



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2212577 C 2010/06/01

(11)(21) **2 212 577**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 1996/02/08
(87) Date publication PCT/PCT Publication Date: 1996/08/15
(45) Date de délivrance/Issue Date: 2010/06/01
(85) Entrée phase nationale/National Entry: 1997/08/07
(86) N° demande PCT/PCT Application No.: DK 1996/000060
(87) N° publication PCT/PCT Publication No.: 1996/024665
(30) Priorités/Priorities: 1995/02/10 (DK0156/95);
1995/03/17 (DK0273/95)

(51) Cl.Int./Int.Cl. *C12N 9/54* (2006.01),
C11D 3/386 (2006.01), *C12N 1/20* (2006.01),
C12P 21/02 (2006.01)
(72) Inventeurs/Inventors:
CONRAD, LARS SPARRE, DK;
OUTTRUP, HELLE, DK
(73) Propriétaire/Owner:
NOVOZYMES A/S, DK
(74) Agent: MCCARTHY TETRAULT LLP

(54) Titre : PROTEASES DE BACILLUS
(54) Title: BACILLUS PROTEASES

(57) **Abrégé/Abstract:**

This invention is in the field of detergent proteases obtainable from a strain of a new *Bacillus* sp. ZI 315. Moreover, the invention is directed towards a process for the preparation of the protease, the use of the protease as detergent enzyme, and detergent compositions comprising the protease of the invention.



PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/54, C11D 3/386	A1	(11) International Publication Number: WO 96/24665 (43) International Publication Date: 15 August 1996 (15.08.96)
(21) International Application Number: PCT/DK96/00060 (22) International Filing Date: 8 February 1996 (08.02.96) (30) Priority Data: 0156/95 10 February 1995 (10.02.95) DK 0273/95 17 March 1995 (17.03.95) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): OUTTRUP, Helle [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). CONRAD, Lars, Sparre [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: BACILLUS PROTEASES (57) Abstract This invention is in the field of detergent proteases obtainable from a strain of a new <i>Bacillus sp.</i> ZI 315. Moreover, the invention is directed towards a process for the preparation of the protease, the use of the protease as detergent enzyme, and detergent compositions comprising the protease of the invention.		

BACILLUS PROTEASES

FIELD OF INVENTION

The present invention relates to detergent proteases obtainable from strains of *Bacillus* sp. More specifically, the invention is directed towards a novel alkaline protease derived from a strain of *Bacillus* sp. ZI 315. Moreover, the invention is directed towards a process for the preparation of the protease, the use of the protease as a detergent enzyme, and detergent compositions comprising the protease of the invention.

BACKGROUND OF THE INVENTION

Detergent enzymes have been marketed for more than 20 years and are now well established as normal detergent ingredients in both powder and liquid detergents all over the world.

Enzymes used in washing formulations comprise many different enzymes such as proteases, lipases, amylases, cellulases, as well as other enzymes, or mixtures hereof. Commercially the most important enzymes are the proteases.

Detergent proteases have been developed by isolation of proteases found in nature followed by testing in detergent formulations. Most detergent proteases are obtained from members of the genus *Bacillus*.

Examples of commercial protease products are AL-CALASE™, ESPERASE™ and SAVINASE™, all supplied by Novo Nordisk A/S, Denmark. The ALCALASE™ protease is produced by strains of the species *Bacillus licheniformis*. The ESPERASE™ and SAVINASE™ proteases are obtained by cultivation of strains of alkalophilic *Bacilli*.

The washing traditions, especially the used washing temperature, the hardness of the used water, and the ingredients of the detergents vary very much from one country to another. Typical conditions are outlined below:

- low pH and low water hardness: liquid detergents in US and Asia;
- low pH and high water hardness: liquid detergents in Europe;
- high pH and low water hardness: powder detergents in US and Asia; and
- high pH and high water hardness: powder detergents in Europe.

(A low pH in detergents is typically a pH in the range 8.0-9.5, in particular around 9; a high pH in detergents is typically a pH in the range 10-11.5, in particular around 10.5. A low water hardness is typically in the range 3-6°dH; a high water hardness is typically in the range 15-20 °dH, in particular around 18°dH).

Furthermore, the compositions of the detergents are changing these years in order to make the washing process more environmental friendly. All these differences and changes within the detergent industry make the field extremely complicated. There is therefore a need all the time to find new proteases which perform optimally at a certain specified set of conditions.

20 SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel detergent proteases with improved washing performance at moderate to low wash temperatures.

Accordingly, in its first aspect, the invention provides a protease characterized by:

- having immunochemical properties identical or partially identical to those of a protease derived from the strain *Bacillus* sp. ZI 315, DSM 9702.

