

TITLE OF THE INVENTION: Improving Productivity and Activity of *Rhizomucour miehei* Lipase

FIELD OF THE INVENTION

5 [0001] The present invention relates to variants of *Rhizomucour miehei* lipase (RML).

BACKGROUND OF THE INVENTION

[0002] Triacylglycerol lipases (EC 3.1.1.3) are hydrolases that can cleave carboxylic acid ester bonds to release carboxylic acids. Under conditions of low water content, the reverse
10 reaction is favoured, allowing lipases to perform esterification and transesterification reactions as well depending on the substrates present. This wide range of functions that lipases possess make them useful in numerous industrial applications.

[0003] For industrial enzymes, their expression levels in the production host is one of the crucial considerations for production. Previous attempts at increasing the expression levels
15 include the optimisation of the signal peptide, the propeptide, the gene copy number and co-expression of protein folding chaperones (Huang *et al.*, 2020, Huang *et al.*, 2014, Xu *et al.*, 2023).

SUMMARY OF THE INVENTION

20 [0004] In a first aspect, there is provided a lipase comprising a peptide sequence with at least 80% sequence similarity to SEQ ID NO: 2 and a F94 mutation of SEQ ID NO: 2. In an embodiment, the peptide sequence similarity is at least 85% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 90% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 95% compared to SEQ
25 ID NO: 2. In another embodiment, the peptide sequence similarity is at least 96% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 97% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 98% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 99% compared to SEQ ID NO: 2. The sequence similarity may be determined by
30 comparing the number of common amino acid residues in the RML variant to SEQ ID NO: 2.

Thus, the lipase contains the peptide sequence with the F94 mutation and possibly other additional components.

[0005] Preferably, the F94 mutation is a substitution of phenylalanine with a hydrophobic amino acid. In an embodiment, the F94 mutation is selected from the group consisting of F94A, F94I, F94L, F94M, F94V, F94W, and F94Y. Preferably, the F94 mutation is selected from the group consisting of F94A, F94M, F94W, and F94Y. More preferably, the F94 mutation is F94W or F94Y.

[0006] In an embodiment, the peptide sequence consists essentially of a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9. In an embodiment, the peptide sequence consists of the sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

[0007] In a second aspect there is provided a method of improving expression levels of a *Rhizomucor miehei* lipase of SEQ ID NO: 2, the method comprising introducing a F94 mutation of SEQ ID NO: 2 to produce a *Rhizomucor miehei* lipase variant comprising a peptide sequence with at least 80% similarity to SEQ ID NO: 2 and the F94 mutation of SEQ ID NO: 2; and expressing the *Rhizomucor miehei* lipase variant in a protein production system.

[0008] Preferably, the F94 mutation is a substitution of phenylalanine with a hydrophobic amino acid. In an embodiment, the F94 mutation is selected from the group consisting of F94A, F94I, F94L, F94M, F94V, F94W, and F94Y. Preferably, the F94 mutation is F94W or F94Y.

[0009] In an embodiment, the protein production system comprises one of the following expression hosts: *Pichia pastoris*, *Escherichia coli*, *Aspergillus niger*, and *Aspergillus oryzae*. Preferably, the protein production system comprises *Pichia pastoris* or *Aspergillus oryzae*.

[0010] In a third aspect there is provided a method of hydrolysing an ester, the method comprising providing a mixture comprising the lipase according to the first aspect and the ester; and forming a reaction product under suitable reaction conditions.

[0011] Preferably, the ester comprises a carboxylic acid component of 4 carbons or more.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure (FIG.) 1 shows the concentration of RML WT and variants expressed in *Pichia pastoris* using shake flasks. All values are determined using Bradford assay and are expressed as mean $\pm$ SD (n=2).

5 DETAILED DESCRIPTION OF THE INVENTION

[0013] In the description herein, numerous specific details are set forth in order to provide a thorough understanding of various illustrative embodiments of the invention. It will be understood, however, to one skilled in the art, that embodiments of the invention may be practiced without some or all of these specific details. Embodiments described in the context of one of the methods or devices are analogously valid for the other methods or devices. Similarly, embodiments described in the context of a method are analogously valid for a device, and vice versa.

[0014] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs.

[0015] As used herein, the articles “a”, “an” and “the” as used with regard to a feature or element include a reference to one or more of the features or elements. As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items. As used herein, the terms “first,” “second,” and “third,” etc. are used merely as labels, and are not intended to impose numerical requirements on their objects.

