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BIBLIOGRAPHIC AND PATENT SEARCH: USE OF ENZYMES IN DETERGENT COMPOSITIONS

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Summary

BIBLIOGRAPHIC AND PATENT SEARCH: USE OF ENZYMES IN DETERGENT COMPOSITIONS

1. Introduction

The **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)** requested a background search regarding the use of enzymes in the production of detergents, with special interest in lipases, with the aim of making those patent and non-patent documents that are part of the state of the art related to this technology available to the Center.

A "STATE OF THE ART" report (Phase I) was sent to the CSIC, which included 93 inventions related to the use of enzymes in the production of detergents.

CSIC reviewed this STATE OF THE ART report (Phase I) and selected 43 patent documents and 14 scientific literature documents for further study. In this study, the particular technical conditions used in the processes described in the selected documents will be analysed, including the enzymes that are used and their conditions.

2. Patent documents

Table 1 shows a summary of the organisms and enzymes described in each patent document, as well as the most important methods and conditions.

Table 1. Enzymes, methods and conditions of each patent document.

	PUBLICATION NUMBER	ENZYME	METHODS / CONDITIONS
1	US2012315689 AA	A lipase in combination with a selected surfactant	(1) Contacting the surface with a lipolytic enzyme and a surfactant. (2) Hydrolyzing fatty acid esters present in the oily stain with the lipolytic enzyme to produce free fatty acids. (3) Solubilizing the free fatty acids produced by the lipolytic enzyme with the surfactant.
2	US2011281324 AA	A polypeptide selected from the group consisting of SriII, ScoIIA, ScoIIB, CefII (lipase enzymatic activity)	Method for cleaning an oily stain on a fabric, comprising contacting the stain with a detergent composition under wash conditions in which the action of the polypeptide on a component of the

			stain facilitates removal of at least a portion of the stain from the fabric. pH range: preferably 5-7. Treatment temperature: 30-40 ° C.
3	WO20041645 A1	CpPelB, CpPelD, CpPelA, Lipase, Cp-Egl, Cbhl, Cbhll, and mannanase.	Lipase, CpPelB, CpPelD, or CpPelA retain activity at 80 °C.The enzymes CpPelD and CpPelA retain activity at pH 10. The treatment is carried out at a temperature between 40 – 80 °C and at a pH between 7.5 and 10.
4	CN108929798 A	Lipase	A new composition: Nitrilotriacetic acid (2 parts), basic zirconium phosphate (4 parts), enzyme (2 parts of lipase), ten carbon alcohol polyoxyethylene ether (3 parts), water (12 parts).
5	CN107338119 A	Lipase	A new composition: Disodium ethylene diamine tetraacetate (15 parts), ammonium perchlorate (13 parts), nitrilotriacetic acid (11 parts), basic zirconium phosphate (16 parts), lipase (22 parts), ten carbon alcohol APEOs (8 parts) and water.
6	US2020205423 AA	A variant polypeptide with lipase activity	Describes a new composition with this polypeptide and at least a second enzyme (See the abstract of the document for more details) pH range: 4-12. Temperature range: 20 °C to 55 °C
7	WO14090573 A1	Protease, lipase or amylase	(1) applying to the substrate a neat or diluted form of the composition. (2) optionally applying to the substrate, a detergent composition. (3) rinsing the substrate.

			pH conditions: 7-10.5
8	US2007202566 AA	Hydrolase	Conditions: temperature 37 °C and pH 4-10.5.
9	WO20009231 A1	Lipase	The polypeptide (lipase) has heat stability at 75 °C - 80°C. The polypeptide has an ability to decompose fats and oils at 15 °C.
10	CN109055044 A	Lipase	Describes a preparation process of a high-efficient cleaning lotion to clean greasy dirt (See the abstract of the document for more details)
11	WO9600292 A1	*Pseudomonas* lipase enzymes	Fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the lipase variant, making a preparation of the lipase variant by separating the lipase variant produced by the micro-organism either from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase either from said broth by physical or chemical methods.
12	US5496490 A	Lipase enzyme (fungus-derived enzyme)	Describes a new composition having significantly improved oily soil removal activity (See the abstract of the document for more details). pH range: 9 to 11.
13	WO9535381 A1	*Pseudomonas* lipases	Describes a process for producing a lipase variant which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the lipase variant, making a preparation of the lipase variant by separating the lipase variant produced by the micro-organism either from the

			fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase either from said broth by physical or chemical methods or purification methods.
14	US2009297495 AA	Hydrolase	The hydrolase specific activity is thermostable or thermotolerant at a temperature in the range from greater than about 37 °C to about 95 °C.
15	US6030933 A	Protease	Describes a detergent composition with a surfactant and: a) from 0.0001% to 2%, by weight of the detergent composition, of one or more enzymes. The enzyme is protease. b) an activated polymer. Each enzyme is immobilized by a covalent binding on the activated polymer and the covalently bound polymer-enzyme does not release more than 5% of any of the starting components of the modified enzyme when exposed to a diluted wash solution of up to pH 11, at a solution temperature up to 90 °C and for at least 30 minutes.
16	US5707950 A	Lipase enzyme variant D96L of the native lipase derived from *Humicola lanuginosa strain D SM 4106* and a protease enzyme	A method for laundering fabrics to maintain whiteness and provide dingy clean up, said method comprising contacting fabrics in need of whiteness and dingy clean-up with an aqueous solution comprising the laundry detergent composition, said aqueous solution comprising the lipase enzyme at a level of from about 150 to about 5000 LU (lipolytic unit) per litre. pH range: 5-9.5

17	WO20046613 A1	Lipolytic enzyme variant	Describes a new composition: One or more calcium ions and/or zinc ions; one or more enzyme stabilizers; from about 0.001% to about 1.0 weight % of said lipolytic enzyme variant(s); one or more bleaching agents; one or more adjunct materials; and/or one or more additional enzymes or enzyme derivatives
18	US5837010 A	Mutated enzyme D96L derived from *Humicola lanuginosa* lipase	Describes a new composition: (a) a mutated enzyme D96L derived from *Humicola lanuginosa* lipase in an amount of 50 to 7500 LU (lipolytic unit) per litre of aqueous solution (b) 10% to 80% of a builder (c) 0.1% to 60% of a surfactant (d) an additional enzyme pH range: 7.5-10.5
19	US4933287 A	Lipolytic enzyme obtained from a bacterial strain selected from *Pseudomonas pseudoalcaligenes* and *Pseudomonas stutzeri*	Conditions: temperature of 60 °C or below and a pH between 7 and 11.
20	WO20238339 A1	Lipase	The concentration of the mutant lipase in the detergent is 10 to 400 U/L. Optimal temperature 40 °C and optimal pH 8.
21	US2012028318 AA	Fungal cutinase	A detergent composition which includes the fungal cutinase cloned from *Magnaporthe grisea* and a non-ionic surfactant. The optimum temperature range for Mgr-C is from about 30 °C to about 40 °C.
22	US2012309063 AA	GeoT1 lipase	The detergent composition is formulated at a pH 8.0 - 10.0 and is effective in

			hydrolyzing a lipid at a temperature of 30 °C – 40 °C.
23	US2012258507 AA	TfuLip2 lipase	The detergent composition is formulated at a pH 8.0 - 10.0 and is effective in hydrolyzing a lipid at a temperature of 30 °C – 40 °C.
24	US2015291944 AA	Lipolytic enzyme variants	Describes a composition with this variant. It could be a laundry detergent composition, a dish detergent composition, or a hard surface cleaning composition and it contains at least one additional enzyme (See the abstract of the document for more details).
25	US2014031272 AA	Alpha/beta hydrolase	Describes a composition which contains the alpha/beta hydrolase. The composition also contains one or more enzymes (See the abstract of the document for more details)
26	US2015203797 AA	Enzyme selected from the group comprising protease, amylase, and lipase	(i)Contacting the textile with a composition comprising about 1 ppb to about 50 ppm of an optical brightener (selected from coumarinic and benzoxazole optical brighteners) having a ClogP from about 1 to about 50, and (ii) rinsing and drying the textile. pH 8-10.5. Temperature 25 °C or below.
27	US2010055085 AA	Hydrolase activity, including lipase activity	The hydrolase specific activity is thermostable or thermotolerant at a temperature in the range from greater than about 100 °C to about 115 °C.
28	JP2077499 A2	Alkaline lipase	Describes a detergent composition containing lipase as an enzyme. (A) 1 to 15% by weight of a non-ionic surfactant having a cloud point of 35 °C

			or less in a 1% by weight aqueous solution. (B) 20 - 70 wt % of Al - Cali - builder 2. (C) Alkaline lipase in which the lipase activity at pH 9 is 30% or more of the lipase activity at pH 7.
29	BRPI9701341 A	Lipases and cellulases	Describes a detergent composition containing lipases and cellulases as an enzyme. (a) 50 to 30000 LU per gram of detergent composition of one or more enzymes of the lipase type (b) 0.3 to 35 S-CEVU (cellulase units) per gram of detergent composition of one or more cellulase-like enzymes; at a pH greater than or equal to 7.
30	JP2077498 A2	Alkaline lipase	Describes a detergent composition containing lipase as an enzyme. A) Non-ionic surfactant: 1 to 15% by weight (B) a non-condensed phosphate-based calcium captured chelating builders 1 to 50% by weight. (C) Alkaline lipase in which the lipase activity at pH = 9 has 30% or more of the lipase activity at pH = 7.
31	JP2008031243 A2	Protease, amylase or lipase	Describes a detergent composition containing lipase, protease or amylase as an enzyme. Comprises Non-ionic surfactant (A) 10% by mass or more of amphoteric surfactant (B), at least one enzyme (C) selected from the group of protease, amylase and lipase
32	WO20178102 A1	2 proteases	Describes a detergent composition containing 2 proteases (See the abstract of the document for more details)

33	JP9249891 A2	Lipase	Describes a detergent composition containing a lipase with excellent oil dirt cleaning power at low temperatures
34	CN108822976 A	Lipase	A preparation method of bio dish washing liquid (6 steps - See the abstract of the document for more details)
35	JP1261499 A2	Lipase	Describes an enzyme-containing detergent which inhibits a decrease in lipase activity due to long-term storage
36	WO20058024 A1	Bacterial lipase derived from *Burkholderia cepacia* or *Psychromonas ingrahamii*	Describes a detergent composition with a bacterial lipase. (i) from 1 to 5 wt.% of a soil release polymer. (ii) from 0.01 to 1 wt.% of a bacterial lipase which is derived from *Burkholderia cepacia* or *Psychromonas ingrahamii* (iii) 8 to 35 wt.% of a surfactant.
37	GB2307695 A1	Protease and lipase	A detergent composition with a protease or lipase. Protease granules in an amount of from 0.1 to 5 wt %, and lipase granules in an amount of from 0.01 to 1 wt %.
38	WO9724426 A1	Hyaluronidase	Describes a detergent composition comprising a hyaluronidase enzyme, which is present at a level of 20 to 200 HU/g composition. This detergent composition also comprises protease (1-5000 PU/g composition), amylase (1-5000 PU/g composition), lipase (1-5000 PU/g composition), cellulase (1-2000 PU/g composition). pH of the solution 7.5-10.5
39	CO4290376 A1	Lipase	Describes a detergent composition including a lipase. (a) from around 1 to 50% by weight of one or more compounds of oily detergents (b) a lipase

			enzyme in an amount effective to remove oily soil from stained fabric; (c) a soil release polymer in an amount effective for removal of oily soil from stained fabric.
40	WO11150157 A2	SgrLip2 lipase	The detergent composition is formulated at a pH of 8.0 - 10.0. and is effective in hydrolyzing a lipid at a temperature of 30 °C – 40 °C.
41	US2012258900 AA	LipA lipase	The detergent composition is formulated at a pH of from 8.0 - 10.0. and is effective in hydrolyzing a lipid at a temperature of 30 °C – 40 °C.
42	EP2987849 A1	Lipid esterase	A detergent composition with lipid esterase and a method of laundering a fabric comprising 4 steps (See the abstract of the document for more details)
43	US2014230155 AA	Lipid esterase	A detergent composition with lipid esterase and a method of laundering a fabric (See the abstract of the document for more details)

Each of the inventions is summarized in detail below, highlighting the microorganisms and enzymes that are involved in the processes, as well as the most important methods and conditions carried out.

1. US2012315689 AA

This document describes compositions and methods related to the removal of oily stains from fabrics and other surfaces using **a lipase in combination with a selected surfactant** to mediate the release of fatty acids generated by the lipase. The compositions and methods have numerous applications, particularly for laundry cleaning, dishwashing, and cleaning other hard surfaces.

The detergent contains (a) a **lipolytic enzyme** for hydrolyzing fatty acid esters present in the oily stain to produce free fatty acids, and (b) a surfactant for solubilizing the free fatty acids in the cleaning composition, thereby releasing the free fatty acids from the stain, wherein the amount of release of fatty acids from the stain is greater than that achieved using an equivalent composition lacking the surfactant.

To enhance the ability of lipases to affect the removal of lipid stains from fabrics, a series of experiments were performed to test the ability of surfactants to remove fatty acids produced by the hydrolysis of triglycerides by a lipase. Surprisingly, of the numerous surfactants tested, only a selected surfactant was effective in mediating the release of fatty acids from fabric, suggesting that the selection of a suitable surfactant is not a straightforward matter.