In a second aspect, the invention relates to an isolated biologically pure culture of a strain of *Bacillus* sp. represented by the strain *Bacillus* sp. ZI 315. In a more specific aspect, the invention relates to a strain of *Bacillus* sp. ZI 315, DSM 9702, or a mutant or a variant thereof.

In a third aspect, the invention provides a process for the preparation of the protease, which process comprises cultivation of a protease producing strain of *Bacillus sp.* ZI 315 in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme. In a more specific aspect, *Bacillus sp.* ZI 315, DSM 9702, or a mutant or a variant thereof, or another host organism carrying the gene encoding a protease having immunochemical properties identical or partially identical to those of the protease derived from *Bacillus sp.* ZI 315, is cultivated.

In a fourth aspect, the use of the enzyme as a detergent enzyme is claimed. In more specific aspects, the invention provides detergent compositions and detergent additives comprising the protease.

In a fifth aspect, the invention provides a washing process comprising addition of the protease.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which

Fig. 1 shows the relation between temperature and the proteolytic activity of an enzyme according to the invention (the enzyme preparation obtained according to Ex. 1, with 1% of casein as substrate and at pH 9.5);

Fig. 2 shows the relation between pH and the proteolytic activity of an enzyme according to the invention (the enzyme preparation obtained according to Ex. 1, with 1% of casein as substrate and at 25°C).

DETAILED DISCLOSURE OF THE INVENTION

The Microorganism

The novel microorganism of the invention, able to produce an enzyme of the invention, is represented by the strain that was isolated from a sample of soil. *Bacillus sp.* ZI 315 has been deposited according to the Budapest Treaty on the

International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 30 January 1995 at DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - under Accession No. DSM 9702.

5 The microorganism of this invention is an aerobic, alkaliphilic, spore forming bacterium belonging to the genus *Bacillus*. Morphologically it can be described as Gram+, motile rods with a diameter of 0.6 - 0.9 micron, and a length of 1.5 - 3 micron. The spores (which occur rarely) are ellipsoid,
10 central to subterminal, swelling the sporangium.

Optimum temperature for growth is within 30 - 40°C, with no growth at 50°C, and optimal pH for growth is within 9 - 10, with good growth at pH 10.0 and no growth at pH 7.0, which makes the strain strictly alkaliphilic.

15 The microorganism forms yellow to orange colonies, round and smooth, on alkaline nutrient agar slants, and no diffusion of pigment into the agar is observed.

Bacillus sp. ZI 315 has been identified as a new species within group 1 of the genus Bacillus. Full 16S rDNA
20 sequence analysis showed that Bacillus sp. ZI 315 is closest related to Bacillus firmus, Bacillus circulans, and Bacillus benzoovorans; it branches further away from other alkaliphilic species of group 1 such as Bacillus cohnii and Bacillus halmapalus. ZI 315 branches away from and shows significant
25 physiological differences to its closest phylogenetic relatives and are for these reasons considered to be a new species within group 1 of the genus Bacillus.

Cultivation of the Microorganism

The microorganism of the invention can be cultivated
30 under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients, the medium being composed in accordance with the principles of the known art.

Suitable carbon sources are carbohydrates such as
35 sucrose, glucose and starch, or carbohydrate containing materials such as cereal grain, malt, rice and sorghum. The carbohydrate concentration incorporated in the medium may vary

widely, e.g. up to 25% and down to 1 - 5%, but usually 8 - 10% will be suitable, the percentages being calculated as equivalents of glucose.

The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources quite a number are used regularly in fermentation processes involving the cultivation of bacteria. Illustrative examples are soybean meal, cotton seed meal, peanut meal, casein, corn, corn steep liquor, yeast extract, urea, and albumin. In addition, the nutrient medium should also contain usual trace substances.

The novel *Bacillus* species of this invention are slightly alkalophilic. Therefore, the cultivation is preferably conducted at alkaline pH values, which can be obtained by addition of suitable buffers such as sodium carbonate, pH 9.0 - 10.5, after sterilization of the growth medium. For cultivation in tank fermentors it is necessary to use artificial aeration. The rate of aeration is similar to that used in conventional tank fermentation.

After fermentation, liquid enzyme concentrates may be produced by removal of coarse material from the broth or, if desired, concentration of the broth by, e.g., evaporation at low temperature or by reverse osmosis. Finally, preservatives may be added to the concentrate.

Solid enzyme preparations may be prepared from the purified and/or concentrated broth by precipitation with salts, such as Na_2SO_4 or water-miscible solvents, such as ethanol or acetone. Removal of the water in the broth by suitable drying methods, such as spray-drying, may also be employed.

Assay for Proteolytic Activity

The proteolytic activity is determined with casein as substrate. One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 mM of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5). A folder AF 228, describing the analytical

method, is available upon request to Novo Nordisk A/S, Denmark.

