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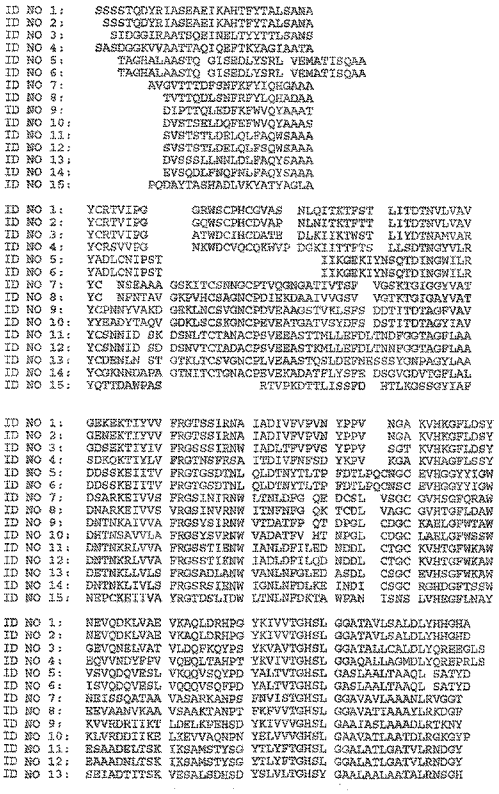
[Continued on next page]

(54) Title: LIPASE VARIANTS

(57) Abstract: The invention provides variant lipases, preferably, variants with reduced tendency to odor generation obtained by introducing mutations in one or more regions identified in a parent lipase.



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LIPASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to lipase variants.

BACKGROUND OF THE INVENTION

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 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.

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

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.

In a preferred embodiment, the present invention relates to lipases having improved properties for use in detergents. For example, the invention provides variants with reduced tendency to odor generation obtained by introducing mutations in one or more regions identified in the parent lipase. In another preferred embodiment, the present invention provides lipase variants which, as compared to the parent lipase, have reduced potential for odor generation without the attachment of a C-terminal extension.

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.

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

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the alignment of lipases.

SEQUENCE LISTINGS

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

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 aligment" to the sequence of the *T. lanuginosus* lipase shown in SEQ ID NO: 2.

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.

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.

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.

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

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

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

a) at least two substitution in Region I, and

b) at least one substitution in Region II, and

c) at least one substitution in Region III, and

d) at least one substitution in Region IV;'

and wherein the variant has lipase activity.

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 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2.

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 vari-

ant lipase 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.

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 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.

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 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 (syno-

nym: Humicola lanuginose), Mucor miehei, Myceliophthora thermophila, Neurospora crassa, Penicillium purpurogenum, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesei, or Trichoderma viride.

In another preferred aspect, the variant is a variant of a Thermomyces lipase.

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.

Identification of regions and substitutions

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

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.

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.

In particular the following substitutions have been identified: X1N/* X4V, X227G, X231R and X233R.

In a preferred embodiment the variant lipase has at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% identity to SEQ ID NO:2

In a most preferred embodiment the variant lipase is a variant of the lipase having the aminio acid sequence of SEQ ID NO: 2.

Substitutions in Region II

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.

The lipase variant may comprise at least one substitution in Region II, such as two, three, four, five or six substitutions in Region II.

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.

In particular the following substitutions have been identified: X202G, X210K/W/A, X255Y/V/A and X256K/R and X259G/M/Q/V.

In a preferred embodiment the variant lipase has at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2

In a most preferred embodiment the variant lipase is a variant of the lipase having the aminio acid sequence of SEQ ID NO: 2.

Substitutions in Region III

Region III consists of amino acid residues that forms 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.

The lipase variant may comprise at least one substitution in Region III, such as two, three, four, five or six substitutions in Region III.

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.

In particular the following substitutions have been identified: X83T, X86V and X90A/R.

In a preferred embodiment the variant lipase has at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2

In a most preferred embodiment the variant lipase is a variant of the lipase having the aminio acid sequence of SEQ ID NO: 2.

Substitutions in Region IV

Region IV consists of amino acid residues that binds 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.

The lipase variant may comprise at least one substitution in Region IV, such as two, three, four, five or six substitutions in Region IV.

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.

In particular the following substitutions have been identified: X27R, X58N/AG/T/P and X60V/S/G/N/R/K/A/L.

