ L227G+ T231 R+ N233R+ P256K
44	Q4N+ L6S+ S58N+ V60S+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
45	E1N+ V60K+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
46	V60K+ I86V+ A150G+ K223N+ G225S+ T231 R+ N233R+ P256K
47	E210V T231R+ N233R+ Q249R
48	S58N+ V60S+ E210V+ T231R+ N233R+ Q249R
49	Q4V+ V60K+ I90R+ T231R+ N233R+ I255V
50	Q4V+ V60K+ A150G+ T231R+ N233R
51	V60K+ S83T+ T231R+ N233R
52	V60K+ A150G+ T231R+ N233R+ I255V
53	T231 R+ N233G+ D234G
54	S58N+ V60S+ I86V+ A150G+ E210K+ L227G+ T231R+ N233R+ Q249R+ P256K
55	S58N+ V60S+ I86V+ A150G+ E210K+ L227G+ T231 R+ N233R+ I255A+ P256K
56	S58N+ V60S+ I86V+ A150G+ G156R+ E210K+ L227G+ T231R+ N233R+ I255A+ P256K
57	S58T+ V60K+ I86V+ N94K+ A150G+ E210V L227G+ T231 R+ N233R+ P256K
58	S58T+ V60K+ I86V+ D102A+ A150G+ L227G+ T231R+ N233R+ P256K
59	S58T+ V60K+ I86V+ D102A+ A150G+ E210V+ L227G+ T231R+ N233R+ P256K
60	S58T+ V60K+ S83T+ I86V+ N94K+ A150G+ E210V+ L227G+ T231R+ N233R+ P256K
61	S58A+ V60S+ I86V+ T143S+ A150G+ L227G+ T231R+ N233R+ P256K
62	G91S+ D96V+ D254R
63	V60L+ G91M+ T231W+ Q249L
64	T37A+ D96A+ T231R+ N233R+ Q249G
65	E56G+E87D+T231R+N233R+D254A
66	E210K+T231R+N233R

Variant	Mutations in SEQ ID NO: 2
Reference	T231R + N233R
67	D27H+E87Q+D96N+T231R+N233R+D254V
68	F181L+E210V+T231R+N233R
69	D27N+ D96G+ T231 R+ N233R
70	D96N+ T231 R+ N233R
71	T231 R+ N233I+ D234G
72	S58K+ V60L+ E210V Q249R
73	S58H+ V60L+ E210V Q249R
74	Q4V+ F55V+ I86V+ T231 R+ N233R+ I255V
75	Q4V+ S58T+ V60K+ T199L+ N200A+ E210K+ T231 R+ N233R+ I255A+ P256K
76	Q4V+ D27N+ V60K+ T231R+ N233R
77	I90F+ I202P+ T231R+ N233R+ I255L
78	S58N+ V60S+ D158N+ T231R+ N233R
79	S58N+ V60S+ S115K+ T231R+ N233R
80	S58N+ V60S+ L147M+ A150G+ F211L+ T231R+ N233R
81	V60K+ A150G+ T231 R+ N233R
82	I90V+L227G+T231R+N233R+ P256K
83	T231R+N233R+ I255S
84	186G+ T231R+ N233R
85	V60K+ I202V+ E210K+ T231 R+ N233R+ I255A+ P256K
86	I90G+ I202L+ T231R+ N233R+ I255S
87	S58G+ V60G+ T231R+ N233R

The reference lipase is described in WO 2000/060063.

SEQUENCE LISTING

[0097]

<110> Novozymes A/S Novozymes North America, Inc. vind, Jesper Knotzel, Jurgen Carsten Franz Borch, Kim Svendsen, Allan Callisen, Thomas Honger Yaver, Debbie Bjornvad, Mads Eskelund Hansen, Peter Kamp Ge, Haiyan Lamsa, Michael

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            20                  25                  30

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Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro Glu Val Glu Lys Ala Asp
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        35                  40                  45

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    50                  55                  60

Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys Leu Ile Val Leu Ser Phe
65                  70                  75                  80

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                85                  90                  95

Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly Cys Arg Gly His Asp Gly
            100                 105                 110

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        35                  40                  45

Ser Asn Leu Gln Ile Thr Lys Thr Phe Ser Thr Leu Ile Thr Asp Thr
    50                  55                  60

Asn Val Leu Val Ala Val Gly Glu Lys Glu Lys Thr Ile Tyr Val Val
65                  70                  75                  80

Phe Arg Gly Thr Ser Ser Ile Arg Asn Ala Ile Ala Asp Ile Val Phe
                85                  90                  95

Val Pro Val Asn Tyr Pro Pro Val Asn Gly Ala Lys Val His Lys Gly
            100                 105                 110

Phe Leu Asp Ser Tyr Asn Glu Val Gln Asp Lys Leu Val Ala Glu Val
        115                 120                 125

Lys Ala Gln Leu Asp Arg His Pro Gly Tyr Lys Ile Val Val Thr Gly
    130                 135                 140

His Ser Leu Gly Gly Ala Thr Ala Val Leu Ser Ala Leu Asp Leu Tyr
145                 150                 155                 160

His His Gly His Ala Asn Ile Glu Ile Tyr Thr Gln Gly Gln Pro Arg
                165                 170                 175

Ile Gly Thr Pro Ala Phe Ala Asn Tyr Val Ile Gly Thr Lys Ile Pro
            180                 185                 190

Tyr Gln Arg Leu Val His Glu Arg Asp Ile Val Pro His Leu Pro Pro
        195                 200                 205

Gly Ala Phe Gly Phe Leu His Ala Gly Glu Glu Phe Trp Ile Met Lys
    210                 215                 220

Asp Ser Ser Leu Arg Val Cys Pro Asn Gly Ile Glu Thr Asp Asn Cys
225                 230                 235                 240
```

```
Ser Asn Ser Ile Val Pro Phe Thr Ser Val Ile Asp His Leu Ser Tyr
                245                 250                 255

Leu Asp Met Asn Thr Gly Leu Cys Leu
            260                 265
```

<210> 4

<211> 264

<212> PRT

<213> Absidia corymbifera

<400> 4

```
Ser Ser Ser Thr Gln Asp Tyr Arg Ile Ala Ser Glu Ala Glu Ile Lys
1               5                  10                  15

Ala His Thr Phe Tyr Thr Ala Leu Ser Ala Asn Ala Tyr Cys Arg Thr
            20                  25                  30

Val Ile Pro Gly Gly Gln Trp Ser Cys Pro His Cys Asp Val Ala Pro
        35                  40                  45

Asn Leu Asn Ile Thr Lys Thr Phe Thr Thr Leu Ile Thr Asp Thr Asn
    50                  55                  60