The Enzyme

The enzyme of the invention is a novel detergent protease. It is obtainable by cultivation of a microorganism of the invention, preferably *Bacillus* sp. ZI 315, DSM 9702, or a mutant or a variant thereof, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts. The enzyme can also be obtained by recombinant DNA-technology.

10 The protease of the invention may be characterized by the physical-chemical properties described below.

Physical-Chemical Properties

A molecular weight of 38 kD, determined by SDS-PAGE. A pI at above 9.3 could be determined by isoelectric focusing 15 on LKB Ampholine® PAG plates. The protease activity is inhibited by PMSF, α -1-antitrypsin, and Turkey-egg-white proteinase inhibitor. EDTA and soybean-protein inhibitor do not influence the protease activity.

The temperature activity relationship was determined 20 with 1% of casein as substrate and at pH 9.5. The assay for proteolytic activity described previously was used with the modification that the incubation temperature was varied in the interval of from 10°C to 70°C.

The result is shown in Fig. 1. It appears from the 25 figure that the enzyme possesses proteolytic activity from temperatures below 10°C to about 50°C, and has a temperature optimum at around 40°C.

The dependence of activity on pH was determined by the same procedure using Britten-Robinson buffers adjusted to 30 predetermined pH values in the pH range of from 6 to 11.

The result is shown in Fig. 2. It appears from this figure that the enzyme possesses proteolytic activity at pH values below 6 to above 11 with a pH optimum in the range of from pH 9 to pH 11.

The protease of the invention possesses especial potentials in detergents with low water hardness and moderate to low wash temperatures.

Immunochemical Properties

5 The protease of the invention has immunochemical properties identical or partially identical (i.e. at least partially identical) to those of a protease derived from the strain *Bacillus* sp. ZI 315, DSM 9702.

 The immunochemical properties for various *Bacillus*
10 proteases are indeed a very distinguishing feature: whereas pH-optimum, temperature-optimum, pI etc. as disclosed above are more or less the same, different immunochemical properties result in very different stability in various detergents.

 The immunochemical properties can be determined
15 immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to N. H. Axelsen; Handbook of Immuno-precipitation-in-Gel Techniques; Blackwell Scientific Publica-
20 tions (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, chapters 5, 19 and 20.

 Monospecific antiserum was generated according to the above mentioned method by immunizing rabbits with the purified
25 protease of the invention. The immunogen was mixed with Freund's adjuvant and injected subcutaneously into rabbits every second week. Antiserum was obtained after a total immunization period of 8 weeks, and immunoglobulin was prepared therefrom as described by N. H. Axelsen, supra.

30 Using the ouchterlony double immunodiffusion test described above the protease of the invention showed no cross reaction to the known serine proteases:

- ALCALASE™ (available from Novo Nordisk A/S)
- SAVINASE™ (available from Novo Nordisk A/S)
- 35 - ESPERASE™ (available from Novo Nordisk A/S)
- subtilisin Novo (available from Novo Nordisk A/S),

- KAZUSASE™ (available from SHOWA DENKO),
- the Bacillus proteases described in WO 92/07067,
- the Bacillus proteases described in WO 92/17576,
- the Bacillus proteases described in WO 92/17577,
- 5 - the Bacillus proteases described in WO 92/17578,
- the Bacillus proteases described in WO 93/18140,
- the Bacillus proteases described in WO 93/24623,
- the Bacillus proteases described in WO 94/01532, and
- the Bacillus proteases described in WO 95/07350.

10 Various Bacillus proteases tolerate various detergents with a **great variety**, and one of the best tools today in differentiating between Bacillus proteases is the immunochemical identity tool.

Detergent Compositions

15 According to the invention, the protease may typically be a component of a detergent composition, e.g., a dishwashing or a laundry detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme.

20 Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000

25 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of

30 film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods.

35 Other enzyme stabilizers are well known in the art. Protected

enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyl dimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as amylase, lipase, cutinase, cellulase, peroxidase, and oxidase, e.g., laccase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetythylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
30	Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
	Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
35	Sodium carbonate (as Na_2CO_3)	14 - 20%
	Soluble silicate (as $Na_2O, 2SiO_2$)	2 - 6%
	Zeolite (as $NaAlSiO_4$)	15 - 22%
	Sodium sulfate (as Na_2SO_4)	0 - 6%

	Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
	Sodium perborate (as $NaBO_3 \cdot H_2O$)	11 - 18%
	TAED	2 - 6%
5	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
10	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

