(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 309 244 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
18.04.2018 Bulletin 2018/16

(51) Int Cl.:
C11D 3/386 *(2006.01)*

(21) Application number: **16193372.6**

(22) Date of filing: **11.10.2016**

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
MA MD

(71) Applicant: **BASF SE**
67056 Ludwigshafen am Rhein (DE)

(72) Inventors:
- **NIELSEN, Jesper**
  92121 San Diego (US)
- **SPANGENBERG, Oliver**
  67056 Ludwigshafen (DE)
- **JENEWEIN, Stefan**
  67056 Ludwigshafen (DE)

(74) Representative: **BASF IP Association**
BASF SE
G-FLP-C006
67056 Ludwigshafen (DE)

(54) **LOW TEMPERATURE PROTEASE**

(57) The present invention is directed to specific subtilisin variants that are improved in their efficiency of removing stains on textile and soils on hard surfaces at low temperature and to methods of using such protease variants to improve wash performance of a washing step on stains and soils at low washing temperature and to methods of using such protease variants to identify protease variants with increased or reduced proteolytic activity in detergents at low temperature.

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Description

Field of the invention

[0001] The present invention is directed to specific subtilisin variants that are improved in their efficiency of removing stains on textile and soils on hard surfaces at low temperature and to methods of using such proteases variants to improve wash performance of a washing step on stains and soils at low washing temperature and to methods of using such proteases variants to identify protease variants with increased or reduced proteolytic activity in detergents at low temperature.

Background of the invention

[0002] In order to obtain optimal cleaning results, washing of stained textiles or soiled hard surfaces is usually performed at high temperature, preferably above 30°C, e.g., between 35-90°C. Washing at 30°C and below is not favored due to reduced stain/soil removal performance at these low temperatures. However, in view of the common trend to reduce energy consumption in laundry and hard surface cleaning - mostly for cost and environmental reasons - there is a strong need to improve cleaning efficiency at low temperature.

[0003] An attempt to improve cleaning efficiency and thereby reducing energy consumption in a washing step is the use of enzymes in detergent compositions. Enzymes are key components for effective stain removal.

[0004] In particular, proteases, especially subtilisin proteases, are used in detergent compositions to improve cleaning efficiency. Subtilisins are a class of proteases widely used in commercial products (for example, in laundry and dish washing detergents, and contact lens cleaners) and for research purposes (catalysts in synthetic organic chemistry). One member of the subtilisin family, a highly alkaline protease for use in detergent formulations has been described in patent application WO9102792 (BLAP, SEQ ID NO: 1). This Bacillus lentus alkaline protease (BLAP) can be obtained in commercial quantities from Bacillus licheniformis ATCC 53926 strain transformed by an expression plasmid harboring the wild type BLAP gene under the control of the B. licheniformis ATCC 53926 alkaline protease gene promoter.

[0005] Various attempts have been made to modify the amino acid sequence of subtilisins in order to improve the biochemical properties of these enzymes, in particular with respect to their wash performance, stability and substrate specificity.

[0006] For instance, it is stated in WO9523221A1 that improved wash performance could be achieved by removal of positively charged amino acid residues or the introduction of negatively charged amino acid residues in the region of the substrate binding pocket. Moreover, WO9523221A1 expresses the intent to provide detergent proteases that remove specific stains on fabric such as egg and blood stains. Modifications at amino acid positions R101, S156 and L217 (according to the numbering of the BPN' subtilisin protease from Bacillus amyloliquefaciens) are emphasised. Also, the exchange of arginine at position 101 by glutamic acid or an aspartic acid residue is mentioned. However, it is proposed that mutants that are particularly effective in removing blood and egg stains from fabrics are those mutants made by making the following replacements in the wild-type Bacillus lentus DSM 5483 protein: R101G, R101A, R101S, S156D, S156E, L217D, and L217E. Moreover, it is taught that mutants having replacement amino acid residues at positions 101 and 156 are particularly effective in removing blood stains. In contrast, mutants having replacement amino acid residues at position 217 are described to be particularly effective in removing egg stains. Furthermore, it is disclosed that a combination of mutant enzymes having replacement amino acid residues at positions 101 and 156 and those having replacement amino acid residues at position 217 are particularly effective in removing a combination of blood and egg stains.

[0007] In WO2011032988A1 it is disclosed that a protease comprising the combination of amino acid residues 101 E, 103A, and 104I is improved in its cleaning activity in liquid detergents in automatic dishwashing on egg yolk stains compared to a protease comprising the mutations 99S, 101S, 103S, 104V, and 159G (Savinase or Subtilisin 309).

[0008] WO2014030097A1 describes that a protease with the combination of amino acid residues 15A, 99D, 101 E, 103A, and 104I improves the wash performance on baked pudding stains compared to a protease comprising the mutations 9R, 15T, 68A, 99S, 101S, 103S, 104V, 159G, 218D, and 245R.

[0009] However, the problem of commonly used detergent proteases is the reduction of washing performance at temperatures at or below 30°C, in particular below 20°C. Thus, there is a strong need for detergent proteases that retain wash performance also at low temperatures.

[0010] WO2011032988A1 shows the wash performance of a protease comprising the amino acid residues 3S, 4V, 101 E, 194A, 199V, 205V, and 217L and the wash performance of a protease comprising the amino acid residues 3T, 4I, 101R, 194P, 199M, 205I, and 217D (according to BPN' numbering) at 20°C compared to 40°C in a detergent composition comprising phosphonate.

[0011] The present inventors have surprisingly found that the introduction of negative charges in the region corresponding to residues 98 to 104 of a protease as shown in SEQ ID NO: 1, in particular the R101E or R101D mutation,

leads to a wash performance that remains constant at lower temperatures, in particular at temperatures below 30°C.

Brief summary of the invention

[0012] The present invention is directed to a method for cleaning a stained textile and/or a soiled hard surface comprising the step of contacting a stained textile and/or a soiled hard surface at low temperature with a detergent composition comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2.

[0013] Furthermore, the present invention is directed to a method for improving the performance of a washing step on protein containing stains at low temperature comprising the step of contacting a textile or a hard surface, comprising one or more protein containing stains, with a detergent composition comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2. Preferably, the wash performance remains constant at low temperature.

[0014] Moreover, the present invention is directed to a method for testing the wash performance of a protease at low temperature comprising the steps of contacting a first one or more protein containing stains on a textile or a hard surface with a first protease and contacting a second one or more protein containing stains with a second protease wherein the first protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the first protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, preferably wherein the first and the second one or more protein containing stains are the same kind of stains for the first and the second protease.

[0015] In addition, the present invention is directed to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 for washing a textile or a hard surface at low temperature.

Detailed description of the invention

[0016] The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the examples included herein.

Definitions

[0017] Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art.

[0018] It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

[0019] Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

[0020] The term "introduction of at least two negative charges" into a particular amino acid sequence refers to the increase of the net charge of the particular amino acid sequence by at least two negative charges. Such increase of the net charge of the particular amino acid sequence by at least two negative charges is achieved by altering the amino acid sequence and can be reached by one or more amino acid sequence alterations selected from the group consisting of substitution, deletion and insertion, preferably by one or more amino acid substitutions. The increase of the net charge of the particular amino acid sequence by at least two negative charges can be achieved by removing positive charges or by introducing negative charges or by combinations thereof. The four amino acids aspartic acid (Asp, D), glutamic acid (Glu, E), lysine (Lys, K) and arginine (Arg, R) have a side chain which can be charged at neutral pH. At pH 7.0, two are negatively charged: aspartic acid (Asp, D) and glutamic acid (Glu, E) (acidic side chains), and two are positively charged: lysine (Lys, K) and arginine (Arg, R) (basic side chains). Thus, the introduction of at least two negative charges in the amino acid sequence can be reached for instance by substituting arginine by glutamic acid, substituting two non-charged leucine residues by two glutamic acid residues, by inserting two aspartic acid residues or by deleting two lysine residues. The introduction of at least two negative charges by modification of the amino acid sequence is evaluated preferably under conditions usually occurring in a washing step, preferably at pH 6-11, preferably at pH 7-9, more preferably at pH 7.5-8.5, further preferred at pH 7.0-8.0, most preferably at pH 7.0 or pH 8.0.

[0021] "Parent" sequence (also called "parent enzyme" or "parent protein") is the starting sequences for introduction

of changes (e.g. by introducing one or more amino acid substitutions) of the sequence resulting in "variants" of the parent sequences. Thus, the term "enzyme variant" or "sequence variant" or "protein variant" are used in reference to parent enzymes that are the origin for the respective variant enzymes. Therefore, parent enzymes include wild type enzymes and variants of wild-type enzymes which are used for development of further variants. Variant enzymes differ from parent enzymes in their amino acid sequence to a certain extent; however, variants at least maintain the enzyme properties of the respective parent enzyme. Preferably, enzyme properties are improved in variant enzymes when compared to the respective parent enzyme. More preferably, variant enzymes have at least the same enzymatic activity when compared to the respective parent enzyme or variant enzymes have increased enzymatic activity when compared to the respective parent enzyme.

