TITLE: VARIANTS OF FUNGAL SERINE PROTEASE

ABSTRACT: The present invention relates to fungal serine protease variants, which comprise an amino acid substitution of valine at position 208 of the parent Fusarium equiseti Fe_RF63 18 serine protease, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF63 8 enzyme defined in SEQ ID NO: 2. The variants have improved thermal stability and/or detergent stability compared to the parent Fe_RF63 18 enzyme. Preferably the substitution is V208I and more preferably the variants comprise additional amino acid changes which further increase the stability. Also disclosed are nucleic acid sequences encoding said protease variants as well as recombinant vectors and host cells for the production of the variants. The serine protease variants are applicable in laundry and dish-washing detergent compositions, in treating fibers, in treating wool, in treating hair, in treating leather, in treating food or feed, or in any application involving modification, degradation or removal of proteinaceous material.
VARIANTS OF FUNGAL SERINE PROTEASE

FIELD OF THE INVENTION

The present invention relates to fungal serine protease variants useful in various applications, particularly in laundry and dish-washing detergents. The invention relates also to a nucleic acid molecule encoding said protease variant, a recombinant vector, a host cell for producing said protease variant, an enzyme composition comprising said protease variant as well as a process for preparing such composition. This invention further relates to various uses of said protease variant or compositions comprising said protease variant.

BACKGROUND

Microbial proteases are among the most important hydrolytic enzymes and find applications in various industrial sectors, such as detergents, food, leather, pharmaceuticals, diagnostics, waste management and silver recovery. Microbial extracellular proteases account for a major part, more than one third, of the total worldwide industrial enzyme sales (Cherry and Fidantsef, 2003). Approximately 90% of the commercial proteases are detergent enzymes (Gupta et al, 2002). The commercial detergent preparations currently in use comprise the naturally occurring alkaline serine proteases (EC 3.4.21) of the subtilisin family or subtilisins, originating from Bacillus, species, or are recombinant protease preparations thereof (Maurer, 2004).

Examples of commercial proteases are such as subtilisin Carlsberg (Alcalase®), subtilisin 309 (Savinase®), Subtilisin 147 (Esperase®), Kannase®, Everlase®, Ovozyme®, and the cold-wash protease Polarzyme® (Novozymes A/S, DK); Purafect®, Purafect® Ox, Purafect® Prime and Properase® (Genencor Int., Inc., USA); and the BLAP S and X series (Henkel, DE).

Several alkaline serine proteases and genes encoding these enzymes have also been isolated from eukaryotic organisms, including yeast and filamentous fungi. US Patent No. 3,652,399 and EP 519229 (Takeda Chemical Industries, Ltd., JP) disclose an alkaline protease from the
genus *Fusarium* (asexual state, teleomorph) or *Gibberella*, (sexual state, anamorph) particularly from *Fusarium* sp. S-19-5 (ATCC 20192, IFO 8884), *F. oxysporum* f. sp. *lini* (IFO 5880) or *G. saubinetti* (ATCC 20193, IFO6608), useful in the formulation of detergent and other cleanser compositions. WO 88/03946 and WO 89/04361 (Novo Industn A/S, DK) disclose an enzymatic detergent additive and a detergent composition comprising a protease and a lipase, wherein the fungal protease is derived from *Fusarium*, particularly *F. oxysporum* or *F. solani*. WO1994025583 (NovoNordisk A/S, DK) discloses an active trypsin-like protease enzyme derivable from a *Fusarium* species, in particular a strain of *F. oxysporum* (DSM 2672), and the DNA sequence encoding the same. The amino acid sequence of a novel protease deriving from *Fusarium* sp. BLB (FERM BP-10493) is disclosed in WO 2006101140 (SODX Co. Ltd, Nakamura). Use of *F. equiseti* and other fungi in reducing organic matter in waste waters is disclosed in the EP 1464626 patent application (Biovitis S.A., FR). The amino acid and nucleotide sequences of the serine proteases from *F. equiseti* and *F. acuminatum* have been disclosed in FI20095497 and FI20095499, respectively (AB Enzymes Oy, Fl). The amino acid and nucleotide sequences of the serine protease derived from several *Trichoderma* species have been disclosed e.g. in WO2008045148A (Catalyst Biosciences, Inc., U.S.A.), WO96/018722A (Centro de Investigacion y de Estudios Avanzados del I.P.N., MX) and WO98/020116A (Novo Nordisk A/S, DK). Also, alkaline proteases from fungal species such as *Tritirachium* and *Conidiobolus* have been reported (reviewed in Anwar and Saleemuddin, 1998).

Different methods have been used for improving the stability of the industrial serine proteases. WO 92/03529 (NovoNordisk A/S, DK) discloses detergent compositions comprising a reversible protease inhibitor of the peptide or protein type. In one preferred embodiment the protease is a subtilisin, preferably derived from *Bacillus* and the inhibitor is a subtilisin inhibitor of family III, VI or VII. The liquid detergent compositions comprising proteases often include protease inhibitors such as boric acid with or without polyols to inhibit the autocatalytic activity of proteases. One of such inhibitors is 4-formyl phenyl boronic acid (4-FPBA) disclosed in US0120649 (Novozymes A/S, DK). EP0352244A2 (NovoNordisk A/S, DK) discloses improvement of stability of *Bacillus* derived enzymes using amphoteric compounds, such as surfactants.
Variants of the natural serine proteases with improved catalytic efficiency and/or better stability towards temperature, oxidizing agents and different washing conditions have been developed through site-directed and/or random mutagenesis. Most of the work has been carried out with *Bacillus* derived subtilisins. Replacement of one or more amino acid residues of *B. amyloliquefaciens* subtilisin is disclosed in WO98/55634 and WO99/20727 (Procter & Gamble Co., Genencor Int., Inc., U.S.A.) and in WO99/20770, US20090011489A and EP1025241B2 (Genencor Int., Inc., U.S.A.). Protease variants of *B. lentus* subtilisin with improved wash performance are disclosed in WO2003/062381 and EP1523553B1 (Genencor Int., Inc., U.S.A.). The substitutions of R170S-A1R, R170S-G61R, R170S-N216R or R170S-G100R do not change the net electrostatic charge of the variant when compared to the precursor protease. Multiply-substituted variants of *B. lentus* protease with altered net charge, resulting in improved efficacy at low, medium or high detergent concentrations is disclosed in EP 1612271 A2 (Genencor Int., Inc. U.S.A.).


Variants of fungal serine proteases have been prepared for trypsin-like proteases. WO95/030743A1 (NovoNordisk A/S, DK) discloses variants of a trypsin-like *Fusarium* protease, in which a naturally occurring amino acid residue (other than proline) is substituted with a proline residue at one or more positions which positions are not located in regions in which the protease is characterized by possessing alpha-helical or beta-sheet structure. The variants have improved proteolytic stability and are less susceptible to oxidation as compared to the parent protease. EP1546318B (Novozymes Inc., U.S.A.) discloses trypsin variants of *Fusarium oxysporum* comprising substitutions, insertions or deletions of one or more amino acids of the precursor protease.
Despite the fact that numerous patent publications, reviews and articles have been published, in which serine proteases from various microorganisms, for example, the low temperature alkaline proteases from actinomycete (*Nocardiopsis dassonvillei*) and fungal (*Paecilomyces marquandii*) microorganisms are disclosed, e.g. in EP 0290567 and EP 0290569 (Novo Nordisk A/S, DK), there is still a great need for alternative serine proteases, which are suitable for and effective in modifying, degrading and removing proteinaceous materials of different stains, particularly in low or moderate temperature ranges and which are stable in the presence of detergents with highly varying properties. Due to autocatalytic property of serine proteases, the stability during storage is also very important.

It is also desirable that the serine protease can be produced in high amounts, and can be cost-effectively down-stream processed, by easy separation from fermentation broth and mycelia.

**SUMMARY OF THE INVENTION**

The present invention provides serine protease variants of the Fe_RF6318 serine protease originating from the filamentous fungus *Fusarium equiseti* RF6318 deposited at Centraalbureau voor Schimmelcultures on 7 April 2006 under accession number CBS 119568. The wild-type Fe_RF6318 serine protease has broad substrate specificity, is active at broad pH ranges and has a broad temperature optimum, i.e. functions both at low and moderate temperatures, as disclosed in the patent application FI20095497 (AB Enzymes Oy, FI), filed on 30 April 2009. Particularly, the present invention provides Fe_RF6318 variants, which have improved thermal stability and are more stable in varying detergent compositions compared to the native precursor protease. The Fe_RF6318 variants have similar or improved wash performance compared to the parent wild-type Fe_RF6318 protease. The Fe_RF6318 variants of the invention are capable of removing proteinaceous material, including stains in washing laundry and dishes, at lower temperatures than the present commercial enzyme preparations, thereby saving energy. The Fe_RF6318 variant proteases can be produced in high-yielding fungal hosts and their down-stream processing, e.g. separation of fermentation broth and mycelia is easy to perform.
The present invention relates to a Fe_RF6318 serine protease variant polypeptides, which have serine protease activity and comprise an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid other than valine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2. Preferably, the Fe_RF6318 variants comprise the substitution V208I relative to the parent mature Fe_RF6318 serine protease.

The Fe_RF6318 variants of the invention comprise a substitution which results in improved thermal stability as well as improved stability in detergents and has retained or improved wash performance compared to the parent mature Fe_RF6318 serine protease of SEQ ID NO:2.

Preferably, the Fe_RF6318 variant, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, comprises one or more amino acid changes, which are selected from the group consisting of a substitution, insertion, and deletion. The preferred changes are selected from the group consisting of an amino acid substitution at position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288; a deletion of asparagine at position 167; a deletion of alanine at position 65 and histidine at position 66; and an amino acid insertion at position 104, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

More preferably, according to increase in thermostability, the changes include, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 14, 24, 29, 33, 34, 47, 52, 61, 63, 65, 83, 91, 100, 103, 106, 111, 121, 144, 153, 157, 158, 164, 175, 176, 185, 210, 234, 236, 256, 268 or 281, and an amino acid insertion at position 104.
Even more preferably, according to increase in thermostability, the changes include, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 24, 29, 33, 47, 100, 103, 106, 111, 185, 210, 268 or 281, and an amino acid insertion at position 104.

Most preferably, according to increase in thermostability, the Fe_RF6318 variant of the invention comprises an amino acid sequence having the substitution V208I (SEQ ID NO:6), V208I-A11 1D (SEQ ID NO:8), V208I-A281L (SEQ ID NO:10), V208I-A11 1D-A281L (SEQ ID NO:12), V208I-A11 D-A281L-A33E (SEQ ID NO:14), V208I-A11 1D-A281L-A47E (SEQ ID NO:16), V208I-A111D-A281L-I185M (SEQ ID NO:20), V208I-A111D-A281L-T268R (SEQ ID NO:22), V208I-A11 1D-A281L-Q103A-ins.G104 (SEQ ID NO:24), V208I-A11 1D-A281L-T3C-T29C (SEQ ID NO:26), V208I-A11 1D-A281L-S6R-T24D (SEQ ID NO:28), V208I-A11 1D-A281L-V100D (SEQ ID NO:30), V208I-A11 1D-A281L-T106N (SEQ ID NO:34), V208I-A11 1D-A281L-T3C-T29C-S6R-T24D (SEQ ID NO:36) or V208I-A11 1D-A281L-I185M-G210A (SEQ ID NO:38) relative to the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease defined in SEQ ID NO:2.

More preferably, according to stability in detergent, the changes include, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 37, 47, 61, 63, 65, 83, 100, 111, 123, 157, 175, 176, 185, 210, 234, 236, 247, 268, 281.

Even more preferably, according to stability in detergent, the changes include, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 47, 63, 100, 111, 123, 157, 175, 176, 185, 210, 236, 268, 281.

Most preferably, according to the increase in detergent stability, the Fe_RF6318 variant of the invention comprises an amino acid sequence having the substitution V208I (SEQ ID NO:6),

The Fe_RF6318 serine protease variant of the invention is encoded by an isolated polynucleotide sequence which encodes the polypeptide comprising the amino acid sequence of SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, and SEQ ID NO: 50.

The present invention relates also to an isolated nucleic acid molecule comprising a nucleotide sequence which encodes the Fe_RF6318 serine protease variant, which has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the mature Fe_RF6318 serine protease with an amino acid other than valine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO: 2.

Preferably, the nucleic acid molecule of the invention comprises a nucleotide sequence which encodes a Fe_RF6318 variant which, in addition to the substitution of valine at position 208 of the Fe_RF6318 serine protease, comprises one or more amino acid changes, which are
selected from the group consisting of a substitution, insertion and deletion. The preferred changes include, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position, 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288, a deletion of asparagine at position 167, deletion of alanine at position 65 and histidine at position 66, and an amino acid insertion at position 104, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

According to a most preferred embodiment of the invention the isolated nucleic acid molecule comprises the nucleotide sequence defined in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, and SEQ ID NO:49.

The invention further relates to recombinant expression vectors comprising the nucleotide sequences of the invention operably linked to regulatory sequences capable of directing expression of the gene encoding the Fe_RF6318 serine protease variant of the invention in a suitable host, such as a filamentous fungus. Suitable hosts include heterologous hosts, preferably microbial hosts of the genus Trichoderma, Aspergillus, Fusarium, Humicola, Chrysosporium, Neurospora, Rhizopus, Penicillium and Mortiriella. Preferably said enzyme is produced in Trichoderma or Aspergillus, most preferably in T. reesei.

The invention relates also to a host cell comprising the recombinant expression vector as described above. Preferred host cells include the microbial hosts, such as filamentous fungi of a genus Trichoderma, Aspergillus, Fusarium, Humicola, Chrysosporium, Neurospora, Rhizopus, Penicillium and Mortiriella. Preferably said host is Trichoderma or Aspergillus, most preferably T. reesei.
The present invention relates to a process of producing a variant polypeptides having serine protease activity, said process comprising the steps of culturing the host cell of the invention and recovering the variant polypeptide.

The invention further relates to a process for obtaining an enzyme preparation, which comprises the serine protease variant of the invention. The process comprises the steps of culturing a host cell of the invention and either recovering the variant polypeptide from the cells or separating the cells from the culture medium and obtaining the supernatant.

The invention relates to an enzyme preparation, which comprises the Fe_RF6318 variant polypeptide of the invention.

The enzyme preparation of the invention may further comprise other enzymes selected from the group of protease, amylase, cellulase, lipase, xylanase, mannanase, cutinase, pectinase or oxidase with or without a mediator as well as suitable additives selected from the group of stabilizers, buffers, surfactants, bleaching agents, mediators, anti-corrosion agents, builders, antiredeposition agents, optical brighteners, dyes, pigments, caustics, abrasives and preservatives, etc.

The spent culture medium of the production host can be used as such, or the host cells may be removed, and/or it may be concentrated, filtrated or fractionated. It may also be dried. The enzyme preparation of the invention may be in the form of liquid, powder or granulate.