In a preferred embodiment the variant lipase has at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO:2

In a most preferred embodiment the variant lipase is a variant of the lipase having the aminio acid sequence of SEQ ID NO: 2.

Amino acids at other positions

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 lipaase. 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.

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.

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.

Parent lipase variants

Variant lipases include parent lipases having the substitutions listed below in table 1 (using SEQ ID NO:2 for numbering).

Region I	Region II	Region III	Region IV	Outside regions
X4V + X227G + X231R + X233R	X210K + X256K	X83T + X86V	X58A + X60S	X150G
X227G + X231R + X233R	X256K	X86V	X58N + X60S	X150G
X231R + X233R	X255Y			
X231R + X233R	X202G			
X227G + X231R + X233R	X256K	X86V		
X4V + X231R + X233R			X58N + X60S	
X231R + X233R		X90R	X58N + X60S	
X231R + X233R	X255V	X90A		
X227G + X231R + X233R	X256K	X86V	X58N + X60S	X150G

X231R + X233R	X211L		X58N + X60S	X147M
X231R + X233R				X150K

Table 1:

In a further particular embodiment, the parent lipase is identical to SEQ ID NO:2, and the variants of Table 1 will thus be:

Region I	Region II	Region III	Region IV	Outside regions
Q4V + L227G + T231R + N233R	E210K + P256K	S83T + I86V	S58A + V60S	A150G
L227G + T231R + N233R	P256K	I86V	S58N + V60S	A150G
T231R + N233R	I255Y			
T231R + N233R	I202G			
L227G + T231R + N233R	P256K	I86V		
Q4V + T231R + N233R			S58N + V60S	
T231R + N233R		I90R	S58N + V60S	
T231R + N233R	I255V	I90A		
L227G + T231R + N233R	P256K	I86V	S58N + V60S	A150G
T231R + N233R	F211L		S58N + V60S	L147M
T231R + N233R				A150K

Table 2: Some particular variants of SEQ ID NO:2

Nomenclature for amino acid modifications

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)

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.

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.

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.

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.

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

Amino acid grouping

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.

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

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

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.

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 se-

quence ("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.

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).

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

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.

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.

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 se-quence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Hybridization

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.

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.

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

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.

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)

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

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

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

EXAMPLES

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

Example 1

Production of enzyme

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

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.

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)

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.

The assay is conducted under the experimental conditions specified below:

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 (EMPA221 used as cotton textile)

Table 3

Cream-turmeric swatches were prepared by mixing 5 g of turmeric (Santa Maria, Denmark) with 100 g cream (38% fat, Arla, 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.

The performance of the enzyme variant is measured as the brightness of the colour 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.

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.

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:

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

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))

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 T231R + N233R.

Example 3

GC – Gas Chromatograph – for calculation of risk factor

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.

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 buturic acid developed at 1 mg enzyme protein / l corrected for blank

$$\text{Alpha}_{\text{test enzyme}} = \text{Odour}_{\text{test enzyme}} - \text{Blank}$$

$$\text{Alpha}_{\text{reference enzyme}} = \text{Odour}_{\text{reference enzyme}} - \text{Blank}$$

$$R = \text{Alpha}_{\text{test enzyme}} / \text{Alpha}_{\text{reference enzyme}}$$

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

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

LU/A280:

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 T231R + N233R.

Example 5

BR – Benefit Risk

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.

Applying the above methods the following results were obtained:

Variant	Mutations in SEQ ID NO: 2	Average RP (RP_{avg})	BR	LU/A280
1	I202G + T231R + 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 + T231R, N233R + Q249R + 270H + 271T + 272P + 273S + 274S + 275G + 276R + 277G + 278G	0.13	not determined	500

Table 4

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

Example 6

BR – Benefit Risk

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.