[0022] In describing the variants of the present invention, the abbreviations for single amino acids used according to the accepted IUPAC single letter or three letter amino acid abbreviation is used.

[0023] "Substitutions" are described by providing the original amino acid followed by the number of the position within the amino acid sequence, followed by the substituted amino acid. For example the substitution of histidine at position 120 with alanine is designated as "His120Ala" or "H120A".

[0024] "Deletions" are described by providing the original amino acid followed by the number of the position within the amino acid sequence, followed by *. Accordingly, the deletion of glycine at position 150 is designated as "Gly150*" or G150*". Alternatively, deletions are indicated by e.g. "deletion of D183 and G184".

[0025] "Insertions" are described by providing the original amino acid followed by the number of the position within the amino acid sequence, followed by the original amino acid and the additional amino acid. For example an insertion at position 180 of lysine next to glycine is designated as "Gly180GlyLys" or "G180GK". When more than one amino acid residue is inserted, such as e.g. a Lys and Ala after Gly180 this may be indicated as: Gly180GlyLysAla or G195GKA.

[0026] In cases where a substitution and an insertion occur at the same position, this may be indicated as S99SD+S99A or in short S99AD. In cases where an amino acid residue identical to the existing amino acid residue is inserted, it is clear that degeneracy in the nomenclature arises. If for example a glycine is inserted after the glycine in the above example this would be indicated by G180GG. Variants comprising multiple alterations are separated by "+", e.g., "Arg170Tyr+Gly195Glu" or "R170Y+G195E" representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively. Alternatively multiple alterations may be separated by space or a comma e.g. R170Y G195E or R170Y, G195E respectively. Where different alterations can be introduced at a position, the different alterations are separated by a comma, e.g. "Arg170Tyr, Glu" and R170T, E, respectively, represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Alternatively different alterations or optional substitutions may be indicated in brackets, e.g., Arg170[Tyr, Gly] or Arg170{Tyr, Gly} or in short R170 [Y, G] or R170 {Y, G}.

[0027] The numbering of the amino acid residues of the subtilisin proteases described herein is according to the numbering of the BPN' subtilisin protease from Bacillus amyloliquefaciens as shown in SEQ ID NO: 2 (i.e., according to the numbering of SEQ ID NO: 2 or according to "BPN' numbering").

[0028] Variants of the parent enzyme molecules may have an amino acid sequence which is at least n percent identical to the amino acid sequence of the respective parent enzyme having enzymatic activity with n being an integer between 50 and 100, preferably 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 compared to the full length polypeptide sequence. Preferably, variant enzymes which are n percent identical when compared to a parent enzyme, have enzymatic activity.

[0029] "Identity" in relation to comparison of two amino acid sequences herein is calculated by dividing the number of identical residues by the length of the alignment region which is showing the shorter sequence over its complete length. This value is multiplied with 100 to give "percent-identity".

[0030] To determine the percent-identity between two amino acid sequences (i.e. pairwise sequence alignment), two sequences have to be aligned over their complete length (i.e. global alignment) in a first step. For producing a global alignment of two sequences, any suitable computer program, like program "NEEDLE" (The European Molecular Biology Open Software Suite (EMBOSS)), program "MATGAT" (Campanella, J.J, Bitincka, L. and Smalley, J. (2003), BMC Bioinformatics, 4:29), program "CLUSTAL" (Higgins, D.G. and Sharp, P.M. (1988), Gene, 73, 237-244) or similar programs may be used. In lack of any program, sequences may also be aligned manually.

[0031] After aligning two sequences, in a second step, an identity value shall be determined from the alignment. Depending on the applied method for percent-identity calculation, different percent-identity values can be calculated from a given alignment. Consequently, computer programs which create a sequence alignment, and in addition calculate percent-identity values from the alignment, may also report different percent-identity values from a given alignment, depending which calculation method is used by the program. Therefore, the following calculation of percent-identity according to the invention applies:

percent-identity = (identical residues / length of the alignment region which is showing the shorter sequence over its complete length) *100.

[0032] The calculation of percent-identity according to the invention is exemplified as follows:

Seq 1: TTTTTTAAAAAAAACCCCHHHCCCCAAARVHHHHHTTTTTTTT - length: 43 amino acids
Seq 2: TTAAAAAAAACCCCHHCCCCAAADLSSHHHHHTTTT - length: 36 amino acids Hence, the shorter sequence is sequence 2.

[0033] Producing a pairwise global alignment which is showing both sequences over their complete lengths results in

```
Seq 1: TTTTTTAAAAAAAACCCCHHHCCCCAAARV--HHHHHTTTTTTTT
           |||||||||||||| |||||||||  :  |||||||||
Seq 2: ----TTAAAAAAAACCCC-HHCCCCAAADLSSHHHHHTTTT----
```

[0034] Producing a pairwise alignment which is showing the shorter sequence over its complete length according the invention consequently results in:

```
Seq 1:  TTAAAAAAAACCCCHHHCCCCAAARV--HHHHHTTTT
        |||||||||||||| |||||||||  :  |||||||||
Seq 2:  TTAAAAAAAACCCC-HHCCCCAAADLSSHHHHHTTTT
```

[0035] The number of identical residues is 32, the alignment length showing the shorter sequence over its complete length is 37 (one gap is present which is factored in the alignment length of the shorter sequence). Therefore, %-identity according to the invention is: (32 / 37) * 100 = 86%

[0036] A special aspect concerning amino acid substitutions are conservative mutations which often appear to have a minimal effect on protein folding resulting in substantially maintained enzyme properties of the respective enzyme variant compared to the enzyme properties of the parent enzyme. Conservative mutations are those where one amino acid is exchanged with a similar amino acid. Such an exchange most probably does not change enzyme properties. Herein and in particular for the determination of percent-similarity the following conservative exchanges are considered:

Amino acid A is similar to amino acids S
Amino acid D is similar to amino acids E; N
Amino acid E is similar to amino acids D; K; Q
Amino acid F is similar to amino acids W; Y
Amino acid H is similar to amino acids N; Y
Amino acid I is similar to amino acids L; M; V
Amino acid K is similar to amino acids E; Q; R
Amino acid L is similar to amino acids I; M; V
Amino acid M is similar to amino acids I; L; V
Amino acid N is similar to amino acids D; H; S
Amino acid Q is similar to amino acids E; K; R
Amino acid R is similar to amino acids K; Q
Amino acid S is similar to amino acids A; N; T
Amino acid T is similar to amino acids S
Amino acid V is similar to amino acids I; L; M
Amino acid W is similar to amino acids F; Y
Amino acid Y is similar to amino acids F; H; W.

Conservative amino acid substitutions may occur over the full length of the sequence of a polypeptide sequence of a functional protein such as an enzyme. Preferably, such mutations are not pertaining the functional domains of an enzyme, more preferably conservative mutations are not pertaining the catalytic centers of an enzyme.

[0037] To take conservative mutations into account, a value for "similarity" of two amino acid sequences may be calculated. "Similarity" in relation to comparison of two amino acid sequences herein is calculated by dividing the number

of identical residues plus the number of similar residues by the length of the alignment region which is showing the shorter sequence over its complete length. This value is multiplied with 100 to give "percent-similarity". Therefore, the following calculation of percent-similarity according to the invention applies:

percent-similarity= [(identical residues + similar residues) / length of the alignment region which is showing the shorter sequence over its complete length] *100.

Using the example above with the pairwise alignment showing the shorter sequence over its complete length according the invention as follows for calculation of percent-similarity:

```
Seq 1:  TTAAAAAAAACCCCHHHCCCCAAARV--HHHHHTTTT
        ||||||||||||||| ||||||||| :  |||||||||
Seq 2:  TTAAAAAAAACCCC-HHCCCCAAADLSSHHHHHTTTT
```

[0038] The number of identical residues is 32, the number of similar amino acids exchanged is 1 (indicated by ":" in the alignment displayed above), the alignment length showing the shorter sequence over its complete length is 37 (one gap is present which is factored in the alignment length of the shorter sequence). Therefore, percent-similarity according to the invention is: [(32 + 1) / 37] * 100 = 89%

[0039] Especially, variant enzymes comprising conservative mutations which are at least m percent similar to the respective parent sequences with m being an integer between 50 and 100, preferably 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 compared to the full length polypeptide sequence, are expected to have essentially unchanged enzyme properties. Preferably, variant enzymes with m percent-similarity when compared to a parent enzyme, have enzymatic activity.