The invention further relates to a detergent composition comprising the Fe_RF6318 serine protease variant of the invention.

Also within the invention is the Fe_RF6318 serine protease variant or the enzyme preparation comprising said variant for use in detergents, in treating fibers, in treating wool, in treating hair, in treating leather, in treating food or feed, or in any application involving modification,
degradation or removal of proteinaceous material. Particularly, the enzyme or enzyme preparation is useful as a detergent additive in detergent liquids and detergent powders.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1A and B show the nucleotide sequence of *Fusarium equiseti* RF6318 *Fe prtS8A* cDNA used for constructing the mutant proteases and the deduced amino acid sequence. The putative signal peptide, analyzed by SignalP V3.0 program is in lower case letters and underlined. The pro sequence and the deduced amino acids of the pro sequence are in lower case letters. The mature nucleotide and the deduced amino acid sequences are in capital letters (the N-terminal sequence has been previously determined from the purified wild type *Fe_RF6318* protein; FI20095497). The stop codon is shown by an asterisk below the sequence.

Figure 2A and B show the nucleotide sequence of a synthetic construction including the *Fusarium equiseti* RF6318 *Fe prtS8A* wild type cDNA sequence, fused at its 5'-end to a partial *cbhl* promoter (from *SacII* site, marked and underlined) and at its 3'-end to the *cbhl* terminator sequence (to *Agel* site, marked and underlined). Analogous synthetic constructions were ordered for each mutant protease cDNA and were used for construction of the expression cassettes. *pcbhl*, partial *cbhl* promoter; *tcbhl* partial *cbhl* terminator.

Figure 3 shows the plasmid map of pALK1910 used as a backbone for constructing the cassettes for m1 - m14 mutant protease production. The DNA fragments encoding the mutant proteases (cDNAs with 5' partial *cbhl* promoter and 3' partial *cbhl* terminator, see Fig. 2) and cleaved with *SacII* and *Agel* were ligated into *SacII* and *Agel* cleaved pALK1910. For the m1 - m14 expression cassettes, the *amdS* marker gene was then ligated to the *EcoKV* site of the constructed plasmids. Only the relevant restriction sites are shown, *pcbhl*, *cbhl* promoter; *tcbhl*, *cbhl* terminator.

Figure 4 shows the map of pALK2777 used as a backbone for constructing the cassettes for m15 - m26 and D1-D68 mutant protease expression. The DNA fragments encoding the mutant proteases (cDNAs with 5' partial *cbhl* promoter and 3' partial *cbhl* terminator, see
Fig. 2) and cleaved with Sacll and Agel were ligated into Sacll-Agel cleaved pALK2777. This plasmid includes a synthetic amdS marker gene for transformant screening. Only the relevant restriction sites are shown, pcbhl, cbhl promoter; tcbhl, cbhl terminator; s_amdS, synthetic amdS marker gene (cDNA).

Figure 5A shows the map of pALK2749 including the m14 expression cassette as an example of a plasmid constructed for mutant protease expression in T. reesei, basing on ligation of the mutant gene to pALK1910. The corresponding plasmids including the cassettes for m1 - m13 production are listed in Table 2. Only the relevant restriction sites are shown, pcbhl, cbhl promoter; tcbhl, cbhl terminator; m14, FeprtS8A mutant protease gene (cDNA) m14; amdS, amdS marker gene.

Figure 5B shows the map of pALK2888 including the m18 expression cassette as an example of a plasmid constructed for mutant protease expression in T. reesei, basing on ligation of the mutant gene to pALK2777. The corresponding plasmids including the cassettes for m15 - m17 and m19 - m23 mutant protease production are listed in Table 2, those for m24 - m26 production in Table 4, for D1 - D30 production in Table 5 and for D31-D68 production in Table 9. Only the relevant restriction sites are shown, pcbhl, cbhl promoter; tcbhl, cbhl terminator; m18, FeprtS8A mutant protease gene m18 (cDNA); s_amdS, synthetic amdS marker gene (cDNA).

Figure 6 shows the thermal stability of m-series mutant proteases determined from fermentation culture supernatants in 20 mM Tris buffer, pH 8.5. Enzyme samples were incubated at 45 °C for 120 minutes and the residual activity was analyzed at definite intervals.

Figure 7 shows the thermal stability of mutant proteases m24, m25 and m26 determined from fermentation culture supernatants in 20 mM Tris buffer, pH 8.5. Enzyme samples were incubated at 45 °C for 100 minutes and the residual activity was analyzed at definite intervals. Wild type protease Fe_RF63 18 was used for comparison.

Figure 8A and 8B show the nucleotide sequence and the deduced amino acid sequence of the m26 mutant protease cDNA used as a starting sequence for the "D-series" mutant proteases.
The putative signal peptide, analyzed by SignalP V3.0 program is in lower case letters and underlined. The pro sequence and the deduced amino acids of the pro sequence are in lower case letters. The mature nucleotide and the deduced amino acid sequences are in capital letters. The stop codon is shown by an asterisk below the sequence. The mutated codons and modified amino acids compared to the wild type sequence (Fig. 1) are boxed.

**Figure 9** shows the thermal stability of chosen D-series mutants. The stabilities were determined from fermentation culture supernatants in 20 mM Tris buffer, pH 8.5. Enzyme samples were incubated at 45 °C for 100 minutes and the residual activity was analyzed at definite intervals. The m26 mutant protease was used for comparison.

**Figure 10A** describes the stain removal performance of selected mutant proteases from m-series with blood/milk/ink stain (Art.117, EMPA) at 30°C, approx. pH 7.4, 60 min in the presence of Liquid Base detergent for colored fabrics with concentration of 5 g/l. Wild type protease Fe_RF63 18 was used for comparison.

**Figure 10B** describes the stain removal performance of selected mutant proteases from m-series with blood/milk/ink stain (Art.117, EMPA) at 45°C, approx. pH 7.4, 60 min in the presence of Liquid Base detergent for colored fabrics with concentration of 5 g/l. Wild type protease was used for comparison.

**Figure 11A** shows the stability of selected mutant proteases from m-series and wild type protease in Liquid Base detergent for colored fabrics at 37°C (pH approx. 8.2).

**Figure 11B** shows the stability of selected mutant proteases from m-series in Liquid Base detergent for colored fabrics with 2 % sodium tetraborate decahydrate and 17 % propylene glycol incubated at 37°C (pH approx. 7).

**Figure 12A** shows the stability of selected mutant proteases from m-series and wild type in Ecolabel Reference Detergent (wfk Testgewebe GmbH) incubated at 37°C for 18 hours (pH approx. 7.2).
Figure 12B shows the stability of selected mutant proteases from m-series and wild type protease in Commercial liquid detergent incubated at 37°C, pH approx. 8.2, for 22 hours and two days (approx. 44 hours).

Figure 13A shows the stability of selected mutant proteases from m-series in Ecolabel Reference Detergent (wfk Testgewebe GmbH) with 2% sodium tetraborate decahydrate and 17% propylene glycol incubated at 37°C (pH approx. 6.5). Wild type protease was used for comparison.

Figure 13B shows the stability of selected mutant proteases from m-series in Commercial liquid detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol incubated at 37°C and pH approx. 7.5. Wild type protease was used for comparison.

Figure 14A describes the stain removal performance of mutant proteases from D-series (D3, D5, D7 and D8) with blood/milk/ink stain (Art. 117, EMPA) at 30°C, approx. pH 7.8, 60 min in the presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26 and wild type protease were used for comparison.

Figure 14B describes the stain removal performance of mutant proteases from D-series (D13, D16, D17 and D18) with blood/milk/ink stain (Art. 117, EMPA) at 30°C, approx. pH 7.8, 60 min in the presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26 was used for comparison.

Figure 14C describes the stain removal performance of mutant proteases from D-series (D3, D5, D7 and D8) with blood/milk/ink stain (Art. 117, EMPA) at 50°C, approx. pH 7.8 60 min in the presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26 and wild type were used for comparison.

Figure 14D describes the stain removal performance of mutants from D-series (D13, D16, D17 and D18) with blood/milk/ink stain (Art. 117, EMPA) at 50°C, approx. pH 7.8 60 min in
the presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26
was used for comparison.

**Figure 15A** describes the stain removal performance of mutants from D-series (D2, D6, D22
and D23) with blood/milk/ink stain (Art. 117, EMPA) at 30°C, approx. pH 7.8, 60 min in the
presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26
was used for comparison.

**Figure 15B** describes the stain removal performance of mutants from D-series (D2, D6, D22
and D23) with blood/milk/ink stain (Art. 117, EMPA) at 50°C, approx. pH 7.8, 60 min in the
presence of Commercial liquid detergent with concentration of 5 g/l. Mutant protease m26
was used for comparison.

**Figure 16A** shows the stability of mutants of D-series (D3, D5, D7, D8, D13, D16, D17 and
D19) in Commercial liquid detergent incubated at 37°C, pH approx. 8.2. Mutant protease m26
was used for comparison.

**Figure 16B** shows the stability of mutants of D-series (D2, D6, D22, and D23) in
Commercial liquid detergent incubated at 37°C, pH approx. 8.2. Mutant protease m26 was used for comparison.

**Figure 17A** shows the stability of mutants from D-series (D3, D5, D7, D8, D13, D16, D17
and D19) in Commercial liquid detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol incubated at 37°C, pH approx. 7.5. Mutant protease m26 was used for comparison.

**Figure 17B** shows stability of mutants from D-series (D2, D6, D22 and D23) in Commercial
liquid detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol
incubated at 37°C, pH approx. 7.5. Mutant protease m26 was used for comparison.
Figure 18 shows stability of mutant of D-series in Ecolabel Reference Detergent incubated at 37°C pH approx. 7.2 for 20 hours. Mutant protease m26 and wild type protease were used for comparison.

SEQUENCE LISTING

SEQ ID NO:1 The nucleotide sequence encoding the amino acid sequence of the mature form of *Fusarium equiseti* RF6318 (Fe_RF6318) protease.

SEQ II) NO:2 The amino acid sequence of the mature form of *Fusarium equiseti* RF6318 (Fe_RF6318) protease.

SEQ ID NO:3 The nucleotide sequence encoding the amino acid sequence of the mature form of *Fusarium acuminatum* RF7182 protease.

SEQ II) NO:4 The amino acid sequence of the mature form of *Fusarium acuminatum* RF7182 protease.

SEQ ID NO:5 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitution V208I.

SEQ ID NO:6 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitution V208I.

SEQ ID NO:7 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I and A111D.

SEQ ID NO:8 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I and A111D.

SEQ ID NO:9 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I and A281L.
SEQ ID NO: 10 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I and A281L.

SEQ ID NO: 11 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID and A281L.

SEQ ID NO: 12 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID and A281L.

SEQ ID NO: 13 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and A33E.

SEQ ID NO: 14 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and A33E.

SEQ ID NO: 15 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and A47E.

SEQ ID NO: 16 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and A47E.

SEQ ID NO: 17 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and G63P.

SEQ ID NO: 18 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, All ID, A281L and G63P.
SEQ ID NO: 19 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and I185M.

SEQ ID NO: 20 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and I185M.

SEQ ID NO: 21 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and T268R.

SEQ ID NO: 22 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and T268R.

SEQ ID NO: 23 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, Q103A, insertion G104.

SEQ ID NO: 24 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, Q103A, insertion G104.

SEQ ID NO: 25 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, T3C and T29C.

SEQ ID NO: 26 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, T3C and T29C.
SEQ ID NO:27 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L, S6R and T24D.

SEQ ID NO:28 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L, S6R and T24D.

SEQ ID NO:29 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and V100D.

SEQ ID NO:30 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and V100D.

SEQ ID NO:31 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and V100K.

SEQ ID NO:32 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and V100K.

SEQ ID NO:33 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and T106N.

SEQ ID NO:34 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L and T106N.

SEQ ID NO:35 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A11ID, A281L, T3C, T29C, S6R and T24D.
SEQ ID NO:36 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, T3C, T29C, S6R and T24D.

SEQ ID NO:37 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, I185M and G210A.

SEQ ID NO:38 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L, I185M and G210A.

SEQ ID NO:39 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and V100Q.

SEQ ID NO:40 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and V100Q.

SEQ ID NO:41 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and K123R.

SEQ ID NO:42 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and K123R.

SEQ ID NO:43 The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and S157T.

SEQ ID NO:44 The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and S157T.
The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and G175S.

The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and G175S.

The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and Q176T.

The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and Q176T.

The nucleotide sequence encoding the amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and C236T.

The amino acid sequence of the mature form of Fe_RF6318 serine protease variant comprising substitutions V208I, A111D, A281L and C236T.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides variants of a fungal Fe_RF6318 serine protease deriving from *Fusarium equiseti* RF6318, deposited at Centraalbureau voor Schimmelcultures on 7 April 2006 under accession number CBS 119568. The Fe_RF6318 wild-type protease shows broad substrate specificity, is stable at high pH ranges and has a broad temperature optimum, i.e. good performance both at low and moderate temperatures as disclosed in the patent application FI20095497, filed on 30 April 2009. The variant enzymes are ideal for detergent applications, withstanding oxidizing and chelating agents and being effective at low
enzyme levels in detergent solutions. Particularly, the variants of the Fe_RF6318 serine protease have improved thermal stability and they stand varying detergents better than the native Fe_RF6318 serine protease having the naturally occurring amino acid sequence. Thus, the present invention provides alternative serine proteases for use in detergent and other applications. The Fe_RF6318 serine protease variants can be produced in high-yielding fungal hosts and their down-stream processing, e.g. separation of fermentation broth and mycelia is easy to perform.

By "serine protease" or "serine endopeptidase" or "serine endoproteinase" is in connection to this invention meant an enzyme classified as EC 3.4.21 by the Nomenclature of the International Union of Biochemistry and Molecular Biology. Based on their structural similarities, serine proteases have been grouped into at least six clans (SA, SB, SC, SE, SF and SG; S denoting serine protease), which have been further subgrouped into families with similar amino acid sequences and three-dimensional structures (see, for example the Serine protease home page at http://www.biochem.wustl.edu/~protease/, Department of Biochemistry and Molecular Biophysics, Washington University of Medicine, St. Louis, MO, USA). These protein hydrolyzing or degrading enzymes are characterized by the presence of a nucleophilic serine group in their active site, and the proteases of clan SA and clan SB are also distinguished by having essential aspartate and histidine residues, which along with the serine, form a catalytic triad.

The major clans of serine proteases include the "chymotrypsin-like", including chymotrypsin, trypsin and elastase (clan SA) and "subtilisin-like" (clan SB) proteases. The characterized "subtilisin-like serine proteases" or "subtilases" of clan SB (EC 3.4.21.62), represented by various Bacillus, like B. amyloliquifaciens, B. licheniformis and B. subtilis (Rao et al, 1998), are specific for aromatic or hydrophobic residues, such as tyrosine, phenylalanine and leucine. The enzymes target different regions of the polypeptide chain, based upon the side chains of the amino acid residues surrounding the site of cleavage.