Generally, the most effective surfactants had a relatively small hydrophilic portion with no net charge. The preferred hydrophobic portions were linear, saturated, and/or included an aliphatic hydrophobic portion. The best surfactants tended to be sugar-based compounds and zwitterionic compounds.

The surfactant is selected from the group consisting of a Triton or oxide non-ionic surfactant, a zwitterionic surfactant, a FOS-choline surfactant or a sulfobetaine surfactant.

The present compositions include one or more lipolytic enzymes (triacylglycerol acylhydrolases, E.C. 3.1.1.3, cutinases (E.C. 3.1.1.50) for use in combination with one or more adjuvants. Lipases include wild- type lipases and variant lipases, including fragments, having lipase activity.

The method for removing an oily stain from a surface, comprising:

- contacting the surface with a lipolytic enzyme and a surfactant.
- hydrolyzing fatty acid esters present in the oily stain with the lipolytic enzyme to produce free fatty acids.
- solubilizing the free fatty acids produced by the lipolytic enzyme with the surfactant.
- removing the oily stain from the surface.

2. US2011281324 AA

The document describes a detergent composition comprising a **polypeptide** selected from the group consisting of SriII, ScoIIA, ScoIIB, CefII (SEQ ID NO. 1 – See Annex I), and a variant, thereof, wherein the detergent composition exhibits improved cleaning of an oily stain compared to an equivalent detergent composition lacking the polypeptide.

The polypeptide has **lipase enzymatic activity** and at least one additional activity selected from phospholipase, lysophospholipase, and acyltransferase activity.

The composition comprises at least one additional polypeptide selected from the group consisting of a protease, an amylase, a cellulase, a laccase, a lipase, a phospholipase, a lysophospholipase, an acyltransferase, a perhydrolase, and an arylesterase.

The composition comprises at least one surfactant.

The document also describes a method for cleaning an oily stain on a fabric, comprising contacting the stain with a detergent composition under wash conditions in which the polypeptide is enzymatically active, wherein catalytic action of the polypeptide on a component of the stain facilitates removal of at least a portion of the stain from the fabric.

3. WO20041645 A1

The document describes an antibiotic free transgenic plant expressing a heterologous enzyme which retains enzymatic function in crude extracts obtained from said plant. The enzyme is selected from one or more of CpPelB, CpPelD, CpPelA, Lipase, Cp-Egl, Cbhl, Cbhll, or mannanase.

The enzymes Lipase, CpPelB, CpPelD, or CpPelA retain activity at 80 °C. The enzymes CpPelD and CpPelA retain activity at pH 10.

The document also provides a detergent composition comprising lipase which is thermostable and retains activity during hot water washing.

It also been described a method for treating cellulosic material, wherein the method comprises reacting the cellulosic material with one or more enzymes. The cellulosic material could be textile material.

The cellulosic material is textile material, plants used in animal feed, or wood-derived pulp, fruit derived pulp, or secondary fiber.

The treatment is carried out at a temperature between 40 – 80 °C and at a pH between 7.5 and 10.

4. CN108929798 A

The present document describes a kind of novel oil stain detergent, characterized because it includes (according to parts by weight): nitrilotriacetic acid 1-3 (2 parts), basic zirconium phosphate 2-5 (4 parts), enzyme 1-3 (2 parts of **lipase**), ten carbon alcohol polyoxyethylene ether 2-4 (3 parts), water 10-15 (12 parts).

This new detergent is useful for cleaning oil stains.

5. CN107338119 A

This document describes a kind of grease dirt cleaning agent. Its composition is disodium ethylene diamine tetraacetate (15 parts), ammonium perchlorate (13 parts), nitrilotriacetic acid (11 parts), basic zirconium phosphate (16 parts), **lipase** (22 parts), ten carbon alcohol APEOs (8 parts) and water.

The basic zirconium phosphate is bedded zirconium phosphate and the lipase is produced by fermentation.

The grease particles are easily washed scattered, that is, makes grease be transformed to soft dirt by hard scale.

The cleaning agent of the invention can suppress bacterium grow and protect the health of people.

6. US2020205423 AA

The document describes a variant polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 (See Annex I), and the variant polypeptide has **lipase activity**.

This variant polypeptide comprises an amino acid substitution is selected from the group consisting of: Y23A, K33N, S82T, S83D, S83H, S83I, S83N, S83R, S83T, S83Y, S84S, S84N, 84'Y, 84'L, 84'S, I85A, I85C, I85F, I85H, I85L, I85M, I85P, I85S, I85T, I85V, I85Y, K160N, P199I, P199V, I254A, I254C, I254E, I254F, I254G, I254L, I254M, I254N, I254R, I254S, I2454V, I254W, I254Y, I255A, I255L, A256D, L258A, L258D; L258E, L258G, L258H, L258N, L258Q, L258R, L258S, L258T, L258V, D263G, D263K, D263P, D263R, D263S; T264A, T264D, T264G, T264I, T264L, T264N, T264S, D265A, D265G, D265K, D265L, D265N, D265S, D265T, T268A, T268G, T268K, T268L, T268N, T268S, D308A, and Y311E.

The document also describes a composition comprising the variant polypeptide. This composition also comprises at least a **second enzyme**. The second enzyme is selected from the group consisting of: a second lipase, an amylase, a xylanase, a protease, a cellulase, a glucoamylase, an oxidoreductase, a phospholipase and a cyclodextrin glucanotransferase.

The composition comprising the variant polypeptide as disclosed herein further comprise a carrier (wheat flour), a stabilizer (calcium acetate, calcium chloride, magnesium chloride, sodium chloride, sodium sulphate, guar gum), a buffer (calcium acetate, sodium acetate, sodium citrate, sodium phosphate, potassium phosphate), a preservative (calcium acetate, sodium acetate, sodium propionate, calcium propionate, propionic acid, potassium sorbate, sorbic acid, sodium benzoate, benzoic acid, acetic acid), or any combination thereof.

7. WO14090573 A1

This document provides a cleaning composition comprising:

(i) hydrogen peroxide or a peroxygen bleach compound capable of yielding hydrogen peroxide in aqueous solution.

(ii) a first bleach activator comprising a precursor of peracetic acid (tetraacetylethylenediamine).

(iii) a second bleach activator comprising a precursor of C8 to Cn. The second bleach activator is hydroxybenzoic acid derivative of the formula I (**Figure 1**).

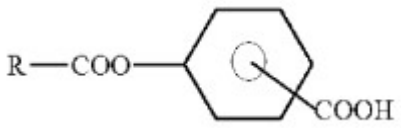


Figure 1. General structure of the second bleach activator, a derivative of hydroxybenzoic acid.

In this formula I, R is C8-Cn alkyl group.

The ratio of first bleach activator to second bleach activator is 5:1 to 1 :5.

The composition comprises:

(i) a **protease**.

(ii) a **lipase or an amylase** or mixtures thereof.

The composition is a granular detergent composition. Such detergent composition may be prepared by mixing and granulating process, for example, using a high-speed mixer or granulator, or other non- tower process.

This document also provides a method of bleaching a bleachable substrate comprising the steps of:

(i) applying to the substrate a neat or diluted form of the composition.

(ii) optionally applying to the substrate, a detergent composition.

(iii) rinsing the substrate. Use of a cleaning composition for bleaching fruity or sebum stains.

8. US2007202566 AA

This document describes an isolated, synthetic or recombinant nucleic acid which encodes at least one polypeptide having a **hydrolase activity** or the nucleic acid encodes an antigen for generating an antibody which binds specifically to a hydrolase or a hydrolase active site. The hydrolase activity comprises a **specific activity at about 37 °C**. The polypeptide retains a hydrolase activity under conditions comprising about **pH 4- 10.5**.

Optionally the hydrolase activity comprises a lipase activity, a protease activity, an esterase activity or a phospholipase activity.

The document also describes a detergent composition comprising the polypeptide with the hydrolase activity, wherein optionally the hydrolase is a no surface-active hydrolase or a surface-active hydrolase, or optionally the hydrolase is formulated in a non-aqueous liquid composition, a cast solid, a granular form, a particulate form, a compressed tablet, a gel form, a paste, or a slurry form.

9. WO20009231 A1

The lipase according to the present disclosure comprises a polypeptide comprising an amino acid sequence, or a polypeptide encoded by a nucleotide sequence (SEQ ID NO:3-See Annex I). Is a polypeptide derived from ***Burkholderia arboris***.

The biological activity of the polypeptide is an ability associated with assimilation of the trans-fatty acid-containing fat or oil, or an ability to degrade the trans-fatty acid-containing fat or oil. 45 to 70 °C is an optimum temperature for decomposing oils and fats. **The polypeptide has heat stability at 75 °C or higher, preferably at 80 °C or lower. The polypeptide has an ability to decompose fats and oils at 15 °C.**

The use of the **lipase** of the present disclosure facilitates the treatment of trans fatty acid-containing fats and oils, which were conventionally difficult to remove. In addition, fats and oils can be removed and oils can be treated in a high-temperature environment or a low-temperature environment, which has been difficult to use with conventional microorganisms and enzymes. Therefore, the novel lipase of the present disclosure can be used for detergents, leather industry, food industry, purification of environmental pollution by fats and oils, garbage treatment, composting treatment, waste treatment and composting such as wastewater treatment, pharmaceuticals such as digestive agents and oily substances.

10. CN109055044 A

The document describes a preparation process of a high efficiency cleaning lotion to clean greasy dirt.

Step 1, according to the mass fraction, take 0.4-1.2 parts of barium sulphate, 1.2-2.5 parts of talcum powder 1.5-2.5 parts of konilite porous, 0.5-1.2 parts of calcium sulphate, 0.2-0.7 parts of diatomite and 0.1-0.2 parts of zinc oxide. Calcination time 2-5h, temperature 950-1050 °C and pressure 200-300 MPa. The mix is ground after cooling. It is ground to 100-300nm of average grain diameter, obtaining nanometer abrasive material.

Step 2, according to the mass fraction, by nanometer abrasive, 9-18 parts of sodium carboxymethyl starch, 15-31 parts of trichloro ethylene, 14- 20 parts of carboxylic acid, 15-25 parts of **lipase** (carboxy-esterase), 12-30 parts of tartaric acid, 14-24 parts of ethyl alcohol, 18-30 parts of glycerol, 70- 140 parts of deionized water are uniformly mixed.

11. WO9600292 A1

The document relates to variants of ***Pseudomonas* lipase enzymes**. The lipase to be used as parent *Pseudomonas* lipase or starting material in the present invention for the modification by means of recombinant DNA techniques, is preferably chosen from ***Pseudomonas glumae*** or ***Pseudomonas pseudoalcaligenes***. When used in certain detergent compositions, these *Pseudomonas* lipases may exhibit some "in-the-wash" effects.

The lipase variant of a parent *Pseudomona*s lipase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved. The electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by uncharged amino acid residues. The less hydrophobic amino acid residues are selected from the group consisting of glycine, serine, alanine, aspartic acid, and threonine.

The modified residues are located at one or more of the following positions in the amino acid sequence of the *Pseudomonas glumae* lipase, or the corresponding amino acids of a different lipase: 4, 8, 22, 43, 70, 165, 177, 196, 244, 277, 296, 298, 316.

The lipase variant is a variant of *Pseudomonas glumae* lipase and comprises one or more of the following mutations: Y4D, K22D, K43D, K70E, R165E, R177D, Y196D, T244E, I277E, T296D, A298D, L316E and is a variant of *Pseudomonas pseudoalcaligenes* lipase and comprises one or more of the following mutations: Y8D, K12D, R41N, K195D, I247E, L286E.

A process for producing a lipase variant which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the lipase variant, making a preparation of the lipase variant by separating the lipase variant produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase either from said broth or from said cells by physical or chemical concentration or purification methods.

12. US5496490 A

This document describes a particulate laundry detergent composition having significantly improved oily soil removal activity comprising, by weight,

(a) from about 1% to 50% of one or more detergent compounds selected from the group consisting of anionic sulphated or sulfonated detergents and C10-C18 alcohol ethylene oxide condensate non-ionic detergents and mixtures thereof.

(b) 0.1% to 1.0% of a **lipase enzyme (fungus-derived enzyme)** in an amount effective for oily soil removal from stained fabric. It has an activity of about 100,000 units of lipase per gram of enzyme.

(c) 0.5% to 10% of a soil release copolymer of polyethylene terephthalate (PET) and polyoxyethylene terephthalate (POET) having a molecular weight in the range of about 15,000 to 50,000 wherein the polyoxyethylene (POET) is of a molecular weight in the range of about 1,000 to 10,000 and the molar ratio of PET to POET units is from 2:1 to 6:1, in an amount effective for oily soil removal from stained fabric.

(d) from about 10% to 75% of a water soluble or water insoluble, inorganic or organic builder for said detergent compound.

Said laundry detergent composition being capable of removing a variety of oily soils from fabric to an extent greater than the additive soil removing effects measured with comparative compositions containing components (a), (b) and (d) or (a), (c) and (d) respectively, each of said comparative compositions being devoid of any combination of said lipase enzyme and said soil release copolymer.