15	Linear alkylbenzenesulfonate (cal- culated as acid)	6 - 11%
	Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
20	Alcohol ethoxylate (e.g. C_{14-15} alco- hol, 7 EO)	5 - 9%
	Sodium carbonate (as Na_2CO_3)	15 - 21%
	Soluble silicate (as $Na_2O, 2SiO_2$)	1 - 4%
25	Zeolite (as $NaAlSiO_4$)	24 - 34%
	Sodium sulfate (as Na_2SO_4)	4 - 10%
	Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
	Carboxymethylcellulose	0 - 2%
30	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
35	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	5	-	9%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO)	7	-	14%
	Soap as fatty acid (e.g. C ₁₆₋₂₂ fatty acid)	1	-	3%
	Sodium carbonate (as Na ₂ CO ₃)	10	-	17%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	3	-	9%
10	Zeolite (as NaAlSiO ₄)	23	-	33%
	Sodium sulfate (as Na ₂ SO ₄)	0	-	4%
	Sodium perborate (as NaBO ₃ ·H ₂ O)	8	-	16%
	TAED	2	-	8%
	Phosphonate (e.g. EDTMPA)	0	-	1%
15	Carboxymethylcellulose	0	-	2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
20	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0	-	5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO)	10	- 25%
	Sodium carbonate (as Na ₂ CO ₃)	14	- 22%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1	- 5%
	Zeolite (as NaAlSiO ₄)	25	- 35%
	Sodium sulfate (as Na ₂ SO ₄)	0	- 10%
10	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
20	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO or C ₁₂₋₁₅ alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
25	Alkenylsuccinic acid (C ₁₂₋₁₄)	0	- 13%
	Aminoethanol	8	- 18%
	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
30	Borate (as B ₄ O ₇)	0	- 2%
	Ethanol	0	- 3%
	Propylene glycol	8	- 14%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	- 5%

6) An aqueous structured liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. oleic acid)	3	- 10%
10	Zeolite (as NaAlSiO ₄)	14	- 22%
	Potassium citrate	9	- 18%
	Borate (as B ₄ O ₇)	0	- 2%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. PEG, PVP)	0	- 3%
15	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
	Glycerol	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	- 5%

25 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Fatty alcohol sulfate	5	- 10%
	Ethoxylated fatty acid monoethanol-amide	3	- 9%
30	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na ₂ CO ₃)	5	- 10%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1	- 4%
	Zeolite (as NaAlSiO ₄)	20	- 40%
	Sodium sulfate (as Na ₂ SO ₄)	2	- 8%
35	Sodium perborate (as NaBO ₃ ·H ₂ O)	12	- 18%
	TAED	2	- 7%

	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
5	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	-	5%

8) A detergent composition formulated as a granulate comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	8	-	14%
	Ethoxylated fatty acid monoethanolamide	5	-	11%
	Soap as fatty acid	0	-	3%
	Sodium carbonate (as Na_2CO_3)	4	-	10%
15	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	1	-	4%
	Zeolite (as NaAlSiO_4)	30	-	50%
	Sodium sulfate (as Na_2SO_4)	3	-	11%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	5	-	12%
20	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0	-	5%

25 9) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	-	12%
	Nonionic surfactant	1	-	4%
	Soap as fatty acid	2	-	6%
30	Sodium carbonate (as Na_2CO_3)	14	-	22%
	Zeolite (as NaAlSiO_4)	18	-	32%
	Sodium sulfate (as Na_2SO_4)	5	-	20%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	3	-	8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	4	-	9%
35	Bleach activator (e.g. NOBS or TAED)	1	-	5%

5	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. polycarboxylate or PEG)	1 - 5%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	15	-	23%
	Alcohol ethoxysulfate (e.g. C ₁₂₋₁₅ alcohol, 2-3 EO)	8	-	15%
15	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ al- cohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	3	-	9%
	Soap as fatty acid (e.g. lauric acid)	0	-	3%
	Aminoethanol	1	-	5%
	Sodium citrate	5	-	10%
20	Hydrotrope (e.g. sodium toluenesulfonate)	2	-	6%
	Borate (as B ₄ O ₇)	0	-	2%
	Carboxymethylcellulose	0	-	1%
	Ethanol	1	-	3%
25	Propylene glycol	2	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
30	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	-	5%

11) An aqueous liquid detergent composition comprising

35	Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	6 - 12%
	Aminoethanol	2 - 6%

	Citric acid	8	- 14%
	Borate (as B_4O_7)	1	- 3%
5	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
	Glycerol	3	- 8%
10	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. hydro-tropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having
 15 a bulk density of at least 600 g/l comprising

20	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
	Sodium carbonate (as Na_2CO_3)	8	- 25%
	Soluble silicates (as Na_2O , $2SiO_2$)	5	- 15%
25	Sodium sulfate (as Na_2SO_4)	0	- 5%
	Zeolite (as $NaAlSiO_4$)	15	- 28%
	Sodium perborate (as $NaBO_3 \cdot 4H_2O$)	0	- 20%
	Bleach activator (TAED or NOBS)	0	- 5%
30	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) wherein all
 or part of the linear alkylbenzenesulfonate is replaced by (C_{12} -
 35 C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having
 a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	9	- 15%
	Alcohol ethoxylate	3	- 6%
	Polyhydroxy alkyl fatty acid amide	1	- 5%
	Zeolite (as NaAlSiO ₄)	10	- 20%
5	Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
	Sodium carbonate (as Na ₂ CO ₃)	3	- 12%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	0	- 6%
	Sodium citrate	4	- 8%
10	Sodium percarbonate	13	- 22%
	TAED	3	- 8%
	Polymers (e.g. polycarboxylates and PVP)	0	- 5%
15	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