By the term "serine protease activity" as used in the invention is meant hydrolytic activity on protein containing substrates, e.g. casein, haemoglobin, keratin and BSA. The methods for
analysing proteolytic activity are well-known in the literature and are referred e.g. in Gupta et al. (2002) and Maurer and Gabler (2005).

Proteases can be classified using group specific inhibitors. The diverse group of "serine protease inhibitors" includes synthetic chemical inhibitors and natural proteinaceous inhibitors. Thus, the serine protease activity can be determined in an assay based on cleavage of a specific substrate or in an assay using any protein containing substrate with or without a specific inhibitor of serine proteases under suitable conditions.

The serine proteases are synthesized as inactive "zymogenic precursors" or "zymogens" in the form of a preproenzyme, which are activated by removal of the signal sequence (secretion signal peptidase or propeptide) and the prosequence (propeptide) to yield an active mature form of the enzyme (Chen and Inouye, 2008). This activation process involves action of proteases and may result from limited self-digestive or autocatalytic processing of the serine protease, e.g. during posttranslational phases of the production or in the spent culture medium or during the storage of the culture medium or enzyme preparation. Activation of the proenzyme may also be achieved by adding a proteolytic enzyme capable of converting the inactive proenzyme into active mature enzyme into the culture medium during or after cultivation of the host organism. The shortening of the enzyme can also be achieved e.g. by truncating the gene encoding the polypeptide prior to transforming it to the production host. The "prepro-form" of the Fe_RF6318 serine protease in the present invention means an enzyme comprising the pre- and propeptides. The "pro-form" means an enzyme, which comprises the propeptide but lacks the propeptide (signal sequence).

The term "mature" means the form of the serine protease enzyme which after removal of the signal sequence (prepeptide) and propeptide comprises the essential amino acids for enzymatic or catalytic activity. In filamentous fungi it is the native form secreted into the culture medium.

The largest group of commercial serine proteases are "alkaline serine proteases", which means that the enzymes are active and stable at pH 9 to pH 11 or even at pH 10 to 12.5
(Shimogaki et al., 1991) and have isoelectric point around pH 9. Determination of the optimal pH of the catalytic activity can be carried out in a suitable buffer at different pH values by following the activity on a protein substrate. The detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pi value for the enzyme. pi can be determined by isoelectric focusing on an immobilized pH gradient gel composed of polyacrylamide, starch or agarose or by estimating the pi from the amino acid sequence, for example by using the pi/MW tool at ExPASy server (http://expasy.org/tools/pi_tool.html; Gasteiger et al., 2003).

The molecular masses of mature alkaline serine proteases range between 15 and 35 kDa, typically from about 25 to 30 kDa. The molecular mass of the serine protease can be determined by mass spectrometry or on SDS-PAGE according to Laemmli (1970). The molecular mass can also be predicted from the amino acid sequence of the enzyme.

The temperature optima of most natural serine proteases are around 60°C (Rao et al., 1998). The temperature optimum of serine protease can be determined in a suitable buffer at different temperatures by using casein as a substrate as described in Example 1a or by using other substrates and buffer systems described in the literature (Gupta et al., 2002).

The parent Fe_RF6318 protease is a subtilisin-like serine protease belonging to clan SB, family 8 of serine proteases. The mature wild-type Fe_RF6318 serine protease has a molecular weight of ca. 29 kDa, an optimal temperature of approximately 60°C at pH 9 using 15 min reaction time and casein as a substrate, a pH optimum at approximately pH 10 at 50°C using 15 min reaction time and casein as a substrate. The wild-type Fe_RF6318 serine protease has a good performance in the presence of detergents with highly varying properties, at broad, i.e. from low to moderate temperature ranges. The wild-type Fe_RF6318 serine protease, depending on the washing conditions and auxiliary ingredients and additives in detergents, is useful particularly in temperatures at or below 50°C as disclosed in FI20095497 (AB Enzymes Oy, FI).
From the properties described above it can be concluded that the wild-type Fe_RF6318 serine protease is capable of satisfying the greatly varying demands of detergent customers and detergent industry and is well suited to the requirements of future regulations and customer habits, e.g. to the need for lower washing temperatures.

To improve the performance of the Fe_RF6318 serine protease in varying industrial applications, such as in detergents, the properties of the native enzyme may be further optimized. These properties include improvement of the storage stability, e.g. by decreasing the autoproteolytic activity of the enzyme. Other properties to be optimized include the stability in the presence or absence of detergent, pH stability, oxidative stability or resistance against bleaching agents and substrate specificity. It is self-evident that e.g. in laundry and dish washing compositions the wash performance of the modified protease may not be impaired in comparison to the parent or precursor protease enzyme. In other words the enzyme variants have similar or even improved wash performance or stain removal property compared to the parent serine protease.

Based on information derived from the crystal structures and sequence similarity comparisons between homologous proteins, variants with improved stability and/or improved performance may be designed.

A variant with improved stability may be obtained e.g. by substitution with proline, introduction of a disulfide bond, altering a hydrogen bond contact, altering charge distribution, introduction of a salt bridge, introduction of metal binding sites, filling an internal structural cavity with one or more amino acids with bulkier side groups (in e.g. regions which are structurally mobile), substitution of histidine residues with other amino acids, removal of a deamidation site, or by helix capping. Stability of the protein may be improved also by substitution of at least one amino acid with cysteine residue or insertion of one or more cysteine residues which create at least one disulfide bridge.

The change in the amino acid sequence may be obtained by constructing a modified nucleotide sequence or DNA sequence by using genetic engineering. As a result a modified nucleotide sequence is obtained, which encodes the variant or mutant polypeptide of the
invention. The methods for modifying the nucleotide sequences include e.g. site-directed and random mutagenesis. For site-directed mutagenesis, a method based on protein structure, a good understanding of the structure-function relationship would be beneficial. In the absence of such deep understanding, methods based on random mutagenesis may be used. For example, as disclosed in Sambrook and Russell (2001) oligonucleotide-directed mutagenesis for changing the base sequence of a segment of the coding DNA may be used to test the role of particular residues in the structure, catalytic activity, and ligand-binding capacity of a protein. In the absence of a three-dimensional structure, this type of protein engineering relies on educated guesses concerning the structure of the protein and contribution of individual residues to protein stability and function.

WO 97/07206 discloses a method for preparing polypeptide variants by shuffling different nucleotide sequences of homologous DNA sequences by in vivo recombination.

The variant or mutant serine proteases of the present invention were designed basing on sequence comparison of the Fusarium equiseti RF6318 and F. acuminatum RF7182 protease (Fe_RF6318 and Fa_RF7182, respectively) amino acid sequences (SEQ ID NO:2 and SEQ ID NO:4, respectively) and their three-dimensional structures. Isolation of the Fe_RF6318 and Fa_RF7182 serine proteases and their amino acid and nucleotide sequences are disclosed in patent applications FI20095497 and FI20095499 (AB Enzymes Oy, FI), both filed on 30 April 2009. Also sequences of other serine proteases of the subtilisin clan were used in comparisons. The modifications of Fe_RF6318 serine protease aimed on increasing the stability of the wild type Fe_RF6318 protease e.g. by reducing flexibility of the protein structure.

The term "variant" or "mutant" polypeptide in the present invention means a change in the amino acid sequence of the parent or precursor Fe_RF6318 enzyme having the amino acid sequence of the wild-type enzyme. The change may be a substitution of a naturally-occurring amino acid with any of the nineteen other naturally occurring L-amino acids, or a deletion or insertion of a naturally-occurring amino acid at the specific amino acid position. Instead of using the full name of the amino acid, also the three letter or one letter codes for amino acids
are used as identified in the literature, e.g. in the standard molecular biology handbooks, such as Sambrook and Russell (2001).

Altogether 94 mutant proteases, named as m1 - m26 and D1 - D68 were designed (Table 1).

Table 1. The mutant proteases m1 - m26 and D1 - D68. The mutant protease code, modifications made on the amino acid and nucleotide sequences (codons) of the parent/naturally-occurring wild type protease polypeptide or protease cDNA and the codes for the expression plasmids are shown. The modified native amino acid, its position in the mature protease sequence and the amino acid replacing the native amino acid in the mutated protease are shown. For the cDNA and amino acid sequences of the mature wild type Fe_RF6318 protease, see SEQ ID NO:1 and SEQ ID NO:2, respectively. For example, the code R17H means that the arginine at position 17 of SEQ ID NO:2 was substituted with histidine. The code ΔA65ΔH66 means that the alanine at position 65 and the histidine at position 66 of SEQ ID NO:2 were deleted, the deletions resulting in renumbering all the subsequent amino acids. The code ins. G104 means that glycine was inserted at position 104 of SEQ ID NO:2, the insertion resulting in renumbering of all the subsequent amino acids.

<table>
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<th>Modification (amino acid sequence)</th>
<th>Modification (cDNA sequence)</th>
<th>Expression cassette Code</th>
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<td>No</td>
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m9  V239L  GTT → CTC  pALK2889
m20  A248V  GCT → GTC  pALK2890
m21  A281L  GCC → CTC  pALK2891
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m23  R17H, R18K, G22S, F284Y, A287N, T288G  GCC → CTC, CGC → GTC, AAC → CCG, AAG → ATC, AAT → GGC  pALK2893
m24  All ID, V208I  GCT → GAC, GTC → ATC  pALK2894
m25  V208I, A281L  GTC → ATC, GCC → CTC  pALK2898
m26  All ID, V208I, A281L  GCT → GAC, GTC → ATC, GCC → CTC  pALK2899
D1  A11ID, V208I, A281L, N7R  GCT → GAC, GTC → ATC, GCC → CTC, AAC → CGC  pALK3051
D2  A11ID, V208I, A281L, A33E  GCT → GAC, GTC → ATC, GCC → CTC, GCC → GAG  pALK3052
D3  A11ID, V208I, A281L, A47E  GCT → GAC, GTC → ATC, GCC → CTC, GCC → GAG  pALK3053
D4  A11ID, V208I, A281L, S56R  GCT → GAC, GTC → ATC, GCC → CTC, TCT → CGC  pALK3054
D5  A11ID, V208I, A281L, A61P  GCT → GAC, GTC → ATC, GCC → CTC, GTG → CCG  pALK3055
D6  A11ID, V208I, A281L, G63P  GCT → GAC, GTC → ATC, GCC → CTC, GGT → CCG  pALK3056
D7  A11ID, V208I, A281L, V76A  GCT → GAC, GTC → ATC, GCC → CTC, GTT → GGC  pALK3057
D8  A11ID, V208I, A281L, N83D  GCT → GAC, GTC → ATC, GCC → CTC, AAC → GAC  pALK3058
D9  A11ID, V208I, A281L, N114R  GCT → GAC, GTC → ATC, GCC → CTC, AAC → CGC  pALK3059
D10  A11ID, V208I, A281L, V155I  GCT → GAC, GTC → ATC, GCC → CTC, GTC → ATC  pALK3060
D11  A11ID, V208I, A281L, V158I  GCT → GAC, GTC → ATC, GCC → CTC, GTG → ATC  pALK3061
D12  A11ID, V208I, A281L, G164A  GCT → GAC, GTC → ATC, GCC → CTC, GGT → GCC  pALK3062
D13  A11ID, V208I, A281L, I185M  GCT → GAC, GTC → ATC, GCC → CTC, ATC → ATG  pALK3063
D14  A11ID, V208I, A281L, T196R  GCT → GAC, GTC → ATC, GCC → CTC, ACT → CGC  pALK3064
D15  A11ID, V208I, A281L, V206L  GCT → GAC, GTC → ATC, GCC → CTC, GTC → ATC  pALK3065
D16  A11ID, V208I, A281L, M234S  GCT → GAC, GTC → ATC, GCC → CTC, ATG → AGC  pALK3066
D17  A11ID, V208I, A281L, Q247L  GCT → GAC, GTC → ATC, GCC → CTC, CAG → CTG  pALK3067
D18  A11ID, V208I, A281L, N260R  GCT → GAC, GTC → ATC, GCC → CTC, AAC → CGC  pALK3068
<p>| D19 | All ID, V208I, A281L, T268R | GCT→GAC, GTC→ATC, GCC→CTC, ACC→CGA | pALK3069 |
| D20 | All ID, V208I, A281L, ΔN 167 | GCT→GAC, GTC→ATC, GCC→CTC, AAAC | pALK3070 |
| D21 | All ID, V208I, A281L, GCT→GAC, GTC→ATC, pALK3070 Δ167 GCC→CTC, ACC→CGA, ins. GGC | pALK3071 |
| D22 | All ID, V208I, A281L, T3C, T29C | GCT→GAC, GTC→ATC, GCC→CTC, ACC→TGC, ACC→GAC | pALK3072 |
| D23 | All ID, V208I, A281L, T121D, Q153R | GCT→GAC, GTC→ATC, GCC→CTC, ACC→GAC, CAA→CGA | pALK3074 |
| D24 | All ID, V208I, A281L, S174R, E205D | GCT→GAC, GTC→ATC, GCC→CTC, TCT→CGA, GAG→GAC | pALK3075 |
| D26 | All ID, V208I, A281L, N214D, S230R | GCT→GAC, GTC→ATC, GCC→CTC, AAC→GAC, AGC→CGA | pALK3077 |
| D27 | All ID, V208I, A281L, A33E, N83D, N114R, T196R, N260R | GCT→GAC, GTC→ATC, GCC→CTC, GCC→GAG, AAC→GAC, AAC→CGC, ACT→CGC, AAC→CGC | pALK3078 |
| D31 | All ID, V208I, A281L, A8V | GCT→GAC, GTC→ATC, GCC→CTC, GCT→GTC | pALK3082 |
| D32 | All ID, V208I, A281L, G34S | GCT→GAC, GTC→ATC, GCC→CTC, GGT→AAC | pALK3083 |
| D33 | All ID, V208I, A281L, G34N | GCT→GAC, GTC→ATC, GCC→CTC, GGT→AAC | pALK3084 |
| D34 | All ID, V208I, A281L, Y36D | GCT→GAC, GTC→ATC, GCC→CTC, TAC→GAC | pALK3085 |</p>
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<td>All ID, V208I, A281L, P138K</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, ACC→AAG</td>
<td>pALK3113</td>
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<td>D44</td>
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<td>GCT→GAC, GTC→ATC, GCC→CTC, GCT→AAC</td>
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<td>D45</td>
<td>All ID, V208I, A281L, A144T</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCT→ACC</td>
<td>pALK3115</td>
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<td>D46</td>
<td>All ID, V208I, A281L, E205N</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→GAG</td>
<td>pALK3116</td>
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<tr>
<td>D47</td>
<td>All ID, V208I, A281L, E205R</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→GAG</td>
<td>pALK3117</td>
</tr>
<tr>
<td>D48</td>
<td>All ID, V208I, A281L, L249T</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→CGC</td>
<td>pALK3118</td>
</tr>
<tr>
<td>D49</td>
<td>All ID, V208I, A281L, L252T</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→ACC</td>
<td>pALK3119</td>
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<tr>
<td>D50</td>
<td>All ID, V208I, A281L, L252A</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→ACC</td>
<td>pALK3120</td>
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<tr>
<td>D51</td>
<td>All ID, V208I, A281L, A256S</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→AGC</td>
<td>pALK3121</td>
</tr>
<tr>
<td>D52</td>
<td>All ID, V208I, A281L, Q247L, L249T</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, CAG→CTC; CTC→ACC</td>
<td>pALK3122</td>
</tr>
<tr>
<td>D53</td>
<td>All ID, V208I, A281L, T3C, T29C, S6R, T24D</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, ACC→TGC; ACC→TGC; AGC→CGC; ACC→GAC</td>
<td>pALK3123</td>
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<tr>
<td>D54</td>
<td>All ID, V208I, A281L, I185C, T259C</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, ATC→TGC; ACC→TGC</td>
<td>pALK3124</td>
</tr>
<tr>
<td>D55</td>
<td>All ID, V208I, A281L, G210A, I185M</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, ATC→ATG</td>
<td>pALK3125</td>
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<tr>
<td>D56</td>
<td>All ID, V208I, A281L, A33E, A47E, N83D</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC, GCC→GAG; GCC→GAG; AAC→GAC</td>
<td>pALK3126</td>
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</table>
The expression cassettes harboring the corresponding modified nucleotide sequences were synthesized as described in Examples 3, 6, 9 and 16. The codons for the amino acids were chosen to be such that are generally used in *T. reesei* cellulase and xylanase genes (Bergquist *et al.*, 2002) and that did not create any additional *SacII, AgeI* or *NotI* restriction sites into the nucleotide sequence. The *T. reesei* host was transformed with the expression cassettes and the transformants were screened for protease activity on haemoglobin plates as described in Example 4. The transformants showing the strongest intensities of dark brown color around the growing mycelia were chosen for shake flask cultivations in a lactose-based cellulase-inducing medium. The protease activity from the culture supernatants was determined according to the method disclosed in Example 1a and the thermal stability according to the method of Example 1b. The protease variants showing the best stability in these preliminary assays were produced in larger amounts by cultivating the corresponding *T. reesei* transformants in laboratory scale bioreactors in a cellulase-inducing complex medium. The spent culture media of the shake flask and/or fermentor cultivations were used for
determination of the thermal stability (Examples 1b, 8, 11 and 17), stain removal performance (Examples 12, 14 and 17) and detergent stability (Examples 13, 15 and 17).