[0016] Where a combination is disclosed, each subcombination of the elements of that combination is also specifically disclosed and is within the scope of the invention. Conversely, where different elements or groups of elements are disclosed, combinations thereof are also disclosed. Where any element of an invention is disclosed as having a plurality of alternatives, examples of that invention in which each alternative is excluded singly or in any combination with the other alternatives are also hereby disclosed; more than one element of an invention can have such exclusions, and all combinations of elements having such exclusions are hereby disclosed.

[0017] Where a range of values is recited, it is to be understood that each intervening integer value, and each fraction thereof, between the recited upper and lower limits of that range is also specifically disclosed, along with each subrange between such values. The upper and lower limits of any range can independently be included in or excluded from the range, and each

range where either, neither or both limits are included is also encompassed within the invention. Where a value being discussed has inherent limits, for example where a component can be present at a concentration of from 0 to 100%, or where the pH of an aqueous solution can range from 1 to 14, those inherent limits are specifically disclosed. Where a value is explicitly recited,
5 it is to be understood that values which are about the same quantity or amount as the recited value are also within the scope of the invention, as are ranges based thereon.

[0018] Although each of these terms has a distinct meaning, the terms “comprising”, “consisting of” and “consisting essentially of” may be interchanged for one another throughout the instant application. The term “having” has the same meaning as “comprising” and may be
10 replaced with either the term “consisting of” or “consisting essentially of”. The terms “consisting essentially of”. means that specific further components can be present, namely those not materially affecting the essential characteristics of the lipase or lipase variant in the context herein.

[0019] The terms “polypeptide” and “protein”, used interchangeably herein, refer to a
15 polymer of amino acids without regard to the length of the polymer; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude chemical or post-expression modifications of the polypeptides of the invention, although chemical or post-expression modifications of these polypeptides may be included or excluded as specific embodiments. Therefore, for example, modifications to
20 polypeptides that include the covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like are expressly encompassed by the term polypeptide. Further, polypeptides with these modifications may be specified as individual species to be included or excluded from the present invention. The natural or other chemical modifications, such as those listed in examples above can occur anywhere in a polypeptide, including the peptide backbone,
25 the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Modifications include acetylation, acylation, ADP-
30 ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or

lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Also included within the definition are polypeptides which contain one or more analogs of an amino acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

[0020] The terms “sequence similarity”, “percentage of sequence identity” and “percentage homology” are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Identity is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, CLUSTAL W, FASTDB [Pearson and Lipman, (1988), Proc. Natl. Acad. Sci. USA 85(8):2444-2448; Altschul et al., (1990), J. Mol. Biol. 215(3):403-410; Thompson et al. (1994), Nucleic Acids Res. 22(2):4673-4680; Higgins et al., (1996), Meth. Enzymol. 266:383-402; Altschul et al., (1993), Nature Genetics 3:266-272; Brutlag et al. (1990) Comp. App. Biosci. 6:237-24], the disclosures of which are incorporated by reference in their entireties.

[0021] It has been found that the introduction of a mutation on the lid hinge of the RML mature peptide (SEQ ID NO: 2) improves the production level of the RML variant compared

to the wild type. The variants of *Rhizomucor miehei* lipase (RML) with amino acid at position 94 mutated to other hydrophobic residues have had their expression levels in *Pichia pastoris* increased by 48% - 161%. Furthermore, mutations to Trp or Tyr also significantly improved the specific activity of RML. The specific activity of the variants RML F94W and RML F94Y, were found to be increased by 39.6%-185.6% when tested on eight different substrates. The concurrent increase in both productivity and activity in RML F94W and RML F94Y makes these two variants particularly useful for industrial production and applications. It is possible that the F94 mutations, especially the F94W and F94Y mutations, may be combined with other mutations to provide RML variants with improved expression levels, substrate selectivity, and/or lipase activity.

[0022] The RML variant comprises a peptide sequence with at least 80% sequence similarity to a base peptide sequence of SEQ ID NO: 2 and a F94 mutation of SEQ ID NO: 2. The F94 mutations is thus one of the differences between the RML variant and the wild type RML. In another embodiment, the peptide sequence similarity is at least 85% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 90% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 95% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 96% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 97% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 98% compared to SEQ ID NO: 2. In another embodiment, the peptide sequence similarity is at least 99% compared to SEQ ID NO: 2. The sequence similarity may be determined by comparing the number of common amino acid residues in the RML variant to SEQ ID NO: 2.