13. WO9535381 A1

The document describes a lipase variant of a parent ***Pseudomonas* lipase** (*Pseudomonas glumae* lipase or a *Pseudomonas pseudoalcaligenes* lipase), wherein the amino acid sequence has been modified in such way that the hydrophobicity at the surface of the enzyme has been increased. The hydrophobicity has been increased by replacing one or more amino acid residues by amino acid residues selected from the group consisting of valine, leucine, isoleucine, phenylalanine, tryptophan and methionine.

The amino acid sequence has been modified in such way that in addition to the increase in hydrophobicity at the surface, one or more positive charges have been introduced. The positive charges have been introduced by introduction of one or more lysine or arginine residues.

One or more of the following modifications have been affected in the amino acid sequence of the *Pseudomonas glumae* lipase, or the corresponding positions in a different lipase: F23R, T129Y, L134R, T148V, T233R, L234R, V239F, T240L, H282R, L292F.

The document also describes a process for producing a lipase variant which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the lipase variant, making a preparation of the lipase variant by separating the lipase variant produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase either from said broth or from said cells by physical or chemical concentration or purification methods.

14. US2009297495 AA

The document describes an isolated, synthetic, or recombinant nucleic acid which encodes at least one polypeptide having a **hydrolase activity**. It also comprises a **lipase activity, a protease activity, an esterase activity or a phospholipase activity** (See Figure 30 of the attached document 14-US2009297495 AA which describes the selected characteristics of exemplary nucleic acids and polypeptides of the invention).

The hydrolase activity is thermostable or thermotolerant. The document also describes a method of increasing thermotolerance or thermostability of a hydrolase polypeptide, the method comprising:

(a) glycosylating a hydrolase polypeptide

(b) the method of (a), wherein the hydrolase specific activity is thermostable or thermotolerant at a temperature in the range from greater than **about 37 °C to about 95 °C.**

15. US6030933 A

The document describes a detergent composition comprising a surfactant and:

a) from 0.0001% to 2%, by weight of the detergent composition, of one or more enzymes. The enzyme is **protease**.

b) an activated polymer, wherein the polymer has a water soluble of at least about 7.10-10 Mol/l. The polymer is polyethylene glycol. The molecular weight of the polymer is from about 5 kD to about 20 kD.

Each enzyme is immobilized by a covalent binding on the activated polymer and wherein the covalently bound polymer-enzyme does not release more than 5% of any of the starting components of the modified enzyme when exposed to a diluted wash solution of up to pH 11, at a solution temperature up to 90 °C and for at least 30 minutes.

16. US5707950 A

The document describes a laundry detergent composition comprising:

(a) from about 0.001% to about 2% of a **lipase enzyme** variant D96L of the native lipase derived from *Humicola lanuginosa* strain D SM 4106. This lipase is available from Amano Pharmaceutical Co. Ltd., Nagoya, Japan, under the trade name Lipase P "Amano," hereinafter referred to as "Amano-P". Lipases include M1 LipaseR and LipomaxR (Gist-Brocades) and LipolaseR (Novo).

(b) a protease enzyme selected from the group consisting of the *Bacillus lentus subtilisin* variant N76D/S103A/V104I according to the numbering of *Bacillus amyloliquefaciens subtilisin* at a level to provide from 0.005 to 0.1 AU per gram of composition (Suitable commercial protealytic enzymes which may be considered for inclusion in the present invention compositions include Alcalase®, Esperase®, Durazym®, Savinase®, Maxatase®, Maxacal®, and Maxapem® 15 (protein engineered Maxacal); Purafect® and subtilisin BPN and BPN').

(c) from about 0.1% to about 60% of a surfactant.

The document also provides a method for laundering fabrics to maintain whiteness and provide dingy clean-up, said method comprising contacting fabrics in need of whiteness and dingy clean-up with an aqueous solution comprising the laundry detergent composition, said aqueous solution comprising the lipase enzyme at a level of from about 150 to about 5000 LU per litre.

17. WO20046613 A1

This document describes a **lipolytic enzyme** variant or active fragment which has an improved performance relative to the parent lipolytic enzyme, wherein the improved performance is selected from the group consisting of an improved wash performance (at a low temperature.), a decreased malodor, an increased detergent stability, an increased thermostability, an increased calcium ion binding stability, an increased protease stability, and any one combination thereof.

The variant or active fragment has a **protease stability** that is greater that the protease stability of the parent lipolytic enzyme.

This composition further comprises one or more calcium ions and/or zinc ions; one or more enzyme stabilizers; from about 0.001% to about 1.0 weight % of said lipolytic enzyme variant(s); one or more bleaching agents; one or more adjunct materials; and/or **one or more additional enzymes** or enzyme derivatives selected from the group consisting of acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, DNase or nuclease, endo-beta-l, 4-glucanases, endo-beta- mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, lysozymes, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, nucleases, additional serine proteases, and combinations thereof.

This document also describes a detergent composition selected from a laundry detergent, a fabric softening detergent, a dishwashing detergent, and a hard- surface cleaning detergent.

18. US5837010 A

This document describes a method of improving the whiteness of a fabric and preventing the dinginess build up on a fabric by preventing redeposition of removed soils in solution from reattaching to the fabric by contacting the fabric to be cleaned with a composition comprising the following:

(a) a mutated enzyme D96L derived from ***Humicola lanuginosa*** **lipase** in an amount of 50 to 7500 LU per litre of aqueous solution (See Annex I – SEQ ID NO: 4)

(b) 10% to 80% of a builder

(c) 0.1% to 60% of a surfactant

(d) an **additional enzyme** selected from the group consisting of xylanase, protease, amylase peroxidase, cellulase, and mixtures thereof.

19. US4933287 A

The document describes an enzymatic detergent additive consisting essentially of a **lipolytic enzyme** obtained from a bacterial strain selected from ***Pseudomonas pseudoalcaligenes*** **and** ***Pseudomonas stutzeri*** and customary ingredients. Said enzyme being further characterized by:

(a) a **pH optimum in the range of 8 to 10.5**.

(b) exhibiting effective lipase activity in an aqueous solution containing a detergent at a concentration up to 10 g/l of solution under washing conditions at **a temperature of 60 °C** or below and at a pH between 7 and 11.

The enzymatic detergent also contains an **amylolytic enzyme**.

20. WO20238339 A1

This document describes a **lipase (Lipase, EC 3.1.1.3) with stability, especially thermal stability and strong pH stability under alkaline conditions**.

The document also describes a detergent characterized in that it contains the lipase mutant, a surfactant and a buffer. The surfactant can be sodium dodecyl sulphate (SDS), sodium ethoxylated alkyl sulphate (AES), fatty alcohol polyoxyethylene Ether (AEO-9) and/or Disodium Lauryl Sulfosuccinate (DLS).

The buffer can be a phosphate buffer, a Tris buffer, a citrate buffer or a sodium carbonate buffer.

The concentration of the mutant lipase in the detergent is 10 to 400 U/L.

21. US2012028318 AA

The document describes a **fungal cutinase cloned from *Magnaporthe grisea***, also known as *Pyricularia grisea* or rice blast fungus.

It describes a recombinant Mgr-C polypeptide having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO: 5 (See Annex I). The recombinant Mgr-C polypeptide is expressed in *Trichoderma reesei* as a secreted polypeptide.

The document also describes a detergent composition which comprises the polypeptide and a non-ionic surfactant (ethoxylate surfactant). This detergent is used to hydrolyze a lipid present in a soil or stain on a surface.

The optimum temperature range for Mgr-C is from about 30 °C to about 40 °C, although the enzyme clearly has significant activity outside this range.

22. US2012309063 AA

The document describes a detergent composition, comprising a **lipase (GeoT1 lipase)** from ***Geobacillus stearothermophilus***, and a surfactant, wherein the detergent composition is more effective in removing oily stains from a surface to be cleaned than the detergent composition in the absence of the lipase

The GeoT1 lipase is a fusion protein comprising a BCE103 cellulase amino-terminal fragment (See Annex I – SEQ ID NO:6).

The surfactant comprises one or more surfactants selected from the group consisting of sodium dodecyl benzene sulfonate, sodium hydrogenated cocoate, sodium laureth sulphate, C12-14 pareth-7, C12-15 pareth-7, sodium C12-15 pareth sulphate, and C14-15 pareth-4.

The detergent composition also comprises a subtilisin protease.

The detergent composition is formulated at a **pH of from about 8.0 to about 10.0** and is effective in hydrolyzing a lipid at a temperature of from about **30 °C to about 40 °C**.

23. US2012258507 AA

The document describes a detergent composition, comprising: a **lipase (TfuLip2 lipase)** from ***Thermobifida fusca*** and a surfactant, wherein the detergent composition is more effective in removing oily stains from a surface to be cleaned than an equivalent detergent composition lacking the lipase (See Annex I – SEQ ID NO:7)

The surfactant comprises one or more surfactants selected from the group consisting of sodium dodecyl benzene sulfonate, sodium hydrogenated cocoate, sodium laureth sulphate, C12-14 pareth-7, C12-15 pareth-7, sodium C12-15 pareth sulphate, and C14-15 pareth-4.

The detergent composition also comprises a subtilisin protease.

The detergent composition is formulated at a **pH of from about 8.0 to about 10.0** and is effective in hydrolyzing a lipid at a temperature of from about **30 °C to about 40 °C**.

24. US2015291944 AA

The document describes lipolytic enzyme variants having one or more modifications, such as a substitution, as compared to a parent lipolytic enzyme. This can be achieved by making improvements to the enzyme by improving wash performance in standard detergent formulations and low surfactant detergent formulations, stability of the enzyme in detergent compositions, thermostability of the enzyme, substrate hydrolysis, expression and/or modified charge/hydrophobicity profiles that improve effectiveness of the enzyme in a wash cycle.

It describes a **lipolytic enzyme variant** or an active fragment thereof comprising an amino acid modification to a parent lipolytic enzyme, wherein the modification could be:

a) a position wherein the minimum performance indices (PI) relative to TLL parent for expression, CS-61 micro-swatch activity at pH 8.2, activity on p-Nitrophenyl ester substrates at pH 6 or pH 8.2, and detergent stability, LAS stability or thermostability are greater than or equal to 0.9, and in addition have a PI for any one of these tests that is greater than or equal to 1.0.

b) a position wherein the minimum performance indices (PI) relative to TLL parent for expression, CS-61 micro-swatch activity at pH 8.2, activity on p-Nitrophenyl ester substrates at pH 6 or pH 8.2, and detergent stability, LAS stability or thermostability are greater than or equal to 0.8, and in addition have a PI for any one of these tests that is greater than or equal to 1.2.

c) a position wherein the minimum performance indices (PI) relative to TLL parent for expression, CS-61 micro-swatch activity at pH 8.2, activity on p-Nitrophenyl ester substrates at pH 6 or pH 8.2, and detergent stability, LAS stability or thermostability are greater than or equal to 0.5, and in addition have a PI for any one of these tests that is greater than or equal to 1.5.

The original sequence is ***Thermomyces lanuginosus*** **lipase TLL** (SEQ ID NO: 8- See Annex I).

The document also describes a cleaning composition comprising at least one lipolytic enzyme variant. It could be a laundry detergent composition, a dish detergent composition, or a hard surface cleaning composition and also it contains **at least one additional enzyme** selected from the group consisting of hemicellulases, cellulases, peroxidases, lipolytic enzymes, metallolipolytic enzymes, xylanases, lipases, phospholipases, esterases, perhydrolases, cutinases, pectinases, pectate lyases, mannanases, keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, β-glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases, and amylases.

25. US2014031272 AA

The document describes a composition comprising:

(a) a lipolytic enzyme belongs to an **alpha/beta hydrolase** superfamily selected from the group consisting of abH23, abH25, and abH15. Is **obtained from a filamentous fungus**.

(b) a hydrophobin having the general formula (I):

(Y1)n-B1-(X1)a-B2-(X2)b-B3-(X3)c-B4-(X4)d-B5-(X5)e-B6-(X6)f-B7-(X7)g-B8-(Y2)m (I)

wherein:

m and n are independently 0 to 2000:

B1, B2, B3, B4, B5, B6, B7 and B8 are each independently amino acids selected from Cys, Leu, Ala, Pro, Ser, Thr, Met or Gly, at least 6 of the residues B1 through B8 being Cys;

X1, X2, X3, X4, X5, X6, X7, Y1 and Y2 independently represent any amino acid:

a is 1 to 50;

b is 0 to 5;

c is 1 to 100;

d is 1 to 100;

e is 1 to 50;

f is 0 to 5; and

g is 1 to 100.

The hydrophobin is a Class II hydrophobin.

The composition also contains **one or more enzymes** selected from the group consisting of a protease, an amylase, a glucoamylase, a maltogenic amylase, a non-maltogenic amylase, a lipase, a cutinase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, a laccase, and a peroxidase, and one or more surfactants.

26. US2015203797 AA

This document describes a method of treating a visibly distinct oily stain on a textile surface, the method comprising the steps of: (i) contacting the textile with a composition comprising about 1 ppb to about 50 ppm of an optical

brightener (selected from coumarinic and benzoxazole optical brighteners) having a ClogP from about 1 to about 50, and ii) rinsing and drying the textile.