The expression "improved thermal stability" or "improved thermostability" means that after incubating the culture supernatants comprising the protease variant of the invention at different temperatures for definitive time intervals, the residual protease activity measured from the culture supernatant is better than the residual activity of the parent wild-type Fe_RF6318 protease incubated in corresponding conditions. The thermal stability may be determined using shake flask or fermentation supernatant as an enzyme source as described in Example 1b. Alternatively a purified enzyme may be used. The residual activity of the sample is measured and calculated following e.g. the assay described in Example 1a or any other assay determining protease activity described in the literature (Gupta et al., 2002).

By the term "stain removal performance" in connection to the present invention is meant that the enzyme or enzyme variant is capable of hydrolyzing or removing proteinaceous stains, in particular the insoluble substances or material on the substrate. Typically, the wash performance in varying conditions and exposed to varying treatments is measured as "stain removal efficiency" or "stain removal effect" or "degree of cleaning property", meaning a visible and measurable increase of lightness or change in colour of the stained material, e.g. in artificially soiled swatches or test cloths. Lightness or change in colour values can be measured, for example by measuring the colour as reflectance values with a spectrophotometer using L*a*b* colour space coordinates as described in Examples 12, 14 and 17. Fading or removal of proteinaceous stain indicating of the protease performance (stain removal efficiency) is calculated for example as AL*, which means lightness value L* of enzyme treated fabric minus lightness value L* of fabric treated with buffer or washing liquor without enzyme (enzyme blank or control). The presence of detergent may improve the performance of the enzyme in removing the stains.

By the term "improved wash performance" is meant that the performance of the serine protease variant of the invention is better or remarkably better than the performance of the parent or precursor serine protease, which does not include amino acid changes, i.e. an
enzyme corresponding to the native serine protease obtainable from the wild-type *F. equiseti* RF63 18 strain. The parent amino acid sequence of the serine protease may also be produced by a recombinant strain expressing and secreting said unmodified serine protease.

The expression "detergent" is used to mean substance or material intended to assist cleaning or having cleaning properties. The term "detergency" indicates presence or degree of cleaning property. The degree of cleaning property can be tested on different proteinaceous or protein containing substrate materials or stains or stain mixtures bound to solid, water-insoluble carrier, such as textile fibers or glass. Typical proteinaceous material includes blood, milk, ink, egg, grass and sauces. For testing purposes mixtures of proteinaceous stains are commercially available. The function of the detergent enzyme is to degrade and remove the protein-containing stains. Test results depend on the type of stain, the composition of the detergent and the nature and status of textiles used in the washing test (Maurer, 2004).

In the present invention the term "detergent stability" means that the enzyme or enzyme variant sufficiently retains its activity in detergent solution, during storage and/or washing. Therefore it is efficient in degrading or removing proteinaceous stains or material in the presence of a detergent such as the Liquid Base detergent for colored fabric (see Table 7 of Example 12), the Ecolabel Reference Detergent, light duty (wfk Testgewebe GmbH) or the Commercial liquid detergent as described in Table 8 of Example 13. The stability may be assayed by determining the residual activity e.g. after one or several days incubation (at 37°C) in the presence of a detergent. The residual protease activity may be determined using the method described in Example 1a or any other method disclosed in the literature (Gupta et al. 2002).

The expression "V208I" means a replacement of a valine (V) at position 208 with an isoleucine (I). For sequence numbering, the amino acid sequence of Fe_RF6318 serine protease is numbered according to the mature sequence shown in SEQ ID NO:2. Similarly, e.g. the expression "A111D" means that the alanine (A) at position 111 of the mature Fe_RF6318 serine protease of SEQ ID NO:2 is substituted with an aspartic acid (D).
The spent culture supernatants of the protease variants m7 (comprising the amino acid substitution A77S), m8 (All ID), m18 (V208I), and m21 (A281L) showed better thermal stability than the parent wild-type Fe_RF6318 protease. The multiply substituted variant m22 (All ID, T46I, A88S, A173V, A208I, A239L, A248V and A281L) was clearly more stable than the parent wild-type protease (Example 5). The thermal stability assays performed with the fermentor culture supernatants confirmed that the protease variants m18 and m21 containing single amino acid substitutions were among the ones that seemed to be the most stable in the assays (Figure 6). Also, they showed good performance in the preliminary stain removal tests.

Three new mutant genes m24 (encoding the amino acid change A11ID, V208I), m25 (V208I, A281L) and m26 (A11ID, V208I, A281L) were designed and constructed as described in Example 6. The thermal stability assays performed with the fermentor culture supernatants showed that the protease variants m24, m25 and m26 comprising two or three amino acid substitutions had better stability than the parent wild-type Fe_RF6318 protease. Also, the protease variants had better stability compared to the m8, m18 and m21 protease variants having only single mutations (Figure 7).

The m26 variant was chosen as a background molecule for designing new protease variants of the D-series. The expression cassettes encoding the designed variant were transformed to T. reesei and the transformants were screened as described in Example 7.

The thermostability of mutant proteases D2 (All ID, A208I, A2818L, A33E), D3 (A11ID, V208I, A281L, A47E), D19 (All ID, V208I, A281L, T268R), D22 (All ID, V208I, A281L, T3C, T29C) and D23 (A11ID, V208I, A281L, S6R, T24D) was better than that of the mutant protease m26 comprising three amino acid substitutions (All ID, V208I, A281L), as shown in Fig. 9. Mutant proteases D5 (All ID, V298I, A281L, A61P), D8 (All ID, V298I, A281L, N83D) and D13 (A11ID, V208I, A281L, I185M) had approximately equal thermal stability as m26 mutant protease. Mutant proteases D7 (A11ID, V208I, A281L, V76A), D16 (A11ID, V208I, A281L, M234S) and D17 (A11ID, V208I, A281L, Q247L) had lower thermal stability than m26 mutant protease but the stability was better than the stability of wild type
Fe_RF6318 protease. The D38 (All ID, V208I, A281L,V100D), D41 (Al l ID, V208I, A281L, T106N), D53 (All ID, V208I, A281L, T3C, T29C, S6R, T24D) and D55 (All ID, V208I, A281L, I185M, G210A) mutant proteases show clearly better thermal stability than m26, as shown in Example 17 (Table 10).

The stain removal performance of the mutant proteases of m-series showing the best thermal stability and the parent Fe_RF63 18 protease produced in T. reesei were tested for their ability to remove blood/milk/ink standard stain at 30°C and 45°C in the presence of Liquid Base detergent for colored fabrics at a concentration of 5 g/l (pH approx. 7.4). As shown in Example 12 with the stabilized fermentation culture supernatants comprising the serine protease variants m8 (All ID), m18 (V208I), m21 (A281L), m22 (All ID, T46I, A88S, A173V, V208I, V239L, A248V, A281L), m24 (Al l ID, V208I), m25 (V208I, A281L), and m26 (All ID, V208I, A281L) of the invention, the mutations have not affected harmfully on the stain removal performance compared to the parent Fe_RF63 18 protease (Figures 10A and 10B). The mutant proteases showed similar wash performance as the parent Fe_RF6318 protease also when tested in the presence of another liquid detergent, Ecolabel Reference Detergent, and with detergent powders, like ECE reference detergent 77 without optical brightener (Art. 601, EMPA) and ECE reference detergent 98 without phosphate (Art. 600, EMPA).

The stain removal performance of the mutant proteases of D-series showing the best thermal stability (Example 11) and the parent Fe_RF6318 protease produced in T. reesei, as described in Example 10, were tested for their ability to remove blood/milk/ink standard stain at 30°C and 50°C in the presence of Commercial liquid detergent at a concentration of 5 g/l (pH approx. 7.8). As shown in Example 14 the stabilized culture supernatants comprising the serine protease variants D2 (All ID, V208I, A281L, A33E), D3 (All ID, V208I, A281L, A47E), D5 (All lD, V208I, A281L, A61P), D6 (All ID, V208I, A281L, G63P), D7 (All lD, V208I, A281L, V76A), D8 (All ID, V208I, A281L, N83D), D13 (All ID, V208I, A281L, I185M), D16 (A11 ID, V208I, A281L, M234S), D17 (A111D, V208I, A281L, Q247L), D18 (All ID, V208I, A281L, N260R), D22 (All ID, V208I, A281L, T3C, T29C), and D23 (All ID, V208I, A281L, S6R, T24D) of the invention had better performance at 30°C than
the parent Fe_RF6318 protease and similar or better performance than the m26 variant comprising three amino acid substitutions (Figures 14A, 14B and 15A). The D19 (All ID, V208I, A281L, T268R) variant had similar performance compared to the parent Fe_RF6318 protease.

At 50°C the variants D2, D3, D5, D6, D8, D13, D16, D22, and D23 had relatively similar stain removal performance compared to the m26 variant comprising three amino acid substitutions (Figures 14C, 14D and 15B). The D17 and D19 variants were slightly better in removing the blood/milk/ink standard stain than the parent Fe_RF6318 protease.

The performance of all tested D31-D68 mutant proteases, except D39, to remove blood/milk/ink standard stain in the presence of Commercial liquid detergent at 30 °C, was similar or better compared to m26 protease. The ability of D35 (All ID, V208I, A281L, G52E), D37 (All ID, V208I, A281L, V100T), D38 (All ID, V208I, A281L, V100D) and D44 (All ID, V208I, A281L, A144N) mutant proteases was shown to be especially good compared to m26 (Example 17).

The serine protease variants of the present invention had improved stability in the presence of various liquid detergents, as shown in Examples 13 and 15. Stability of the m18 (V208I), m22 (All ID, T46I, A88S, A173V, V208I, V239L, A248V, A281L), m24 (All ID, V208I), m25 (V208I, A281L), and m26 (All ID, V208I, A281L) variants was considerably improved compared to the parent Fe_RF6318 serine protease in Ecolabel Reference Detergent and in Commercial liquid detergent (Figures 12-13).

(Al1ID, V208I, A281L, V76A) was lower compared to m26. However, the mutants D16 and D17 were more stable than the wild type (Fig. 12B) in previous test.

In Commercial liquid detergent with added sodium tetraborate decahydrate and propylene glycol (Figs 17A and B), stability of mutants D2 (All ID, V208I, A281L, A33E), D3 (All ID, V208I, A281L, A47E), D6 (All ID, V208I, A281L, G63P), D13 (All ID, V208I, A281L, 1185M), D22 (All ID, T46l, A88S, A173V, V208I, V239L, A248V, A281L) and especially D19 (Al1ID, V208I, A281L, T268R), was better compared to m26 and mutant D5 (All ID, V298I, A281L, A61P) had relatively similar stability The stability of mutants D8 (All ID, V208I, A281L, N83D), D16 (All ID, V208I, A281L, M234S), D17 (Al1ID, V208I, A281L, Q247L), D32 (Al1ID, V208I, A281L, S6R, T24D) and especially D7 (All ID, V208I, A281L, V76A) was lower compared to m26. However, all the D-series mutants having lower stability compared to m26, except D7 were more stable than the wild type (Fig. 13B).


The results show that mutant proteases were constructed that have improved stability and also similar or even better stain removal performance compared to the wild type protease.
According to a preferred embodiment the Fe_RF63 18 serine protease variant of the invention is a polypeptide, which has serine protease activity and comprises an amino acid sequence having a substitution of valine at position 208 of the parent wild type Fe_RF6318 serine protease with an amino acid other than valine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF63 18 enzyme as defined in SEQ ID NO:2.

Preferably, the serine protease variant of the invention comprises a substitution which results in improved thermal stability and/or improved stability in liquid detergents and has retained or improved wash performance in liquid and powdered detergents compared to the parent mature Fe_RF6318 serine protease of SEQ ID NO:2.

Further, the serine protease variant of the invention has good performance in the presence of detergent, i.e. is capable of degrading or removing proteinaceous stains or material in the presence of detergent at low to moderate temperature ranges, such as from 30°C to 50°C. Specifically the protease variant of the invention has similar or better wash performance than the parent wild-type Fe_RF63 18 protease.

Preferably, the serine protease variant has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid selected from the group of isoleucine, leucine and methionine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2. The substitutions with isoleucine, leucine and methionine change rigidity of the polypeptide.