[0023] In an embodiment, the peptide sequence consists essentially of the F94 mutation described herein. In an embodiment, the peptide sequence consists of the F94 mutation described herein. Thus the F94 mutation is the essential mutation in the RML variant, but the RML variant could still contain other components.

[0024] In an embodiment, the RML variant consists essentially of the F94 mutation described herein. In an embodiment, the RML variant consists of the F94 mutations described herein.

[0025] **RML mutagenesis and expression**

[0026] To generate RML variants, site directed mutagenesis was used to introduce substitution into wild-type (WT) RML. The site directed mutagenesis was performed to introduce a mutation at amino acid residue 94 of the mature peptide (SEQ ID NO: 2) of RML that is located on the lid hinge. In total, seven variants, which included mutations (or a substitution) to other possible hydrophobic natural amino acids (alanine (A), valine, (V), isoleucine (I), leucine (L), methionine (M), tyrosine (Y), and tryptophan (W)), were generated. It is possible that other hydrophobic amino acids may also be used to substitute phenylalanine (F) at residue 94 of SEQ ID NO: 2. The variants include F94A (SEQ ID NO: 3), F94I (SEQ ID NO: 4), F94L (SEQ ID NO: 5), F94M (SEQ ID NO: 6), F94V (SEQ ID NO: 7), F94W (SEQ ID NO: 8), and F94Y (SEQ ID NO: 9). The lipase variants described herein thus each have a sequence with SEQ ID NO: 2 being the base (or original) sequence with at least one amino acid mutation or substitution of F94 in SEQ ID NO: 2. The expressed lipase variants may contain only the mature peptide.

[0027] The mutations listed above is with respect to the RML mature peptide (SEQ ID NO: 2). The mutations are listed with the first alphabet indicating the single letter abbreviation of the original amino acid residue in SEQ ID NO: 2, the number indicating amino acid position in SEQ ID NO: 2, and the second alphabet indicating the single letter abbreviation of the new amino acid residue.

[0028] The codon optimized sequence (SEQ ID NO:1) for WT RML was synthesised based on its amino acid sequence (Uniprot Identifier P19515) for optimal expression in *Pichia pastoris* (also known as *Komagataella pastoris*). The propeptide region had been modified for improved expression in *Pichia pastoris* by the addition of a linker sequence between the two copies of the propeptide. Mutated RML were amplified from pAO815W-WT RML construct using AOX promoter (SEQ ID NO: 10) and terminator primers (SEQ ID NO: 11) paired with primers designed to substitute the amino acid sequence at the targeted sites (Table 1) and cloned into the pAO815W vector at the HindIII and EcoRI sites. The same forward primer RML F94X_F' (SEQ ID NO: 12) was used for all variants along with different specific reverse primers (SEQ ID NO: 13 to 19). The pAO815W vector was modified from pAO815 with the removal of the HindIII site within the 5'-AOX promoter region and its reintroduction downstream of the 5'-AOX promoter. The resulting pAO815W-RML variant constructs were transformed into *P. pastoris* GS115 by electroporation as described previously (Wu *et al.*,

2004). For the expression of RML variants, cells from a single colony were cultured overnight at 30°C in shake flasks containing buffered glycerol-complex medium (BMGY; 1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10⁻⁵% biotin and 1% glycerol). On the following day, the cells were pelleted and resuspended in equal volumes of buffered methanol-complex medium (BMMY; 1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10⁻⁵% biotin and 0.5% methanol) with OD600 normalized to that of the slowest growing culture, which is between 8-9 per ml of BMMY. For the next three days, methanol (0.5% v/v) was supplemented to the cultures twice daily. On the 6th day, culture media containing the secreted RML were collected and clarified by centrifugation. The protein concentration was determined from Bradford assays using bovine serum albumin as a reference. The RML variants may possibly be expressed in other host expression systems including *Escherichia coli*, *Aspergillus niger*, and *Aspergillus oryzae*. In an embodiment, the host expression system of the RML variants may be *Pichia pastoris* or *Aspergillus oryzae*.