Val Leu Val Ala Val Gly Glu Asn Glu Lys Thr Ile Tyr Val Val Phe
65                  70                  75                  80

Arg Gly Thr Ser Ser Ile Arg Asn Ala Ile Ala Asp Ile Val Phe Val
                85                  90                  95

Pro Val Asn Tyr Pro Pro Val Asn Gly Ala Lys Val His Lys Gly Phe
            100                 105                 110

Leu Asp Ser Tyr Asn Glu Val Gln Asp Lys Leu Val Ala Glu Val Lys
        115                 120                 125

Ala Gln Leu Asp Arg His Pro Gly Tyr Lys Ile Val Val Thr Gly His
    130                 135                 140

Ser Leu Gly Gly Ala Thr Ala Val Leu Ser Ala Leu Asp Leu Tyr His
145                 150                 155                 160

His Gly His Asp Asn Ile Glu Ile Tyr Thr Gln Gly Gln Pro Arg Ile
                165                 170                 175

Gly Thr Pro Glu Phe Ala Asn Tyr Val Ile Gly Thr Lys Ile Pro Tyr
            180                 185                 190

Gln Arg Leu Val Asn Glu Arg Asp Ile Val Pro His Leu Pro Pro Gly
        195                 200                 205

Ala Phe Gly Phe Leu His Ala Gly Glu Glu Phe Trp Ile Met Lys Asp
    210                 215                 220

Ser Ser Leu Arg Val Cys Pro Asn Gly Ile Glu Thr Asp Asn Cys Ser
225                 230                 235                 240

Asn Ser Ile Val Pro Phe Thr Ser Val Ile Asp His Leu Ser Tyr Leu
                245                 250                 255
```

```
Asp Met Asn Thr Gly Leu Cys Leu
            260
```

<210> 5

<211> 269

<212> PRT

<213> Rhizomucor miehei

<400> 5

```
Ser Ile Asp Gly Gly Ile Arg Ala Ala Thr Ser Gln Glu Ile Asn Glu
1               5                  10                  15

Leu Thr Tyr Tyr Thr Thr Leu Ser Ala Asn Ser Tyr Cys Arg Thr Val
            20                  25                  30

Ile Pro Gly Ala Thr Trp Asp Cys Ile His Cys Asp Ala Thr Glu Asp
        35                  40                  45

Leu Lys Ile Ile Lys Thr Trp Ser Thr Leu Ile Tyr Asp Thr Asn Ala
    50                  55                  60

Met Val Ala Arg Gly Asp Ser Glu Lys Thr Ile Tyr Ile Val Phe Arg
65                  70                  75                  80

Gly Ser Ser Ser Ile Arg Asn Trp Ile Ala Asp Leu Thr Phe Val Pro
                85                  90                  95

Val Ser Tyr Pro Pro Val Ser Gly Thr Lys Val His Lys Gly Phe Leu
            100                 105                 110

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

Gln Phe Lys Gln Tyr Pro Ser Tyr Lys Val Ala Val Thr Gly His Ser
    130                 135                 140

Leu Gly Gly Ala Thr Ala Leu Leu Cys Ala Leu Asp Leu Tyr Gln Arg
145                 150                 155                 160

Glu Glu Gly Leu Ser Ser Ser Asn Leu Phe Leu Tyr Thr Gln Gly Gln
                165                 170                 175

Pro Arg Val Gly Asp Pro Ala Phe Ala Asn Tyr Val Val Ser Thr Gly
            180                 185                 190

Ile Pro Tyr Arg Arg Thr Val Asn Glu Arg Asp Ile Val Pro His Leu
        195                 200                 205

Pro Pro Ala Ala Phe Gly Phe Leu His Ala Gly Glu Glu Tyr Trp Ile
    210                 215                 220

Thr Asp Asn Ser Pro Glu Thr Val Gln Val Cys Thr Ser Asp Leu Glu
225                 230                 235                 240

Thr Ser Asp Cys Ser Asn Ser Ile Val Pro Phe Thr Ser Val Leu Asp
                245                 250                 255

His Leu Ser Tyr Phe Gly Ile Asn Thr Gly Leu Cys Thr
            260                 265
```

<210> 6

<211> 271

<212> PRT

<213> Rhizopus oryzae

<400> 6

```
Ser Ala Ser Asp Gly Gly Lys Val Val Ala Ala Thr Thr Ala Gln Ile
1               5                  10                  15

Gln Glu Phe Thr Lys Tyr Ala Gly Ile Ala Ala Thr Ala Tyr Cys Arg
            20                  25                  30

Ser Val Val Pro Gly Asn Lys Trp Asp Cys Val Gln Cys Gln Lys Trp
        35                  40                  45

Val Pro Asp Gly Lys Ile Ile Thr Thr Phe Thr Ser Leu Leu Ser Asp
    50                  55                  60

Thr Asn Gly Tyr Val Leu Arg Ser Asp Lys Gln Lys Thr Ile Tyr Leu
65                  70                  75                  80

Val Phe Arg Gly Thr Asn Ser Phe Arg Ser Ala Ile Thr Asp Ile Val
                85                  90                  95

Phe Asn Phe Ser Asp Tyr Lys Pro Val Lys Gly Ala Lys Val His Ala
            100                 105                 110

Gly Phe Leu Ser Ser Tyr Glu Gln Val Val Asn Asp Tyr Phe Pro Val
        115                 120                 125

Val Gln Glu Gln Leu Thr Ala His Pro Thr Tyr Lys Val Ile Val Thr
    130                 135                 140

Gly His Ser Leu Gly Gly Ala Gln Ala Leu Leu Ala Gly Met Asp Leu
145                 150                 155                 160

Tyr Gln Arg Glu Pro Arg Leu Ser Pro Lys Asn Leu Ser Ile Phe Thr
                165                 170                 175

Val Gly Gly Pro Arg Val Gly Asn Pro Thr Phe Ala Tyr Tyr Val Glu
            180                 185                 190

Ser Thr Gly Ile Pro Phe Gln Arg Thr Val His Lys Arg Asp Ile Val
        195                 200                 205

Pro His Val Pro Pro Gln Ser Phe Gly Phe Leu His Pro Gly Val Glu
    210                 215                 220

Ser Trp Ile Lys Ser Gly Thr Ser Asn Val Gln Ile Cys Thr Ser Glu
225                 230                 235                 240

Ile Glu Thr Lys Asp Cys Ser Asn Ser Ile Val Pro Phe Thr Ser Ile
                245                 250                 255

Leu Asp His Leu Ser Tyr Phe Asp Ile Asn Glu Gly Ser Cys Leu
            260                 265                 270
```

<210> 7

<211> 267

<212> PRT

<213> Aspergillus niger

<400> 7