Thus, one preferred embodiment of the invention is a Fe_RF6318 variant, which has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF63 18 serine protease with an amino acid isoleucine (V208I), with an amino acid leucine (V208L) or with an amino acid methionine (V208M), wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF63 18 enzyme as defined in SEQ ID NO:2.
Preferably, the serine protease variant of the invention comprises an amino acid sequence having the substitution V208I relative to the parent mature Fe_RF6318 serine protease, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2.

Another preferred embodiment of the invention is a serine protease variant which has serine protease activity and, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, comprises one or more amino acid changes, which are selected from the group consisting of a substitution, insertion and deletion.

The term "substitution" in the present invention means replacement of one or more amino acids in the naturally-occurring amino-acid sequence of a protein or polypeptide with another amino acid. If a functionally equivalent amino acid is substituted, the protein may retain wild-type activity. Substitution may also diminish or eliminate protein function or modify the wild-type activity. Substitutions are indicated by the term corresponding to "V208I" which means that valine at position 208 of the parent Fe_RF6318 is substituted with isoleucine, wherein the amino acid position corresponds to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

**Insertion** of one or more amino acids to the wild-type amino acid sequence may result in an enzyme which is more resistant to the action of other proteases, including also autocatalysis. The present invention provides stabilization of the Fe_RF6318 serine protease by insertion of glycine at position 104 of the parent Fe_RF6318 serine protease. The insertion is indicated by the term "G104".

Stabilization may be improved also by introducing one or more cysteines into the amino acid sequence, thus creating additional disulphide bonds and stabilizing the three-dimensional structure of the protein. The invention provides e.g. variants which include one additional cysteine bridge between cysteines at positions 3 and 29 of the parent Fe_RF6318 protease, indicated by the term "T3C-T29C". Stability of the three-dimensional structure may also be
improved by insertion of additional salt bridges between the amino acids. The present invention provides e.g. variants which include a salt bridge between arginine at position 6 and aspartic acid at position 24 of the parent Fe_RF6318 protease, indicated by the term "S6R-T24D".

Deletion of a naturally-occurring amino acid is indicated by the symbol "Δ". Thus, the term "ΔN167" means that the asparagine at position 167 of the parent Fe_RF6318 protease is deleted. Similarly, the terms "ΔA65" and "ΔH66" mean that the alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 protease are deleted.

In one preferred embodiment of the invention the serine protease variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288 of the parent Fe_RF6318 serine protease, a deletion of asparagine at position 167 of the parent Fe_RF6318 serine protease, a deletion of alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

In another preferred embodiment of the invention, according to increase in thermostability, the serine protease variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 14, 24, 29, 33, 34, 47, 52, 61, 63, 65, 83, 91, 100, 103, 106, 111, 121, 144, 153, 157, 158, 164, 175, 176, 185, 210, 234, 236, 256, 268 or 281 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.
In another preferred embodiment of the invention, according to increase in thermostability, the serine protease variant comprises, in addition to the substitution of valine at position 208 of the mature Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 24, 29, 33, 47, 100, 103, 106, 111, 185, 210, 268 or 281 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.


In another preferred embodiment of the invention, according to stability in detergent, the serine protease variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 37, 47, 61, 63, 65, 83, 100, 111, 123, 157, 175, 176, 185, 210, 234, 236, 247, 268 or 281 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.
In another preferred embodiment of the invention, according to stability in detergent, the serine protease variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 47, 63, 100, 111, 123, 157, 175, 176, 185, 210, 236, 268 or 281 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.


According to the most preferred embodiment of the invention the serine protease variant polypeptide is encoded by an isolated polynucleotide sequence which encodes the polypeptide comprising the amino acid sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, and SEQ ID NO:50.
Thus, within the scope of the invention is the polypeptide encoded by the nucleic acid molecule, which includes the nucleotide sequence comprising the "coding sequence" for the enzyme. The expression "coding sequence" means the nucleotide sequence which initiates from the translation start codon (ATG) and stops at the translation stop codon (TAA, TAG or TGA). The translated full-length polypeptide starts usually with methionine and comprises intron regions. Thus, within the scope of the invention are the serine protease variants which comprise the mature form of the Fe_RF6318 enzyme, as well as the pro-form and prepro-form of the enzyme. The prepro-form is the preferred construction since this facilitates the expression, secretion and maturation of the serine protease variants.

The present invention relates also to an isolated nucleic acid molecule comprising a nucleotide sequence encoding the fungal serine protease (Fe_RF6318) variant according to the invention.

Thus, according to a preferred embodiment of the invention the isolated nucleic acid molecule comprises a nucleotide sequence encoding the Fe_RF6318 serine protease variant, which has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid other than valine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2.

Preferably, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a fungal serine protease (Fe_RF6318) variant which carries a substitution resulting in improved thermal stability and/or improved stability in detergents when compared to the parent mature Fe_RF6318 serine protease of SEQ ID NO:2. The variant has retained or improved wash performance in liquid or powdered detergents compared to the parent mature Fe_RF6318 serine protease of SEQ ID NO:2. Thus, the fungal serine protease variant of the invention has good performance in the presence of detergent, i.e. is capable of degrading or removing proteinaceous stains or material in the presence of detergent at low to moderate temperature ranges, such as from 30°C to 50°C.
One preferred embodiment of the invention is an isolated nucleic acid molecule which comprises a nucleotide sequence encoding a serine protease variant, which has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid selected from the group of isoleucine, leucine and methionine wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2. The substitutions with isoleucine, leucine and methionine change the rigidity of the polypeptide.

Thus, one preferred embodiment of the invention is an isolated nucleic acid molecule comprising a nucleotide sequence encoding a serine protease variant, which has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid isoleucine (V208I), with an amino acid leucine (V208L), or with an amino acid methionine (V208M), wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2.

Preferably, the encoded serine protease variant of the invention comprises an amino acid sequence having the substitution V208I relative to the parent mature Fe_RF6318 serine protease, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2.

Another preferred embodiment of the invention is an isolated nucleic acid molecule which comprises a nucleotide sequence encoding a serine protease variant, which has serine protease activity and, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, comprises one or more amino acid changes, which are selected from the group consisting of a substitution, insertion, deletion or insertion.

In one preferred embodiment of the invention the isolated nucleic acid molecule comprises a nucleotide sequence which encodes a serine protease variant comprising, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at
position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288 of the parent Fe_RF6318 serine protease, a deletion of asparagine at position 167 of the parent Fe_RF6318 serine protease, a deletion of alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

In another preferred embodiment of the invention, according to increase in thermostability, the isolated nucleic acid molecule comprises a nucleotide sequence, which encodes a serine protease variant comprising, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 14, 24, 29, 33, 34, 47, 52, 61, 63, 65, 83, 91, 100, 103, 106, 111, 121, 144, 153, 157, 158, 164, 175, 176, 185, 210, 234, 236, 256, 268 or 281 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

In another preferred embodiment of the invention, according to increase in thermostability, the isolated nucleic acid molecule comprises a nucleotide sequence which encodes a serine protease variant comprising, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 24, 29, 33, 47, 100, 103, 106, 111, 185, 210, 268 or 281 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

In the most preferred embodiment of the invention, according to increase in thermostability, the encoded serine protease variant comprises an amino acid sequence having substitution

In another preferred embodiment of the invention, according to stability in detergent, the isolated nucleic acid molecule comprises a nucleotide sequence which encodes a serine protease variant comprising, in addition to substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 37, 47, 61, 63, 65, 83, 100, 111, 123, 157, 175, 176, 185, 210, 234, 236, 247, 268, 281 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

In another preferred embodiment of the invention, according to stability in detergent, the encoded serine protease variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 47, 63, 100, 111, 123, 157, 175, 176, 185, 210, 236, 268, 281 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

A11 1D-A281L-C236T relative to the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease defined in SEQ ID NO:2.

According to the most preferred embodiment of the invention the isolated nucleic acid molecule comprises the nucleotide sequence defined in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, and SEQ ID NO:49.

The nucleic acid molecule of the invention may be RNA or DNA, wherein the DNA may constitute of the genomic DNA or cDNA.

Standard molecular biology methods can be used in construction of the polynucleotide sequence encoding the fungal serine protease variant of the invention, including isolation of genomic and plasmid DNA, digestion of DNA to produce DNA fragments, sequencing, *E. coli* transformations etc. The basic methods are described in the standard molecular biology handbooks, e.g. Sambrook and Russell, 2001.

Constructions of the *FeprtS8A* mutant genes encoding the Fe_RF6318 polypeptide variants of the invention are described in Examples 3, 6, 9 and 16. Briefly, the synthetic cDNAs encoding the wild type Fe_RF6318 protease and the protease variants of the invention were ordered from GenScript Corporation (NJ, U.S.A.). The inserts from the GenScript plasmids were isolated from agarose gel after *SacU-Agel* digestion and ligated to pALK1919 or pALK2777 vector backbone. The expression cassettes contained the cDNAs encoding the full-length wild-type Fe_RF6318 serine protease or the protease variants of the invention. The cassettes contained also the *T. reesei cbhl* (*cellA*) promoter and terminator regions and the *amdS* marker gene (Hynes *et al.* 1983) for selecting the transformants. The sequences of the gene fusions and the mutations in the cDNA sequences were confirmed from the genetic
constructions by sequencing. Examples of the plasmid constructions carrying the mutant protease expression cassette are provided in Figure 5.

The nucleic acid molecule of the invention may also be an analogue of the nucleotidic sequence characterized above. For example, due to degeneracy of the nucleotidic sequence. The "degeneracy" means analogues of the nucleotidic sequence, which differ in one or more nucleotides or codons, but which encode the Fe_RF63 18 protease variant of the invention.

The present invention relates also to a recombinant expression vector or recombinant expression construct, which can be used to propagate or express the nucleic acid sequence encoding the chosen serine protease variant in a suitable prokaryotic or eukaryotic host. The recombinant expression vector comprises a nucleotide sequence encoding the serine protease variant of the invention operably linked to regulatory sequences which facilitate or direct expression and preferably, secretion of a sequence encoding the serine protease variant of the invention in a suitable host, such as promoters, enhancers, terminators (including transcription and translation termination signals) and signal sequences operably linked the polynucleotide sequence encoding said serine protease variant. The expression vector may further comprise marker genes for selection of the transformant strains or the selection marker may be introduced to the host in another vector construct by co-transformation. Said regulatory sequences may be homologous or heterologous to the production organism or they may originate from the organism, from which the gene encoding the serine protease is isolated.

Examples of promoters for expressing the serine protease variant of the invention in filamentous fungal hosts are the promoters of A. oryzae TAKA amylase, alkaline protease ALP and triose phosphate isomerase, Rhizopus miehei lipase, Aspergillus niger or A. awamori glucoamylase (glaA), Fusarium oxysporum trypsin-like protease, Chrysosporium lucknowense cellobiohydrolase I promoter, Trichoderma reesei cellobiohydrolase I (Cel7A) etc.
In yeast, for example promoters of *S. cerevisiae* enolase (ENO-1), galactokinase (GAL1), alcohol dehydrogenase (ADH2) and 3-phosphoglycerate kinase can be used to provide expression.

Examples of promoter sequences for directing the transcription of the serine protease variant of the invention in a bacterial host are the promoter of *lac* operon of *Escherichia coli*, the *Streptomyces coelicolor* agarase *dagA* promoter, the promoter of the *B. licheniformis* alpha-amylase gene (*amyL*), the promoter of the *B. stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *B. subtilis* *xylA* and *xylB* genes, etc.

Suitable terminators include those of the above mentioned genes or any other characterized terminator sequences.

Suitable transformation or selection markers include those which complement a defect in the host, for example the *dal* genes from *B. subtilis* or *B. licheniformis* or *Aspergillus* *amdS* and *niaD*. The selection may be based also on a marker conferring antibiotic resistance, such as ampicillin, kanamycin, chloramphenicol, tetracycline, phleomycin or hygromycin resistance.

Extracellular secretion of the serine protease variant of the invention is preferable. Thus, the recombinant vector comprises sequences facilitating secretion in the selected host. The signal sequence of the serine protease of the invention or the presequence or prepeptide may be included in the recombinant expression vector or the natural signal sequence may be replaced with another signal sequence capable of facilitating secretion in the selected host. Thus, the chosen signal sequence may be homologous or heterologous to the expression host.

Examples of suitable signal sequences are those of the fungal or yeast organisms, e.g. signal sequences from well expressed genes. Such signal sequences are well known from the literature.

The recombinant vector may further comprise sequences facilitating integration of the vector into the host chromosomal DNA to obtain stable expression.
The Fe_RF6318 protease variant of the invention was expressed with its own signal sequence from the *T. reesei* cbhl (*celVA*) promoter as described in Examples 3, 6, 9 and 16. The expression constructs used to transform the *T. reesei* host included also cbhl terminator and *amdS* marker for selecting the transformants from the untrasformed cells.

The present invention relates also to host cells comprising the recombinant expression vector as described above. Suitable hosts for production of the fungal serine protease variant are homologous or heterologous hosts, such as the microbial hosts including bacteria, yeasts and fungi. Production systems in plant or mammalian cells are also possible.

Filamentous fungi, such *Trichoderma, Aspergillus, Fusarium, Humicola, Chrysosporium, Neurospora, Rhizopus, Penicillium* and *Mortiriella*, are preferred production hosts due to the ease of down-stream processing and recovery of the enzyme product. Suitable expression and production host systems are for example the production system developed for the filamentous fungus host *Trichoderma reesei* (EP 244234), or *Aspergillus* production systems, such as *A. oryzae ox A. niger* (WO 9708325, US 5,843,745, US 5,770,418), *A. awamori, A. sojae* and *A. japonicus-type* strains, or the production system developed for *Fusarium*, such as *F. oxysporum* (Malardier et al., 1989) or *F. venenatum*, and for *Neurospora crassa, Rhizopus miehei, Mortiriella alpinis, H. lanuginosa* or *H. insolens* or for *Chrysosporium lucknowense* (US 6,573,086). Suitable production systems developed for yeasts are systems developed for *Saccharomyces, Schizosaccharomyces* or *Pichia pastoris*. Suitable production systems developed for bacteria are a production system developed for *Bacillus*, for example for *B. subtilis, B. licheniformis, B. amyloliquefaciens*, for *E. coli*, or for the actinomycete *Streptomyces*.

Preferably the serine protease variant of the invention is produced in a filamentous fungal host of the genus *Trichoderma* or *Aspergillus*, such as *T. reesei*, or *A. niger, A. oryzae, A. sojae, A. awamori* or *A. japonicus-type* strains. According the most preferred embodiment of the invention the fungal serine protease (Fe_RF6318) variant is produced in *T. reesei*. 
The production host cell may be homologous or heterologous to the serine protease variant of the invention. The host may be free of homogenous proteases due to removal of proteases either by inactivation or removal of one or more host proteases, e.g. by deletion of the gene(s) encoding such homogenous or homologous proteases.