[0040] "Enzyme properties" include, but are not limited to catalytic activity as such, substrate/cofactor specificity, product specificity, increased stability in the course of time, thermostability, pH stability, chemical stability, and improved stability under storage conditions.

[0041] The term "substrate specificity" reflects the range of substrates that can be catalytically converted by an enzyme.

[0042] "Enzymatic activity" means the catalytic effect exerted by an enzyme, expressed as units per milligram of enzyme (specific activity) or molecules of substrate transformed per minute per molecule of enzyme (molecular activity). Enzymatic activity can be specified by the enzymes actual function, e.g. proteases exerting proteolytic activity by catalyzing hydrolytic cleavage of peptide bonds, lipases exerting lipolytic activity by hydrolytic cleavage of ester bonds, etc.

[0043] The term "protease" (or alternatively "peptidase" or "proteinase") is used for an enzyme with proteolytic activity, i.e., an enzyme that hydrolyses peptide bonds that link amino acids together in a polypeptide chain.

[0044] Enzymatic activity might change during storage or operational use of the enzyme. The term "enzyme stability" according to the current invention relates to the retention of enzymatic activity as a function of time during storage or operation. Retention of enzymatic activity as a function of time during storage is called "storage stability" and is preferred within the context of the invention.

[0045] To determine and quantify changes in catalytic activity of enzymes stored or used under certain conditions over time, the "initial enzymatic activity" is measured under defined conditions at time zero (100%) and at a certain point in time later (x%). By comparison of the values measured, a potential loss of enzymatic activity can be determined in its extent. The extent of enzymatic activity loss determines an enzyme's stability or non-stability.

[0046] "Half-life of enzymatic activity" is a measure for time required for the decaying of enzymatic activity to fall to one half (50%) of its initial value.

[0047] "Enzyme inhibitors" slow down the enzymatic activity by several mechanism as outlined below. Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually bind covalently to an enzyme by modifying the key amino acids necessary for enzymatic activity. Reversible inhibitors usually bind non-covalently (hydrogen bonds, hydrophobic interactions, ionic bonds). Four general kinds of reversible inhibitors are known:

(1) substrate and inhibitor compete for access to the enzymes active site (competitive inhibition),
(2) inhibitor binds to substrate-enzyme complex (uncompetitive inhibition),
(3) binding of inhibitor reduces enzymatic activity but does not affect binding of substrate (non-competitive inhibition),
(4) inhibitor can bind to enzyme at the same time as substrate (mixed inhibition).

[0048] As used herein, "wash performance" (also called herein "cleaning performance") of an enzyme refers to the contribution of the enzyme to the cleaning performance of a detergent composition, i.e. the cleaning performance added to the detergent composition by the performance of the enzyme. Wash performance is compared under relevant washing

conditions. The term "relevant washing conditions" is used herein to indicate the conditions, particularly washing temperature, time, washing mechanics, sud concentration, type of detergent and water hardness, actually used in households in a dish detergent market segment. The term "improved wash performance" is used to indicate that a better end result is obtained in stain removal under relevant washing conditions, or that less enzyme, on weight basis, is needed to obtain the same end result relative to the corresponding control conditions. The control conditions are preferably the same conditions but with using a protease that comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease does not comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2. The term "constant wash performance" refers to a wash performance that does not or does not significantly change when the wash conditions, e.g., the temperature, changes. Preferably, "constant wash performance" refers to a wash performance that is within +/- 1 %, +/- 2%, +/- 5%, +/- 10%, +/-15%, or +/- 20% of the wash performance under control conditions (e.g., compared to a control protease at higher temperature or compared to the improved protease described herein at temperature).

[0049] As used herein, the term "specific performance" refers to the cleaning of specific stains or soils per unit of active enzyme. In some embodiments, the specific performance is determined using stains or soils such as egg, egg yolk, milk, grass, minced meat blood, chocolate sauce, baby food, sebum, etc.

[0050] A detergent composition and/or detergent solution of the invention comprises one or more detergent components. The term "detergent component" is defined herein to mean the types of chemicals, which can be used in detergent compositions and / or detergent solutions.

[0051] Detergent compositions and / or detergent solutions according to the invention include detergent compositions and / or detergent solutions for different applications such as laundry and hard surface cleaning.

[0052] The term "laundry" relates to both household laundering and industrial laundering and means the process of treating textiles and/or fabrics with a solution containing a detergent composition of the present invention. The laundering process may be carried out by using technical devices such as a household or an industrial washing machine. Alternatively, the laundering process may be done by hand.

[0053] The term "textile" means any textile material including yarns (thread made of natural or synthetic fibers used for knitting or weaving), yarn intermediates, fibers, non-woven materials, natural materials, synthetic materials, as well as fabrics made of these materials such as garments, cloths and other articles. The terms "fabric" (a textile made by weaving, knitting or felting fibers) or "garment" (any article of clothing made of textile) as used herein, mean to include the broader term textile as well.

[0054] The term "fibers" includes natural fibers, synthetic fibers, and mixtures thereof. Examples of natural fibers are of plant (such as flax, jute and cotton) or animal origin, comprising proteins like collagen, keratin and fibroin (e.g. silk, sheeps wool, angora, mohair, cashmere). Examples for fibers of synthetic origin are polyurethane fibers such as Spandex® or Lycra®, polyester fibers, polyolefins such as elastofin, or polyamide fibers such as nylon. Fibers may be single fibers or parts of textiles such as knitwear, wovens, or nonwovens.

[0055] The term "hard surface cleaning" is defined herein as cleaning of hard surfaces wherein hard surfaces may include any hard surfaces in the household, such as floors, furnishing, walls, sanitary ceramics, glass, metallic surfaces including cutlery or dishes. A particular form of hard surface cleaning is automatic dishwashing (ADW).

[0056] The term "dish wash" refers to all forms of washing dishes, e.g. by hand or automatic dish wash. Washing dishes includes, but is not limited to, the cleaning of all forms of crockery such as plates, cups, glasses, bowls, all forms of cutlery such as spoons, knives, forks and serving utensils as well as ceramics, plastics such as melamine, metals, china, glass and acrylics.

[0057] In the technical field of the present invention, usually the term "stains" is used with reference to laundry, e.g., cleaning for textiles, fabric, or fibers, whereas the term "soils" is usually used with reference to hard surface cleaning, e.g., cleaning of dishes and cutlery. However, herein the terms "stain" and "soil" shall be used interchangeably.

Detailed description of the invention

[0058] The present invention is directed to a method for cleaning a textile or a hard surface comprising the step of contacting a textile or a hard surface at low temperature with a detergent composition comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2. Preferably, the detergent composition is free of phosphonate.

[0059] In a preferred embodiment, the low temperature is equal or below 30°C, preferably the low temperature is equal or below 29°C, equal or below 28°C, equal or below 27°C, equal or below 26°C, equal or below 25°C, equal or below 24°C, equal or below 23°C, equal or below 22°C, equal or below 21°C, equal or below 20°C, equal or below 19°C, equal or below 18°C, equal or below 17°C, equal or below 16°C, equal or below 15°C, equal or below 14°C, equal or below 13°C, equal or below 12°C, equal or below 11°C, equal or below 10°C, or equal or below 9°C. Preferably, the low

temperature is between 10°C and 31°C, between 10°C and 30°C, preferably between 10°C and 29°C, preferably between 10°C and 28°C, preferably between 10°C and 27°C, preferably between 10°C and 26°C, preferably between 10°C and 25°C, preferably between 10°C and 24°C, preferably between 10°C and 23°C, preferably between 10°C and 22°C, preferably between 10°C and 21°C, preferably between 10°C and 20°C, preferably between 10°C and 19°C, preferably between 10°C and 18°C, preferably between 10°C and 17°C, preferably between 10°C and 16°C, or preferably between 10°C and 15°C. Further preferred is low temperature between 10°C and 25°C, preferably between 11°C and 25°C, preferably between 12°C and 25°C, preferably between 13°C and 25°C, preferably between 14°C and 25°C, or preferably between 15°C and 25°C. Further preferred is low temperature between 10°C and 15°C, preferably between 10°C and 14°C, preferably between 10°C and 13°C, preferably between 10°C and 12°C, or preferably between 10°C and 11°C. Preferably, the low temperature is 30°C, 29°C, 28°C, 27°C, 26°C, 25°C, 24°C, 23°C, 22°C, 21°C, 20°C, 19°C, 18°C, 17°C, 16°C, 15°C, 14°C, 12°C, 11°C, or 10°C, preferred low temperature is 20°C or 15°C. In particular, preferred low temperature is equal or below 20°C or equal or below 15°C, preferably, between 10°C and 19°C or between 10°C and 16°C.