```
Thr Ala Gly His Ala Leu Ala Ala Ser Thr Gln Gly Ile Ser Glu Asp
1               5                  10                  15

Leu Tyr Ser Arg Leu Val Glu Met Ala Thr Ile Ser Gln Ala Ala Tyr
            20                  25                  30

Ala Asp Leu Cys Asn Ile Pro Ser Thr Ile Ile Lys Gly Glu Lys Ile
        35                  40                  45

Tyr Asn Ser Gln Thr Asp Ile Asn Gly Trp Ile Leu Arg Asp Asp Ser
    50                  55                  60

Ser Lys Glu Ile Ile Thr Val Phe Arg Gly Thr Gly Ser Asp Thr Asn
65                  70                  75                  80

Leu Gln Leu Asp Thr Asn Tyr Thr Leu Thr Pro Phe Asp Thr Leu Pro
                85                  90                  95

Gln Cys Asn Gly Cys Glu Val His Gly Gly Tyr Tyr Ile Gly Trp Val
            100                 105                 110

Ser Val Gln Asp Gln Val Glu Ser Leu Val Lys Gln Gln Val Ser Gln
        115                 120                 125

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

Leu Ala Ala Leu Thr Ala Ala Gln Leu Ser Ala Thr Tyr Asp Asn Ile
145                 150                 155                 160

Arg Leu Tyr Thr Phe Gly Glu Pro Arg Ser Gly Asn Gln Ala Phe Ala
                165                 170                 175

Ser Tyr Met Asn Asp Ala Phe Gln Ala Ser Ser Pro Asp Thr Thr Gln
            180                 185                 190

Tyr Phe Arg Val Thr His Ala Asn Asp Gly Ile Pro Asn Leu Pro Pro
        195                 200                 205

Val Glu Gln Gly Tyr Ala His Gly Gly Val Glu Tyr Trp Ser Val Asp
    210                 215                 220

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

Cys Cys Glu Ala Gln Gly Gly Gln Gly Val Asn Asn Ala His Thr Thr
                245                 250                 255

Tyr Phe Gly Met Thr Ser Gly Ala Cys Thr Trp
            260                 265
```

<210> 8

<211> 266

<212> PRT

<213> Aspergillus tubingensis

<400> 8

```
Thr Ala Gly His Ala Leu Ala Ala Ser Thr Gln Gly Ile Ser Glu Asp
1               5                  10                  15

Leu Tyr Ser Arg Leu Val Glu Met Ala Thr Ile Ser Gln Ala Ala Tyr
            20                  25                  30

Ala Asp Leu Cys Asn Ile Pro Ser Thr Ile Ile Lys Gly Glu Lys Ile
        35                  40                  45

Tyr Asn Ser Gln Thr Asp Ile Asn Gly Trp Ile Leu Arg Asp Asp Ser
    50                  55                  60

Ser Lys Glu Ile Ile Thr Val Phe Arg Gly Thr Gly Ser Asp Thr Asn
65                  70                  75                  80

Leu Gln Leu Asp Thr Asn Tyr Thr Leu Thr Pro Phe Asp Thr Leu Pro
                85                  90                  95

Gln Cys Asn Ser Cys Glu Val His Gly Gly Tyr Tyr Ile Gly Trp Ile
            100                 105                 110

Ser Val Gln Asp Gln Val Glu Ser Leu Val Gln Gln Gln Val Ser Gln
        115                 120                 125

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

Leu Ala Ala Leu Thr Ala Ala Gln Leu Ser Ala Thr Tyr Asp Asn Ile
145                 150                 155                 160

Arg Leu Tyr Thr Phe Gly Glu Pro Arg Ser Asn Gln Ala Phe Ala Ser
                165                 170                 175

Tyr Met Asn Asp Ala Phe Gln Ala Ser Ser Pro Asp Thr Thr Gln Tyr
            180                 185                 190

Phe Arg Val Thr His Ala Asn Asp Gly Ile Pro Asn Leu Pro Pro Ala
        195                 200                 205

Asp Glu Gly Tyr Ala His Gly Val Val Glu Tyr Trp Ser Val Asp Pro
    210                 215                 220

Tyr Ser Ala Gln Asn Thr Phe Val Cys Thr Gly Asp Glu Val Gln Cys

225                 230                 235                 240

Cys Glu Ala Gln Gly Gly Gln Gly Val Asn Asn Ala His Thr Thr Tyr
                245                 250                 255

Phe Gly Met Thr Ser Gly His Cys Thr Trp
            260                 265
```

<210> 9

<211> 276

<212> PRT

<213> Fusarium oxysporum

<400> 9

```
Ala Val Gly Val Thr Thr Thr Asp Phe Ser Asn Phe Lys Phe Tyr Ile
1               5                  10                  15

Gln His Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala Ala Ala Gly Ser
            20                  25                  30

Lys Ile Thr Cys Ser Asn Asn Gly Cys Pro Thr Val Gln Gly Asn Gly
        35                  40                  45

Ala Thr Ile Val Thr Ser Phe Val Gly Ser Lys Thr Gly Ile Gly Gly
    50                  55                  60

Tyr Val Ala Thr Asp Ser Ala Arg Lys Glu Ile Val Val Ser Phe Arg
65                  70                  75                  80

Gly Ser Ile Asn Ile Arg Asn Trp Leu Thr Asn Leu Asp Phe Gly Gln
                85                  90                  95

Glu Asp Cys Ser Leu Val Ser Gly Cys Gly Val His Ser Gly Phe Gln
            100                 105                 110

Arg Ala Trp Asn Glu Ile Ser Ser Gln Ala Thr Ala Ala Val Ala Ser
        115                 120                 125

Ala Arg Lys Ala Asn Pro Ser Phe Asn Val Ile Ser Thr Gly His Ser
    130                 135                 140

Leu Gly Gly Ala Val Ala Val Leu Ala Ala Ala Asn Leu Arg Val Gly
145                 150                 155                 160

Gly Thr Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn
                165                 170                 175

Ala Gln Leu Ser Ala Phe Val Ser Asn Gln Ala Gly Gly Glu Tyr Arg
            180                 185                 190

Val Thr His Ala Asp Asp Pro Val Pro Arg Leu Pro Pro Leu Ile Phe
        195                 200                 205

Gly Tyr Arg His Thr Thr Pro Glu Phe Trp Leu Ser Gly Gly Gly Gly
    210                 215                 220

Asp Lys Val Asp Tyr Thr Ile Ser Asp Val Lys Val Cys Glu Gly Ala
225                 230                 235                 240