Table 1: List of primers used

Name	Sequence
AOX_Promoter SEQ ID NO: 10	GACTGGTTCCAATTGACAACG
AOX_Terminator SEQ ID NO: 11	GCAAATGGCATTCTGACATCC
RML F94X_F' SEQ ID NO: 12	GTTCCAGTCTCTTACCCTCCAG
RML F94A_R' SEQ ID NO: 13	GAGGGTAAGAGACTGGAACAGCTGTCAAATCAGCGATC CAATTAC
RML F94I_R' SEQ ID NO: 14	GAGGGTAAGAGACTGGAACAATTGTCAAATCAGCGATC CAATTAC
RML F94L_R' SEQ ID NO: 15	GAGGGTAAGAGACTGGAACCAATGTCAAATCAGCGATC CAATTAC
RML F94M_R' SEQ ID NO: 16	GAGGGTAAGAGACTGGAACCATTGTCAAATCAGCGATC CAATTAC

RML F94V_R' SEQ ID NO: 17	GAGGGTAAGAGACTGGAACAACTGTCAAATCAGCGATC CAATTAC
RML F94W_R' SEQ ID NO: 18	GAGGGTAAGAGACTGGAACCCATGTCAAATCAGCGATC CAATTAC
RML F94Y_R' SEQ ID NO: 19	GAGGGTAAGAGACTGGAACGTATGTCAAATCAGCGATC CAATTAC

[0029] All variants showed significant improvements in expression levels in *Pichia pastoris* when cultured in shake flask. Average protein concentration among these variants ranged from the lowest of 18.1 mg/ml for RML F94I to the highest level of 31.9 mg/ml for RML F94A compared to the 12.2 mg/ml of protein for RML wild type (WT) (Figure 1). This represents an improvement of between 48% - 161% in production levels of the variants compared to the RML WT (Figure 1). It is postulated that by substituting different hydrophobic residues at position 94, its interactions with Pro 209 across the substrate binding pocket could have been improved and enhanced the stability of the lid in its closed form. Additionally, in the crystal structure published by Moroz et al. (2019), residue 94 is in interaction with Tyr 77 on the propeptide. Thus, this substitution by residues of differing hydrophobicity could also have changed the interactions between the propeptide and mature protein in a way that facilitated improved folding. With improved structural stability and folding efficiency, this may have reduced ER stress during protein synthesis to promote greater productivity. The improved expression levels of the modified RML lipase is an unexpected technical advantage and effect over the natural RML and other modified RML variants.

[0030] **Lipase activity assay**

[0031] Subsequently, a lipase activity assay was performed with these variants. The specific activity for the different RML variants was determined with different substrates including olive oil, castor oil, glyceryl trioctanoate, tributyrin, C12 methyl ester, C10 methyl ester, C8 methyl ester and vinyl laurate emulsion as substrate. The emulsion was prepared by homogenizing the oil with 4% (w/v) polyvinyl alcohol 30 000 solution in a ratio of 1:3 using a knife homogenizer and used immediately. For the assay, 2 ml of the oil emulsion, 1.5 ml of distilled water and 1 ml of 0.2 M Tris-HCl buffer, pH 8.0 was first added to a 100 ml flat bottom flask and incubated in a 40°C water-bath with shaking at 150 rpm. After 5 min, 500 µl of the enzyme solution (10

µg/ml) was added to the reaction mixture and incubated for a further 15 mins. 5 ml of absolute ethanol was then added to terminate the reaction. The amount of free fatty acid released during the reaction was determined through titration with 50 mM NaOH using phenolphthalein as an indicator.

- 5 [0032] The specific activity (U/mg) of each RML variant was calculated using the following equation:

$$\text{Specific activity (U/mg)} = \frac{(v - v_0) * C}{t * A}$$

v: Volume NaOH required for neutralization of reaction mix (ml)

v₀: Volume of NaOH required for neutralization of blank (ml)

- 10 C: Concentration of NaOH (µmol/ml)

t: Reaction time (min)

A: Amount of RML added (mg)

1U corresponds to 1 µmol of free fatty acid released from the hydrolysis of the substrate in 1 min.

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[0033] RML F94W and RML F94Y were found to have consistent improvements in specific activity across all the eight substrates tested, namely olive oil, castor oil, glyceryl trioctanoate, tributyrin, C12 methyl ester, C10 methyl ester, C8 methyl ester and vinyl laurate. For RML F94W, the improvement in hydrolytic activity was between 58.7% to 185.6% where the smallest increase was seen on glyceryl trioctanoate and the largest increase was on vinyl laurate (Table 2). For RML F94Y, the increase in activity was the smallest on olive oil at 39.6% and the largest on C8 methyl ester at 101.0% (Table 2).