[0060] The method of the present invention comprise the use of a protease as described herein. The protease is preferably a variant protease of the parent protease shown in SEQ ID NO: 1, preferably a subtilisin protease. Preferably the variant protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 (BPN' numbering, i.e., wherein the positions are numbered by their correspondence to the amino acid sequence of subtilisin BPN' of B. amyloliquefaciens, established as SEQ ID NO: 2), wherein the protease is preferably a subitilisin protease.

[0061] Preferably, the protease has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 and comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2.

[0062] Preferably, the protease has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 and comprises compared to SEQ ID NO: 1 the amino acid substitution R101E according to the numbering of SEQ ID NO: 2.

[0063] Preferably, the protease has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 and comprises compared to SEQ ID NO: 1 the amino acid substitution R101E and the amino acid substitutions S3T, V4I, and V205I according to the numbering of SEQ ID NO: 2.

[0064] Preferably, the protease has at least 80% sequence identity to SEQ ID NO: 1 and comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, wherein compared to SEQ ID NO: 1 the protease comprises one or more conservative amino acid exchanges as described herein. Preferably, compared to SEQ ID NO: 1 the protease comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 10, at least 15, at least 20, at least 30 or at least 40 conservative amino acid exchanges.

[0065] Preferably, compared to SEQ ID NO: 1 a protease described herein can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative amino acid exchanges in addition to the modifications resulting in at least two additional negative charges in the loop region of residues 98 to 104.

[0066] Preferably, the protease has at least 80% sequence identity to SEQ ID NO: 1 and comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, wherein compared to SEQ ID NO: 1 the remaining difference in amino acid sequence is due to conservative amino acid exchanges as described herein.

[0067] In a preferred embodiment, the protease comprises compared to SEQ ID NO: 1 one or more substitutions at positions according the numbering of SEQ ID NO: 2 selected from the group consisting of 3, 4, 9, 15, 24, 27, 33, 36, 57, 68, 76, 77, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 118, 120, 123, 128, 129, 130, 131, 154, 160, 167, 170, 194, 195, 199, 205, 206, 217, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274.

[0068] Preferably the variant protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 and protease comprises compared to SEQ ID NO: 1 one or more substitutions at positions according the numbering of SEQ ID NO: 2 selected from the group consisting of 3, 4, 9, 15, 24, 27, 33, 36, 57, 68, 76, 77, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 118, 120, 123, 128, 129, 130, 131, 154, 160, 167, 170, 194, 195, 199, 205, 206, 217, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274.

[0069] Preferably, the protease has at least 80% sequence identity to SEQ ID NO: 1 as described herein and comprises compared to SEQ ID NO: 1 at least two, three, or four additional negative charges, more preferably three additional negative charges, most preferably two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 compared to the region of SEQ ID NO: 1 corresponding to residues 98 to 104 of SEQ

ID NO: 2.

[0070] Preferably, the at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 are obtained by one or more amino acid alterations selected from the group consisting of substitutions, deletions and insertions, preferably by substitutions. Preferably, the at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 are obtained by one or more amino acid alterations selected from the group consisting of D99E, R101 D and R101 E.

[0071] Preferably, in the protease the at least two additional negative charges compared to SEQ ID NO: 1 in the loop region of residues 98 to 104 are caused by one or more amino acid substitutions at amino acid position according the numbering of SEQ ID NO: 2 selected from the group consisting of 98, 99, 100, 101, 102, 103, and 104, preferably at position 101.

[0072] In a preferred embodiment, the protease comprises an amino acid sequence which comprises compared to SEQ ID NO: 1 the amino acid substitution R101 E or R101 D according to the numbering of SEQ ID NO: 2. In another preferred embodiment, the at least two additional negative charges compared to SEQ ID NO: 1 in the loop region of residues 98 to 104 are not caused by the amino acid substitution R101 E or R101 D.

[0073] In a preferred embodiment, the loop sequence 98-104 has compared to SEQ ID NO: 1 two additional negative charges with the following sequence ADGEGAI, ADGDGAI, ADGDGSV, ADGEGSV, AADGEGSV, or ASEGEGSV with longer sequences having an insertion in the loop sequence.

[0074] In another embodiment of the present invention, the protease comprising as described herein an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, comprises according to the numbering of SEQ ID NO: 2 at least one of the amino acid residues selected from the group consisting of

a. threonine or serine at position 3 (3T or 3S),
b. isoleucine or valine at position 4 (4I or 4V),
c. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T or 63R),
d. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E),
e. serine or proline at position 194 (194S or 194P),
f. serine, valine, or methionine at position 199 (199S, 199V, or 199M)
g. isoleucine or valine at position 205 (205I or 205V); and
h. aspartic acid, glutamic acid, glutamine, glycine at position or leucine at position 217 (217D, 217E, 217Q, 217G or 217L).

[0075] In a preferred embodiment, the protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 the amino acid substitution R101 E or R101 D according to the numbering of SEQ ID NO: 2 and wherein the protease according to the numbering of SEQ ID NO: 2 comprises at least one of the amino acid residues selected from the group consisting of

a. threonine or serine at position 3 (3T or 3S),
b. isoleucine or valine at position 4 (4I or 4V),
c. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T or 63R),
d. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E),
e. serine or proline at position 194 (194S or 194P),
f. serine, valine, or methionine at position 199 (199S, 199V, or 199M)
g. isoleucine or valine at position 205 (205I or 205V); and
h. aspartic acid, glutamic acid, glutamine, glycine at position or leucine at position 217 (217D, 217E, 217Q, 217G or 217L).

[0076] Preferably, the protease described herein comprises compared to SEQ ID NO: 1 the amino acid substitution R101E or R101D and the amino acid substitutions S3T, V4I, and V205I according to the numbering of SEQ ID NO: 2.

[0077] In a another embodiment, the protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and the protease comprises compared to SEQ ID NO: 1 the amino acid substitution R101E or R101D and one or more substitutions selected from the group consisting of S156D, L262E, Q137H, S3T, R45E,D,Q, P55N, T58W,Y,L, Q59D,M,N,T, G61 D,R, S87E, G97S, A98D,E,R, S106A,W, N117E, H120V,D,K,N, S125M, P129D, E136Q, S144W, S161T, S163A,G, Y171 L, A172S, N185Q, V199M, Y209W, M222Q, N238H, V244T, N261T,D and L262N,Q,D according to the numbering of SEQ ID NO: 2.

[0078] Preferably, the protease has an additional mutation at position 217 according to the numbering of SEQ ID NO: 2, preferably L217Q, L217D, L217E, or L217G.

[0079] In a preferred embodiment, the protease comprises an amino acid sequence selected from the group consisting of

a) amino acid sequence of SEQ ID NO: 3,
b) amino acid sequence of SEQ ID NO: 3, wherein the amino acid sequence comprises at least one additional amino acid substitution selected from the group consisting of

i. threonine at position 3 (3T);
ii. isoleucine at position 4 (4I);
iii. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T, or 63R);
iv. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E);
v. serine or proline at position 194 (194S or 194P);
vi. methionine or serine at position 199 (199M or 199S);
vii. isoleucine at position 205 (205I); and
viii. aspartic acid, glutamic acid, glutamine or glycine at position 217 (217D, 217E, 217Q or 217G);

c) amino acid sequence of SEQ ID NO: 4, and
d) amino acid sequence of SEQ ID NO: 4, wherein the amino acid sequence comprises at least one additional amino acid substitution selected from the group consisting of

i. serine at position 3 (3S);
ii. valine at position 4 (4V);
iii. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T, or 63R);
iv. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E);
v. serine or proline at position 194 (194S or 194P);
vi. methionine or serine at position 199 (199M or 199S);
vii. valine at position 205 (205V); and
viii. aspartic acid, glutamic acid, glutamine or glycine at position 217 (217D, 217E, 217Q or 217G).

[0080] Preferably, the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 alanine at position 103 (103A) and isoleucine at position 104 (104I) according to the numbering of SEQ ID NO: 2, more preferably, 101R, 104I, and 103A.

[0081] In a further preferred embodiment, the amino acid sequence of the protease compared to SEQ ID NO: 1 does not comprises an additional amino acid residue in the loop region from position 98 to 104 according to the numbering of SEQ ID NO: 2. Preferably, the amino acid sequence of the protease compared to SEQ ID NO: 1 does not comprises an additional amino acid residue between positions 42-43, 51-55, 155-165, 187-189, 217-218, or 218-219 according to the numbering of SEQ ID NO: 2.