Ala Asn Leu Gly Cys Asn Gly Gly Thr Leu Gly Leu Asp Ile Ala Ala
                245                 250                 255

His Leu His Tyr Phe Gln Ala Thr Asp Ala Cys Asn Ala Gly Gly Phe
            260                 265                 270

Ser Trp Arg Arg
        275
```

<210> 10

<211> 273

<212> PRT

<213> Fusarium heterosporum

<400> 10

```
Thr Val Thr Thr Gln Asp Leu Ser Asn Phe Arg Phe Tyr Leu Gln His
1               5                  10                  15

Ala Asp Ala Ala Tyr Cys Asn Phe Asn Thr Ala Val Gly Lys Pro Val
            20                  25                  30

His Cys Ser Ala Gly Asn Cys Pro Asp Ile Glu Lys Asp Ala Ala Ile
        35                  40                  45

Val Val Gly Ser Val Val Gly Thr Lys Thr Gly Ile Gly Ala Tyr Val
    50                  55                  60

Ala Thr Asp Asn Ala Arg Lys Glu Ile Val Val Ser Val Arg Gly Ser
65                  70                  75                  80

Ile Asn Val Arg Asn Trp Ile Thr Asn Phe Asn Phe Gly Gln Lys Thr
                85                  90                  95

Cys Asp Leu Val Ala Gly Cys Gly Val His Thr Gly Phe Leu Asp Ala
            100                 105                 110

Trp Glu Glu Val Ala Ala Asn Val Lys Ala Ala Val Ser Ala Ala Lys
        115                 120                 125

Thr Ala Asn Pro Thr Phe Lys Phe Val Val Thr Gly His Ser Leu Gly
    130                 135                 140

Gly Ala Val Ala Thr Ile Ala Ala Ala Tyr Leu Arg Lys Asp Gly Phe
145                 150                 155                 160

Pro Phe Asp Leu Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn Asp Phe

                165                 170                 175

Phe Ala Asn Phe Val Thr Gln Gln Thr Gly Ala Glu Tyr Arg Val Thr
            180                 185                 190

His Gly Asp Asp Pro Val Pro Arg Leu Pro Pro Ile Val Phe Gly Tyr
        195                 200                 205

Arg His Thr Ser Pro Glu Tyr Trp Leu Asn Gly Gly Pro Leu Asp Lys
    210                 215                 220

Asp Tyr Thr Val Thr Glu Ile Lys Val Cys Glu Gly Ile Ala Asn Val
225                 230                 235                 240

Met Cys Asn Gly Gly Thr Ile Gly Leu Asp Ile Leu Ala His Ile Thr
                245                 250                 255

Tyr Phe Gln Ser Met Ala Thr Cys Ala Pro Ile Ala Ile Pro Trp Lys
            260                 265                 270

Arg
```

<210> 11

<211> 278

<212> PRT

<213> Aspergillus oryzae

<400> 11

```
Asp Ile Pro Thr Thr Gln Leu Glu Asp Phe Lys Phe Trp Val Gln Tyr
1               5                  10                  15

Ala Ala Ala Thr Tyr Cys Pro Asn Asn Tyr Val Ala Lys Asp Gly Glu
            20                  25                  30

Lys Leu Asn Cys Ser Val Gly Asn Cys Pro Asp Val Glu Ala Ala Gly
        35                  40                  45

Ser Thr Val Lys Leu Ser Phe Ser Asp Asp Thr Ile Thr Asp Thr Ala
    50                  55                  60

Gly Phe Val Ala Val Asp Asn Thr Asn Lys Ala Ile Val Val Ala Phe
65                  70                  75                  80

Arg Gly Ser Tyr Ser Ile Arg Asn Trp Val Thr Asp Ala Thr Phe Pro
                85                  90                  95

Gln Thr Asp Pro Gly Leu Cys Asp Gly Cys Lys Ala Glu Leu Gly Phe
            100                 105                 110

Trp Thr Ala Trp Lys Val Val Arg Asp Arg Ile Ile Lys Thr Leu Asp
        115                 120                 125

Glu Leu Lys Pro Glu His Ser Asp Tyr Lys Ile Val Val Val Gly His
    130                 135                 140

Ser Leu Gly Ala Ala Ile Ala Ser Leu Ala Ala Ala Asp Leu Arg Thr
145                 150                 155                 160

Lys Asn Tyr Asp Ala Ile Leu Tyr Ala Tyr Ala Ala Pro Arg Val Ala
                165                 170                 175

Asn Lys Pro Leu Ala Glu Phe Ile Thr Asn Gln Gly Asn Asn Tyr Arg
            180                 185                 190

Phe Thr His Asn Asp Asp Pro Val Pro Lys Leu Pro Leu Leu Thr Met
        195                 200                 205

Gly Tyr Val His Ile Ser Pro Glu Tyr Tyr Ile Thr Ala Pro Asp Asn
    210                 215                 220

Thr Thr Val Thr Asp Asn Gln Val Thr Val Leu Asp Gly Tyr Val Asn
225                 230                 235                 240

Phe Lys Gly Asn Thr Gly Thr Ser Gly Gly Leu Pro Asp Leu Leu Ala
                245                 250                 255

Phe His Ser His Val Trp Tyr Phe Ile His Ala Asp Ala Cys Lys Gly
            260                 265                 270

Pro Gly Leu Pro Leu Arg
        275
```

<210> 12

<211> 278

<212> PRT

<213> Penicillium camemberti

<400> 12

```
Asp Val Ser Thr Ser Glu Leu Asp Gln Phe Glu Phe Trp Val Gln Tyr
1               5                  10                  15

Ala Ala Ala Ser Tyr Tyr Glu Ala Asp Tyr Thr Ala Gln Val Gly Asp
            20                  25                  30

Lys Leu Ser Cys Ser Lys Gly Asn Cys Pro Glu Val Glu Ala Thr Gly
        35                  40                  45

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

Gly Tyr Ile Ala Val Asp His Thr Asn Ser Ala Val Val Leu Ala Phe
65                  70                  75                  80

Arg Gly Ser Tyr Ser Val Arg Asn Trp Val Ala Asp Ala Thr Phe Val

                85                  90                  95

His Thr Asn Pro Gly Leu Cys Asp Gly Cys Leu Ala Glu Leu Gly Phe
            100                 105                 110

Trp Ser Ser Trp Lys Leu Val Arg Asp Asp Ile Ile Lys Glu Leu Lys
        115                 120                 125

Glu Val Val Ala Gln Asn Pro Asn Tyr Glu Leu Val Val Val Gly His
    130                 135                 140

Ser Leu Gly Ala Ala Val Ala Thr Leu Ala Ala Thr Asp Leu Arg Gly
145                 150                 155                 160