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[0034] As for the other variants, the mutation either maintained or affected the hydrolytic activity in a negative manner (Table 2). The RML F94A and RML F94M variants have mixed results with different substrates having increased and decreased activity relative to the RML WT. It is believed that since residue 94 lies in the region of the substrate binding pocket, it is likely to contribute to interactions with the substrate and could therefore influence the activity and substrate preference of the enzyme.

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[0035] **Table 2:** Specific activity of RML WT and the eight RML F94 variants on eight different substrates measured using lipase activity assay. The values are presented as a relative percentage to the activity of RML WT.

	Relative activity of RML F94 variants (%)							
	Olive oil	Castor oil	Glyceryl Trioctanoate	Tributyrin	C12 Methyl Ester	C10 Methyl Ester	C8 Methyl Ester	Vinyl Laurate
RML WT	100	100	100	100	100	100	100	100
RML F94A	107.6	134.5	103.6	88.4	83.5	127.4	123.0	100.6
RML F94I	64.7	24.1	69.2	40.4	16.1	9.9	79.5	38.1
RML F94L	65.7	45.9	63.3	59.4	16.1	18.4	69.7	36.3
RML F94M	96.6	65.4	100.2	76.3	32.6	54.0	127.7	78.1
RML F94V	81.2	44.4	78.3	61.7	42.2	42.1	86.7	64.0
RML F94W	188.4	264.9	158.7	252.4	237.5	211.4	242.1	286.5
RML F94Y	139.6	163.2	156.9	163.1	178.4	184.9	201.0	182.4

[0036] The RML F94 variants may thus be used to react with an ester to form a reaction product under suitable conditions. The ester may have a carboxylic acid component of 4 carbons or more and may be substituted or unsubstituted. Non-limiting examples of the ester include a fatty acid ester, a vegetable oil, a monoglyceride, a diglyceride, and a triglyceride.

5 Non-limiting examples of a vegetable oil include saturated and unsaturated oils, olive oil, castor oil, palm oil, coconut oil, canola oil, soybean oil, sunflower oil, rapeseed oil, peanut oil and cottonseed oil. Numerous other vegetable oils are also known and may be used. The alcohol component of the ester may be short chained alcohols or glycerol, other alcohols may also be used.

10 [0037] The RML F94 variants described herein show improved production levels of the synthesis of the RML variant enzymes and leads to improved cost efficiency. Some of the variants also exhibit improved activities against certain substrates.

[0038] **Sequences**

[0039] Gene sequence of RML WT mature peptide (SEQ ID NO: 1):

15 TCCATCGACGGAGGTATTAGAGCCGCTACTTCTCAGGAAATCAACGAACTTACTT
ACTATACAACCTTTGTCAGCTAATTCTTACTGTAGAACTGTTATTCCTGGTGCTACT
TGGGATTGCATACATTGTGACGCCACTGAAGATTTAAAGATAATTAACCTGGT
CTACTTTGATTTACGACACTAACGCTATGGTTGCTAGAGGAGATTCCGAGAAGAC
TATTTATATCGTGTTTAGAGGTTCTTCATCTATTTCGTAATTGGATCGCTGATTTGA
20 CATTCGTTCCAGTCTCTTACCCTCCAGTTTCTGGTACTAAGGTTACAAAGGATTT
CTTGATTCTTATGGTGAAGTTCAAACGAGTTGGTTGCTACTGTCTTGGATCAGTT
TAAACAATACCCATCTTATAAGGTTGCTGTCACTGGTCACTCTTTGGGAGGTGCT
ACTGCCTTGCTGTGTGCTTTAGATTTATACCAGAGAGAGGAAGGATTGTCTTCAA
GTAACCTATTCTTGTACACTCAAGGTCAGCCTAGAGTTGGAGATCCAGCATTTC
25 TAATTATGTGGTTTCTACTGGTATTCCATATAGACGTACTGTAAACGAAAGAGAC
ATAGTACCACACTTGCCTCCAGCTGCCTTCGGATTTCTGCATGCCGGTGAAGAGT
ACTGGATCACAGATAATTCTCCTGAAACCGTTCAAGTGTGTACATCTGATTTAGA
GACTTCCGACTGCTCTAACAGTATTGTTCCATTTACTTCAGTTCTTGATCATTGT
CTTATTTTGAATTAACACCGGTTTGTGTACTTAA