The textile surface is contacted with the composition at a temperature of about 30° C, or about 25° C or below.

The composition contacting the textile surface is an aqueous wash liquor or an aqueous rinse liquor in a domestic treatment process. It additionally comprises from about 0.0001 to about 0.5 g/l an optionally substituted alkyl phthalimide.

The composition additionally comprises an **enzyme selected** from the group comprising **protease, amylase, and lipase** enzymes and mixtures thereof.

Proteases selected:

(a) subtilisins (EC 3.4.21.62), including those derived from Bacillus, such as Bacillus lentus, B. alkalophilus, B. subtilis, B. amyloliquefaciens, Bacillus pumilus and Bacillus gibsonii described in U.S. Pat. No. 6,312,936 B1, U.S. Pat. No. 5,679,630, U.S. Pat. No. 4,760,025, U.S. Pat. No. 7,262,042 and WO09/021867.

(b) trypsin-type or chymotrypsin-type proteases, such as trypsin (e.g., of porcine or bovine origin), including the Fusarium protease described in WO 89/06270 and the chymotrypsin proteases derived from Cellumonas described in WO 05/052161 and WO 05/052146.

(c) metalloproteases, including those derived from Bacillus amyloliquefaciens described in WO 07/044993A2.

Amylases selected:

(a) the variants described in WO 94/02597, WO 94/18314, WO96/23874 and WO 97/43424, especially the variants with substitutions in one or more of the following positions versus the enzyme listed as SEQ ID No. 2 in WO 96/23874: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

(b) the variants described in U.S. Pat. No. 5,856,164 and WO99/23211, WO 96/23873, WO00/60060 and WO 06/002643, especially the variants with one or more substitutions in the following positions versus the AA560 enzyme listed as SEQ ID No. 12 in WO 06/002643:

26, 30, 33, 82, 37, 106, 118, 128, 133, 149, 150, 160, 178, 182, 186, 193, 203, 214, 231, 256, 257, 258, 269, 270, 272, 283, 295, 296, 298, 299, 303, 304, 305, 311, 314, 315, 318, 319, 339, 345, 361, 378, 383, 419, 421, 437, 441, 444, 445, 446, 447, 450, 461, 471, 482, 484, preferably that also contain the deletions of D183* and G184*.

(c) variants exhibiting at least 90% identity with SEQ ID No. 4 in WO06/002643, the wild-type enzyme from Bacillus SP722, especially variants with deletions in the 183 and 184 positions and variants described in WO 00/60060, which is incorporated herein by reference.

(d) variants exhibiting at least 95% identity with the wild-type enzyme from Bacillus sp.707 (SEQ ID NO:7 in U.S. Pat. No. 6,093, 562), especially those comprising one or more of the following mutations M202, M208, S255, R172, and/or M261. Preferably said amylase comprises one or more of M202L, M202V, M202S, M202T, M202I, M202Q, M202W, S255N and/or R172Q. Particularly preferred are those comprising the M202 L or M202T mutations.

(e) variants described in WO 09/149130, preferably those exhibiting at least 90% identity with SEQ ID NO: 1 or SEQ ID NO:2 in WO 09/149130, the wild-type enzyme from *Geobacillus stearophermophilus* or a truncated version thereof;

(f) variants as described in EP2540825 and EP2357220;(g) variants as described in WO2009100102 and WO2010115028.

Lipase of the document: US PA 2009/0217464 (See Annex I – SEQ ID NO:9)

The composition additionally comprises from about 1 ppb to about 1000 ppm of a dye transfer inhibitor selected from the group comprising polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinyloxazolidones and polyvinylimidazoles and mixtures thereof.

The composition is formed by mixing a detergent composition with water.

27. US2010055085 AA

This document describes polypeptides having **hydrolase activity**, including **lipase activity**. In one aspect, provided herein are novel classes of lipases termed “saturases”, “palmitases” and “stearatases”. Also provided are polynucleotides encoding polypeptides having saturase, e.g., palmitase and/or stearatase activity, and methods of making and using these polynucleotides and polypeptides. In one aspect, provided herein are polypeptides, e.g., enzymes, having a hydrolase activity, e.g., lipase, saturase, palmitase and/or stearatase activity having thermostable and/or thermotolerant enzyme (catalytic) activity. The enzymatic activities of the polypeptides and peptides as provided herein include (comprise or consist of) a saturase activity or a lipase activity, including hydrolysis of lipids, acidolysis reactions (e.g., to replace an esterified fatty acid with a free fatty acid), transesterification reactions (e.g., exchange of fatty acids between triacylglycerides), ester synthesis, ester interchange reactions and lipid acyl hydrolase (LAH) activity.

See the SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, or SEQ ID NO:19 of the attached document (27-US2010055085 AA).

The **hydrolase specific activity is thermostable or thermotolerant at a temperature in the range from greater than about 100 °C to about 115 °C**.

The hydrolase is a no surface-active hydrolase or a surface-active hydrolase, or optionally the hydrolase is formulated in a non-aqueous liquid composition, a cast solid, a granular form, a particulate form, a compressed tablet, a gel form, a powder, a gel, a hydrogel, a liposome, an aerosol, a paste, or a slurry form.

28. JP2077499 A2

The present document relates to an automatic dishwashing detergent composition containing lipase as an enzyme.

The detergent composition is characterized by containing the following components:

(A) 1 to 15% by weight of a non-ionic surfactant having a cloud point of 35 °C or less in a 1% by weight aqueous solution.

(B) 20 - 70 wt % of Al - Cali - builder 2.

(C) **Alkaline lipase** in which the lipase activity at pH = 9 is 30% or more of the lipase activity at pH = 7.

To the detergent composition of the present invention, in addition to the above essential components, various optional components can be added as necessary.

As optional components, an anionic surfactant other than the component (a), a non-ionic surfactant, a surfactant such as a cationic surfactant, a zeolite, a water-soluble calcium-free calcium chelate builder; bleaching agents such as enzymes, hypochlorite and percarbonate; reducing agents such as sulphite; anti-fouling agent such as carboxymethyl cellulose recolour; defoaming agent such as silicone; inorganic substance such as white carbon; filler such as Glauber's salt etc.; additives such as perfume or pigment.

29. BRPI9701341 A

The document describes a laundry detergent composition, preferably of cotton, characterized in that it comprises at least one blend of:

(a) 50 to 30000 LU (units of lipases) per gram of detergent composition of one or more enzymes of the **lipase** type

(b) 0.3 to 35 S-CEVU (cellulase units) per gram of detergent composition of one or more **cellulase**-like enzymes; at a pH greater than or equal to 7.

This composition also comprises surfactants.

This document also provides a process for washing fabrics, preferably cotton.

Commercially available lipase preparations suitable for example of the present invention are: Lipolase (Novo Nordisk A / S), Lipolase Ultra (Novo Nordisk A / S), Lipomax (Genencor Inc.).

Suitable cellulases in the present invention are for example those commercially available: Carezyme (Novo Nordisk A / S), Endolase (Novo Nordisk A / S), Celluzyme (Novo Nordisk A / S) and Clazinase (Genencor Inc).

30. JP2077498 A2

The present invention is characterized by containing the following components A) Non-ionic surfactant: 1 to 15% by weight (B) a non-condensed phosphate-based calcium captured chelating builders: 1 to 50% by weight (C) **alkaline lipase** in which the lipase activity at pH = 9 has 30% or more of the lipase activity at pH = 7.

The non-ionic surfactant used as component (A) can be appropriately selected from conventionally known non-ionic surfactants, but it is desirable that it is low foaming property, and 1 wt % a non-ionic surfactant having an intersection point in an aqueous solution of 35 °C or less, preferably 25 °C or less is suitable.

Examples of non-phosphate type calcium capturing chelate builder used as component (b) in the present invention include aminopolyacetate salts such as nitrilotriacetate and ethylenediamine tetraacetate; citrate; a polyvalent carboxylate such as a cohadate salt; a polyacrylate, a hydroxypolyacrylate, a polyacetal carboxylate, a salt of a copolymer of acrylic acid and maleic anhydride, a combination of maleic anhydride and methyl vinyl ether, a salt of a polymer, a salt of a copolymer of maleic anhydride and an olefin, a polymer electrolyte such as a salt of a copolymer of acrylic acid and methacrylic acid, which may be used alone , and they may be mixed in combination of two or more.

31. JP2008031243 A2

The document describes a detergent composition which comprises non-ionic surfactant (A) 10% by mass or more, amphoteric surfactant (B), at least one enzyme (C) selected from the group of **protease, amylase and lipase**, and the following general formula I (**Figure 2**).

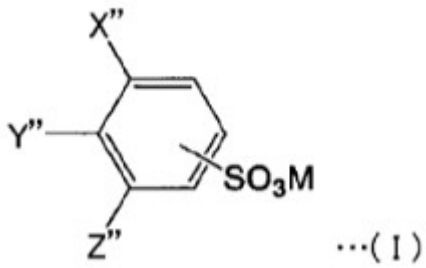


Figure 2. General formula of the detergent component described in JP2008031243 A2.

In formula I, X ", Y ", Z " are each independently a hydrogen atom, Cn " H_2n" +1 (n is an integer of 1 to 3) or (CH_3) 2CH; M represents a hydrogen atom, an alkali metal atom, an alkaline earth metal atom, NH_4^+ or an alkanolamine.

Also, in the liquid detergent composition the amphoteric surfactant (B) contains a compound (B1) represented by the following general formula II (**Figure 3**).

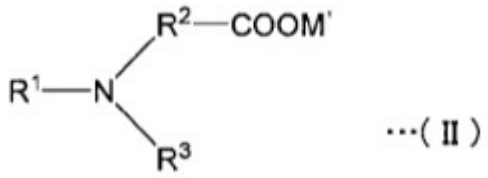


Figure 3. General formula of the compound B1 described in JP2008031243 A2.

In the formula II, R1 is a linear or branched alkyl group or alkenyl group having 10 to 20 carbon atoms; R2 is a divalent hydrocarbon group having 1 to 5 carbon atoms. M' represents a hydrogen atom, an alkali metal atom, an alkaline earth metal atom, NH_4^+ or an alkanolamine.

32. WO20178102 A1

This document describes a detergent composition comprising a first protease and a second protease, wherein:

1) the first **protease** has a net formal charge of -1, -3 or -4 relative to the protease of the sequence (SEQ ID NO: 10 (See Annex I), and the second protease has a net formal charge of -2 relative to the protease of SEQ ID NO: 10; or

2) the first protease has a net formal charge of -3, -4 or -5 relative to the protease of SEQ ID NO: 10, and the second protease has a net formal charge of -1, 0 or +1 relative to the protease of SEQ ID NO: 10.

The document also describes a detergent composition which includes the two proteases. The composition has an improved wash performance in laundry compared to the same composition comprising either the first protease or the second protease.

33. JP9249891 A2

This document describes a detergent composition containing a **lipase** with excellent oil dirt cleaning power at low temperatures. The composition is 0.05 of anionic surfactant 10 to 50 wt %, 20 to 50 wt % of an alkali builder, 1-10% of sulphite, and the lipase having the amino acid sequence of SEQ ID NO: 11 (See Annex I).

Lipase represented by the SEQ ID NO: 11 (hereinafter, Lipase EX) is excellent in alkali resistance. When this is added to the detergent composition, the removal of oil stains at low temperatures it is remarkably improved. The Lipase EX (SEQ ID NO: 11) is 0.05 wt % to 5 wt % in the final detergent composition, preferably formulated to 2 wt % 0.1 wt %. If it is less than 0.05 wt %, one detergency at low temperature is insufficient, since it exceeds 5 wt % detergency reaches saturation.

In addition, the Lipase EX (SEQ ID NO: 11) can be used in combination with various enzymes. For example, hydrolytic enzymes, oxidase, or the like reductase.

34. CN108822976 A

This document describes a preparation method of the compound bio dish washing liquid containing paratoluenesulfonic acid sodium salt, which is characterized in that include the following steps:

(1) Cellulose powder, ammonium persulfate, lauryl methacrylate, cetearyl trimethyl ammonium chloride, coconut palm oleyl amine polyoxyethylene ether and dimethyl ethylene glycol acrylate are added in deionized water, stir 30 at 60-100 °C 60 min and then, washing is dried to obtain modified cellulose powder.

(2) Starch is added in the buffer solution of disodium hydrogen phosphate and citric acid (pH 5), configuration concentration is 30-40 wt %. Starch solution heats in 40-50 °C of water-bath, and 1-2 wt % carbohydrase is then added, reacts 10-15h, and then hydroxide is added. Drying is washed in centrifugal filtration, and ground 500 mesh obtains micropore starch.

(3) Components (weighed by weight): 4-8 parts of modified cellulose powder, 3-8 parts of micropore starch, 0.2-1 parts of alkyl glycosides, 0.2-1 parts of paratoluenesulfonic acid sodium salt, 2-5 parts of sodium citrate, 0.1-0.4 parts of carboxymethyl cellulose, 0.2-1 parts of **lipase**, 0.1-0.2 parts fragrance, 10-20 parts of ethyl alcohol, 100-120 parts of water.