Lys Gly Tyr Pro Ser Ala Lys Leu Tyr Ala Tyr Ala Ser Pro Arg Val
                165                 170                 175

Gly Asn Ala Ala Leu Ala Lys Tyr Ile Thr Ala Gln Gly Asn Asn Phe
            180                 185                 190

Arg Phe Thr His Thr Asn Asp Pro Val Pro Lys Leu Pro Leu Leu Ser
        195                 200                 205

Met Gly Tyr Val His Val Ser Pro Glu Tyr Trp Ile Thr Ser Pro Asn
    210                 215                 220

Asn Ala Thr Val Ser Thr Ser Asp Ile Lys Val Ile Asp Gly Asp Val
225                 230                 235                 240

Ser Phe Asp Gly Asn Thr Gly Thr Gly Leu Pro Leu Leu Thr Asp Phe
                245                 250                 255

Glu Ala His Ile Trp Tyr Phe Val Gln Val Asp Ala Gly Lys Gly Pro
            260                 265                 270

Gly Leu Pro Phe Lys Arg
        275
```

<210> 13

<211> 270

<212> PRT

<213> Aspergillus foetidus

<400> 13

```
Ser Val Ser Thr Ser Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp
1               5                  10                  15

Ser Ala Ala Ala Tyr Cys Ser Asn Asn Ile Asp Ser Lys Asp Ser Asn
            20                  25                  30

Leu Thr Cys Thr Ala Asn Ala Cys Pro Ser Val Glu Glu Ala Ser Thr
        35                  40                  45

Thr Met Leu Leu Glu Phe Asp Leu Thr Asn Asp Phe Gly Gly Thr Ala
    50                  55                  60

Gly Phe Leu Ala Ala Asp Asn Thr Asn Lys Arg Leu Val Val Ala Phe
65                  70                  75                  80

Arg Gly Ser Ser Thr Ile Glu Asn Trp Ile Ala Asn Leu Asp Phe Ile
                85                  90                  95

Leu Glu Asp Asn Asp Asp Leu Cys Thr Gly Cys Lys Val His Thr Gly
            100                 105                 110

Phe Trp Lys Ala Trp Glu Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile
        115                 120                 125

Lys Ser Ala Met Ser Thr Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly
    130                 135                 140

His Ser Leu Gly Gly Ala Leu Ala Thr Leu Gly Ala Thr Val Leu Arg
145                 150                 155                 160

Asn Asp Gly Tyr Ser Val Glu Leu Tyr Thr Tyr Gly Cys Pro Arg Ile
                165                 170                 175

Gly Asn Tyr Ala Leu Ala Glu His Ile Thr Ser Gln Gly Ser Gly Ala
            180                 185                 190

Asn Phe Arg Val Thr His Leu Asn Asp Ile Val Pro Arg Val Pro Pro
        195                 200                 205

Met Asp Phe Gly Phe Ser Gln Pro Ser Pro Glu Tyr Trp Ile Thr Ser
    210                 215                 220

Gly Asn Gly Ala Ser Val Thr Ala Ser Asp Ile Glu Val Ile Glu Gly
225                 230                 235                 240

Ile Asn Ser Thr Ala Gly Asn Ala Gly Glu Ala Thr Val Ser Val Leu
                245                 250                 255

Ala His Leu Trp Tyr Phe Phe Ala Ile Ser Glu Cys Leu Leu
            260                 265                 270
```

<210> 14

<211> 270

<212> PRT

<213> Aspergillus niger

<400> 14

```
Ser Val Ser Thr Ser Thr Leu Asp Glu Leu Gln Leu Phe Ser Gln Trp
1               5                   10                  15

Ser Ala Ala Ala Tyr Cys Ser Asn Asn Ile Asp Ser Asp Asp Ser Asn

            20                  25                  30

Val Thr Cys Thr Ala Asp Ala Cys Pro Ser Val Glu Glu Ala Ser Thr
        35                  40                  45

Lys Met Leu Leu Glu Phe Asp Leu Thr Asn Asn Phe Gly Gly Thr Ala
    50                  55                  60

Gly Phe Leu Ala Ala Asp Asn Thr Asn Lys Arg Leu Val Val Ala Phe
65                  70                  75                  80

Arg Gly Ser Ser Thr Ile Lys Asn Trp Ile Ala Asp Leu Asp Phe Ile
                85                  90                  95

Leu Gln Asp Asn Asp Asp Leu Cys Thr Gly Cys Lys Val His Thr Gly
            100                 105                 110

Phe Trp Lys Ala Trp Glu Ala Ala Ala Asp Asn Leu Thr Ser Lys Ile
        115                 120                 125

Lys Ser Ala Met Ser Thr Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly
    130                 135                 140

His Ser Leu Gly Gly Ala Leu Ala Thr Leu Gly Ala Thr Val Leu Arg
145                 150                 155                 160

Asn Asp Gly Tyr Ser Val Glu Leu Tyr Thr Tyr Gly Cys Pro Arg Val
                165                 170                 175

Gly Asn Tyr Ala Leu Ala Glu His Ile Thr Ser Gln Gly Ser Gly Ala
            180                 185                 190

Asn Phe Pro Val Thr His Leu Asn Asp Ile Val Pro Arg Val Pro Pro
        195                 200                 205

Met Asp Phe Gly Phe Ser Gln Pro Ser Pro Glu Tyr Trp Ile Thr Ser
    210                 215                 220

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

Ile Asn Ser Thr Ala Gly Asn Ala Gly Glu Ala Thr Val Asp Val Leu
                245                 250                 255

Ala His Leu Trp Tyr Phe Phe Ala Ile Ser Glu Cys Leu Leu
            260                 265                 270
```

<210> 15

<211> 269

<212> PRT

<213> Aspergillus oryzae

<400> 15

```
Asp Val Ser Ser Ser Leu Leu Asn Asn Leu Asp Leu Phe Ala Gln Tyr
1               5                   10                  15
```

```
1               5                  10                  15

Ser Ala Ala Ala Tyr Cys Asp Glu Asn Leu Asn Ser Thr Gly Thr Lys
            20                  25                  30

Leu Thr Cys Ser Val Gly Asn Cys Pro Leu Val Glu Ala Ala Ser Thr
        35                  40                  45

Gln Ser Leu Asp Glu Phe Asn Glu Ser Ser Ser Tyr Gly Asn Pro Ala
    50                  55                  60

Gly Tyr Leu Ala Ala Asp Glu Thr Asn Lys Leu Leu Val Leu Ser Phe
65                  70                  75                  80

Arg Gly Ser Ala Asp Leu Ala Asn Trp Val Ala Asn Leu Asn Phe Gly
                85                  90                  95