15) A detergent composition formulated as a granulate having
 20 a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
25	Sodium carbonate (as Na ₂ CO ₃)	2	- 8%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	0	- 4%
	Sodium percarbonate	13	- 22%
	TAED	1	- 8%
	Carboxymethyl cellulose	0	- 3%
30	Polymers (e.g. polycarboxylates and PVP)	0	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	- 3%

- 16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.
- 5 17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.
- 18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds
10 described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.
- 19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxyated primary alcohol, a builder system (e.g.
15 phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The protease of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the
20 invention, the protease may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of protease per liter of wash liquor.

The invention is further illustrated in the following examples, which are not intended to be in any way limiting to
25 the scope of the invention as claimed.

EXAMPLE 1

Bacillus sp. ZI 315, DSM 9702, was cultivated at 30°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of medium of the following
30 composition (per litre):

Potato starch	100	g
Ground barley	50	g

20

Soybean flour	20	g
Na ₂ HPO ₄ x 12 H ₂ O	9	g
Pluronic®	0.1	g
Sodium caseinate	10	g

5 The starch in the medium is liquified with α -amylase, and the medium is sterilized by heating at 120°C for 45 minutes.

 After sterilization the pH of the medium is adjusted to 9.7 by addition of 10 ml of a 1 M solution of sodium
10 sesquicarbonate.

 After cultivation (3 days) and separation of the solid material the protease was purified by a conventional chromatographic method.

 Yield from 1.5 l of culture broth was 50 ml with 70
15 CPU/l. Purity was more than 90% as judged by SDS-PAGE.

 The characteristics of the preparation prepared in accordance with this Example have been referred to earlier in this specification, and reference is made hereto.

EXAMPLE 2

20 Wash Performance of Bacillus sp. ZI 315 protease (at 20°C)

 The wash performance tests were accomplished on grass juice soiled cotton, in a model wash system at 20°C, at a constant temperature for 10 minutes.

 The tests were performed at protease concentrations
25 of 1.6, 3.2, 8, 16, 32, 64 and 160 nM.

 2.0 g/l of an American type powder detergent composition were used in the test. The detergent did not contain any enzymes prior to the addition of the protease of the invention. The detergent was dissolved in approx. 6°dH (German Hardness)
30 water. The pH of the wash liquor was 10. The textile/wash liquor ratio was approximately 5 g of textile per litre of wash liquor. For each enzyme concentration two independent tests were performed.

 Subsequent to the fabric washing, the cloths were
35 flushed in running tap water for 20 minutes and then air-dried.

The performance of the protease of the invention and of Savinase™ was assessed by the change (ΔR) of the remission (%R) at 460 nm measured on a Datacolor Elrephometer 2000, ΔR being the remission after wash with protease added, minus the remission after wash with no protease added.

The results of these tests are shown in Table 1 below (mean of 2 tests).

Table 1

10	Protease Concentration (nM)	ΔR	
		ZI 315	SAVINASE™
	1.6	4.6	0.8
	3.2	7.7	2.7
	8	12.0	6.6
15	16	17.7	8.5
	32	18.2	14.8
	64	21.9	17.0

It can be seen from Table 1 that ΔR (Bacillus sp. ZI 315) is higher than ΔR (Savinase™) at all the measured protease concentrations, i.e. the protease of the invention has a better wash performance at all the measured concentrations at 20°C.

EXAMPLE 3

Wash Performance of Bacillus sp. ZI 315 protease (at 25°C)

The wash performance tests were accomplished on grass juice soiled cotton, in a model wash system at 25°C, at a constant temperature for 10 minutes.

The tests were performed at protease concentrations of 1, 2, 7.5, and 20 nM.

2.0 g/l of a detergent with the following composition

Linear alkylbenzenesulfonate	0.3	g/l
Alcohol ethoxylate	0.04	g/l
Soap	0.1	g/l
Na ₂ SO ₄	0.3	g/l

Na ₂ CO ₃	0.4 g/l
Zeolith	0.6 g/l
Na ₃ -citrate	0.08 g/l
Carboxymethylcellulose	0.006 g/l
5 Polycarboxylate	0.083 g/l

were used in the test. The detergent was dissolved in approx. 6°dH (German Hardness) water. The pH of the wash liquor was adjusted to pH 10. The textile/wash liquor ratio was approximately 5 g of textile per litre of wash liquor. For each enzyme
10 concentration two independent tests were performed.