Variant	Mutations in SEQ ID NO: 2
Reference	T231R + N233R
9	L97V+ T231R+N233R
10	A150G+T231R+N233R
11	I90R+T231R+N233R
12	I202V+T231R+N233R
13	L227G+ T231R+ N233R+ P256K
14	I90A+ T231R+ N233R
15	T231R+N233R+ I255P
16	I90V+I255V+T231R+N233R
17	F211L+ L227G+ T231R+ N233R+ I255L+ P256K
18	S58N+ V60S+ T231R+ N233R+ Q249L

19	S58N+ V60S+ T231R+ N233R+ Q249I
20	A150G+ L227G+ T231R+ N233R+ P256K
21	K46L+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254I
22	Q4L+ E43T+ K46I+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254I
23	Q4L+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254I
24	K46I+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254L
25	K46L+ S58N+ V60S+ K223I+ T231R+ N233R+ D254I
26	E43T+ K46I+ S58N+ V60S+ T231R+ N233R+ Q249L+ D254I
27	S58N+ V60S+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
28	K24R+ K46R+ K74R+ I86V+ K98R+ K127R+ D137K+ A150G+ K223R+ T231R+ N233R
29	S58A+V60A+ I86V+T231R+N233R
30	K24R+ K46R+ S58N+ V60S+ K74R+ I86V+ K98R+ K127R+ D137K+ K223R+ T231R+ N233R
31	S58A+ V60A+ I86V+ A150G+ T231R+ N233R
32	S58N+ V60V+ D62G+ T231R+ N233R
33	Q4V+ S58N+ V60S+ I86V+ T231R+ N233R+ Q249L
34	Q4V+ S58N+ V60S+ I86V+ A150G+ T231R+ N233R+ I255V
35	Q4V+ S58N+ V60S+ I90A+ A150G+ T231R+ N233R+ I255V
36	Y53A+ S58N+ V60S+ T231R+ N233R+ P256L
37	I202L+ T231R+ N233R+ I255A
38	S58A+ V60S+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
39	D27R+ S58N+ V60S+ I86V+ A150G+ L227G+ T231R+ N233R+ P256K
40	V60K+ I86V+ A150G+ L227G+ T231R+ 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+ T231R+ 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+ T231R+ 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	T231R+ N233G+ D234G
54	S58N+ V60S+ I86V+ A150G+ E210K+ L227G+ T231R+ N233R+ Q249R+ P256K
55	S58N+ V60S+ I86V+ A150G+ E210K+ L227G+ T231R+ N233R+ I255A+ P256K
56	S58N+ V60S+ I86V+ A150G+ G156R+ E210K+ L227G+ T231R+ N233R+ I255A+ P256K
57	S58T+ V60K+ I86V+ N94K+ A150G+ E210V+ L227G+ T231R+ 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
67	D27H+E87Q+D96N+T231R+N233R+D254V
68	F181L+E210V+T231R+N233R
69	D27N+ D96G+ T231R+ N233R
70	D96N+ T231R+ N233R
71	T231R+ N233I+ D234G
72	S58K+ V60L+ E210V+ Q249R
73	S58H+ V60L+ E210V+ Q249R
74	Q4V+ F55V+ I86V+ T231R+ N233R+ I255V
75	Q4V+ S58T+ V60K+ T199L+ N200A+ E210K+ T231R+ 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+ T231R+ N233R
82	I90V+L227G+T231R+N233R+ P256K
83	T231R+N233R+ I255S
84	I86G+ T231R+ N233R
85	V60K+ I202V+ E210K+ T231R+ N233R+ I255A+ P256K
86	I90G+ I202L+ T231R+ N233R+ I255S
87	S58G+ V60G+ T231R+ N233R

Table 5

The reference lipase is described in WO 2000/060063.

CLAIMS

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):

a) at least two substitutions in Region I, and

b) at least one substitution in Region II, and

c) at least one substitution in Region III, and

d) at least one substitution in Region IV;

and wherein the variant has lipase activity.

2. A lipase variant according to claim 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).

3. A lipase variant according to claim 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).

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

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

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

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

8. A lipase variant according to claim 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).

9. A lipase variant according to claim 8, wherein the at least one substitution in the parent lipase is selected from the group consisting of X202G, X210K, X211L, X255Y/V and X256K (using SEQ ID NO:2 for numbering).