(4) Deionized water is heated to 20-30 °C, micropore starch is added and obtains micropore starch aqueous solution after mixing evenly.

(5) Modified cellulose powder is added in ethyl alcohol, after mixing, obtains modified cellulose powder ethanol solution.

(6 Modified cellulose powder ethanol solution is added in micropore starch aqueous solution, after being mixed evenly, is added other remaining ingredient first stirs 10-20min at revolving speed 100-200r/min. The ultrasonic wavelength-division is 500-1000W, 2-4 min is dissipated, compound bio dish washing liquid is obtained.

35. JP1261499 A2

This document describes an enzyme-containing detergent which inhibits a decrease in lipase activity due to long-term storage.

The present invention describes the combined use of a specific surfactant system and a lipase, which has significantly improved the oil stain detergency. Composition:

(i)10 to 50% by weight of a non-ionic surfactant

(ii) **Alkali lipase** having a lipase activity at pH 9 of 30% or more of the lipase activity at pH 7

Alternatively, instead of the component (i), the following components can be used:

- 5 to 30% by weight of a sulfonic acid salt of a saturated fatty acid having 8 to 22 carbon atoms in the fatty acid residue.
- a primary or secondary alcohol having 8 to 18 carbon atoms with ethylene oxide.

This document describes a detergent composition comprising:

(i) from 1 to 5 wt.% of a soil release polymer

(ii) from 0.01 to 1 wt.% of a **bacterial lipase** derived from ***Burkholderia cepacia* or *Psychromonas ingrahamii***

(iii) 8 to 35 wt % of a surfactant.

wherein the soil release polymer is a polyester based soil released polymer.

The detergent composition additionally comprising a further enzyme selected from the group consisting of proteases, cellulases, alpha- amylases, peroxidases/oxidases, pectate lyases, and/or mannanases.

The document also describes a method of treatment of a substrate with a detergent composition comprising incorporate a bacterial lipase enzyme into a detergent composition and subsequent treatment of a substrate, preferably textiles, with said composition.

36. GB2307695 A1

This document describes a detergent composition for washing fabrics, comprising: (a) from 2 to 50 wt % of an organic surfactant system comprising one or more anionic, non-ionic, cationic, amphoteric or zwitterionic surfactants, (b) from 0 to 80 wt % of a builder component comprising one or more inorganic or organic detergency builders, (c) a soil release effective amount of a water-soluble or water-dispersible sulphonated polyester comprising monomer units of

(i) an unsulphonated aromatic diacidic monomer (A),

(ii) a sulphonated aromatic diacidic monomer (SA),

(iii) optionally a hydroxylated aromatic or aliphatic diacidic monomer (HA), in an amount replacing up to 50 mole % of (A) and/or (SA),

(iv) a polyol (P) selected from ethylene glycol, propylene glycol, isopropylene glycol, glycerol, 1,2, 4-butanetriol and 1,2,3-butanetriol, and oligomers of these having from 1 to 8 monomer units,

(v) an **enzyme system comprising a protease, a lipase, optionally an amylase, and optionally a cellulase**. The enzyme system comprises protease granules in an amount of from 0.1 to 5 wt %, and lipase granules in an amount of from 0.01 to 1 wt %.

Examples of suitable proteolytic enzymes are the subtilisins, which are obtained from particular strains of *B. subtilis* and B. licheniformis, such as the commercially available subtilisins Maxatase (Trade Mark), as supplied by Gist-Brocades N.V., Delft, Holland, and Alcalase (Trade Mark), as supplied by Novo Industri A/S,

An example of a lipase suitable for use in the present invention is Lipolase (Trade Mark) ex Novo Industri A/S.

An example of an amylase suitable for use in the present invention is Termamyl (Trade Mark) ex Novo Industri A/S.

Examples of cellulases suitable for use in the present invention include Celluzyme (Trade Mark) ex Novo IndustriA/S and Carezyme (Trade Mark).

37. WO9724426 A1

This document describes a detergent composition comprising a **hyaluronidase enzyme** (Hyaluronidase can be a carbohydrase from the following EC categories: EC 3.2.1.35, EC 3.2.1.36 and EC 4.2.2.1 and are commercialised by Fluka, Sigma and Biozyme.), which is present at a level of 20 to 200 HU/g composition. This detergent composition also comprises **protease** (1-5000 PU/g composition), **amylase** (1-5000 PU/g composition), **lipase** (1-5000 PU/g composition) and **cellulase** (1-2000 PU/g composition).

This detergent composition also comprises a bleach system comprising a bleach activator and a bleaching agent. Said bleach activator is tetraacetylethylenediamine at a level of 0.1 to 10% by weight of composition and said bleaching agent is percarbonate or perborate or a mixture thereof at a level from 0.1 to 30% by weight of composition. Also, the composition includes surfactants.

38. CO4290376 A1

This document describes a detergent composition for washing particular clothes having significantly improved oily dirt removal activity, including (a) from around 1 to 50% by weight, of one or more compounds of oily and no mis detergents (b) a **lipase enzyme** in an amount effective to remove oily soil from stained fabric; (c) a soil release polymer in an amount effective for removal of oily soil from stained fabric.

Such detergent composition to wash clothes can remove a variety of oily dirt from a fabric.

39. WO11150157 A2

This document describes a detergent composition, comprising a **lipase (SgrLip2 lipase)** from ***Streptomyces griseus***, and a surfactant, wherein the detergent composition is more effective in removing oily stains from a surface to be cleaned than an equivalent detergent composition lacking the lipase. (See Annex I – SEQ ID NO: 12).

The surfactant comprises one or more surfactants selected from the group consisting of sodium dodecyl benzene sulfonate, sodium hydrogenated cocoate, sodium laureth sulphate, C12-14 pareth-7, C12- 15 pareth-7, sodium C12-15 pareth sulphate, and C14-15 pareth-4.

The detergent composition also comprises subtilisin protease.

The detergent composition is formulated at a **pH of from about 8.0 to about 10.0**. and is effective in hydrolyzing a lipid at a temperature of from about **30 °C to about 40 °C**.

40. US2012258900 AA

This document describes a detergent composition, comprising a **lipase (LipA lipase)** from ***Bacillus subtilis*,** and a surfactant, wherein the detergent composition is more effective in removing oily stains from a surface to be cleaned than the detergent composition in the absence of the lipase (See Annex I – SEQ ID NO:13).

The surfactant comprises one or more surfactants selected from the group consisting of sodium dodecyl benzene sulfonate, sodium hydrogenated cocoate, sodium laureth sulphate, C12-14 pareth-7, C12-15 pareth-7, sodium C12-15 pareth sulphate, and C14-15 pareth-4.

The detergent composition also comprises subtilisin protease.

The detergent composition is formulated at a **pH of from about 8.0 to about 10.0.** and is effective in hydrolyzing a lipid at a temperature of from about **30 °C. to about 40 °C**.

41. EP2987849 A1

The document describes a method of laundering a fabric comprising the steps of:

(i) contacting the fabric with a **lipid esterase** selected from class E.C. 3.1.1.3, class E.C. 3.1.1.1 or a combination thereof (80 and 1600 ng enzyme/g fabric). The lipid esterase in step (i) is a variant having at least 90% sequence identity to wild-type lipase from *Thermomyces lanuginosus* and having sequence substitutions T231R and N233R.

The wild-type sequence is the 269 amino acids (amino acids 23 - 291) of the Swissprot accession number Swiss-Prot 059952 (derived from Thermomyces lanuginosus (Humicola lanuginosa)). Suitable lipases would include those sold under the tradenames Lipex®, Lipolex® and Lipoclean® by Novozymes, Bagsvaerd, Denmark.

(ii) contacting the fabric from step (i) with a soil

(iii) contacting the fabric from step (ii) with a surfactant composition, comprising from 5 to 100 wt. % detersive surfactant and optionally additionally comprising **a lipid esterase**; and

(iv) contacting the fabric from step (iii) with an aqueous wash liquor comprising a detergent composition.

The laundry detergent composition comprises a surfactant composition comprising an anionic surfactant, a non-ionic surfactant and optionally additionally a zwitterionic surfactant.

The aqueous liquor is contacted with the fabric at a temperature of between 10 °C and 30 °C.

42. US2014230155 AA

This document describes a method of laundering a fabric comprising the steps of

(i) contacting the fabric with a lipid esterase (80 and about 1600 ng enzyme/g fabric.) selected from class E.C. 3.1.1.3, class E.C. 3.1.1.1 or a combination thereof.

(ii) contacting the fabric from step (i) with a soil

(iii) contacting the fabric from step (ii) with a laundry detergent composition, wherein the laundry detergent composition optionally comprises a detersive surfactant, and optionally comprises a **lipid esterase.**

The lipid esterase is a variant having at least about 90% sequence identity to wild-type lipase from *Thermomyces lanuginosus* and having sequence substitutions T231R and N233R.

The wild-type sequence is the 269 amino acids (amino acids 23-291) of the Swissprot accession number Swiss-Prot 059952 (derived from Thermomyces lanuginosus (Humicola lanuginosa)). Suitable lipases would include those sold under the tradenames Lipex®, Lipolex® and Lipoclean® by Novozymes, Bagsvaerd, Denmark.

The temperature is between 10 °C and 30 °C.

3. Scientific literature

Table 2 shows a summary of the organisms and enzymes described in each scientific document, as well as the most important methods and conditions.

Table 2. Enzymes, methods and conditions of each scientific document.

	REFERENCE (TITLE)	**ENZYME**	**METHODS/CONDITIONS**
1	Biochemical properties of the alkaline lipase of *Bacillus flexus XJU-1* and its detergent compatibility	Alkaline lipase	The lipase showed optimum activity at pH 10.0 and was 100% stable for 24 h at pH 10.0 and 11.0. It exhibited maximum activity at 70 °C and retained more than 70% of the initial activity at 60, 70 and 80 °C for 24 hours.
2	Lipase immobilized on walls of plastic beaker: Kinetic properties and application in washing of oil-stained cloth	Porcine pancreas lipase	(See the conditions in the abstract of the document)
3	A strategic approach of enzyme engineering by attribute ranking and enzyme immobilization on zinc oxide nanoparticles to attain thermostability in mesophilic *Bacillus subtilis* lipase for detergent formulation	Mesophilic *Bacillus subtilis* lipase. Three lipase mutants, bsl_the1 (V149K, Q150E), bsl_the2 (F41K, W42E, V149K, Q150E) and bsl_the3	bsl_wt showed optimum activity at 35 °C and pH 8 ZnO-bsl_the3 showed optimum activity at 60 °C and pH 10

		(F41K, W42E, P119E, Q121K, V149K, Q150E	
4	Partially purified rubber seed lipase for efficient removal of fatty soil	Rubber seed lipase	Conditions: pH 8 and 40 °C
5	Microbial detergent compatible lipases	Alkaline lipases	All the detergent lipases have optimum in the alkaline region between pH 8.0 and 12.0. The bacterial detergent lipases exhibited a significant lipolytic activity in the temperature range of 30 to 60 °C
6	A thiol-activated lipase from *Trichosporon asahii MSR 54*: detergent compatibility and presoak formulation for oil removal from soiled cloth at ambient temperature	Lipase from *Trichosporon asahii MSR 54*	Alkaline lipase having pH activity in the range of pH 8.0–10.0 and temperature in the range of 25–50 °C.
7	Partial purification and characterization of lipase from locally produced edible oil-seed sand its relevance in industries	Lipase	Conditions: 37 °C and pH 8
8	Evaluation of alkali and thermotolerant lipase from an indigenous isolated *Bacillus* strain for detergent formulation	*Bacillus* lipase	Lipase has an optimal activity at pH 8.0 and at 37 °C
9	Purification and characterization of a surfactant-compatible lipase from *Aspergillus tamarii JGIF06* exhibiting energy-efficient removal of oil stains from polycotton fabric	Lipase from *Aspergillus tamarii JGIF06*	The optimal lipase activity was recorded at pH 4 and at 37 °C
10	Purification and characterisation of a thermostable alkaline lipase from a new thermophilic *Bacillus sp. RSJ-1*	Alkaline lipase from a new thermophilic *Bacillus sp. RSJ-1*	The purified enzyme exhibited maximum activity at 50 °C and pH 8.0–9.0

11	Study on the potential of cold-active lipases from psychrotrophic fungi for detergent formulation	Lipases from BPF4 and BPF6 identified as *Penicilium canesense* and *Pseudogymnoascus roseus*	BPF4 and BPF6 lipases showed maximum activity at pH 11 and 9 respectively and at 40 °C.
12	A high-detergent-performance, cold-adapted lipase from *Pseudomonas stutzeri PS59* suitable for detergent formulation	Lipase from *Pseudomonas stutzeri PS59*	The lipase showed optima activity at pH 8.5 and 20 °C.
13	Two step purification of *Acinetobacter sp*. lipase and its evaluation as a detergent additive at low temperatures	*Acinetobacter sp*. lipase	The purified lipase exhibited pH and temperature optima of 8.5 and 37 °C, respectively.
14	Life cycle assessment supports cold-wash enzymes	Proteases and amylases: Polarzyme® and Stainzyme®	Enzymes are active in the range 30–100 °C and between pH 7–11

Each of the scientific documents is summarized in detail below, highlighting the microorganisms and enzymes involved in the processes, as well as the methods and conditions carried out.