Subsequent to the fabric washing, the cloths were flushed in running tap water for 20 minutes and then air-dried. The performance of the protease of the invention and of Savinase™ was assessed by the change (ΔR) of the remission (%R)
15 at 460 nm measured on a Datacolor Elrephometer 2000, ΔR being the remission after wash with protease added, minus the remission after wash with no protease added.

The results of these tests are shown in Table 2 below (mean of 2 tests).

20 Table 2

Protease Concentration (nM)	ΔR	
	ZI 315	SAVINASE™
1	3.7	2.7
25 2	7.5	2.9
7.5	14.8	7.8
20	19.6	13.7

It can be seen from Table 2 that ΔR (Bacillus sp. ZI
30 315) is higher than ΔR (Savinase™) at all the measured protease concentrations, i.e. the protease of the invention has a better wash performance at all the measured concentrations at 25°C.

EXAMPLE 4

Improvement factor/Model detergents

An improvement factor (defined below) for the protease of the invention was established at low and high water hardness, using a model detergent at pH 9 with Savinase as reference.

5 The improvement factor was determined in the following way:

Measurement of remission (R) on a test material (grass on cotton) was done at 460 nm using an Elrepho 2000 photometer (without UV). The measured values were fitted to the
10 expression:

$$R = (a \cdot \Delta R_{\max} \cdot c) / (\Delta R_{\max} + a \cdot c) + b.$$

The improvement factor (IF) is then calculated by use of the initial slope of the curve:

$$IF = a/a_{\text{ref}};$$

15 wherein

R: is the wash effect of the enzyme in remission units,
a: is the initial slope of the fitted curve,
a_{ref}: is the initial slope for the reference enzyme,
b: is the intersection of the fitted curve and the y-
20 axis,
c: is the enzyme concentration in nanomoles active enzyme per liter, and
ΔR_{max}: is the theoretical maximum wash effect of the enzyme in remission units.

25 The following experimental conditions were used:

Detergent:	25% STP (Na ₅ P ₃ O ₁₀)
	25% Na ₂ SO ₄
	10% Na ₂ CO ₃
	20% LAS (Nansa 80S)
30	5% NI (Dobanol 25-7)
	5% Na ₂ Si ₂ O ₅
	0.5% CMC (carboxymethylcellulose)
	9.5% water

Detergent dose: 3 g/l
pH: 9.0
Washing time: 15 min.
Washing temperature: 15°C
5 Water hardness: 6°dH and 18°dH
Enzyme concentrations: 0, 3, 6, 9, 15, 30 and 60 nM
Swatch/volume: 5 swatches (Diameter: 2.5 cm) per
50 ml washing solution
Test material: Grass on cotton.

10 The following results were obtained:

IF = 4.8, when the water hardness was 6°dH, and
IF = 1.4, when the water hardness was 18°dH.

This example shows that the protease of the invention
would be very useful in a liquid detergent (low pH) for use in
15 US and Asia, (where the water hardness is low; around or less
than 6°dH).

EXAMPLE 5

Improvement factor/Commercial detergents

Improvement factors, obtained as explained in Example
20 4, were also established for the commercial detergents Koso Top
and Omo Powder China, again with Savinase as the reference
enzyme.

The following experimental conditions were used:

Detergent dose: 1 g/l
25 pH: 10.5 (Koso Top) and
10.2 (Omo Powder China)
Washing time: 15 min.
Washing temperature: 15°C
Water hardness: 3°dH
30 Enzyme concentrations: 3, 6, 9, 15, 30 and 60 nM
Swatch/volume: 5 swatches (Diameter: 2.5 cm) per
50 ml washing solution

Test material: Grass on cotton.

The following results were obtained:

IF = 2.1 (Koso Top), and

IF = 3.1 (Omo Powder China)

5 This example shows that the protease of the invention would be very useful in detergents with high pH for use in Asia, (where the water hardness is low, around 3°dH).

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>2</u> , line <u>27-28</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐	
Name of depositary institution DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL- KULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b, D-38124 Braunschweig, Federal Re- public of Germany	
Date of deposit 30 January 1995	Accession Number DSM 9702
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet ☐	
During the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. e.g. Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71) in those designated states providing for such "expert solution".	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only ☒ This sheet was received with the international application Authorized officer 	For International Bureau use only ☐ This sheet was received by the International Bureau on: Authorized officer
--	---