10. A lipase variant according to claim 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).

11. A lipase variant according to claim 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).

12. A lipase variant according to claim 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).

13. A lipase variant according to claim 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).

14. A lipase variant according to claim 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).

15. A lipase variant according to claim 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).

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

a) T231R + N233R + I255Y
b) I202G + T231R + N233R
c) I86V + L227G + T231R + N233R + P256K
d) Q4V + S58N + V60S + T231R + N233R
e) S58N + V60S + I90R + T231R + N233R
f) I90A + T231R + N233R + I255V
g) S58N + V60S + I86V + A150G + L227G + T231R + N233R + P256K
h) S58N + V60S + L147M + F211L + T231R + N233R
i) Q4V + S58A + V60S + S83T + I86V + A150G + E210K + L227G + T231R + N233R + P256K
j) S58N + V60S + I86V + A150G + L227G + T231R + N233R + P256K

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

18. An expression vector harbouring the DNA sequence of claim 17.

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

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

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.

```
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:             DVETSELDQFEFWVQYAAAS
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  LIYDTNANVAR
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 GTKLTCSVGNCPLVEAASTQSLDEFNESSSYGNPAGYLAA
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 VIDATFP 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
ID NO 6:   ISVQDQVESL VQQQVSQFPD YALTVTGHSL GASLAALTAAQL SATYD
ID NO 7:   NEISSQATAA VASARKANPS FNVISTGHSL GGAVAVLAAANLRVGGT
ID NO 8:   EEVAANVKAA VSAAKTANPT FKFVVTGHSL GGAVATIAAAYLRKDGF
ID NO 9:   KVVRDRTIKT LDELKFEHSD YKIVVVGHSL GAAIASLAAADLRTKNY
ID NO 10:  KLVRDDIIKE LKEVVAQNPN YELVVVGHSL GAAVATLAATDLRGKGYP
ID NO 11:  ESAADELTSK IKSAMSTYSG YTLYFTGHSL GGALATLGATVLRNDGY
ID NO 12:  EAAADNLTSK IKSAMSTYSG YTLYFTGHSL GGALATLGATVLRNDGY
ID NO 13:  SEIADTITSK VESALSDHSD YSLVLTGHSV GAALAALAATALRNSGH
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Figure 1 (cont.)

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ID NO 14:  RSVADTLRQK VEDAVREHPD YRVVFTGHSL GGALATVAGADLRGNGY
ID NO 15:  LVSMQQVQEA VDSLLAKCPD ATISFTGHSL GGALACISMVDTAQRHRGI

ID NO 1:     NIEIYTQG QPRIGTPAFA NYVIGT        KIPYQRLVNERDIVPHL
ID NO 2:     NIEIYTQG QPRIGTPEFA NYVIGT        KIPYQRLVNERDIVPHL
ID NO 3:   SSNLFLYTQG QPRVGDPAFA NYVVST        GIPYRRTVNERDIVPHL
ID NO 4:   FKNLSIFTVG GPRVGNPTFA YYVEST        GIPFQRTVHKRDIVPHV
ID NO 5:     NIRLYTFG EPRSGNQAFA SYMNDAFQASSPDTTQYFRVTHANDGIPNL
ID NO 6:     NIRLYTFG EPRS NQAFA SYMNDAFQASSPDTTQYFRVTHANDGIPNL
ID NO 7:     PVDIYTYG SPRVGNAQLS AFVSNQ        AGGEYRVTHADDPVPRL
ID NO 8:     PFDLYTYG SPRVGNDFFA NFVTQQ        TGAEYRVTHGDDPVPRL
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 QPRLGNEALA TYITDQ       NKGGNYRVTHTNDIVPKL
ID NO 14:    DIDVFSYG 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:   PPLIPGYRHT TPEFWLSGGGGDKVDYTISDVKVCEGAANLG CNGGTL
ID NO 8:   PPIVFGYRHT SPEYWLNG GPLDKDYTVTEIKVCEGIANVM CNGGTI
ID NO 9:   PLLTMGYVHI SPEYYITA  PDNTTVTDNQVTVLDGYVNFK GNTGTS
ID NO 10:  PLLSMGYVHV SPEYWITS  PNNATVSTSDIKVIDGDVSFD GNTGTG
ID NO 11:  PPMDFGFSQP SPEYWITS  GNGASVTASDIEVIEGINSTA GNAGEA
ID NO 12:  PPMDFGFSQP SPEYWITS  GTGASVTASDIELIEGINSTA GNAGEA
ID NO 13:  PPTLLGYHHF SPEYYISS  ADEATVTTTDVTEVTGIDATG GNDGTD
ID NO 14:  PPREFGYSHS SPEYWIKS  GTLVPVTRNDIVKIEGIDATG 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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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

Figure 1 (cont.)

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.)