Leu Glu Asp Ala Ser Asp Leu Cys Ser Gly Cys Glu Val His Ser Gly
            100                 105                 110

Phe Trp Lys Ala Trp Ser Glu Ile Ala Asp Thr Ile Thr Ser Lys Val
        115                 120                 125

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

His Ser Tyr Gly Ala Ala Leu Ala Ala Leu Ala Ala Thr Ala Leu Arg
145                 150                 155                 160

Asn Ser Gly His Ser Val Glu Leu Tyr Asn Tyr Gly Gln Pro Arg Leu
                165                 170                 175

Gly Asn Glu Ala Leu Ala Thr Tyr Ile Thr Asp Gln Asn Lys Gly Gly
            180                 185                 190

Asn Tyr Arg Val Thr His Thr Asn Asp Ile Val Pro Lys Leu Pro Pro
        195                 200                 205

Thr Leu Leu Gly Tyr His His Phe Ser Pro Glu Tyr Tyr Ile Ser Ser
    210                 215                 220

Ala Asp Glu Ala Thr Val Thr Thr Thr Asp Val Thr Glu Val Thr Gly
225                 230                 235                 240

Ile Asp Ala Thr Gly Gly Asn Asp Gly Thr Asp Gly Thr Ser Ile Asp
                245                 250                 255

Ala His Arg Trp Tyr Phe Ile Tyr Ile Ser Glu Cys Ser
            260                 265
```

<210> 16

<211> 251

<212> PRT

<213> Landerina penisapora

<400> 16

```
Pro Gln Asp Ala Tyr Thr Ala Ser His Ala Asp Leu Val Lys Tyr Ala
1               5                   10                  15

Thr Tyr Ala Gly Leu Ala Tyr Gln Thr Thr Asp Ala Trp Pro Ala Ser
            20                  25                  30
```

```
Arg Thr Val Pro Lys Asp Thr Thr Leu Ile Ser Ser Phe Asp His Thr
        35                  40                  45

Leu Lys Gly Ser Ser Gly Tyr Ile Ala Phe Asn Glu Pro Cys Lys Glu
    50                  55                  60

Ile Ile Val Ala Tyr Arg Gly Thr Asp Ser Leu Ile Asp Trp Leu Thr
65                  70                  75                  80

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

Leu Val His Glu Gly Phe Leu Asn Ala Tyr Leu Val Ser Met Gln Gln
            100                 105                 110

Val Gln Glu Ala Val Asp Ser Leu Leu Ala Lys Cys Pro Asp Ala Thr
        115                 120                 125

Ile Ser Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Cys Ile Ser
    130                 135                 140

Met Val Asp Thr Ala Gln Arg His Arg Gly Ile Lys Met Gln Met Phe
145                 150                 155                 160

Thr Tyr Gly Gln Pro Arg Thr Gly Asn Gln Ala Phe Ala Glu Tyr Val
                165                 170                 175

Glu Asn Leu Gly His Pro Val Phe Arg Val Val Tyr Arg His Asp Ile
            180                 185                 190

Val Pro Arg Met Pro Pro Met Asp Leu Gly Phe Gln His His Gly Gln
        195                 200                 205

Glu Val Trp Tyr Glu Gly Asp Glu Asn Ile Lys Phe Cys Lys Gly Glu
    210                 215                 220

Gly Glu Asn Leu Thr Cys Glu Leu Gly Val Pro Phe Ser Glu Leu Asn
225                 230                 235                 240

Ala Lys Asp His Ser Glu Tyr Pro Gly Met His
                245                 250
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<210> 17