1. Biochemical properties of the alkaline lipase of *Bacillus flexus XJU-1* and its detergent compatibility

The enzyme was monomeric protein as confirmed by liquid chromatography-mass spectrometry and its molecular weight was 15.95 kDa. The **lipase showed optimum activity at pH 10.0 and was 100% stable for 24 h at pH 10.0 and 11.0.** It exhibited **maximum activity at 70 °C** and retained more than 70% of the initial activity at 60, 70 and 80 °C for 24 h. The activity was stimulated by Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+}, whereas 50% of the initial activity was lost with Fe^{3+} and Hg^{2+}. The activity was inhibited by 10 mM N-bromosuccinimide and tosyl-L-lysylchloromethylketone, while N-ethylmaleimide, phenylmethylsulphonylfluoride and urea did not show any effect. The enzyme significantly hydrolysed olive, cottonseed, sunflower, groundnut, and gingelly oils. With p-nitrophenyl palmitate, Vmax and Km were 62.5 U/mL and 2.25 mM, respectively. The lipase maintained its stability in Tween-80, Triton-100 and H_2O_2 at 1%, but an activation of 10% and a reduction of 15% in relative activity were observed with NaClO and sodium dodecyl sulphate, respectively. The enzyme retained maximum storage stability for 20 days at 20, 4 and 30 °C. In the presence of 0.7% (w/v) Ariel, Henko, Super wheel, Tide plus and Rin, a retention of more than 84.90% initial activity was recorded after 24 h at 60 °C. The supplementation of the lipase to the detergents improved the olive oil stain removal. These properties suggested the present enzyme as a potential additive for detergent preparations.

2. Lipase immobilized on walls of plastic beaker: Kinetic properties and application in washing of oil-stained cloth

Commercial **porcine pancreas lipase** was immobilized covalently onto alkylamine glass-beads affixed on the inner wall of a plastic beaker by an adhesive. The immobilized enzyme retained 10.8% of the initial activity of free enzyme with a conjugation yield of 52 mg/g support. The optimum pH and incubation temperature were decreased, while time for linearity and Km for triolein of enzyme were increased after immobilization (See the following Table). The utility of immobilized enzymes in removal of oil stain from cotton cloth by various detergents was tested by chemical method. All the detergents gave better washing in presence of immobilized lipase than that by detergent alone. Further, the washing by cheaper (non-enzymic) detergents in presence of immobilized lipase was almost like that by expensive (enzymic) detergents. The immobilized enzyme was used about 100 times without any considerable loss of activity.

Parameters	Free lipase	Lipase conjugated to free alkylamine glass-beads	Lipase conjugated to affixed alkylamine glass-beads
Optimum pH	7.5	7.1	6.8
Temperature for maximum activity	35°C	30°C	30°C
E_a (Kcal/mole)	2.05	5.404	5.556
Time of incubation	15 min	20 min	50 min
Saturating concentration of triolein substrate (mM)	50 mM	—	100 mM
K_m for triolein	4.242×10^{-3} M	—	17.728×10^{-3} M
V_{max}	1.515 mol/min	—	1.33 mol/min

Data are the mean of three replicates

Table 3. Kinetic parameters of free porcine pancreas lipase and lipase bound to free and affixed alkylamine glass beads.

3. A strategic approach of enzyme engineering by attribute ranking and enzyme immobilization on zinc oxide nanoparticles to attain thermostability in mesophilic *Bacillus subtilis* lipase for detergent formulation

Contributing amino acids for thermostability were analyzed from homologous thermophilic-mesophilic protein dataset through relative abundance and generated ranking model. Analyses divulged priority of charged amino acids for thermostability. Ranking model was used to predict thermostabilizing mutations. **Three lipase mutants, bsl_the1 (V149K, Q150E), bsl_the2 (F41K, W42E, V149K, Q150E) and bsl_the3 (F41K, W42E, P119E, Q121K, V149K, Q150E)** were generated and validated through in silico and in vitro approaches for improved activity and thermostability. ZnO nanoparticles were synthesized by precipitation method and functionalized using polyethylenimine, APTES and glutaraldehyde for lipase immobilization. The immobilization was confirmed through various analytical techniques. **Analysis revealed bsl_wt showed optimum activity at 35 °C and pH 8 which was increased to 60 °C and pH 10 in case of ZnO-bsl_the3**. The ZnO-bsl_the3 showed 80% of their initial activity after 60 days of storage stability and retained 78% of activity after 20 cycles of reuse. Lipases were applied for oil and grease stain removal from fabric. ZnO-bsl_the3 removed 90% and 82% of oil and grease stains, respectively. Conclusively, it revealed a promising perspective of low-cost nanobiocatalysts in detergent formulation.

4. Partially purified rubber seed lipase for efficient removal of fatty soil

Lipase was extracted from rubber seeds and partially purified 7.59-fold with a yield of 62.14% recovery using $(NH_4)2SO_4$ fractionation followed by batch adsorption on diethylaminoethyl (DEAE) cellulose**. The extracted enzyme exhibited**

optimum activity as well as high stability at a temperature of 40 °C and pH 8. The kinetics of product formation continued for 30 minutes and was linear for the first 10 minutes. The enzyme exhibited good stability with sodium dodecyl sulphate (SDS), $CaCl_2$ and Triton X-100 whereas poor stability was observed with sodium perborate and hydrogen peroxide. The enzyme was found to be less stable in the presence of commercial detergents. Hence, rather than supplementing the enzyme with detergents, pre-incubation of the enzyme with fatty soil and subsequent washing with detergents was shown to be more successful in removing fatty soil. Further studies showed that fatty stain caused by coconut oil (1mL) can be completely removed by stirring the fabric with 2% SDS 5.00 mL for 15 minutes, followed by incubation with lipase (4.00 mL, 1 mg/mL) for 30 minutes at 40 °C and subsequent washing with detergent.

These findings indicated that rubber seed lipase, with its ability to function in alkaline pH (~ 8.0) and temperature ~ 40 °C and its capability to remove fatty soil efficiently, is suitable for use as a potential bio-detergent.

5. Microbial detergent compatible lipases

The use of **alkaline lipase** in detergent formulations can reduce or substitute the use of these harmful ingredients in higher amounts. The detergent lipases active at ambient temperature are now preferred as the quality of the cleaned fabric is maintained and energy saved. Review papers on the production, purification, characterization, and application of lipases in various industries are available, but no specific review on the microbial alkaline lipases or detergent compatible lipases. In the present review, screening, production, and properties of detergent compatible lipases are reported with emphasis on the stability and compatibility of alkaline lipases in detergent and detergent constituents and the methods for examination of oil stain removal.

The detergent lipases are mainly produced by the microorganisms belonging to the following genera: *Acinetobacter, Bacillus, Burkholderia, Streptomyces, Rhodococcus, Pseudomonas, Staphylococcus, Aspergillus, Cryptococcus, Fusarium, Talaromyces* and *Trichosporon*. Low-cost medium is preferred to make the fermentation cost-effective.

All the detergent lipases have optimum pH values in the alkaline region between pH 8.0 and 12.0. The optimum pH values of 8.0, 11.0, and 12.0 were pH reported for the detergent lipases of *B. smithii*, *S. arlettae*, and *Staphylococcus sp*., respectively. Similarly, the detergent lipase of the *Fusarium globulosum* acted maximally at pH 10.0 and exhibited marked activation at the alkaline pH of 9 to11.0.

Any detergent compatible lipase must have a broad temperature adaptability. The bacterial detergent lipases exhibited a significant lipolytic activity in the **temperature range of 30 to 60 °C**. It was reported that the temperature of 30, 40, 50, and 60 °C were the optimum for the detergent lipases of *S. arlettae JPBW-111, A. calcoaceticus1-710, B. mithii BTMS*, and *B. licheniformis*, respectively. Similarly, **the optimum temperature for various fungal detergent lipases was in the range of 30 to 50 °C.**

6. A thiol-activated lipase from *Trichosporon asahii MSR 54*: detergent compatibility and presoak formulation for oil removal from soiled cloth at ambient temperature

An **alkaline lipase from *Trichosporon asahii MSR 54*** was used to develop presoak formulation for removing oil stains at ambient temperature. The lipase was produced in a reactor followed by concentration by ultrafiltration and then it was dried with starch. The biochemical characteristics of enzyme showed that it was an alkaline lipase having **pH activity in the range of pH 8.0–10.0 and temperature in the range of 25–50 °C.** The present lipase was active >80% at 25 °C. The lipase was cysteine activated with fourfold enhancement in presence of 5 mM cysteine and likewise the activity was also stimulated in presence of papain hydrolysate which served as source of cysteine. The presoak formulation consisted of two components A and B, component A was enzyme additive and B was a mixture of carbonate/bicarbonate source of alkali and papain hydrolysate as source of cysteine. The results indicated that the presoaking in enzyme formulation followed by detergent washing was a better strategy for stain removal than direct washing with detergent in presence of lipase. Further, it was observed that 0.25% presoak component B in presence of 100 U enzyme component A (0.1 g) was the best formulation in removing maximum stain from mustard oil/triolein soiled clothes as indicated by increase in reflectance which was found equal to that of control cloth. The lipase action in presoaked formulation was clearly indicated by quantitated fatty acid release and the TLC results of wash water, where oil hydrolytic products were visible only in presence of enzyme in the treatment. The wash performance carried at 25 °C indicated that washing at 25 °C was at par with that at 40 °C as indicated by similar reflectance of the washed cloth piece though qualitative fatty acid release was higher at 40 °C.

7. Partial purification and characterization of lipase from locally produced edible oil-seeds and its relevance in industries

Lipase was extracted from germinating seeds of *Helianthus annus* (Sunflower), *Zea mays* (Maize), and *Brassica compastris* (Mustard). The lipolytic activity was assessed using olive oil as substrate at different germination-time and the maximum-activity was obtained after 120 hr. Partial-purification was executed by precipitating the seed-homogenate with varying concentration of ammonium sulphate solution. 80% ammonium sulphate solution showed maximum lipase activity of 5320 IU/mL, 3500 IU/mL, 3080 IU/mL with 9.6, 6.9, and 4.8-fold purification and total protein content of 162, 84, and 60 mg for partially purified enzyme extracts namely SN5, BN5, and MN5, respectively. **The optimum temperature and pH observed for hydrolysis of olive oil were 37 °C, and 8.0 respectively**. Enzyme was found to be stable up to 6 days at 4 °C and its activity was stimulated by Ca^{2+} ions. Oil-stains removal from cotton fabric was observed to be superior in the presence of lipase and detergent. Moreover, the SN5, BN5, and MN5 lipase increased free fatty acid release up to 4.2, 4.3, and 3.8 mg, respectively than wastewater without treatment of lipase (0.21 mg) and promoted fat hydrolysis to approximately 40, 42, and 48% mass reduction after 6 hr incubation of fat particle at a concentration of 20 mg/ml. Biodiesel produced by catalyzing transesterification of vegetable oil with SN5, BN5, and MN5 lipase provided an acid value of 0.8, 1.08, and 0.5 mg/g, viscosity 5.50, 5.7, and 5.53 mm^2/s and density 0.87, 0.88, and 0.79 g/mL, respectively.

8. Evaluation of alkali and thermotolerant lipase from an indigenous isolated *Bacillus* strain for detergent formulation

A **lipase-producing indigenous *Bacillus subtilis*** strain [accession no. KT985358] was isolated from the foothills of Trikuta mountain in Jammu and Kashmir, India. The lipase (BSK-L) produced by this strain expressed alkali and thermotolerance. **Lipase has an optimal activity at pH 8.0 and temperature 37 °C, whereas it is stable at pH 6.0–9.0 and showed active lipolytic activity at temperatures 30 to 60 °C.** Furthermore, lipase activity was found to be stimulated in the presence of the metal ions Mn^{2+}, K^{+}, Zn^{2+}, Fe^{2+} and Ca^{2+}. This lipase was resistant to surfactants, oxidising agents and commercial detergents, suggesting it as a potential candidate for detergent formulation. BSK-L displayed noticeable capability to remove oil stains when used in different washing solutions containing buffer, lipase and commercial detergent. The maximum olive oil removal percentage obtained was 68% when the optimum detergent concentration (Fena) was 0.3%. The oil removal percentage from olive oil-soiled cotton fabric increased with 40 U/mL of lipase.