CLAIMS

1. A protease having immunochemical properties identical to those of a protease derived from the strain *Bacillus sp.* ZI 315, DSM 9702, and further having:
 - (a) an apparent molecular weight of 38 kD;
 - (b) a pI above 9.3;
 - (c) a pH optimum in the range of from pH 9 to pH 11, at 25°C and with casein as substrate;
 - (d) a temperature optimum at around 40°C, at pH 9.5 and with casein as substrate; and
 - (e) a relative activity of around 60% at 25°C, at pH 9.5 and with casein as substrate.
2. The protease of claim 1, obtained from the strain *Bacillus sp.* ZI 315, DSM 9702.
3. An isolated biologically pure culture of a strain of *Bacillus sp.*, wherein the strain is *Bacillus sp.* ZI 315, DSM 9702.
4. A process for the preparation of a protease according to any one of claims 1-2, which process comprises cultivation of a protease producing strain of *Bacillus sp.* according to claim 3, in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme.
5. A process according to claim 4, in which *Bacillus sp.* ZI 315, DSM 9702, carrying the gene encoding the protease according to claim 1, is cultivated.
6. Use of a protease according to any one of claims 1-2 as a detergent enzyme.
7. A detergent composition comprising a surfactant and a protease according to any one of claims 1-2.
8. A detergent composition according to claim 7, which further comprises at least one other enzyme.

- 28 -

9. A detergent composition according to claim 8, wherein the at least one other enzyme is selected from the group consisting of an amylase, lipase, a cellulase, a peroxidase, and an oxidase.

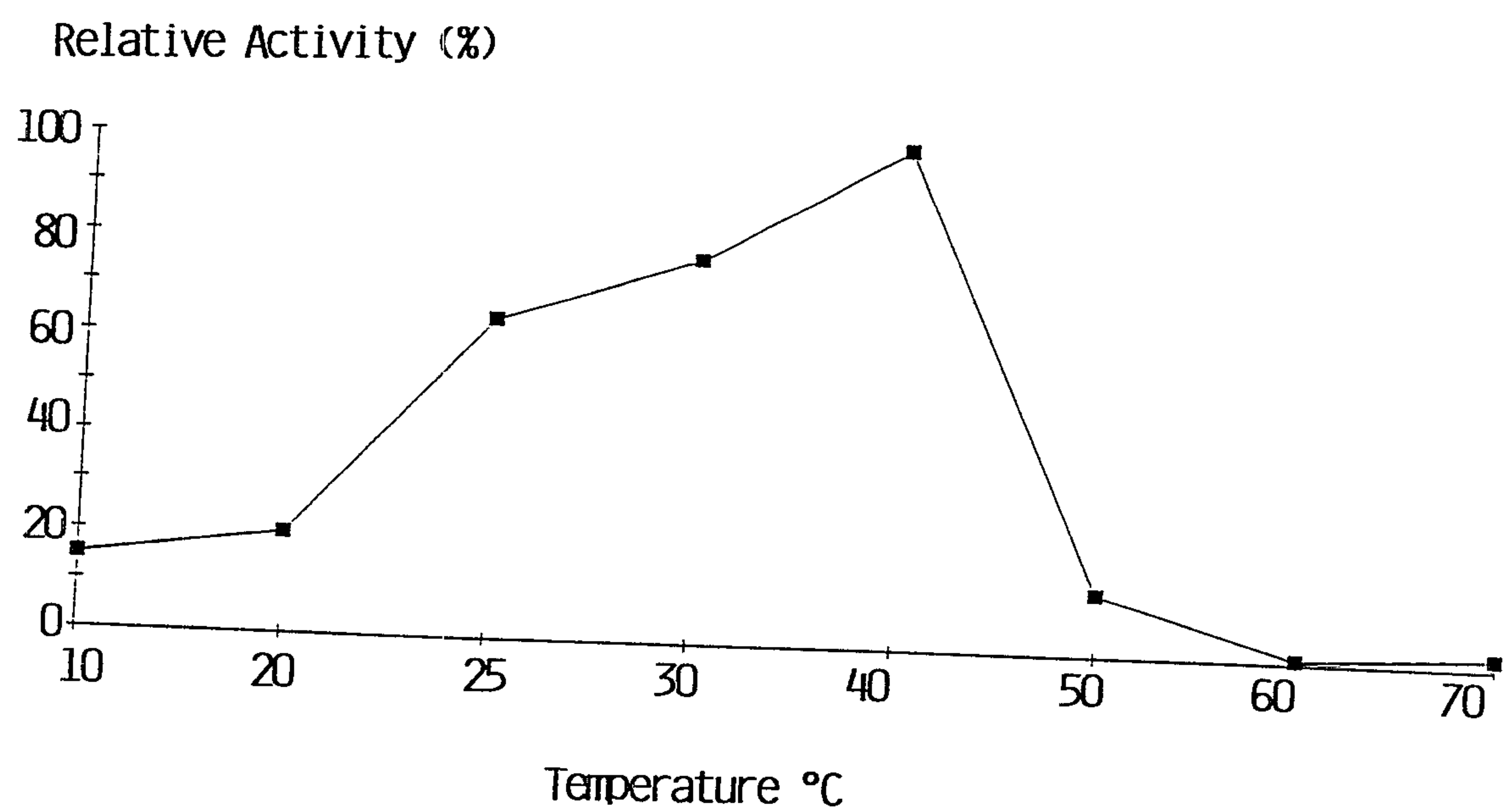
10. A detergent additive comprising a protease according to any one of claims 1-2, provided in the form of a non-dusting granulate, a liquid, a slurry, or a protected enzyme.