The serine protease enzyme variant of the invention derives from a filamentous fungi belonging to a genus *Fusarium*. Fungal alkaline proteases are advantageous to the bacterial proteases due to the ease of down-stream processing to produce a microbe-free enzyme or enzyme composition. Mycelium can be easily removed through filtration techniques prior to the purification of the enzyme.

The present invention relates also to a process for producing a polypeptide having serine protease activity, said process comprising the steps of culturing the natural or recombinant host cell carrying the recombinant expression vector for a serine protease variant of the invention under suitable conditions and optionally isolating said variant enzyme. The production medium may be a medium suitable for growing the host organism and containing inducers for efficient expression. Suitable media are well-known from the literature.

The invention further relates to a process for obtaining an enzyme preparation comprising a variant polypeptide, which has serine protease activity, said process comprising the steps of culturing a host cell carrying the expression vector of the invention and either recovering the variant polypeptide from the cells or separating the cells from the culture medium and obtaining the supernatant having serine protease activity.

The produced variant enzymes can be purified by using conventional methods of enzyme chemistry, such as salt preparation, ultrafiltration, ion exchange chromatography, affinity chromatography, gel filtration and hydrophobic interaction chromatography. Purification can be monitored by protein determination, enzyme activity assays and by SDS polyacrylamide gel electrophoresis. The enzyme activity and stability of the purified enzyme at various temperature and pH values as well as the molecular mass and the isoelectric point can be determined.
Naturally, it is possible to separate the enzyme of the present invention by using other known purification methods instead, or in addition to the methods described herein.

The present invention relates also to an enzyme preparation, which comprises the serine protease variant characterized above. Within the invention is an enzyme preparation which comprises the fungal serine protease variant of the invention, obtained by culturing a host cell, which carries the recombinant expression vector of the invention.

Said enzyme preparation may further comprise different types of enzymes in addition to the serine protease variant of this invention, for example another protease, an amylase, a lipase, a cellulase, cutinase, a pectinase, a mannanase, a xylanase and/or an oxidase such as a laccase or peroxidase with or without a mediator. These enzymes are expected to enhance the performance of the serine protease variants of the invention by removing the carbohydrates and oils or fats present in the material to be handled. Said enzymes may be natural or recombinant enzymes produced by the host strain or may be added to the culture supernatant after the production process.

Said enzyme preparation may further comprise a suitable additive selected from the group of surfactants or surface active agent, buffers, anti-corrosion agents, stabilizers, bleaching agents, mediators, builders, caustics, abrasives and preservatives, optical brighteners, antiredeposition agents, dyes, pigments, perfumes, etc.

Surfactants are useful in emulsifying grease and wetting surfaces. The surfactant may be a non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic.

Buffers may be added to the enzyme preparation to modify pH or affect performance or stability of other ingredients.
Suitable stabilizers include polyols such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or boric or boronic acid derivatives, peptides, lyotropic salts, etc.

Bleaching agent is used to oxidize and degrade organic compounds. Examples of suitable chemical bleaching systems are H₂O₂ sources, such as perborate or percarbonate with or without peracid-forming bleach activators such as tetraacetylenediamine, or alternatively peroxyacids, e.g. amide, imide or sulfone type. Chemical oxidizers may be replaced partially or completely by using oxidizing enzymes, such as laccases or peroxidases. Many laccases do not function effectively in the absence of mediators.

Builders or complexing agents include substances, such as zeolite, diphosphate, triphosphate, carbonate, citrate, etc. The enzyme preparation may further comprise one or more polymers, such as carboxymethylcellulose, poly(ethylene glycol), poly(vinyl alcohol), poly(vinylpyrrolidone), etc. Also, softeners, caustics, preservatives for preventing spoilage of other ingredients, abrasives and substances modifying the foaming and viscosity properties can be added.

According to one preferred embodiment of the invention said enzyme preparation is in the form of liquid, powder or granulate.

The Fe_RF6318 serine protease variant of the present invention may like other proteases, particularly alkaline proteases be used in the detergent, protein, brewing, meat, photographic, leather, dairy and pharmaceutical industries (Kalisz, 1988; Rao et al, 1998). For example, it may be used as an alternative to chemicals to convert fibrous protein waste (e.g. horn, feather, nails and hair) to useful biomass, protein concentrate or amino acids (Anwar and Saleemuddin, 1998). The use of fungal serine protease variant of the present invention may like other enzymes prove successful in improving leather quality and in reducing environmental pollution and saving energy and it may like alkaline proteases be useful in synthesis of peptides and resolution of the mixture of D,L-amino acids. The fungal serine protease variants of the present invention may find use in pharmaceutical industry and may
also like other alkaline proteases be applicable in removal of blood on surgical equipments and cleaning contact lenses or dentures. Like alkaline protease from *Conidiobolus coronatus*, the serine protease variant of the present invention may be used for replacing trypsin in animal cell cultures. The protease variants of the invention can also be used in cleaning of membranes and destruction of biofilms. In baking the protease variants can be used e.g. in destruction of the gluten network and in other food applications in hydrolysis of food proteins, e.g proteins in milk. They can also be used e.g. in treating yeast, rendering (extracting more protein from animal bones), creating new flavours, reducing bitterness, changing emulsifying properties, generating bioactive peptides and reducing allergenicity of proteins. The substrates include animal, plant and microbial proteins.

The present invention relates to the use of the serine protease variant or the enzyme preparation comprising said variant for detergents, treating textile fibers, for treating wool, for treating hair, for treating leather, for treating feed or food, or for any application involving modification, degradation or removal of proteinaceous material.

One preferred embodiment of the invention is therefore the use of the serine protease variant as characterized above as a detergent additive useful for laundry detergent and dish wash compositions, including automatic dish washing compositions.

The serine protease of the present invention degrades various kinds of proteinaceous stains under conditions of neutral and alkaline pH and even in the presence of liquid or powdered detergents with different compositions (as shown in Examples 12 and 14).

One preferred embodiment of the invention is a detergent composition comprising the Fe_RF63 18 variant protease as an ingredient. The detergent composition may also comprise the enzyme composition of the invention. The serine protease variant of the invention was shown to have an improved stability in different liquid detergents. Thus, its stability against the destabilizing agents present in the detergent compositions such as surfactants, complexing
agents or bleaching agents is better than the stability of the wild-type Fe_RF6318 serine protease.

The "detergent composition" includes an effective amount of a serine protease variant, which has serine protease activity and comprises an amino acid sequence having a substitution of valine at position 208 of the parent Fe_RF6318 polypeptide with an amino acid other than valine, and one or more amino acid changes selected from the group consisting of a substitution at position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288 of the parent Fe_RF6318 serine protease, a deletion of asparagine at position 167 of the parent Fe_RF6318 serine protease, a deletion of alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO: 2.

The term "effective amount" of a serine protease refers to the quantity of the protease enzyme necessary to achieve the enzymatic activity in the specific detergent composition. Preferably the detergent composition of the invention comprises from about 0.0001% to about 10% by weight of the detergent composition of a protease variant of the invention, more preferably from 0.001% to about 1%, more preferably from 0.001% to about 0.1%.

According to the experimental results provided below serine protease variants were constructed which have improved thermal stability compared to the parent Fe_RF6318 serine protease. The variants have also improved stability in the presence of different liquid detergents. Similar effects may be observed in any detergent composition wherein the serine protease variant of the invention is an ingredient. The disclosed serine protease variants have similar or even better stain removal performance than the parent Fe_RF6318 serine protease. The protease variant of the invention or the enzyme preparation comprising said variant may
be formulated for use in a hand or machine laundry or may be formulated for use in household hard surface cleaning or preferably in hand or machine dishwashing operations.

EXAMPLE 1

a. Protease activity assay

Protease activity was measured using casein as substrate. Rate of casein degradation by a protease was measured by monitoring the release of acid-soluble peptide fragments as a function of time. Acid-soluble peptides were quantified spectrophotometrically. The result was expressed as 1 µg of tyrosine per min per ml (or g).

First all reagent solutions needed in the assay were prepared in deionized water, Milli-Q or equivalent as follows.

(STW) Synthetic Tap Water:
The following stock solutions were prepared:

- (A) 5.8g CaCl$_2$ x 2 H$_2$O / 200 ml H$_2$O
- (B) 2.8g MgCl$_2$ x 6 H$_2$O / 200 ml H$_2$O
- (C) 4.2g NaHCO$_3$ / 200 ml H$_2$O

10 ml of these solutions were added in the given order to 300 ml of H$_2$O with stirring, then made up to 1 liter with H$_2$O. The resulting solution was called as synthetic tap water.

Tris solution, 0.3 M in synthetic tap water:
36.3 g of Trizma base (SIGMA T-1503) was dissolved in synthetic tap water and made up to 1 liter.

Casein solution:
6 g of Casein Hammarstein grade (Cat. No. 101289 (MP Biomedicals, LCC, US) was added to 350 ml synthetic tap water and dissolved with magnetic stirring for 10 min. 50 ml of Tris solution was added and the solution was stirred for another 10 min. Then, the solution was heated up to 70 °C. After that the temperature was let to decrease or cooled to 50 °C and the
pH was adjusted to 8.5 with 0.1M NaOH. Stirring was continued until room temperature was reached. The solution was made up to 500 ml with synthetic tap water. The substrate solution was stored for maximum of 3 days in refrigerator or stored as frozen.

**110 mM Trichloroacetic acid reagent (reaction stop solution):**
18 g of TCA (Merck 807) was dissolved in H₂O and made up to 1 liter.

**0.5 M Na₂C₀₃:**
53 g of Na₂C₀₃ was dissolved in H₂O and made up to 1 liter.

**Folin solution:**
25 ml of 2N Folin-Ciocalteu's phenol reagent (SIGMA, F 9252) was diluted up to 100 ml with H₂O.

**Sample dilution buffer:**
The sample was diluted in 50 mM Tris-HCl buffer pH 8.5.
The most suitable dilution will yield an absorbance of 0.4 - 0.8 in the reaction.

**Assay:**
The assay was started by temperating 2.5 ml of substrate solution in test tubes for 5 min at 50 °C. After that 0.5 ml of diluted enzyme solution was added, mixed with vortex mixer and the reaction was conducted at 50°C for exactly 30 min. The enzyme blank was prepared like the sample but the reaction stop solution (110 mM TCA) was added in test tube before the sample. After the reaction 2.5 ml of stop solution was added in tubes (not for blank), the contents were mixed and allowed to stand for 30 minutes at room temperature. Tubes were centrifuged 4000 rpm for 10 minutes (Hettich Rotanta 460). One ml of clear supernatant was mixed with 2.5 ml of 0.5 M Na₂C₀₃ and 0.5 ml diluted Folin reagent. After waiting for 10 min (colour development) the absorbance of the mixture (colour) was measured at 660 nm against an enzyme blank. The activity was read from standard curve and the result was multiplied by the dilution factor. At least two parallel samples were used in each measurement.
Standard:

A stock solution of tyrosine was prepared by dissolving 100 mg of tyrosine (Merck 8371) in 0.2 M HCl and made the volume up to 500 ml. The following dilutions were made from the stock solution in 0.2 M HCl:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Tyrosine concentration (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>200</td>
</tr>
<tr>
<td>3:4</td>
<td>150</td>
</tr>
<tr>
<td>1:2</td>
<td>100</td>
</tr>
<tr>
<td>1:3</td>
<td>66.7</td>
</tr>
<tr>
<td>1:4</td>
<td>50</td>
</tr>
<tr>
<td>1:6</td>
<td>33.3</td>
</tr>
<tr>
<td>1:8</td>
<td>25</td>
</tr>
<tr>
<td>1:12</td>
<td>16.7</td>
</tr>
</tbody>
</table>

Duplicate assays of each standard dilution were made. 1 ml of each standard dilution was mixed with 2.5 ml 0.5 M Na₂CO₃ and 0.5 ml of diluted Folin reagent in test tubes. After waiting for 10 min (colour development) the absorbance of the mixture (colour) was measured at 660 nm against an reagent blank. The reagent blank was prepared by adding 0.2 M HCl instead of standard dilution. The corresponding enzyme activity was obtained by dividing tyrosine concentration (g/ml) by the time of hydrolysis, 30 min.

b. Analysis of thermal stability of proteases

The thermal stability of proteases (protease mutants) was determined using shake flask culture or fermentation supernatant as an enzyme source. Culture supernatants were diluted in 20 mM Tris buffer (Trizma base) pH 8.5. Samples were incubated in test tubes at 40 °C, 45 °C or 50 °C in a water bath, for definite time intervals (0-140 min) before residual activity of samples was measured and calculated following standard assay procedure described in Example 1a except that the reaction temperature in the analysis was 40 °C, 45 °C or 50 °C.
EXAMPLE 2. Isolation of DNA and molecular biology methods used

Standard molecular biology methods were used in the isolation and enzyme treatments of DNA (e.g. isolation of plasmid DNA, digestion of DNA to produce DNA fragments, ligations), in *E. coli* transformations, sequencing etc. The basic methods used were either as described by the enzyme, reagent or kit manufacturer or as described in the standard molecular biology handbooks, e.g. Sambrook and Russell (2001). Isolation of genomic DNA from *T. reesei* strains was done as described in detail by Raeder and Broda (1985). The oligonucleotides used for sequencing were ordered from Sigma-Aldrich.

EXAMPLE 3. Design of the m-series mutant proteases ml - m23 and construction of their expression cassettes

Mutant proteases were designed basing on sequence comparison of the *Fusarium equiseti* RF6318 (Fe_RF6318) and *F. acuminatum* RF7182 (Fa_RF7182) protease amino acid sequences (SEQ ID NO:2/FI20095497 and SEQ ID NO:14/FI20095499, respectively) and protease structures. The mature amino acid sequences of above *Fusarium* proteases are included as SEQ ID NO:2 and SEQ ID NO:4 in this application. The modifications aimed on increasing the stability of the wild type Fe_RF6318 protease e.g. by reducing the flexibility of the protein structure. First, altogether 23 mutant proteases named as ml - m23 were designed. For construction of the expression cassettes, synthetic genes encoding the wild type Fe_RF6318 protease and mutant proteases were ordered from GenScript Corporation (NJ, US). The mutations were designed and made on the wild type *Fe prtS8A* cDNA sequence (the cDNA sequence encoding the full-length protease is shown in Fig. 1 and the cDNA sequence encoding the mature polypeptide is included as SEQ ID NO:1). The codons for the amino acids were chosen to be such that are generally used in *T. reesei* cellulase and xylanase genes (Bergquist *et al*, 2002) and that did not create any additional *SacII, AgeI or NotI* sites into the sequence. The codes for the mutant proteases designed and the modifications made are listed in Table 2. In addition to the protease cDNA sequence, the synthetic genes ordered
included at their 5'-end a partial *Trichoderma reesei cbhl* (cel7A) promoter sequence from *Sacill* site (position -16 from ATG) to position -1 and in their 3’-end the partial *cbhl*-terminator sequence to *Agel* site (the partial terminator includes 30 nucleotides after the STOP codon). One example of such a synthetic construction (includes the wild type Fe *prtS8A* cDNA) is shown in Fig. 2. The above additional 5’- and 3’-sequences enabled exact fusions of the synthetic protease cDNAs to the *T. reesei cbhl* promoter and terminator in the expression cassettes pALK1910 and pALK2777 (Figs. 3 and 4, respectively). The *cbhl* sequences can be found e.g. from JGI’s *T. reesei* genome sequence v2.0 (gene ID 123989; [http://genome.jgi-psf.org/Trire2/Trire2.home.html](http://genome.jgi-psf.org/Trire2/Trire2.home.html)).