<211> 12

<212> PRT

<213> Artificial

<220>

<223> Sequence used for alignment

<400> 17

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Ala Cys Met Ser His Thr Trp Gly Glu Arg Asn Leu
1               5                   10
```

<210> 18

<211> 14

<212> PRT

<213> Artificial

<220>
<223> Sequence used for alignment

<400> 18
His Gly Trp Gly Glu Asp Ala Asn Leu Ala Met Asn Pro Ser
1 5 10

REFERENCES CITED IN THE DESCRIPTION

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- WO2006125437A **[0084]**
- WO2000060063A **[0095]** **[0096]**

Non-patent literature cited in the description

- **NEEDLEMAN, S. B.WUNSCH, C. D.**J. Mol. Biol., 1970, vol. 48, 443-453 [0061]
- **NEEDLEMAN, S.B.WUNSCH, C.D.**Journal of Molecular Biology, 1970, vol. 48, 443-45 [0065] [0067]
- **J. SAMBROOKE.F. FRITSCHT. MANIATUS**Molecular Cloning, A Laboratory ManualCold Spring Harbor19890000 [0068]

PATENTKRAV

1. Variant af en stamlipase, hvor varianten har lipaseaktivitet, er mindst 80% identisk med SEQ ID NO: 2 og omfatter substitutionerne Q4V +S58A +V60S +S83T +I86V +A150G +E210K +L227G +T231R +N233R +P256K (under anvendelse af SEQ ID NO: 2 til nummerering).

2. Lipasevariant ifølge krav 1, hvor stamlipasen er lipasen med aminosyresekvensen ifølge SEQ ID NO: 2.

3. Lipasevariant ifølge krav 1, hvor lipasevarianten endvidere omfatter substitutioner valgt fra gruppen bestående af substitutioner i positioner svarende til positionerne 202, 211 og 255 (under anvendelse af SEQ ID NO: 2 til nummerering).

4. Lipasevariant ifølge krav 3, hvor substitutionerne er X202G, X211L og X255Y/V.

5. Lipasevariant ifølge krav 1, hvor lipasevarianten endvidere omfatter en substitution i en position svarende til position 90 (under anvendelse af SEQ ID NO: 2 til nummerering).

6. Lipasevariant ifølge krav 5, hvor substitutionen er X90A/R.

7. Lipasevariant ifølge krav 1, hvor lipasevarianten endvidere omfatter en substitution i en position svarende til position 27 (under anvendelse af SEQ ID NO: 2 til nummerering).

8. Lipasevariant ifølge krav 7, hvor substitutionen er X27R.

9. Lipasevariant ifølge krav 1, hvor lipasevarianten endvidere omfatter mindst én substitution i stamlipasen valgt fra gruppen bestående af substitutioner i positioner svarende til position 81, 147 og 249 (under anvendelse af SEQ ID NO: 2 til nummerering).

10. Lipasevariant ifølge krav 9, hvor substitutionerne er X81Q/E, X147M/Y og X249R/I/L.

11. DNA-sekvens, som koder for lipasevarianten ifølge krav 1-10.

12. Ekspressionsvektor, som huser DNA-sekvensen ifølge krav 11.

13. Transformeret værtscelle, som indeholder DNA-sekvensen ifølge krav 11.

14. Fremgangsmåde til fremstilling af en lipasevariant, hvilken fremgangsmåde omfatter dyrkning af den transformerede værtscelle ifølge krav 13 under betingelser, som er befordrende for fremstilling af lipasevarianten, og indvinding af lipasevarianten fra det resulterende medium.

DRAWINGS

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ID NO 1:     SSSSTQDYRIASEAEIKAHTFYTALSANA
ID NO 2:      SSSTQDYRIASEAEIKAHTFYTALSANA
ID NO 3:       SIDGGIRAATSQEINELTYYTTLSANS
ID NO 4:     SASDGGKVVAATTAQIQEFTKYAGIAATA
ID NO 5:        TAGHALAASTQ GISEDLYSRL VEMATISQAA
ID NO 6:        TAGHALAASTQ GISEDLYSRL VEMATISQAA
ID NO 7:            AVGVTTTDFSNFKFYIQHGAAA
ID NO 8:              TVTTQDLSNFRFYLQHADAA
ID NO 9:              DIPTTQLEDFKFWVQYAAAT
ID NO 10:             DVSTSELDQFEFWVQYAAAS
ID NO 11:             SVSTSTLDELQLFAQWSAAA
ID NO 12:             SVSTSTLDELQLFSQWSAAA
ID NO 13:             DVSSSLLNNLDLFAQYSAAA
ID NO 14:             EVSQDLFNQFNLFAQYSAAA
ID NO 15:           PQDAYTASHADLVKYATYAGLA

ID NO 1:    YCRTVIPG      GRWSCPHCGVAS  NLQITKTFST  LITDTNVLVAV
ID NO 2:    YCRTVIPG      GQWSCPHCDVAP  NLNITKTFTT  LITDTNVLVAV
ID NO 3:    YCRTVIPG      ATWDCIHCDATE  DLKIIKTWST  LIYDTNAMVAR
ID NO 4:    YCRSVVPG      NKWDCVQCQKWVP DGKIITTFTS  LLSDTNGYVLR
ID NO 5:    YADLCNIPST                     IIKGEKIYNSQTDINGWILR
ID NO 6:    YADLCNIPST                     IIKGEKIYNSQTDINGWILR
ID NO 7:    YC  NSEAAA GSKITCSNNGCPTVQGNGATIVTSF  VGSKTGIGGYVAT
ID NO 8:    YC  NFNTAV GKPVHCSAGNCPDIEKDAAIVVGSV  VGTKTGIGAYVAT
ID NO 9:    YCPNNYVAKD GEKLNCSVGNCPDVEAAGSTVKLSFS DDTITDTAGFVAV
ID NO 10:   YYEADYTAQV GDKLSCSKGNCPEVEATGATVSYDFS DSTITDTAGYIAV
ID NO 11:   YCSNNID SK DSNLTCTANACPSVEEASTTMLLEFDLTNDFGGTAGFLAA
ID NO 12:   YCSNNID SD DSNVTCTADACPSVEEASTKMLLEFDLTNNFGGTAGFLAA
ID NO 13:   YCDENLN ST GTKLTCSVGNCPLVEAASTQSLDEFNESSSYGKPAGYLAA
ID NO 14:   YCGKNNDAPA GTNITCTGNACPEVEKADATFLYSFE DSGVGDVTGFLAL
ID NO 15:   YQTTDAWPAS             RTVPKDTTLISSFD  HTLKGSSGYIAF


ID NO 1:    GEKEKTIYVV FRGTSSIRNA IADIVFVPVN YPPV   NGA KVHKGFLDSY
ID NO 2:    GENEKTIYVV FRGTSSIRNA IADIVFVPVN YPPV   NGA KVHKGFLDSY
ID NO 3:    GDSEKTIYIV FRGSSSIRNW IADLTFVPVS YPPV   SGT KVHKGFLDSY
ID NO 4:    SDKQKTIYLV FRGTNSFRSA ITDIVFNFSD YKPV   KGA KVHAGFLSSY