[0082] Proteases, including serine proteases, according to the invention have "proteolytic activity" (also referred to as "protease activity"). This property is related to hydrolytic activity of a protease (proteolysis, which means hydrolysis of peptide bonds linking amino acids together in a polypeptide chain) on protein containing substrates, e.g. casein, haemoglobin and BSA. Quantitatively, proteolytic activity is related to the rate of degradation of protein by a protease or proteolytic enzyme in a defined course of time. The methods for analyzing proteolytic activity are well-known in the literature (see e.g. Gupta et al. (2002), Appl. Microbiol. Biotechnol. 60: 381-395).

[0083] For instance, proteolytic activity and thereby the effect of an inhibitor on the proteolytic activity as such can be determined by using Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA, short AAPF; see e.g. DelMar et al. (1979), Analytical Biochem 99, 316-320) as substrate. pNA is cleaved from the substrate molecule by proteolytic cleavage, resulting in release of yellow color of free pNA which can be quantified by measuring OD_{405}.

[0084] To determine changes in proteolytic activity over time, the "initial enzymatic activity" of a protease is measured under defined conditions at time zero and at a certain point in time later. By dividing the latter activity with the activity at time point zero the residual activity can be calculated (x%). The x% value measured shall preferably equal the 100%-value indicating no loss in activity.

[0085] By comparison of the 100%-value with the x%-value, a potential loss of proteolytic activity can be determined in its extent. The extent of loss of proteolytic activity reflects depending on the experimental setting the stability of a protease and/or the degree of inhibition of the protease.

[0086] The present invention is refers to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 as described herein in a detergent composition or detergent solution.

[0087] In the solutions, detergent compositions, detergent solutions or in the methods of the present invention the protease is preferably in a concentration with measurable protease activity. Thus, a proteolytically active amount of the protease is used.

[0088] Preferably, the protease is present in the solutions and / or detergent compositions described herein in a concentration of at least 1.0 μg/L, preferably, at least 5.0 μg/L, at least 10.0 μg/L, at least 50 μg/L, at least 100 μg/L, at least 200 μg/L, at least 500 μg/L, at least 750 μg/L, at least 900 μg/L, at least 1 mg/L, at least 0.5 mg/L, at least 1.0 mg/L, at least 2.0 mg/L, at least 3.0 mg/L, at least 5 mg/L, at least 10 mg/L, or at least 20 mg/L. Preferably, the protease is present in the solutions and / or detergent compositions solution in a concentration of between 1.0 μg/L and 100 mg/L, preferably between 1 μg/L and 50 mg/L, preferably between 50 μg/L and 50 mg/L, preferably between 500 μg/L and 25 mg/L, preferably between 0.5 mg/L and 50 mg/L, preferably between 0.5 mg/L and 10 mg/L, preferably between 0.5 mg/L and 5 mg/L, between 1.0 mg/L to 5 mg/L, or between 1.0 mg/L and 3.0 mg/L.

[0089] In a preferred embodiment, the solutions comprising the protease described herein and the detergent compositions described herein have a pH value of between pH5 and pH13, preferably between pH6 and pH 11, preferably between pH7 and pH10, preferably between pH8 and pH 11, preferably between pH7 and pH8, more preferably between pH9 and pH10, preferably at pH 7.0 or pH 8.0.

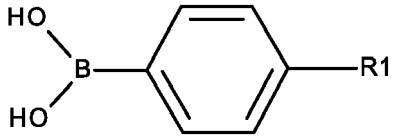
[0090] Preferably, the solution and / or detergent composition comprising the protease described herein is an aqueous phase of a washing step in a washing process for a textile or a hard surface.

[0091] The aqueous solution, detergent solutions or detergent compositions described herein can comprise one or more detergent components. Preferred detergent components include but are not limited to surfactants, hydrotropes, building agents, sequstrants, bleaching systems, polymers, fabric hueing agents, fabric conditioners, foam boosters, suds suppressors, dispersants, fillers, salts, antiredeposition agents, dye transfer inhibitors, fluorescent whitening agents, corrosion inhibitors, perfume, dye, optical brighteners, bactericides, fungicides, soil suspending agents, soil release polymers, enzyme activators, enzyme stabilizer, enzyme inhibitor, preferably non-naturally occurring enzyme inhibitor, antioxidants, solubilizers and other enzymes, preferably detergent enzymes different from the protease described herein. Detergent components vary in type and/or amount in a detergent composition depending on the desired application, as it is known by the skilled person.

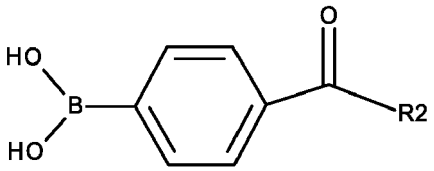
[0092] Preferably, the aqueous solutions, detergent solutions or detergent compositions are free of phosphonate. The aqueous solutions, detergent solutions or detergent compositions described herein can comprise one or more detergent enzymes different from the protease described herein. Furthermore, the methods described herein can comprise the use of one or more detergent enzymes different from the protease. Preferably, a detergent component is a detergent enzyme different from the protease. Preferably, the one or more enzymes different from the protease are selected from the group consisting of protease, amylase, lipase, cellulase, mannanase, peroxidases/oxidases, perhydrolases, lyases, mannanases, pectinase, arabinase, galactanase, and xylanase.

[0093] The aqueous solutions, detergent solutions or detergent compositions described herein can comprise one or more stabilizing agents. Furthermore, the methods described herein can comprise the use of one or more stabilizing agents. In a preferred embodiment, the protease described herein is used in combination with a stabilizing agent, preferably a protease inhibitor, more preferably a reversible protease inhibitor. Preferably, the protease is stabilized with one or more stabilizing agents selected from the group consisting of a diol, preferably, propanediol, calcium, polyethylene glycol, boric acid and its derivatives, and peptide aldehyde or its derivatives.

[0094] The boric acid derivative is preferably a boronic acid derivative. Preferably, the boronic acid is selected from the group consisting of aryl boronic acids and its derivatives. Preferably, the boronic acid is selected from the group consisting of benzene boronic acid (BBA) which is also called phenyl boronic acid (PBA), derivatives thereof, and mixtures thereof. In one embodiment, phenyl boronic acid derivatives are selected from the group consisting of the derivatives of formula (I) and (II):



formula (I)



formula (II)

[0095] Wherein R1 is selected from the group consisting of hydrogen, hydroxy, non-substituted or substituted C_1-C_6 alkyl, and non-substituted or substituted C_1-C_6 alkenyl; in a preferred embodiment, R1 is selected from the group consisting of hydroxy, and non-substituted C_1 alkyl; wherein R2 is selected from the group consisting of hydrogen, hydroxy, non-substituted or substituted C_1-C_6 alkyl, and non-substituted or substituted C_1-C_6 alkenyl; in a preferred embodiment, R2 is selected from the group consisting of hydrogen, hydroxy, and substituted C_1 alkyl.

[0096] In one embodiment phenyl-boronic acid derivatives are selected from the group consisting of 4-formyl phenyl boronic acid (4-FPBA), 4-carboxy phenyl boronic acid (4-CPBA), 4-(hydroxyl-methyl) phenyl boronic acid (4-HMPBA), and p-tolylboronic acid (p-TBA).

In a preferred embodiment, component (a) is selected from the group consisting of benzene boronic acid (BBA) and 4-formyl phenyl boronic acid (4-FPBA).

Other suitable derivatives include: 2-thienyl boronic acid, 3-thienyl boronic acid, (2-acetamido-phenyl) boronic acid, 2-benzofuranyl boronic acid, 1-naphthyl boronic acid, 2-naphthyl boronic acid, 2-FPBA, 3-FBPA, 1-thianthrenyl boronic acid, 4-dibenzofuran boronic acid, 5-methyl-2-thienyl boronic acid, 1-benzothiophene-2 boronic acid, 2-furanyl boronic acid, 3-furanyl boronic acid, 4,4 biphenyl-diboronic acid, 6-hydroxy-2-naphthaleneboronic acid, 4-(methylthio) phenyl boronic acid, 4-(trimethylsilyl) phenyl boronic acid, 3-bromothiophene boronic acid, 4-methylthiophene boronic acid, 2-naphthyl boronic acid, 5-bromothiophene boronic acid, 5-chloro-thiophene boronic acid, dimethylthiophene boronic acid, 2-bromophenyl boronic acid, 3-chlorophenyl boronic acid, 3-methoxy-2-thiophene boronic acid, p-methyl-phenylethyl boronic acid, 2-thianthrenyl boronic acid, di-benzothiophene boronic acid, 9-anthracene boronic acid, 3,5 dichlorophenyl boronic, acid, diphenyl boronic acid anhydride, o-chlorophenyl boronic acid, p-chlorophenyl boronic acid, m-bromophenyl boronic acid, p-bromophenyl boronic acid, p-fluorophenyl boronic acid, octyl boronic acid, 1,3,5 trimethylphenyl boronic acid, 3-chloro-4-fluorophenyl boronic acid, 3-aminophenyl boronic acid, 3,5-bis-(trifluoromethyl) phenyl boronic acid, 2,4 dichlorophenyl boronic acid, 4-methoxyphenyl boronic acid, and mixtures thereof.