The synthetic gene constructions were, at GenScript, ligated to pUC57 vector (*EcoKV* site). For construction of the protease expression cassettes, the inserts from the GenScript plasmids were isolated from agarose gel after *Sacill* - *Agel* digestion. The inserts including the m1 - m14 protease cDNAs were ligated to pALK1910 (Fig. 3) and those including the m15 - m23 proteases to pALK2777 (Fig. 4) plasmid digested with *Sacill* and *Agel*. Then, the *amdS* marker (wild type *Aspergillus* gene with its native promoter and terminator; the 4470 bp *EcoKl* - *SpeI* fragment from p3SR2; Hynes *et al.*, 1983) was ligated to the *EcoKV* site of the plasmids basing on pALK1910 backbone. The pALK2777 plasmid already contains a synthetic *amdS* marker gene and thus the expression plasmids that were constructed using this backbone were ready after the above one-step ligation of the insert to *Sacill* - *Agel* digested plasmid. The synthetic *amdS* in pALK2777 contains a shortened terminator (to *Xbal* site) compared to the native *amdS* in pALK1910. Also, the introns of the native *amdS* gene have been removed and chosen restriction sites from the *amdS* promoter and gene have been modified to ease the construction and isolation of the expression cassettes. However, the amino acid sequence encoded by the synthetic *amdS* gene is identical to that encoded by the wild type *amdS* gene.

The sequences of the gene fusions and the mutations in the cDNA sequences were confirmed from the genetic constructions by sequencing.
The final expression cassettes contain the \textit{Fe prtS8A} wild type cDNA or mutant protease cDNA (with the protease gene's own signal and pro sequences) were fused to the \textit{T. reesei cbhl} promoter and terminator (exact fusions) and the \textit{amdS} marker gene (either native or synthetic, see above) following the \textit{cbhl} terminator region. For examples of the plasmids including mutant protease expression cassettes, see Fig. 5.

**Table 2. The mutant proteases m1 - m23.** The mutant protease code, modifications made on the amino acid and nucleotide sequences (codons) of the wild type protease/protease cDNA and the codes for the expression plasmids are shown. The modified native amino acid, its position in the mature protease sequence (SEQ ID NO:2) and the amino acid replacing the native amino acid in the mutated protease are shown.

<table>
<thead>
<tr>
<th>Mutant code</th>
<th>Modification (amino acid sequence)</th>
<th>Modification (cDNA sequence)</th>
<th>Expression cassette Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>No</td>
<td>No</td>
<td>pALK2764</td>
</tr>
<tr>
<td>m1</td>
<td>R17H</td>
<td>CGC→CAC</td>
<td>pALK2765</td>
</tr>
<tr>
<td>m2</td>
<td>Y25L</td>
<td>TAC→CTC</td>
<td>pALK2766</td>
</tr>
<tr>
<td>m3</td>
<td>D28R</td>
<td>GAC→CGC</td>
<td>pALK2767</td>
</tr>
<tr>
<td>m4</td>
<td>D28Y</td>
<td>GAC→TAC</td>
<td>pALK2768</td>
</tr>
<tr>
<td>m5</td>
<td>ΔA65ΔH66</td>
<td>ΔGCC, ΔCAC</td>
<td>pALK2740</td>
</tr>
<tr>
<td>m6</td>
<td>T69N</td>
<td>ACC→AAC</td>
<td>pALK2741</td>
</tr>
<tr>
<td>m7</td>
<td>A77S</td>
<td>GCT→AGC</td>
<td>pALK2742</td>
</tr>
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<td>m8</td>
<td>A111D</td>
<td>GCT→GAC</td>
<td>pALK2743</td>
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<tr>
<td>m9</td>
<td>N167L</td>
<td>AAC→CTC</td>
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<tr>
<td>m10</td>
<td>R169N</td>
<td>CGT→AAC</td>
<td>pALK2745</td>
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<td>m11</td>
<td>E205G</td>
<td>GAG→GGC</td>
<td>pALK2746</td>
</tr>
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<td>m12</td>
<td>Q216P</td>
<td>CAG→CCC</td>
<td>pALK2747</td>
</tr>
<tr>
<td>m13</td>
<td>M282V</td>
<td>ATG→GTC</td>
<td>pALK2748</td>
</tr>
<tr>
<td>m14</td>
<td>R17H, R169N (m1, m10)</td>
<td>CGC→CAC, CGT→AAC</td>
<td>pALK2749</td>
</tr>
<tr>
<td>m15</td>
<td>T46I</td>
<td>ACC→ATC</td>
<td>pALK2885</td>
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<tr>
<td>m16</td>
<td>A88S</td>
<td>GCC→AGC</td>
<td>pALK2886</td>
</tr>
<tr>
<td>m17</td>
<td>A173V</td>
<td>GCC→GTC</td>
<td>pALK2887</td>
</tr>
<tr>
<td>m18</td>
<td>V208I</td>
<td>GTC→ATC</td>
<td>pALK2888</td>
</tr>
<tr>
<td>m19</td>
<td>V239L</td>
<td>GTT→CTC</td>
<td>pALK2889</td>
</tr>
<tr>
<td>m20</td>
<td>A248V</td>
<td>GCT→GTC</td>
<td>pALK2890</td>
</tr>
<tr>
<td>m21</td>
<td>A281L</td>
<td>GCC→CTC</td>
<td>pALK2891</td>
</tr>
<tr>
<td>m22</td>
<td>m8, m15-m21 combined (altogether 8 mutations)</td>
<td>as in m8 and m15-m21</td>
<td>pALK2892</td>
</tr>
</tbody>
</table>
EXAMPLE 4. Production of the m-series mutant proteases ml - m23 in *Trichoderma reesei*

The expression cassettes were isolated from the vector backbones from agarose gel after *Not*I digestion and were used for transforming *T. reesei* protoplasts. The host strain used in the transformations did not produce any of the four major *T. reesei* cellulases (CBFfl, CBFflII, EGI, EGII). The transformations were performed as in Penttila *et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The transformants producing the highest protease activities were screened by streaking spores from the transformant colonies on haemoglobin test plates. The isolates showing strongest intensity of dark brown color around the growing mycelia were chosen for further cultivations. The haemoglobin plates contained (per 1 liter): 15 g KH₂PO₄, 5 g (NH₄)₂S0₄, 20 g lactose, 10 ml of trace element stock solution, 20 g haemoglobin (BD BBL Bovine, freeze-dried, REF212392), 10 ml Triton X-100 (10 % stock solution) and 10 g Bacto agar. The pH of the plates was adjusted to 7 by KOH prior to autoclaving. After autoclaving (121 °C, 15 min) and cooling of the media to about 50 °C, 2.4 ml 1M MgSO₄ x 7 H₂O and 5.4 ml 1M CaCl₂ x 2 H₂O (per litre) were added. The trace element stock solution contained (per liter): 0.5 g FeSO₄ x 7H₂O, 0.156 g MnSO₄ x H₂O, 0.14 g ZnSO₄ x 7H₂O and 0.49 g CoCl₂ x 6H₂O.

The chosen transformants from each transformation were purified on selection plates through single conidia and sporulated on PD slants prior to their cultivations in liquid medium.

From the PD slants the transformants were inoculated to shake flasks containing 50 ml of complex lactose-based cellulase inducing medium (Joutsjoki *et al.*, 1993) buffered with 5% KH₂PO₄ at pH 6.0. The protease production of the transformants was analyzed from the culture supernatants after growing them for 5 to 7 days at 30 °C, 250 rpm. The culture
supernatants were analysed by running samples on SDS-PAGE gels and by determining the protease activity against casein, as described in Example 1a. In SDS-PAGE gels, a major protein band of about 29 kDa corresponding to the mass of recombinant Fe_RF63 18 protease was detected from most of the spent culture supernatants. Also, most of the transformants produced clearly increased protease activity compared to host.

The integration of the expression cassettes into the fungal genomes was confirmed from the chosen transformants by using Southern blot analysis in which several genomic digests were included. A protease expression cassette was used as a probe in the analysis.

The properties of the mutant proteases were analysed from the culture supernatants as described in Example 5. The mutant proteases showing the best stability in the assays were produced in larger amounts by cultivating the corresponding T. reesei transformants in laboratory scale bioreactors. Cellulase inducing complex medium was used in the cultivations. The spent culture medium obtained from the fermentor cultivations was used for more detailed characterization of the mutant proteases, thermal stability tests (Example 5), stain removal performance (Example 12) and detergent stability tests (Example 13).

**EXAMPLE 5. Characterisation of the m1 - m23 series mutant proteases**

The thermal stability of protease mutants were determined as described in Example 1b using culture supernatants from shake flask cultivations as enzyme source (Example 4). The data obtained from the tests is shown on Table 3. The data indicated that the mutant proteases ml, m8, m18 and m21 showed better thermal stability than the wild type enzyme (wt). The multiply substituted protease variant m22 was clearly more stable than the wild type protease.

**Table 3. Stability of the mutant proteases.** Results are a summary from the thermal stability tests performed at 40 and 45 °C. The shake flask culture supernatants were used in the assays. The symbol "-" indicates that the stability of the mutant protease is lower compared to the wild type enzyme, those marked with "0" show similar stability as the wild
type enzyme, "+" show better stability and "++" clearly better stability than the wild type enzyme. ND, not determined.

<table>
<thead>
<tr>
<th>Mutant code (see Table 2)</th>
<th>Stability compared to wild type Fe_RF6318</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>-</td>
</tr>
<tr>
<td>m2</td>
<td>-</td>
</tr>
<tr>
<td>m3</td>
<td>-</td>
</tr>
<tr>
<td>m4</td>
<td>0</td>
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<tr>
<td>m5</td>
<td>ND</td>
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<td>m6</td>
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<td>m7</td>
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<td>m8</td>
<td>+</td>
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<td>m20</td>
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<td>m21</td>
<td>+</td>
</tr>
<tr>
<td>m22</td>
<td>++</td>
</tr>
<tr>
<td>m23</td>
<td>-</td>
</tr>
</tbody>
</table>
Transformants that according to data obtained from the shake flask culture supernatants produced mutant proteases with improved properties were cultivated in laboratory scale bioreactors (Example 4) to obtain more material for further analysis. The temperature stabilities of the mutant proteases were analysed from the fermentation culture supernatants as described in Example 1b. The temperature stability curves of m8, m18, m21 and m22 mutant proteases compared to that of the wild type protease are shown in Fig 6.

The data indicates that mutant proteases m18 and m21 have equal or better thermal stability than the wild type protease. The multiply substituted protease variant m22 is clearly more stable than the wild type protease. The thermal stability of m8 protease in fermentation sample was lower compared to wild type protease. This was contrary to previous results obtained from the analysis of shake flask samples.

**EXAMPLE 6. Combination of the chosen m-series mutations into one molecule, construction of mutant proteases m24 - m26**

According to the results obtained for the m1 - m23 mutant proteins (analysis of the shake flask culture supernatants, Table 3), m8, m18 and m21 including single mutations were among the ones that seemed to be the most stable in the assays (Example 5). Also, they showed good performance in the preliminary stain removal tests (not shown).

Thus, three new mutant genes m24, m25 and m26 were designed and constructed that included two (m24 and m25) or three (m26) of the m-series mutations combined into one molecule. The m24 mutant gene (cDNA) contained m8 and m18 mutations, m25 contained the m18 and m21 mutations and m26 contained the m8, m18 and m21 mutations (see Tables 2 and 4).

The m8 and m18 mutations were combined into one molecule (m24) by using the protease gene's internal Bsu361 site in the construction. The Bsu361 is located between the m8 and m18 mutation sites (position 979-985 in Fig. 1; see also Fig. 5 for the position of Bsu361 site) and is a single cutter in the GenScript pUC57 based plasmids including the synthetic protease
genes. The GenScript plasmid with m18 mutant cDNA (mutation at 3' side from the Bsu36I site) was cleaved with Bsu36I and EcoRI (at the linker). The DNA fragment with the pUC57 vector (from EcoRI site in the linker) and the end of the synthetic gene construction (from the Bsu36I site) was isolated from agarose gel and used as a vector in the ligation. The insert for the ligation was isolated from the GenScript plasmid including the m8 mutant cDNA by EcoRI - Bsu36I digestion. The insert contained the pUC57 linker from EcoRI and the beginning of synthetic gene to the Bsu36I site. As a result, a pUC57 based plasmid was obtained with the m24 mutant protease cDNA as an insert (with the partial cbhl promoter and terminator sequences at the 5'- and 3'-ends, respectively).

The m18 and m21 mutations were combined into one molecule (m25) by using the internal Xhol site in the construction. The Xhol is located between the m18 and m21 mutation sites (position 1114-1119 in Fig. 1; see also Fig. 5 for position of the Xhol site) and is, like Bsu36I, a single cutter for the GenScript pUC57 based plasmids with the synthetic genes. The GenScript plasmid with m21 mutant cDNA (mutation at 3' side from the Xhol site) was cleaved with Xhol and EcoRI (at the linker). The DNA fragment with the pUC57 vector (from EcoRI site in the linker) and the end of the synthetic gene construction (from the Xhol site) was isolated from agarose gel and used as a vector in the ligation. The EcoRI - Xhol insert for the ligation (pUC57 linker from EcoRI and beginning of the synthetic gene to the Xhol site) was isolated from the GenScript plasmid including the m18 mutation. The resulting pUC57 based plasmid contained the m25 mutant cDNA as an insert (with the partial cbhl promoter and terminator sequences at the 5'- and 3'-ends, respectively).

The m8, m18 and m21 mutations were combined into one molecule (m26) by combining the end of the m25 synthetic gene (including m18 and m21 mutations) and the beginning of the m8 gene with each other, by using the p4436I site in the construction as described above.

The sequences of the fusions and mutations in the cDNA sequences were confirmed by sequencing.
For construction of the mutant protease expression cassettes, the m24, m25 and m26 inserts were isolated from the pUC57 vectors from agarose gels after SacII and Agel digestion and ligated to pALK2777 (Fig. 4) cleaved with SacII and Agel (like m15 - m23 in Example 3).