ID NO 5:    DDSSKEIITV FRGTGSDTNL QLDTNYTLTP FDTLPQCNGC EVHGGYYIGW
ID NO 6:    DDSSKEIITV FRGTGSDTNL QLDTNYTLTP FDTLPQCNSC EVHGGYYIGW
ID NO 7:    DSARKEIVVS FRGSINIRNW LTNLDFG QE DCSL  VSGC GVHSGFQRAW
ID NO 8:    DNARKEIVVS VRGSINVRNW ITNFNFG QK TCDL  VAGC GVHTGFLDAW
ID NO 9:    DNTNKAIVVA FRGSYSIRNW VTDATFP QT DPGL  CDGC KAELGFWTAW
ID NO 10:   DHTNSAVVLA FRGSYSVRNW VADATFV HT NPGL  CDGC LAELGFWSSW
ID NO 11:   DNTNKRLVVA FRGSSTIENW IANLDFILED NDDL  CTGC KVHTGFWKAW
ID NO 12:   DNTNKRLVVA FRGSSTIKNW IADLDFILQD NDDL  CTGC KVHTGFWKAW
ID NO 13:   DETNKLLVLS FRGSADLANW VANLNFGLED ASDL  CSGC EVHSGFWKAW
ID NO 14:   DNTNKLIVLS FRGSRSIENW IGNLNFDLKE INDI  CSGC RGHDGFTSSW
ID NO 15:   NEPCKEIIVA YRGTDSLIDW LTNLNFDKTA WPAN  ISNS LVHEGFLNAY

ID NO 1:    NEVQDKLVAE VKAQLDRHPG YKIVVTGHSL GGATAVLSALDLYHHGHA
ID NO 2:    NEVQDKLVAE VKAQLDRHPG YKIVVTGHSL GGATAVLSALDLYHHGHD
ID NO 3:    GEVQNELVAT VLDQFKQYPS YKVAVTGHSL GGATALLCALDLYQREEGLS
ID NO 4:    EQVVNDYFPV VQEQLTAHPT YKVIVTGHSL GGAQALLAGMDLYQREPRLS
ID NO 5:    VSVQDQVESL VKQQVSQYPD YALTVTGHSL GASLAALTAAQL SATYD
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Figure 1 (cont.)

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ID NO 6:   ISVQDQVESL VQQQVSQFPD YALTVIGHSL GASLAALTAAQL SATYD
ID NO 7:   NEISSQATAA VASARKANPS FNVISIGHSL GGAVAVLAAANLRVGGT
ID NO 8:   EEVAANVKAA VSAAKTANPT FKFVVIGHSL GGAVATIAAAYLRKDGF
ID NO 9:   KVVRDRIIKT LDELKPEHSD YKIVVVGHSL GAAIASLAAADLRTKNY
ID NO 10:  KLVRDDIIKE LKEVVAQNPN YELVVVGHSL GAAVACLAATDLRGKGYP
ID NO 11:  ESAADELTSK IKSAMSTYSG YTLYFIGHSL GGALATLGATVLRNDGY
ID NO 12:  EAAADNLTSK IKSAMSTYSC YTLYFIGHSL GCALATLGATVLRNDGY
ID NO 13:  SEIADTITSK VESALSDHSD YSLVLIGHSY GAALAALAATALRNSGH
ID NO 14:  RSVADTLRQK VEDAVREHPD YRVVFIGHSL GGALATVAGADLRGNGY
ID NO 15:  LVSMQQVQEA VDSILAKCPD ATISFIGHSL GGALACISMVDTAQRHRGI

ID NO 1:     NTEIYTQG QPRIGTPAFA NYVIGI       KIPYQRLVHERDIVPHL
ID NO 2:     NTEIYTQG QPRIGTPEFA NYVIGI       KIPYQRLVNERDIVPHL
ID NO 3:   SSNLFLYTQC QPRVCDPAFA NYVVSI       GIPYRRTVNERDIVPHL
ID NO 4:   PKNLSIFTVG GPRVGNPTFA YYVESI       GIPFQRTVHKRDIVPHV
ID NO 5:     NTRLYTFG EPRSGNQAFA SYMNDAFQASSPDTTQYFRVTHANDGIPNL
ID NO 6:     NTRLYTFG EPRS NQAFA SYMNDAFQASSPDTTQYFRVTHANDGIPNL
ID NO 7:     PVDIYTYG SPRVGNAQLS AFVSNQ       AGGEYRVTHADDPVPRL
ID NO 8:     PFDLYTYC SPRVGNDFFA NFVTQQ       TGAEYRVTHCDDPVPRL
ID NO 9:     DAILYAYA APRVANKPLA EFITNQ        GNNYRFTHNDDPVPKL
ID NO 10:    SAKLYAYA SPRVGNAALA KYITAQ        GNNFRFTHTNDPVPKL
ID NO 11:    SVELYTYG CPRIGNYALA EHITSQ       GSGANFRVTHLNDIVPRV
ID NO 12:    SVELYTYG CPRVGNYALA EHITSQ       GSGANFPVTHLNDIVPRV
ID NO 13:    SVELYNYG QPRIGNEALA TYITDQ       NKGGNYRVTHTNDIVPKL
ID NO 14:    DTDVFSYG APRVGNRAFA EFLTVQ       TGGTLYRITHTNDIVPRL
ID NO 15:    KMQMFTYG QPRTGNQAFA EYVENL        GHPVFRVVYRHDIVPRM

ID NO 1:   PPGAFGFLHA GEEFWIMK          DSSLRVCPNGIETDNCSNSIV
ID NO 2:   PPGAFGFLHA GEEFWIMK          DSSLRVCPNGIETDNCSNSIV
ID NO 3:   PPAAFGFLHA GEEYWITD        NSPETVQVCTSDLETSDCSNSIV
ID NO 4:   PPQSFGFLHP GVESWIKS         GTSNVQICTSEIETKDCSNSIV
ID NO 5:   PPVEQGYAHG GVEYWSV     DPYSAQNTFVCTGDEVQCCE AQGGQG
ID NO 6:   PPADEGYAHG VVEYWSV     DPYSAQNTFVCTGDEVQCCE AQGGQG
ID NO 7:   PPLTFGYRHT TPEFWLSGGCCDKVDYIISDVKVCEGAANLG CNGGTL
ID NO 8:   PPIVFGYRHT SPEYWLNG GPLDKDYIVTEIKVCEGIANVM CNGGTI
ID NO 9:   PLLTMGYVHI SPEYYITA  PDNTTVIDNQVIVLDGYVNFK GNTGTS
ID NO 10:  PLLSMGYVHV SPEYWITS  PNNATVSTSDIKVIDGDVSFD GNTGTG
ID NO 11:  PPMDFGFSQP SPEYWITS  GNGASVIASDIEVIEGINSTA GNAGEA
ID NO 12:  PPMDFGFSQP SPEYWITS  CTGASVIASDIELIEGINSTA GNAGEA
ID NO 13:  PPTLLGYHHF SPEYYISS  ADEATVITTDVIEVTGIDATG GNDGTD
ID NO 14:  PPREFGYSHS SPEYWIKS  GTLVPVIRNDIVKIEGIDATG GNNQPN
ID NO 15:  PPMDLGFQHH GQEVWYEG          DENIKFCKGEGENLTCELGVP

ID NO 1:   PFT  SVIDHLSYLDMNTGL CL
ID NO 2:   PFT  SVIDHLSYLDMNTGL CL
ID NO 3:   PFT  SVLDHLSYFGINTGL CT
ID NO 4:   PFT  SILDHLSYFDINEGS CL
ID NO 5:   VN     NAHTTYF GMTSGACTW
ID NO 6:   VN     NAHTTYF GMTSGHCTW
ID NO 7:   GL   DIAAHLHYF QATDA CNAGGFSWR R
ID NO 8:   GL   DILAHITYF QSMAT CAPIAIPWK R
ID NO 9:   GGLPDLLAFHSHVWYFIHADACKGPGLPLR
ID NO 10:  LPLLTDFEAHIWYF VQVDA GKGPGLPFK R
ID NO 11:  TV   SVLAHLWYF FAISE CLL
ID NO 12:  TV   DVLAHLWYF FAISE CLL
ID NO 13:  GT   SIDAHRWYF IYISE CS
ID NO 14:  IP   DIPAHLWYF GLIGT CL
ID NO 15:  FSEL NAKDHSEYP GMH
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Figure 1 (cont.)

ID NO:	Micro organism	SEQ ID NO.:
1.	*Absidia reflexa*	3
2.	*Absidia corymbifera*	4
3.	*Rhizmucor miehei*	5
4.	*Rhizopus delemar (oryzea)*	6
5.	*Aspergillus niger*	7
6.	*Aspergillus tubingensis*	8
7.	*Fusarium oxysporum*	9
8.	*Fusarium heterosporum*	10
9.	*Aspergillus oryzae*	11
10.	*Penicilium camembertii*	12
11.	*Aspergillus foetidus*	13
12	*Aspergillus niger*	14
13.	*Aspergillus oryzea*	15
14.	*Thermomyces lanuginosus*	2
15.	*Landerina penisapora*	16

Figure 1. Alignment of lipase sequences.

Figure 1 (cont.)