30 [0040] RML WT mature peptide (SEQ ID NO: 2):
SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY

DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

5 [0041] RML F94A variant (SEQ ID NO: 3):

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTAVPVSYPPVSGTKVHKGFLDSYGE
VQNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYT
QGQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPE
10 TVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0042] RML F94I variant (SEQ ID NO: 4):

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTIVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
15 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0043] RML F94L variant (SEQ ID NO: 5):

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTLVPVSYPPVSGTKVHKGFLDSYGEV
20 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0044] RML F94M variant (SEQ ID NO: 6):

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTMVPVSYPPVSGTKVHKGFLDSYGE
25 VQNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYT
QGQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPE
TVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0045] RML F94V variant (SEQ ID NO: 7):

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
30 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTVVPVSYPPVSGTKVHKGFLDSYGE

VQNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYT
 QGQPRVGDPAFANYVVSTGIPYRRTVN ERDIVPHLPPAAFGFLHAGEEYWITDNSPE
 TVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0046] RML F94W variant (SEQ ID NO: 8):

5 SIDGGIRAATSQEINELTYTTLSANSYC RTVIPGATWDCIHCDATEDLKIIKTWSTLIY
 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTWVPVSYPPVSGTKVHKGFLDSYGE
 VQNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYT
 QGQPRVGDPAFANYVVSTGIPYRRTVN ERDIVPHLPPAAFGFLHAGEEYWITDNSPE
 TVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

10 [0047] RML F94Y variant (SEQ ID NO: 9):

SIDGGIRAATSQEINELTYTTLSANSYC RTVIPGATWDCIHCDATEDLKIIKTWSTLIY
 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLT YVPVSYPPVSGTKVHKGFLDSYGE
 VQNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYT
 QGQPRVGDPAFANYVVSTGIPYRRTVN ERDIVPHLPPAAFGFLHAGEEYWITDNSPE
 15 TVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

[0048] **References**

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CLAIMS

[Claim 1] A lipase comprising a peptide sequence with at least 80% sequence similarity to SEQ ID NO: 2 and a F94 mutation of SEQ ID NO: 2.

5 [Claim 2] The lipase according to claim 1, wherein the F94 mutation is a substitution of phenylalanine with a hydrophobic amino acid.

[Claim 3] The lipase according to claim 1, wherein the F94 mutation is selected from the group consisting of F94A, F94I, F94L, F94M, F94V, F94W, and F94Y.

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[Claim 4] The lipase according to claim 1, wherein the F94 mutation is selected from the group consisting of F94A, F94M, F94W, and F94Y.

[Claim 5] The lipase according to claim 1, wherein the F94 mutation is F94W or F94Y.

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[Claim 6] The lipase according to any one of claims 1 to 5, wherein the peptide sequence consists essentially of a sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

20 [Claim 7] The lipase according to claim 6, wherein the peptide sequence consists of the sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

[Claim 8] A method of improving expression levels of a *Rhizomucor miehei* lipase of
25 SEQ ID NO: 2, the method comprising introducing a F94 mutation of SEQ ID NO: 2 to produce a *Rhizomucor miehei* lipase variant comprising a peptide sequence with at least 80% similarity to SEQ ID NO: 2 and the F94 mutation of SEQ ID NO: 2; and expressing the *Rhizomucor miehei* lipase variant in a protein production system.

30 [Claim 9] The method according to claim 8, wherein the F94 mutation is a substitution of phenylalanine with a hydrophobic amino acid.

[Claim 10] The method according to claim 8, wherein the F94 mutation is selected from the group consisting of F94A, F94I, F94L, F94M, F94V, F94W, and F94Y.

5 [Claim 11] The method according to claim 8, wherein the F94 mutation is F94W or F94Y.

[Claim 12] The method according to any one of claims 8 to 11, wherein the protein production system comprises one of the following expression hosts: *Pichia pastoris*, *Escherichia coli*, *Aspergillus niger*, and *Aspergillus oryzae*.

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[Claim 13] The method according to claim 12 wherein the protein production system comprises *Pichia pastoris* or *Aspergillus oryzae*.

[Claim 14] A method of hydrolysing an ester, the method comprising providing a mixture
15 comprising the lipase according to any one of claims 1 to 7 and the ester; and forming a reaction product under suitable reaction conditions.

[Claim 15] The method according to claim 14, wherein the ester comprises a carboxylic acid component of 4 carbons or more.

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