[0097] In a preferred embodiment, the stabilizing agent is a peptide aldehyde or a derivative thereof, preferably a non-naturally occurring peptide aldehyde or a derivative thereof. Preferably, the peptide aldehyde is preferably specially designed for each protease active site. The peptide aldehyde may comprise 2, 3, 4, 5 or 6 amino acid residues. The N-terminal of the peptide aldehyde may be H or protected by an N-terminal protection group, preferably selected from formyl, acetyl, benzoyl, trifluoroacetyl, fluoromethoxy carbonyl, methoxysuccinyl, aromatic and aliphatic urethane protecting groups, benzyloxycarbonyl, t-butyloxycarbonyl, adamantyloxycarbonyl, pmethoxybenzyl carbonyl (MOZ), benzyl (Bn), p-methoxybenzyl (PMB) or p-methoxyphenyl (PMP), methyl carbamate or a methyl urea group. Thus, the peptide aldehyde may have the formula BrBrB0-R wherein:

R is hydrogen, CH3, CX3, CHX2, or CH2X, wherein X is a halogen atom;
B0 is a single amino acid residue;
B1 is a single amino acid residue; and
B2 consists of one or more amino acid residues (preferably one or two), optionally comprising an N-terminal protection group.

In the above formula, B0 may be an L or D-amino acid with an optionally substituted aliphatic or aromatic side chain, preferably D- or L-Tyr (p-tyrosine), m-tyrosine, 3,4-dihydroxyphenylalanine, Leu, Phe, Val, Met, Nva or Nie.

B1 may be a residue with a small optionally substituted aliphatic side chain, preferably Ala, Cys, Gly, Pro, Ser, Thr, Val, Nva, or Nie.

B2 may be either one residue B2 with either a small aliphatic side chain (preferably, Gly, Ala, Thr, Val or Leu) or Arg or Gin; optionally comprising a N-terminal protection group as described in WO2011036153; or B2 may be two residues B3-B2' where B2' is like B2 above and B3 is a residue with an hydrophobic or aromatic side chain (preferably Phe, Tyr, Trp, m-tyrosine, 3,4-dihydroxyphenylalanine, phenylglycine, Leu, Val, Nva, Nie or Ile) optionally comprising a N-protection group as described in WO2011036153.

[0098] Preferred peptide aldehydes are described in WO2011036153. Alternatively the peptide aldehyde may have the formula as described in WO98/13459. A preferred tripeptide aldehydes is Z-GAY-H, preferably wherein Z is benzyloxycarbonyl. A preferred peptide aldehyde derivative is a peptide aldehyde hydrosulfite adduct, preferably a peptide aldehyde hydrosulfite adduct as described in EP2726592B1. A preferred tripeptide aldehyde hydrosultife adduct is Z-

GAY-SO3, preferably wherein Z is benzyloxycarbonyl.

[0099] Preferably, the concentration of the protease inhibitor is below its inhibitory concentration, preferably below IC50 value, preferably below half, below third, below quarter, below eighth, below ninth or below tenth of the IC50 value of the inhibitor.

[0100] The present invention is also directed to a method for cleaning an object, preferably a textile or a hard surface, at low temperatures. Preferably, the method for cleaning comprises the following steps

a) providing a detergent composition / solution comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2; preferably, the protease comprises the mutation R101E or R101D, preferably wherein the detergent composition / solution is free of phosphonate;
b) providing an object, preferably, textile, fabric, fibre and/or hard surface comprising one or more protein containing stains;
c) contacting the object comprising one or more stains from step b) with the detergent composition / solution of step a);
d) incubating the object comprising the one or more stains from step b) at low temperature as described herein with the detergent composition / solution of step a) for a time and under conditions sufficient to allow the protease to proteolytically act on the one or more stains comprising proteins; and
e) optionally analyzing and/or comparing the wash performance of the washing step d).

[0101] In a preferred embodiment of this method, the contacting of the one or more stains with the detergent composition / solution described herein comprising the protease is for at least 1 min, at least 5 min, at least 15 min, at least 30 min, at least 45 min, at least 1 h, at least 1.5 h, at least 2 h, at least 3 h, or at least 4 h.

[0102] The use of the protease described herein being more effective at low temperature improves the wash performance on stains comprising protein. Thus, in another embodiment, the present invention is directed to a method for improving the wash performance of the protease by introducing the respective mutations described herein into the amino acid sequence. Further, the present invention is directed to a method for improving the wash performance of detergent solution or detergent composition by using the protease described herein. Moreover, the present invention is directed to a method for improving the wash performance of a washing step by contacting stains comprising protein with the detergent composition / solution described herein comprising the protease described herein. Preferably, the protease comprises the mutation R101E or R101D. Preferably, the method is for maintaining the wash performance in a washing step or of a detergent composition / solution constant.

[0103] Thus, the present invention is directed to a method for improving the wash performance of a washing step on protein containing stains at low temperature comprising the step of contacting a textile or a hard surface, comprising one or more protein containing stains, with a detergent composition comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2.

[0104] The fact, that the protease described herein shows improved performance at low temperature can be used to test other enzymes for their efficiency at low temperature. A preferred embodiment is a screening method for identifying a protease with increased cleaning efficiency at low temperature. Hence, in a preferred embodiment the present invention refers to a method for testing the wash performance of a protease at low temperature comprising the steps of contacting a first one or more protein containing stains on a textile or a hard surface with a first protease and contacting a second one or more protein containing stains with a second protease wherein the first protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the first protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, preferably wherein the first and the second one or more protein containing stains are the same kind of stains for the first and the second protease.

[0105] Preferably, the method for testing enzyme variants, preferably protease variants, for increased wash performance at low temperature comprises the following steps

a) testing the proteolytic activity of a first protease in a detergent wash solution, detergent solution or detergent composition at low temperatures as described herein, wherein the first protease comprises an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2; preferably the proteolytic activity is tested by measuring wash performance, preferably on stains on textile, fabric, fiber and/or hard surface;
b) testing the proteolytic activity of a second protease in the same way as described in step a) for the first protease;

c) comparing the proteolytic activity and / or wash performance of the first and the second protease;
d) determining whether the proteolytic activity of the second protease is increased or reduced compared the protease activity of the first protease;
e) optionally determining and comparing the amino acid sequence of first and second protease and optionally identifying amino acid residues responsible for the difference in proteolytic activity measured in step a) and b).

Another embodiment of the present invention is directed to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 for washing a textile or a hard surface at low temperature. Preferred low temperature is 20°C or 15°C. In particular, preferred low temperature is below 20°C or below 15°C, preferably, between 10°C and 19°C or between 10°C and 15°C.

[0106] Another embodiment of the present invention is directed to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 in a detergent composition for washing a textile or a hard surface at low temperature, preferably wherein the detergent composition is free of phosphonate.

[0107] A further embodiment of the present invention is directed to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 for improving the wash performance of a washing step at low temperature. Preferred low temperature is 20°C or 15°C. In particular, preferred low temperature is below 20°C or below 15°C, preferably, between 10°C and 19°C or between 10°C and 15°C.

A further embodiment of the present invention is directed to the use of a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 for improving the wash performance of a detergent composition at low temperature, preferably wherein the detergent composition is free of phosphonate.

[0108] A further embodiment of the present invention is directed to the use of a protease or a method comprising the use of a protease, wherein the protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and which comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 for maintaining the wash performance of a washing step or a detergent composition constant at low temperature, preferably compared to a washing step under the same conditions but at higher temperature using a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease compared to SEQ ID NO: 1 does not comprise at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2 and/or preferably compared to a washing step under the same conditions but at higher temperature using a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2, preferably, the low temperature is 20°C or 15°C, preferably the low temperature is below 20°C or below 15°C, preferably, between 10°C and 19°C or between 10°C and 15°C, preferably wherein the detergent composition is free of phosphonate.

[0109] It is readily understood that all features of the protease with increased resistance thereto and the detergent composition or detergent solutions described above are equally applicable to the methods and uses described herein.

[0110] The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Examples

[0111] The following examples only serve to illustrate the invention. The numerous possible variations that are obvious to a person skilled in the art also fall within the scope of the invention.