9. Purification and characterization of a surfactant-compatible lipase from *Aspergillus tamarii JGIF06* exhibiting energy-efficient removal of oil stains from polycotton fabric

An **extracellular lipase** with 23,666.66 U/mL/min activity was produced by ***Aspergillus tamarii JGIF06*** under submerged fermentation in mineral salt medium containing coconut oil (2.5 % v/v), tryptone (2 % w/v) and ammonium chloride (2 % w/v), with initial pH of 5 ± 0.2, incubated at 25 °C for 7 days on a rotary shaker at 120 rpm. A 7.9-fold increase in lipase-specific activity was recorded after purification by DEAE Sepharose ion exchange and Sephadex G200 column chromatography. The apparent molecular mass of this enzyme was revealed as 50 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The optimal lipase activity was recorded at pH 4 and 37 °C. The enzyme revealed broad specificity towards different vegetable oils. The K m and V max of the lipase on olive oil was found to be 330.4 mg and 53,690 U/mL/min, respectively. The lipase activity was stable in the presence of surfactants such as cetrimonium bromide, sodium dodecyl sulphate and Tween 80, and metal ions and reagents such as Ca^{2+}, Ba^{2+} and 2-mercaptoethanol. However, the activity was greatly reduced in the presence of organic solvents such as chloroform. The stain removal potential of the crude lipase was determined on polycotton fabric pieces stained with peanut oil. Lipase added to cold water alone significantly enhanced the removal of stain by 152 %. The addition of lipase also improved the stain removal efficiency of a commercially available detergent in the presence of either cold (25 ± 2 °C) or hot (65 ± 2 °C) water. The current findings suggest the potentiality of this enzyme for energy-efficient biocatalytic application.

Lipase production was carried out in 1000 mL Erlenmeyer flask containing 500 mL of supplemented mineral salts medium containing (g/l) tryptone, 20; NH_4Cl, 20; K_2HPO_4, 3; KH_2PO_4, 1; $MgSO_4$, 0.1; $MgCl_2$, 0.12; cetrimonium bromide (CTAB), 0.5 % (w/v); coconut oil, 2.5 % (v/v) and distilled water, adjusted to an initial pH of 5 ± 0.2 and autoclaved. The sterile medium was aseptically inoculated with 1.5 % (v/v) fungal conidial suspension prepared in mineral salt solution and incubated at 25 °C for 7 days on a rotary shaker at 120 rpm. Post-incubation, the fungal broth was filtered using Whatman's No. 1 filter paper and centrifuged at 5000 rpm for 30 min at 4 °C. The clear supernatant was subjected to lipase assay and further purification.

The optimum pH for lipase activity was measured using olive oil as the substrate, at 27 °C in buffers (200 mM) of different pH values such as citrate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0). The optimum temperature for the lipase activity was determined at different temperatures (4, 27, 37, 45, 60, 80 and

100 °C) using 200 mM citrate buffer of pH 4. Lipase substrate specificity was analysed using different vegetable oils (coconut oil, peanut oil, sesame oil, sunflower oil, soybean oil and olive oil). The effect of substrate concentration on lipase activity was evaluated by incubating 1 mL of the enzyme with varying concentrations (460–9200 mg) of substrate (olive oil) at 37 °C. K m and V max values were calculated from Lineweaver–Burk plot using Hyper32 software.

10. Purification and characterisation of a thermostable alkaline lipase from a new thermophilic *Bacillus sp. RSJ-1*

An **extracellular alkaline lipase** from a new thermophilic ***Bacillus sp. RSJ-1*** was purified to homogeneity by ultrafiltration, followed by ammonium sulphate precipitation, dialysis, Q-Sepharose ion exchange chromatography and Sephacryl S-200 SF gel filtration chromatography. This purification protocol resulted in a 201-fold purification of lipase with 19.7% final yield and the relative molecular weight of the enzyme was determined to be 37 kDa by SDS-PAGE. The kinetic characterisation of the purified enzyme exhibited **maximum activity at 50 °C and pH 8.0–9.0.** It was stable at 50 °C for 60 min and retained >90% of its original activity for 120 min. The half-lives at 55, 60, 65, 70 and 75 °C were 240, 150, 90, 45 and 30 min, respectively. The enzyme was also highly stable in a pH range of 8.0–9.0 for 120 min. The enzyme activity was promoted in the presence of Ca^{2+}, Na^{+}, Mg^{2+} and Ba^{2+} and was strongly inhibited by Cs^{+}, K^{+}, Co^{2+} and Zn^{2+}. EDTA did not affect the enzyme activity, whereas the presence of various oxidizing agents, reducing agents and some surfactants, reduced the enzyme activity. The enzyme was highly stable in the presence of some commercial detergent formulations. The values of Km and Vmax, as calculated from the Lineweaver–Burk plot, were 2.2 mg/mL and 1429 U/mL, respectively.

11. Study on the potential of cold-active lipases from psychrotrophic fungi for detergent formulation

Lipases from psychrotrophic fungal isolates BPF4 and BPF6 identified as ***Penicilium canesense*** and ***Pseudogymnoascus roseus*** respectively were characterized for their compatibility towards laundry detergent. **BPF4 and BPF6 lipases showed maximum activity at pH 11 and 9 respectively and at 40 °C.** The residual activities at 20 °C and 4 °C of BPF4 lipase were 35% and 20% and of BPF6 lipase were 70% and 20 °C respectively. **Both the enzymes were stable at 4 °C, 20 °C and 40 °C for 2 h losing at the most 20% of activities.** Both the enzymes were metalloenzymes with activity enhancement by nearly threefold by Ca^{2+}. Contrary to BPF6 lipase, BPF4 enzyme was not stimulated by EDTA nor inhibited, rather stimulated by SDS and Triton X-100 by 125% and 330% respectively. Both the lipases showed minor to moderate inhibition by $NaClO_3$ and H_2O_2, and exhibited nearly 90% residual activity after 1 h of incubation in selected detergent brands thus indicating potential for their inclusion in detergent formulation thereby facilitating cold washing as a step towards mitigation of climate change.

12. A high-detergent-performance, cold-adapted lipase from *Pseudomonas stutzeri PS59* suitable for detergent formulation

A high-detergent-performance and cold-adapted **lipase** was purified and characterised from ***Pseudomonas stutzeri PS59***, which was isolated from Daqing oil fields (Heilongjiang, PR China). The lipase was purified to homogeneity using ammonium sulphate precipitation, dialysis, freeze–drying, ion exchange chromatography and gel filtration chromatography. The molecular weight of the lipase was approximately 55 kDa, as measured by SDS-PAGE. **The lipase**

showed optima activity at pH 8.5 and 20 °C. The lipase activity was activated by metal ions, such as Ca^{2+} and Mn^{2+} and surfactants, such as Tween 80, Tween 20, sodium dodecyl benzene sulfonate and urea. Oxidising agents, such as H_2O_2 and NaClO, were found to have little effect on the activity of the lipase, and most organic solvents can enhance the activity of the lipase. The broad substrate specificity and the compatibility of the lipase in the presence of surfactants, oxidising agents, and other detergent additives clearly indicate its potential application in the laundry industry. The hydrolysis resolution of (R,S)-ethyl 2-methylbutyrate by *P. stutzeri PS59* lipase was carried out with the yield of 31.2% for R-ethyl 2-methylbutyrate, the enantiomeric excess of residual was 85.7%. Thus, the lipase also showed an attractive potency for application in biocatalysis.

13. Two step purification of *Acinetobacter sp*. lipase and its evaluation as a detergent additive at low temperatures

Acinetobacter sp. **lipase** was purified to homogeneity by a two-step process. The crude enzyme (along with biomass) was subjected to partial purification by aqueous two-phase system (ATPS), avoiding centrifugation and filtration steps. Conditions for lipase partitioning by ATPS were optimized by response surface methodology (RSM) and a combination of 29.45% polyethylene glycol 8000, 15.5% phosphate, and a pH of 7.0 resulted in an optimal partition coefficient. Partially pure lipase was further purified by a modified batch process using Octyl Sepharose CL-4B in a vacuum filtration apparatus. This two-step process resulted in a purified lipase with a yield of 74.6% having a specific activity of 88.8 U/mg of protein and a purification fold of 14.92. The homogeneity of the lipase preparation obtained by the purification process was confirmed by reversed phase high performance liquid chromatography profile. The molecular weight of the purified lipase was found to be around 32 kDa as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. **The purified lipase exhibited pH and temperature optima of 8.5 and 37 °C, respectively**. The lipase was active at low temperatures and it retained 86.8% activity at 10 °C. It also displayed other features such as stability over a broad range of pH (3.0–9.0) as well as stability in the presence of hydrogen peroxide and commercial detergents. Based on these characteristics, the potential of this lipase as an additive in laundry detergent formulation was evaluated under low temperature wash conditions. The results indicated that *Acinetobacter sp*. lipase increased the washing efficiency of the detergent Nirma by 21–24% at 15 °C–20 °C, respectively.

14. Life cycle assessment supports cold-wash enzymes

Both proteases and amylases are now represented by cold-water enzymes, enabling manufacturers to brand detergents specifically for cold wash. A Life Cycle Assessment (LCA) for two specific cold-water enzymes –**Polarzyme® and Stainzyme® (Novozymes)** –quantifies the environmental impact of switching to these enzymes. LCAs offer an objective decision-making tool for evaluating initiatives to promote sustainable development.

Stainzyme® is a liquid enzyme preparation containing a thermostable α-amylase suitable for use in detergent preparations. Stainzyme® is produced by submerged fermentation of a genetically modified microorganism. The enzyme protein is not itself modified. After fermentation, the enzyme is separated from the production organism and purified.

Stainzyme® degrades gelatinized starch to dextrins and oligosaccharides. **It is active in the range 30–100 °C and between pH 7–11. Stainzyme® is not very sensitive to pH, but the maximum activity is reached at pH 9.** It is suitable for low-temperature, short cycle washes. Note: Animal α-amylases (but NOT microbial ones such as Stainzyme®) require chloride ions to activate them.

4. Annex

SEQ ID NO: 1 (DOCUMENT 2 – US 2011281324 AA)

Srill2

SAPRIQATDYVALGDSYSSGVGAGSYDSSSGSCKRSTKSYPALWAASHTGTRFNFTACSGARTGDVLAKQLTPVNSGTDLV
SITIGGNDAGFADTMTTCNLQGESACLARIAKARAYIQQTLPAQLDQVYDAIDSRAPAAQVVVLGYPRFYKLGGSCAVGLSE
KSRAAINAAADDINAVTAKRAADHGFAFGDVNTTFAGHELCSGAPWLHSVTLPVENSYHPTANGQSKGYLPVLNSAT

ScollA

APAQATPTLDYVALGDSYSAGSGVLPVDPANLLCLRSTANYPHVIADTTGARLTDVTCGAAQTADFTRAQYPGVAPQLDAL
GTGTDLVTLTGGNDNSTFINAITACGTAGVLSGGKGSPCKDRHGTSFDDEIEANTYPALKEALLGVRARAPHARVAALGYP
WITPATADPSCFLKLPLAAGDVPYLRAIQAHLNDAVRRAAEETGATYVDFSGVSDGHDACEAPGTRWIEPLLFGHSLVPVH
PNALGERRMAEHTMDVLGLD

ScollB

AQPAAADGYVALGDSYSSGVGAGSYISSSGDCKRSTKAHPYLWAAAHSPSTFDFTACSGARTGDVLSGQLGPLSSGTGLVS
ISIGGNDAGFADTMTTCVLQSESSCLSRIATAEAYVDSTLPGKLDGVYSAISDKAPNAHVVVIGYPRFYKLGTTCIGLSETKRT
AINKASDHLNTVLAQRAAAHGFTFGDVRTTFTGHELCSGSPWLHSVNWLNIGESYHPTAAGQSGGYLPVLNGAA

CefII

REETAGAPPGESSGGIREEGAEASTSITDVYIALGDSYAAMGGRDQPLRGEPFCLRSSGNYPELLHAEVTDLTCQGAVTGDL
LEPRTLGERTLPAQVDALTEDTTLVTLSIGGNDLGFGEVAGCIRERIAGENADDCVDLLGETIGEQLDQLPPQLDRVHEAIRD
RAGDAQVVVTGYLPLVSAGDCPELGDVSEADRRWAVELTGQINETVREAAERHDALFVLPDDADEHTSCAPPQQRWADI
QGQQTDAYPLHPTSAGHEAMAAAVRDALGLEPVQP

SEQ ID NO: 2 (DOCUMENT 6 – US 2020205423 AA)

AITASQLDYENFKFYIQHGAAAYCNSETASGQKITCSDNGCKGVEANNAIIVASFVGKGTGIGGYVSTDNVRKEIVLSIRGSS
NIRNWLTNVDFGQSSCSYVRDCGVHTGFRNAWDEIAQRARDAVAKARTMNPSYKVIATGHSLGGAVATLGAADLRSKGT
AVDIFTFGAPRVGNAELSAFITAQAGGEFRVTHGRDPVPRLPPIVFGYRHTSPEYWLAGGASTKTDYTVNDIKVCEGAANLA
CNGGTLGLDIIAHLRYFQDTDACTAGGISWKRGDKAKRDEIPKRQEGMTDEELEQKLNDYVAMDKEYVESNKM

SEQ ID NO: 3 (DOCUMENT 9 – WO 20009231 A1)

ADNYAATRYPIILVHGLTGTDKYAGVLEYWYGIQEDLQQHGATVYVANLSGFQSDDGPNGRGEQLLAYVKTVLAATGATK
VNLVGHSQGGLTSRYVAAVAPDLVASVTTIGTPHRGSEFADFVQSVLAYDPTGLSSTVIAAFVNLASLKTLTTAQAATYNQN

YPSAGLGAPGSCQTGAPTETVGGNTHLLYSWAGTAIQPTLSLFGVTGAKDTSTIPLVDPANALDPSTLALFGTGTVMINRGS
GQNDGLVSKCSALYGKVLSTSYKWNHIDEINQLLGVRGAYAEDPVAVIRTHANRLQLAGV