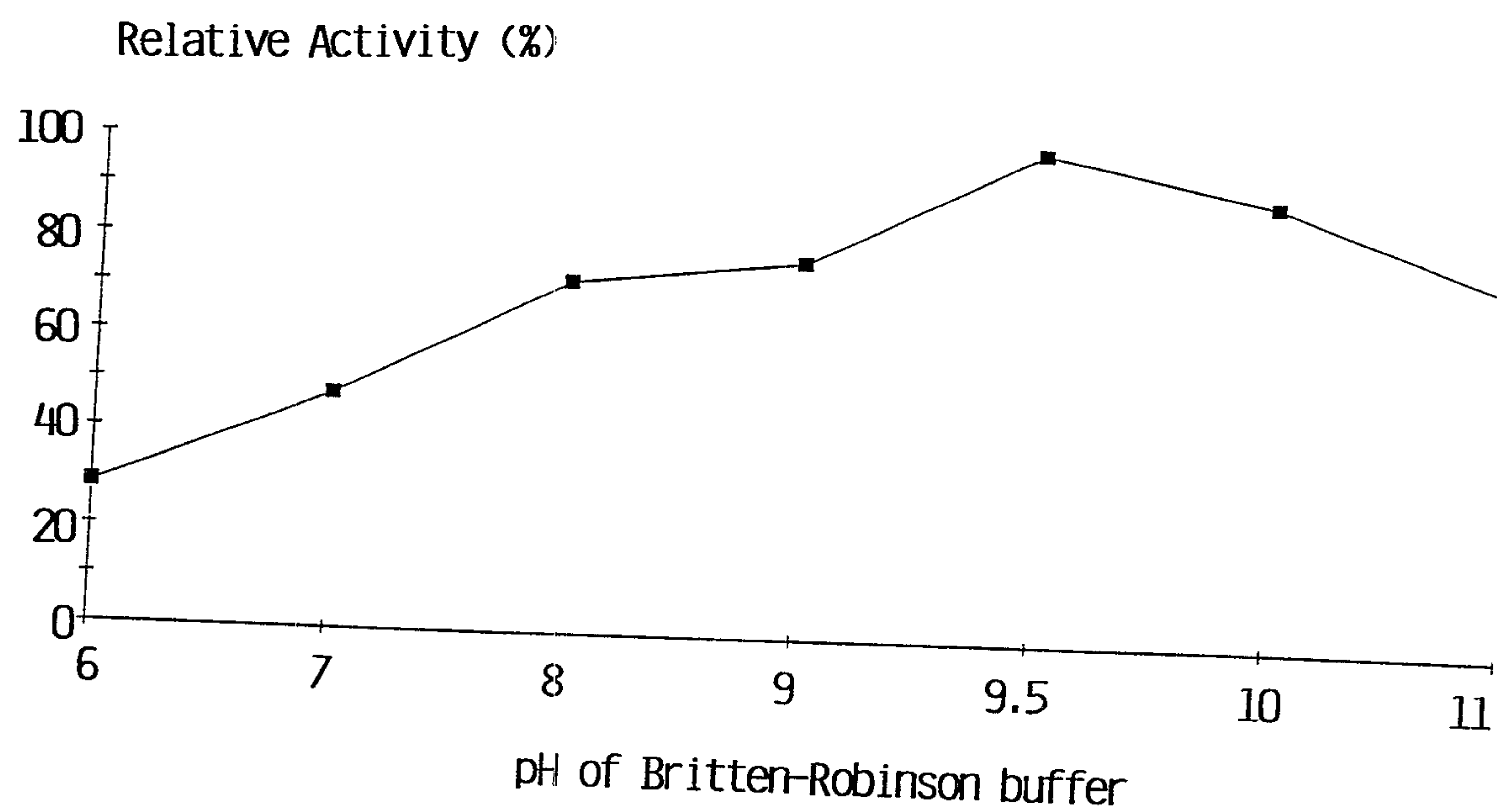
11. A detergent additive according to claim 10, wherein the liquid is a stabilized liquid.

12. A washing process comprising addition of a protease according to any one of claims 1-2.

13. A washing process according to claim 12, comprising addition of a detergent composition according to either of claims 7-8.

14. A washing process according to claim 12, comprising addition of a detergent additive according to claim 10.

1/2**Fig. 1**

2/2**Fig. 2**