[0112] Unless otherwise stated the following experiments have been performed by applying standard equipment, methods, chemicals, and biochemicals as used in genetic engineering and fermentative production of chemical compounds by cultivation of microorganisms. See also Sambrook et al. (Molecular Cloning: A Laboratory Manual. 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) and Chmiel et al. (Bioprocesstechnik 1. Einführung in die Bioverfahrenstechnik, Gustav Fischer Verlag, Stuttgart, 1991).

Example 1

[0113] In order to test the wash performance of a protease across various temperatures, the following model detergents where prepared for testing the washing performance of various proteases:

Table 1: Detergent compositions

	Detergent 1	**Detergent 2**	**Detergent 3**
water	To 100%	To 100%	To 100%
KOH	1.5%	1.5%	1.5%
Linear $C_{10}C_{13}$ alkylbenzolsulfonic acid	5.6%	5.6%	5.6%
$C_{12}C_{18}$ Coconut fatty acid	2.4%	2.4%	2.4%
$C_{12}C_{14}$ Fatty alcohol ether sulfate with 2 EO	5.4%	5.4%	5.4%
$C_{13}C_{15}$ Oxoalcohol ethoxylate with 7 EO	5.4%	5.4%	5.4%
1,2 Propandiol	6%	6%	6%
Ethanol pure	2%	2%	2%
SEQ ID NO: 3	0	0.024%	
SEQ ID NO: 5	0		0.024%

[0114] The wash performance of the proteases SEQ ID NO: 3 and SEQ ID NO: 5 was tested in the following setup. 50g detergent per wash cycle was used to wash a multistain monitor with with 10 stains in a European standard laundry wash using 3.5 kg standard ballast load and standard cotton program at 20°C and 30°C, respectively, in Miele Novotronic W1614 WPS full scale washing machines under the following conditions.

Table 2: Washing conditions

Washing Machine	Miele W1614 WPS
Washing program	Cotton 20 °C or 30°C, 1200 U/min.
Dosage	50 g detergent as described above
Washing cycles	1
Water hardness	2.5 mmol/l Ca^{2+} : Mg^{2+} : HCO^{3-} 4:1:8
pH	8.5
Ballast fabric	3.5 kg cotton towels
Stained fabric	CFT C-05 [1)] Blood, milk, ink CFT C-11 [1)] Milk with carbon black CFT C-S-25 [1)] Spinach concentrate CFT C-S-80 [1)] Grass/mud EMPA 117 [2)] Blood, milk Ink
	EMPA 164 [2)] Grass CFT C-03 [1)] Chocolate Milk with carbon black CFT C-S-06 [1)] Salad dressing CFT C-S-38 [1)] Egg yolk CFT C-S-39 [1)] Full egg
[1)] Producer: Center for Testmaterials BV, 3130 AC Vlaardingen, the Netherlands [2)] Producer: EMPA Testmaterialien AG, Sankt Gallen, Schweiz	

[0115] After the wash cycle, the ballast and stained fabric were removed from the washing machine, stained fabrics were removed from the ballast fabric and dried overnight before being measured for light reflectance on a Mach 5 (Multi Area Color Measurement Hardware, CFT, 3130 AC Vlaardingen, The Netherlands, CIELab*). Results are reported as

the delta L value between a fabric washed without protease (Detergent 1) and with protease (Detergent 2 and Detergent 3).

Table 3: Results of performance testing SEQ ID NO: 3 and SEQ ID NO: 5 at 20°C and 30°C The sum of delta L across all stains clearly shows that SEQ ID NO: 3 surprisingly retains the same performance across both temperatures while SEQ ID NO: 5 as expected show decreased performance at 20°C.

	Delta L		% of 30°C performance	
	30°C	20°C	30°C	20°C
SEQ ID NO: 3	106.9	103.2	100%	97%
SEQ ID NO: 5	97.3	77.1	100%	79%

SEQUENCE LISTING

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<170> BiSSAP 1.3.6

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            20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Asp Gly Arg Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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<213> Bacillus amyloliquefaciens

<220>
<223> wildtype

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35 40 45
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
50 55 60
Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
65 70 75 80
Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
85 90 95
Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
100 105 110
Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
115 120 125
Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
130 135 140
Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145 150 155 160
Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
165 170 175
Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
180 185 190
Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195 200 205
Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
210 215 220
Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225 230 235 240
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
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Ala Ala Gln
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<213> Artificial Sequence

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20 25 30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
35 40 45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
50 55 60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65 70 75 80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
85 90 95

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Asp Gly Glu Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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<213> Artificial Sequence

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            20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Asp Gly Glu Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Ile Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
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Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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<223> subtilisin 309 variant described in WO9820115A1

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1               5                  10                  15
His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
            20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
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    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
145                 150                 155                 160
Ala Pro Ala Ser Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Pro Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
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Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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                20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
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            20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Asp Gly Arg Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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<213> Bacillus amyloliquefaciens

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35 40 45
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
50 55 60
Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
65 70 75 80
Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
85 90 95
Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
100 105 110
Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
115 120 125
Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
130 135 140
Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
145 150 155 160
Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
165 170 175
Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
180 185 190
Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
195 200 205
Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
210 215 220
Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
225 230 235 240
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
245 250 255
Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
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Ala Ala Gln
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20 25 30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
35 40 45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
50 55 60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65 70 75 80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
85 90 95
Asp Gly Glu Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
100 105 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser

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        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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            20                  25                  30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
        35                  40                  45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
    50                  55                  60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Asp Gly Glu Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Ile Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
                245                 250                 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
            260                 265
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Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
35 40 45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
50 55 60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
65 70 75 80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
85 90 95
Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
100 105 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
115 120 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
130 135 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
145 150 155 160
Ala Pro Ala Ser Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
165 170 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Pro Gly Leu Asp Ile
180 185 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
195 200 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
210 215 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225 230 235 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
245 250 255
Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
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<213> Artificial Sequence

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Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala
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20 25 30
Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
35 40 45
Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
50 55 60
His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu

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65                  70                  75                  80
Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
                85                  90                  95
Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
            100                 105                 110
Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
        115                 120                 125
Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
    130                 135                 140
Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
145                 150                 155                 160
Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
                165                 170                 175
Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
            180                 185                 190
Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
        195                 200                 205
Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
    210                 215                 220
Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
225                 230                 235                 240
Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
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Claims

1. Method for cleaning a textile or a hard surface comprising the step of contacting a textile or a hard surface at low temperature with a detergent composition comprising a protease comprising an amino acid sequence which is at least 80% identical to SEQ ID NO: 1 and wherein the amino acid sequence of the protease comprises compared to SEQ ID NO: 1 at least two additional negative charges in the loop region of residues 98 to 104 according to the numbering of SEQ ID NO: 2.

2. The method of claim 1, wherein the detergent composition is free of phosphonate.

3. The method of any of the proceeding claims, wherein the low temperature is equal or below 30°C, preferably equal or below 15°C, preferably between 10°C and 25°C, preferably at 15°C or 20°C.

4. The method of any of the proceeding claims, wherein the protease comprises an amino acid sequence which comprises compared to SEQ ID NO: 1 the amino acid substitution R101 E or R101 D, preferably R101 E, according to the numbering of SEQ ID NO: 2.

5. The method of any of the proceeding claims, wherein the protease according to the numbering of SEQ ID NO: 2 comprises at least one of the amino acid residues selected from the group consisting of

   a. threonine or serine at position 3 (3T or 3S),
   b. isoleucine or valine at position 4 (4I or 4V),
   c. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T or 63R),

d. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E),
e. serine or proline at position 194 (194S or 194P),
f. serine, valine, or methionine at position 199 (199S, 199V, or 199M)
g. isoleucine or valine at position 205 (205I or 205V); and
h. aspartic acid, glutamic acid, glutamine, glycine at position or leucine at position 217 (217D, 217E, 217Q, 217G or 217L).

6. The method of any of the proceeding claims, wherein the protease comprises compared to SEQ ID NO: 1 the amino acid substitution R101E or R101D and the amino acid substitutions S3T, V4I, and V205I according to the numbering of SEQ ID NO: 2.

7. The method of any of the proceeding claims, wherein the protease comprises compared to SEQ ID NO: 1 the amino acid substitution R101 E or R101 D and one or more substitutions selected from the group consisting of S156D, L262E, Q137H, S3T, R45E,D,Q, P55N, T58W,Y,L, Q59D,M,N,T, G61 D,R, S87E, G97S, A98D,E,R, S106A,W, N117E, H120V,D,K,N, S125M, P129D, E136Q, S144W, S161T, S163A,G, Y171 L, A172S, N185Q, V199M, Y209W, M222Q, N238H, V244T, N261T,D and L262N,Q,D according to the numbering of SEQ ID NO: 2.