OR

NVTYHVAGIPTAVTAQQLLYRTNNAQNQPVVNVTSVIRSQVSNGQAISYQSAYDSLNPYDEPSQVIAGDRDVTKVINVGTL
LYSAESIPLSTLLLLGYNIIVPDTEGQTADFAAGPEYGMTTLDSIRAALNTPSTGLNPSSKVAMIGYSGGAIATNWAAQLAPSY
APDINKQLVGAAEGGVLVDPAHNLRYVDGSIVWGGVAAAALAGLSRGYAFDLTPYLSDTGVAVFKDIQNQSLAYILPKYTG
LHWSTLFKPQYANDINSIPAYVTYANKVNAGLAASPTIPMFIGQGTAGALDGTFSSQVGDGVMLAYDVRALAQKFCASGT
PVTYTEYPLEHAGAIVPWVAGMLPWLYDRFNGKTAPSNCWLTSLLPSNSLAPETLH

OR

SDDYATTRYPIILVHGLTGTDKYAGVLEYWYGIQEDLQQHGATVYVANLSGFQSDDGPNGRGEQLLAYVKTVLAATGATKV
NLVGHSQGGLTSRYVAAVAPDLVASVTTIGTPHRGSEFADFVQSVLAYDPTGLSSSVIAAFVNVFGILTSSSHNTNQDALASL
KTLTTAQAATYNQNYPSAGLGAPGSCQTGAPTETVGGNTHLLYSWAGTAIQPTLSVFGVTGATDTSTIPLVDPANALDLST
LALFGTGTVMINRGSGQNDGLVSKCSALYGQVLSTSYKWNHIDEINQLLGVRGAYAEDPVAVIRTHANRLKLAGV

OR

NVTYHVAGIPTALTAQQLLYRTNNALNQPVVNVTSVIRSQVSNGRAISYQSAYDSLNPYDEPSQVIAGDRDVTKIINVGTLLY
SAESIPLSTLLLLGYNVIVPDTEGQTADFAAGPEYGMTTLDSIRAALNTPSTGLSPSSKVAMIGYSGGAIATNWAAQLAPSYA
PEINRQLVGAAEGGVLVDPAHNLRYVDGSIVWGGVAAAALAGLSRGYGFDLTPYLSDTGVAVFNDIQSQSLAYILPKYTGL
RWGTLFKPQYANDINSIPAYVTYANKVNAGLAASPTIPMFIGQGTAGALDGTFSSQVGDGVMLAYDVRALAQKFCASGTP
VTYNEYPLEHAGAIVPWVAGMLPWLYDRFNGKAAPSNCWLTSLLPSNSLAPETLH

SEQ ID NO: 4 (DOCUMENT 18 – WO 20046613 A1)

MRSSLVLFFVSAWTALASPIRREVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNITCTGNACPEVEKADATFLYSFEDSG
VGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVV
FTGHSLGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIK
SGTLVPVTRNDIVKIEGIDATGGNNQPNIPDIPAHLWYFGLIGTCL

SEQ ID NO: 5 (DOCUMENT 21 – US 2012028318 AA)

MQFITVALTLIALASASPIATNVEKPSELEARQLNSVRNDLISGNAAACPSVILIFARASGEVGNMGLSAGTNVASALENEFR
DIWVQGVGDPYDAALSPNFLPAGTTQGAIDEAKRMFTLANTKCPNAAVVAGGYSQGTAVMFNAVSEMPAAVQDQIKG
VVLFGYTKNLQNRGRIPDFPTEKTEVYCNASDAVCFGTLFLLPAHFLYTTESSIAAPNWLIRQIRAA

SEQ ID NO: 6 (DOCUMENT 22 – US 2012309063 AA)

ASLRANDAPIVLLHGFTGWGREEMFGFKYWGGVRGDIEQWLNGYRTFTLAVGPLSSNWDRACEAYAQLVGGTVDYGAA
HAAKHGHARFGTYPGLLPELKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYAKAHNVSLSPLFEGGHHFVLSVTTIAT
PHDGTTLVNMVDFTDRFFDLQKAVLEAAAVSNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTDTARYD

LSVSGAEKLNQWVQASPNTYYLSFSTERTYRGALTGNHYPELGMNAFSAVVCAPFLGSYRNPTLGIDDRWLENDGIVNTVS
MNGPKRGSSDRIVPYDGTLKKGVWNDMGTYNVDHLEIIGVDPNPSFDIRAFYLRLAEQLASLQP

OR

DDYSVVEEHGQLSISNGELVNERGEQVQLKGMSSHGLQWYGQFVNYESMKWLRDDWGITVFRAAMYSSGGYIDDPSVK
EKVKETVEAAIDLGIYVIIDWHILSDNDPNIYKEEAKDFFDEMSELYGDYPNVIYEIANEPNGSDVTWDNQIKPYAEEVIPVIR
DNDPNNIVIVGTGTWSQDVHHAADNQKADPNVMYAFHFYAGTHGQNLRDQVDYALDQGAAIFVSEWGTSAATGDGG
VFLDEAQVWIDFMDERNKSWANWSLTHKDESSAALMPGANPTGGWTEAELSPSGTFVREKIRESASDNNDPIPDPDDE
ASLRANDAPIVLLHGFTGWGREEMFGFKYWGGVRGDIEQWLNDNGYRTFTLAVGPLSSNWDRACEAYAQLVGGTVDY
GAAHAAKHGHARFGRTYPGLLPELKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYAKAHNVSLSPLFEGGHHFVLSV
TTIATPHDGTTLVNMVDFTDRFFDLQKAVLEAAAVASNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTD
TARYDLSVSGAEKLNQWVQASPNTYYLSFSTER

SEQ ID NO: 7 (DOCUMENT 23 – US 2012258507 AA)

ANPYERGPNPTDALLEASSGPFSVSEENVSRLSASGFGGGTIYYPRENNTYGAVAISPGYTGTEASIAWLGERIASHGFVVITI
DTITTLDQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVMGHSMGGGGTLRLASQRPDLKAAIPLTPWHLNKNWSSV
TVPTLIIGADLDTIAPVATHAKPFYNSLPSSISKAYLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCPGPRDGLF
GEVEEYRSTCPF

OR

AGKANANPYERGPNPTDALLEASSGPFSVSEENVSRLSASGFGGGGTIYYPRENNTYGAVAISPGYTGTEASIAWLGERIAS
HGFVVITIDTITTLDQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVMGHSMGGGTLRLASQRPDLKAAIPLTPWHLN
KNWSSVTVPTLIIGADLDTIAPVATHAKPFYNSLPSSISKAYLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCP
RDGLFGEVEEYRSTCPF

SEQ ID NO: 8 (DOCUMENT 24 – US 2015291944 AA)

EVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNITCTGNACPEVEKADATFLYSFEDSGVGDVTGFLALDNTNKLIVLSFR
GSRSIENVVIGNLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVVFTGHSLGGALATVAGADLRG
NGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIKSGTLVPVTRNDIVKIEGIDATG
GNNQPNIPDIPAHLWYFGLIGTCL

SEQ ID NO: 9 (DOCUMENT 26 – US 2015203797 A)

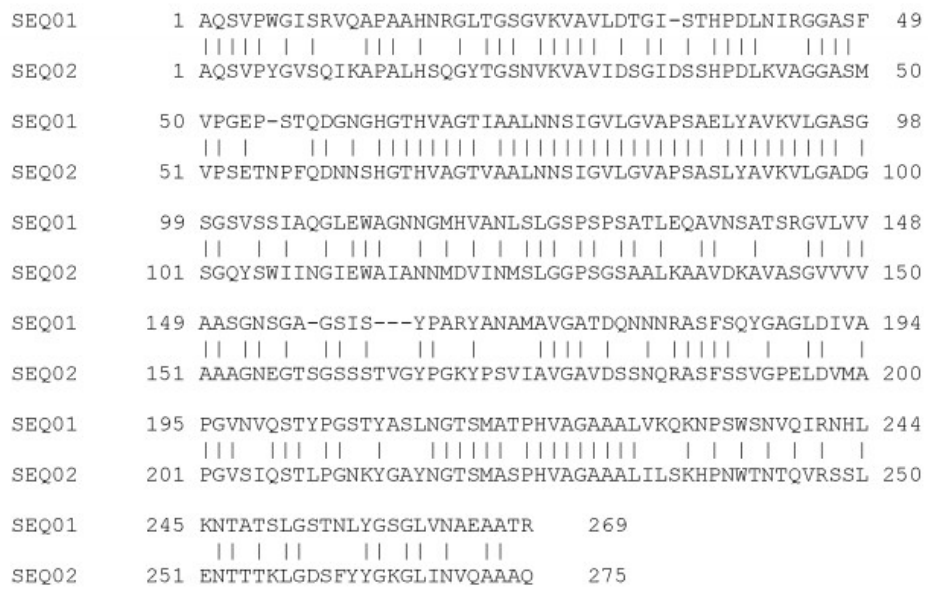
MRSSLVLFFVSAWTALASPIRREVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNITCTGNACPEVEKADATFLYSFEDSG
VGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVV
FTGHSLGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIK
SGTLVPVTRNDIVKIEGIDATGGNNQPNIPDIPAHLWYFGLIG

SEQIDNO:10(DOCUMENT32-WO20178102A1)

It shows an alignment of the amino acid sequences of subtilisin 309 (SEQ01) and subtilisin BPN' (SEQ02).

SEQ01 is the sequence of the Savinase® protease polypeptide from *Bacillus lentus*.

SEQ02 is the sequence of the BPN' protease polypeptide from *Bacillus amyloliquefaciens*.



SEQ01 Savinase® protease polypeptide from *Bacillus lentus*:

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDGNGHGTHVAGTIAALNNSIGVL
GVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSIS
YPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSN
VQIRNHLKNTATSLGSTNLYGSGLVNAEAATR

SEQ02 BPN' protease polypeptide from *Bacillus amyloliquefaciens*:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSHGTHVAGTVAALNNSIG
VLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVVVVAAAGNEGTS
GSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPN
WTNTQVRSSLENTTTKLGDSFYYGKGLINVQAAAQ

SEQ ID NO: 11 (DOCUMENT 33 - JP9249891 A2)

MRSSLVLFFVSAWTALASPIRREVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNITCTGNACPEVEKADATFLYSFEDSG
VGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVV
FTGHSLGGALATVAGADLRGNGYDIDVFSYGAPRVGNRAFAEFLTVQTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIK
SGTLVPVTRNDIVKIEGIDATGGNNQPNIPDIPAHLWYFGLIGTCL

SEQ ID NO: 12 (DOCUMENT 39 - WO11150157 A2)

MLPWIHAARVPRTRGLFAALLLALTVLVAPAAAAAPAAAEATTSRGWNDYSCKPSAAHPRPVVLVHGTFGNSIDNWLVLA
PYLVNRGYCVFSLDYGQLPGVPFFHGLGPIDKSAEQLDVFVDKVLDAGAPKADLVGHSQGGMMPNYYLKFLGGADKVNA
LVGLAPDNHGTTLLGLTKLLPFFPGVEKFITDTTPGLADQIAGSPFITKLTAGGDTVPGVRYTVIATKYDQVVTPYRTQFLDGP
NVRNVLLQDLCPLDLSEHVAIGTVDRIAFHEVANALDPARATPTTCSSVIG

OR

AAPAAAEATTSRGWNDYSCKPSAAHPRPVVLVHGTFGNSIDNWLVLAPYLVNRGYCVFSLDYGQLPGVPFFHGLGPIDKS
AEQLDVFVDKVLDATGAPKADLVGHSQGGMMPNYYLKFLGGADKVNALVGLAPDNHGTTLLGLTKLLPFFPGVEKFITDT
TPGLADQIAGSPFITKLTAGGDTVPGVRYTVIATKYDQVVYPYRTQFLDGPNVRNVLLQDLCPLDLSEHVAIGTVDRIAFHEV
ANALDPARATPTTCSSVIG

SEQ ID NO: 13 (DOCUMENT 40 - US2012258900 AA)

AEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSM
GGANTLYYIKNLDGGNKVANVVTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQIHGVGHIGL
LYSSQVNSLIKEGLNGGGQNTN

OR

DDYSVVEEHGQLSISNGELVNERGEQVQLKGMSSHGLQWYGQFVNYESMKWLRDDWGITVFRAAMYTSSGGYIDDPSV
KEKVKETVEAAIDLGIYVIIDWHILSDNDPNIYKEEAKDFFDEMSELYGDYPNVIYEIANEPNGSDVTWDNQIKPYAEEVIPVI
RDNDPNNIVIVGTGTWSQDVHHAADNQLADPNVMYAFHFYAGTHGQNLRDQVDYALDQGAAIFVSEWGTSAATGDG
GVFLDEAQVWIDFMDERNLSWANWSLTHKDESSAALMPGANPTGGWTEAELSPSGTFVREKIRESASDNNDPIPDPDDE
AEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSM
GGANTLYYIKNLDGGNKVANVVTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQIHGVGHIGL
KSQVNSLIKEQGGGQNTN