8. The method of any of the proceeding claims, wherein the protease has an additional mutation at position 217 according to the numbering of SEQ ID NO: 2, preferably L217Q, L217D, L217E, or L217G.

9. The method of any of the proceedings claims, wherein the protease comprises an amino acid sequence selected from the group consisting of

a) amino acid sequence of SEQ ID NO: 3,
b) amino acid sequence of SEQ ID NO: 3, wherein the amino acid sequence comprises at least one additional amino acid substitution selected from the group consisting of

i. threonine at position 3 (3T);
ii. isoleucine at position 4 (4I);
iii. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T, or 63R);
iv. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E);
v. serine or proline at position 194 (194S or 194P);
vi. methionine or serine at position 199 (199M or 199S);
vii. isoleucine at position 205 (205I); and
viii. aspartic acid, glutamic acid, glutamine or glycine at position 217 (217D, 217E, 217Q or 217G);

c) amino acid sequence of SEQ ID NO: 4, and
d) amino acid sequence of SEQ ID NO: 4, wherein the amino acid sequence comprises at least one additional amino acid substitution selected from the group consisting of

i. serine at position 3 (3S);
ii. valine at position 4 (4V);
iii. serine, alanine, threonine or arginine at position 63 (63S, 63A, 63T, or 63R);
iv. threonine, aspartic acid or glutamic acid at position 156 (156T, 156D, or 156E);
v. serine or proline at position 194 (194S or 194P);
vi. methionine or serine at position 199 (199M or 199S);
vii. valine at position 205 (205V); and
viii. aspartic acid, glutamic acid, glutamine or glycine at position 217 (217D, 217E, 217Q or 217G).

10. The method of any of the proceedings claims, wherein the protease is present in the detergent composition in a concentration of at least 1.0 μg/l.

11. The method of any of the proceedings claims, wherein the detergent composition comprises one or more components selected from the group consisting of surfactants, hydrotropes, building agents, bleaching system, polymers, fabric hueing agents, fabric conditioners, foam boosters, suds suppressors, dispersants, fillers, antiredeposition agents, dye transfer inhibitors, fluorescent whitening agents, corrosion inhibitors, perfume, dye, optical brighteners, bactericides, fungicides, soil suspending agents, soil release polymers, enzyme activators, enzyme stabilizer, enzyme inhibitor, preferably non-naturally occurring enzyme inhibitor, antioxidants, solubilizers and other enzymes, prefer-

ably detergent enzymes different from the protease described in claims 1-9.

12. Method for improving the wash performance of a washing step on protein containing stains at low temperature comprising the step of contacting a textile or a hard surface, comprising one or more protein containing stains, with a detergent composition comprising a protease as described in any of claims 1-10.

13. The method of claim 12, wherein the detergent composition is a detergent composition in a laundry cleaning process or in an automatic dishwashing process.

14. Use of a protease as described in any of claims 1-10 for washing a textile or a hard surface at low temperature.

15. Use of a protease as described in any of claims 1-10 for improving the wash performance of a washing step at low temperature.

16. Use of a protease as described in any of claims 1-10 for maintaining the wash performance of a washing step constant at low temperature.

17. Use of any of claims 14 to 16, wherein the protease is used in a detergent composition, preferably wherein the detergent composition is free of phosphonate.

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EUROPEAN SEARCH REPORT

Application Number
EP 16 19 3372

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	DE 10 2014 212642 A1 (HENKEL AG & CO KGAA [DE]) 31 December 2015 (2015-12-31) * paragraph [0043] - paragraph [0046] * * abstract; sequence 3 *	1-7,9, 11-17	INV. C11D3/386
X	WO 2015/055477 A1 (HENKEL AG & CO KGAA [DE]) 23 April 2015 (2015-04-23) * abstract * * p23 last paragraph *	1,2,4,5, 7,9-17	
X	DE 10 2013 207933 A1 (HENKEL AG & CO KGAA [DE]) 30 October 2014 (2014-10-30) * paragraph [0009] * * abstract *	1-7,9, 11-17	
X	WO 2014/030097 A2 (BASF SE [DE]; BASF SCHWEIZ AG [CH]; BASF CHINA CO LTD [CN]) 27 February 2014 (2014-02-27) * p2 second paragraph; claim 1 *	1-4,9, 11-17	
X	DE 10 2012 215642 A1 (HENKEL AG & CO KGAA [DE]) 6 March 2014 (2014-03-06) * abstract *	1,2,4-7, 11-17	TECHNICAL FIELDS SEARCHED (IPC) C11D
X	WO 2016/096711 A2 (NOVOZYMES AS [DK]) 23 June 2016 (2016-06-23) * p44, p55; sequence 3 *	1-9, 11-17	
X	WO 95/23221 A1 (COGNIS INC [US]) 31 August 1995 (1995-08-31) * claims 1-8 *	1-17	
X	DE 10 2010 028951 A1 (HENKEL AG & CO KGAA [DE]) 17 November 2011 (2011-11-17) * claims 1-3 *	1-17	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	3 March 2017	Rosin, Oliver

CATEGORY OF CITED DOCUMENTS

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03-03-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 102014212642 A1	31-12-2015	DE 102014212642 A1	31-12-2015
		WO 2016000973 A1	07-01-2016
WO 2015055477 A1	23-04-2015	AU 2014336474 A1	02-06-2016
		DE 102013221206 A1	23-04-2015
		EP 3058057 A1	24-08-2016
		KR 20160068950 A	15-06-2016
		US 2016237418 A1	18-08-2016
		WO 2015055477 A1	23-04-2015
DE 102013207933 A1	30-10-2014	DE 102013207933 A1	30-10-2014
		EP 2992093 A1	09-03-2016
		KR 20160003086 A	08-01-2016
		US 2016097021 A1	07-04-2016
		WO 2014177430 A1	06-11-2014
WO 2014030097 A2	27-02-2014	CA 2882331 A1	27-02-2014
		CN 104736703 A	24-06-2015
		DE 102012215107 A1	27-02-2014
		EP 2888362 A2	01-07-2015
		JP 2015532670 A	12-11-2015
		KR 20150048777 A	07-05-2015
		RU 2015110083 A	10-10-2016
		US 2015291917 A1	15-10-2015
		WO 2014030097 A2	27-02-2014
DE 102012215642 A1	06-03-2014	DE 102012215642 A1	06-03-2014
		EP 2892990 A1	15-07-2015
		EP 2893011 A1	15-07-2015
		US 2015166939 A1	18-06-2015
		US 2015175939 A1	25-06-2015
		WO 2014037344 A1	13-03-2014
		WO 2014037345 A1	13-03-2014
WO 2016096711 A2	23-06-2016	NONE	
WO 9523221 A1	31-08-1995	AT 390487 T	15-04-2008
		AT 512226 T	15-06-2011
		AT 512227 T	15-06-2011
		DE 69535736 T2	30-04-2009
		DK 0701605 T3	28-07-2008
		DK 1921147 T3	19-09-2011
		DK 1921148 T3	26-09-2011
		EP 0701605 A1	20-03-1996
		EP 1921147 A2	14-05-2008
		EP 1921148 A2	14-05-2008

EPO FORM P0459

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 19 3372

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03-03-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		ES 2302330 T3	01-07-2008
		JP 3922391 B2	30-05-2007
		JP H10507901 A	04-08-1998
		WO 9523221 A1	31-08-1995
DE 102010028951 A1	17-11-2011	DE 102010028951 A1	17-11-2011
		EP 2569409 A2	20-03-2013
		ES 2559882 T3	16-02-2016
		US 2013071910 A1	21-03-2013
		WO 2011141358 A2	17-11-2011

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 9102792 A **[0004]**
- WO 9523221 A1 **[0006]**
- WO 2011032988 A1 **[0007] [0010]**
- WO 2014030097 A1 **[0008]**
- WO 2011036153 A **[0097] [0098]**
- WO 9813459 A **[0098]**
- EP 2726592 B1 **[0098]**

Non-patent literature cited in the description

- **CAMPANELLA, J.J ; BITINCKA, L. ; SMALLEY, J.** *BMC Bioinformatics,* 2003, vol. 4, 29 **[0030]**
- **HIGGINS, D.G. ; SHARP, P.M.** *Gene,* 1988, vol. 73, 237-244 **[0030]**
- **GUPTA et al.** *Appl. Microbiol. Biotechnol.,* 2002, vol. 60, 381-395 **[0082]**
- **DELMAR et al.** *Analytical Biochem,* 1979, vol. 99, 316-320 **[0083]**
- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0112]**
- **CHMIEL et al.** Bioprocesstechnik 1. Einführung in die Bioverfahrenstechnik. Gustav Fischer Verlag, 1991 **[0112]**