Table 4. The mutant proteases m24 - m26. The mutant protease code, modifications made on the amino acid and nucleotide sequences (codons) compared to the wild type protease/gene and the codes for the expression plasmids are shown. The modified native amino acid, its position in the mature protease sequence (SEQ ID NO:2) and the amino acid replacing the native amino acid after mutation are shown.

<table>
<thead>
<tr>
<th>Mutant code</th>
<th>Modification (amino acid sequence)</th>
<th>Modification (cDNA sequence)</th>
<th>Expression cassette code</th>
</tr>
</thead>
<tbody>
<tr>
<td>m24</td>
<td>A111D, V208I (m8 and m18 combined)</td>
<td>GCT→GAC, GTC→ATC</td>
<td>pALK2897</td>
</tr>
<tr>
<td>m25</td>
<td>V208I, A281L (m18 and m21 combined)</td>
<td>GTC→ATC, GCC→CTC</td>
<td>pALK2898</td>
</tr>
<tr>
<td>m26</td>
<td>A111D, V208I, A281L (m8, m18 and m21 combined)</td>
<td>GCT→GAC, GTC→ATC, GCC→CTC</td>
<td>pALK2899</td>
</tr>
</tbody>
</table>

EXAMPLE 7. Production of the m-series mutant proteases in Trichoderma reesei

The NotI expression cassettes were isolated from the plasmids pALK2897 - pALK2899 and transformed into T. reesei as described in Example 4. The purification, screening and cultivations of the T. reesei transformants was performed as described in Example 4.

The culture supernatants obtained from the shake flask cultivations were analysed by running samples on SDS-PAGE gels and by determining the protease activity against casein, as described in Example 1a. In SDS-PAGE gels, a major protein band of about 29 kDa corresponding to the mass of recombinant Fe_RF6318 protease was detected from the spent culture supernatants. Also, the transformants produced clearly increased protease activities compared to host.
The integration of the expression cassette into the fungal genomes was confirmed from chosen transformants by using Southern blot analysis in which several genomic digests were included. A protease expression cassette was used as a probe in the analysis.

The transformants producing the best protease activities in the shake flask cultivations were cultivated in laboratory scale fermentors (as in Example 4). The properties of the mutant proteases were analysed from the spent culture supernatants as described in Example 1b, Example 12 and Example 13.

**EXAMPLE 8. Characterisation of the m24 - m26 mutant proteases**

The thermal stability of the m24, m25 and m26 protease mutants was determined as described in Example 1b using the culture supernatants from fermentor cultivations as an enzyme source (Example 7). The results obtained are shown in Fig. 7. The data obtained indicates that the stability of protease mutants m24 (combining the m8 and m18 mutations), m25 (combining the m18 and m21 mutations) and m26 (combining the m8, m18 and m21 mutations) had clearly better stability than the wild type protease. Also, these mutant proteases had better stability compared to the m8, m18 and m21 protease mutants having single mutations (Fig 6).

**EXAMPLE 9. Design of the D1 - D30 mutant proteases and construction of their expression cassettes**

New mutations were designed to further improve the stability of protease. The m26 protease was chosen as a background molecule as m26 had, in the previous tests (Example 8) shown better thermal stability compared to the wild type protease.

The mutant proteases designed and the modifications made to the m26 protease cDNA sequence are listed in Table 5. For the mutations already included in m26, see Table 4. The m26 nucleotide (cDNA) sequence encoding the mature protease amino acid sequence and
mature protein sequence are included as SEQ ID NO: 11 and SEQ ID NO: 12, respectively. The m26 cDNA sequence encoding the full-length m26 protease amino acid sequence is included in Fig. 8. The synthetic genes were ordered from GenScript as described in Example 3. The D-series mutant genes (with the 5′- and 3′-additional cbhl promoter and terminator sequences, see Fig. 2) were isolated from the pUC57 backbone by SacII - Agel digestion as described in Example 3. The inserts were ligated to pALK2777, digested with SacII and Agel as described in Example 3.

Table 5. The mutant proteases D1 - D30. The mutant protease code, modifications made on the amino acid and nucleotide sequences (codons) of the m26 mutant protease and the codes for the expression plasmids are shown. The modified native amino acid, its position in the mature m26 protease sequence (SEQ ID NO: 12) and the amino acid replacing the native amino acid after mutation are shown. The cDNA encoding the mature m26 amino acid sequence and mature m26 amino acid sequences are included as SEQ ID NO: 11 and SEQ ID NO: 12, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Modification (amino acid sequence)</th>
<th>Modification (cDNA sequence)</th>
<th>Expression cassette code</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>N7R</td>
<td>AAC→CGC</td>
<td>pALK3051</td>
</tr>
<tr>
<td>D2</td>
<td>A33E</td>
<td>GCC→GAG</td>
<td>pALK3052</td>
</tr>
<tr>
<td>D3</td>
<td>A47E</td>
<td>GCC→GAG</td>
<td>pALK3053</td>
</tr>
<tr>
<td>D4</td>
<td>S56R</td>
<td>TCT→CGC</td>
<td>pALK3054</td>
</tr>
<tr>
<td>D5</td>
<td>A61P</td>
<td>GCT→CCC</td>
<td>pALK3055</td>
</tr>
<tr>
<td>D6</td>
<td>G63P</td>
<td>GGT→CCC</td>
<td>pALK3056</td>
</tr>
<tr>
<td>D7</td>
<td>V76A</td>
<td>GTT→GCC</td>
<td>pALK3057</td>
</tr>
<tr>
<td>D8</td>
<td>N83D</td>
<td>AAC→GAC</td>
<td>pALK3058</td>
</tr>
<tr>
<td>D9</td>
<td>N114R</td>
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<td>pALK3061</td>
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<td>D12</td>
<td>G164A</td>
<td>GGT→GCC</td>
<td>pALK3062</td>
</tr>
<tr>
<td>D13</td>
<td>I185M</td>
<td>ATC→ATG</td>
<td>pALK3063</td>
</tr>
<tr>
<td>D14</td>
<td>T196R</td>
<td>ACT→CGC</td>
<td>pALK3064</td>
</tr>
<tr>
<td>D15</td>
<td>V206L</td>
<td>GTC→CTC</td>
<td>pALK3065</td>
</tr>
<tr>
<td>D16</td>
<td>M234S</td>
<td>ATG→AGC</td>
<td>pALK3066</td>
</tr>
<tr>
<td>D17</td>
<td>Q247L</td>
<td>CAG→CTC</td>
<td>pALK3067</td>
</tr>
<tr>
<td>D18</td>
<td>N260R</td>
<td>AAC→CGC</td>
<td>pALK3068</td>
</tr>
<tr>
<td>D19</td>
<td>T268R</td>
<td>ACC→CGA</td>
<td>pALK3069</td>
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</tbody>
</table>
EXAMPLE 10. Production of the mutant proteases in *Trichoderma reesei* (D-series of mutants)

The *NotI* expression cassettes were isolated from the plasmids and transformed into *T. reesei* as described in Example 4. The screening and cultivation of the *T. reesei* transformants (in shake flasks and in fermentors) was performed as described in Example 4.

The properties of the mutant proteases were analysed from the culture supernatants as described in Example 5. The mutant proteases showing the best thermal stability in these
assays and good performance in the preliminary stain removal tests (not shown) were produced in larger amounts by cultivating the corresponding *T. reesei* transformants in laboratory scale bioreactors as described in Example 4. The spent culture medium obtained from the fermentor cultivations was used for more detailed characterization of the mutant proteases, thermal stability tests (Example 11), stain removal performance (Example 14) and detergent stability tests (Example 15).

**EXAMPLE 11. Characterisation of the D-series mutant proteases**

The thermal stability of protease mutants was determined as described in Example 1b using culture supernatants from shake flask and fermentor cultivations as enzyme sources (Example 10).

The thermal stability of D mutants was compared to m26, which is the background molecule for the D series of mutants. A summary of the results obtained from the shake flask culture supernatants is shown in Table 6. The data indicates that the thermal stability of mutant proteases D2, D3, D13, D19, D21, D22 and D23 was better than that of m26 protease.

**Table 6. Stability of the D1 - D30 mutant proteases.** Results are a summary from the thermal stability tests performed at 45 °C. The shake flask culture supernatants were used in the assays. The symbol "-" indicates that the stability of the mutant protease is lower compared to the wild type recombinant enzyme, those marked with "0" show similar stability as the wild type enzyme, and "+" show better stability than the wild type enzyme. The mutants with "++" has similar stability as m26 mutant protease and those with "+++" symbol show better stability than m26. ND, not determined.

<table>
<thead>
<tr>
<th>Mutant code (see Table 2)</th>
<th>Stability compared to Fe RF6318 and m26</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>+</td>
</tr>
<tr>
<td>D2</td>
<td>+++</td>
</tr>
<tr>
<td>D3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
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<td>---</td>
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</tr>
<tr>
<td>D4</td>
<td>+</td>
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</tr>
<tr>
<td>D30</td>
<td>0</td>
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</tbody>
</table>

The thermal stabilities of mutant proteases were determined in more detail from the fermentation culture supernatants. Transformants producing the mutant protease D21 were
not cultivated in bioreactors because the specific activity of D21 was decreased compared to
the wild type enzyme. The results of a selection of mutant proteases are shown in Fig. 9.

The results obtained indicate that mutant proteases D2, D3, D19, D22 and D23 had better
thermal stability than mutant protease m26. Mutant proteases D5, D8 and D13 had
approximately equal thermal stability as m26 mutant protease. Mutant proteases D7, D16 and
D17 had lower thermal stability than m26 mutant protease but the stability was better than the
stability of wt protease.

EXAMPLE 12. Stain removal performance of mutants of m-series with liquid
detergents

Mutant proteases of m-series showing the best thermal stability (Examples 5 and 8) and wild
type protease produced in Trichoderma, as described in Examples 4 and 7, were chosen for
application tests. Fermentation culture supernatants were first stabilized with 50 % propylen
glycol and were then tested for their ability to remove blood/milk/ink standard stain at 30°C
and 45°C in the presence of Liquid Base detergent for colored fabrics (Table 7), at
concentration of 5 g/l. Standard stain, artificially soiled test cloth Art. 117 (blood/milk/ink,
polyester + cotton, EMPA Testmaterialen AG, Switzerland) was used as test material. Each
enzyme preparation was dosed 0, 0.2, 0.4, 0.8, 1.6, 4, and 8 activity units (µmol tyrosine/min)
per ml wash liquor. Activity was measured as described in Example 1a.

An amount of 5 g of Liquid base detergent was dissolved in 1 liter of tap water (dH ≤ 4),
mixed well with magnetic stirrer and tempered to 30°C and 45°. The pH in the wash liquor
was approx. 7.4. The stain fabric was first cut in to 1.5 cm x 1.5 cm swatches and the pieces
were made rounder by cutting the corners. Pieces were placed in wells of microtiter plates
(Nunc 150200). Into each well having a diameter of 2 cm, 1.5 ml wash liquor containing
detergent and enzyme dilution in water (below 60 µl) was added on top of the fabric. The
plates with samples were in incubated in Infors Ecotron incubator shaker at 30°C and 45°C
for 60 min with 130 rpm. After that the swatches were carefully rinsed under running water
(appr. at washing temperature) and dried overnight at indoor air, on a grid, protected against daylight.

Table 7. Composition of Liquid Base detergent for colored fabric.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaLES (sodium lauryl ether sulphate)</td>
<td>4.9</td>
</tr>
<tr>
<td>Nonionic C12-15 7EO (ethylene oxide)</td>
<td>15</td>
</tr>
<tr>
<td>Na-Soap (Palm Kernel FA)</td>
<td>4.4</td>
</tr>
<tr>
<td>Coco Glucoside</td>
<td>1</td>
</tr>
<tr>
<td>&lt;Total Surfactant&gt;</td>
<td>&lt;25,30&gt;</td>
</tr>
<tr>
<td>Polyoil (Glycerin)</td>
<td>5</td>
</tr>
<tr>
<td>Phosphonate (32%) (ThermPhos)</td>
<td>2</td>
</tr>
<tr>
<td>PVP-Sokalan HP 53 (BASF)</td>
<td>1</td>
</tr>
<tr>
<td>Sokalan PA 15 (BASF)</td>
<td>1.56</td>
</tr>
<tr>
<td>Sorez -100 (ISP)</td>
<td>0.4</td>
</tr>
<tr>
<td>Water upto 100 %</td>
<td></td>
</tr>
</tbody>
</table>

The stain removal effect was evaluated by measuring the colour as reflectance values with Minolta CM 2500 spectrophotometer using L*a*b* colour space coordinates (illuminant D65/2°). The colour from both sides of the swatches was measured after the treatment. Each value was the average of at least 2 parallel fabric samples measured from both side of the fabric. Fading of blood/milk/ink stain, indicating the protease performance (stain removal efficiency), was calculated as AL*, which means lightness value L* of enzyme treated fabric minus lightness value L* of fabric treated with washing liquor without enzyme (enzyme blank, control).

The results obtained with mutants m8, m18, m21, m22, m24, m25 and m26 are shown in Figures 10A and 10B. The mutations have not affected harmfully on the stain removal performance.
Some mutants of m-series were tested at 30°C also with another liquid detergent Eco label Reference Detergent, light duty (Ch. Nr. 196-391, wfk Testgewebe GmbH) and detergent powders, like ECE reference detergent 77 without optical brightener (Art. 601, EMPA) and ECE reference detergent 98 without phosphate (Art. 600, EMPA) at concentrations 3.3 g/l and/or 5 g/l. The tested mutant protease preparations showed similar performance compared to wild type protease preparation.

**EXAMPLE 13. Stability of mutants of m-series in liquid detergents**

Stability of mutants m8, m18, m21, m22, m24, m25 and m26 at 37°C was tested in liquid detergents mentioned in Example 12, Liquid Base detergent for colored fabric and Ecolabel Reference detergent, and in addition to these Commercial liquid detergent (both without enzymes) described in Table 8.

**Table 8. Composition of Commercial liquid detergent.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic surfactants</td>
<td>15-30</td>
</tr>
<tr>
<td>Nonionic surfactants, soap</td>
<td>5-15</td>
</tr>
<tr>
<td>Phosphonate, polycarboxylate</td>
<td>5</td>
</tr>
<tr>
<td>Optical brighteners and perfumes</td>
<td></td>
</tr>
<tr>
<td>pH 8.2 -8.6</td>
<td></td>
</tr>
</tbody>
</table>

Two types of test systems were used:

A) 0.4 g enzyme preparation (containing 50 % propylene glycol) and 9.6 g of detergent solution were mixed well in Sarstedt's test tubes (13 ml).

B) 0.4 g enzyme preparation (containing 50 % propylene glycol) and 2.5 g of solution containing 8 % sodium tetraborate decahydrate and 8 mM CaCl₂ (pH adjusted to 6 with HC1) in distilled water, 1.5 g propylene glycol and 5.6 g of detergent solution
were mixed well in Sarsted's test tubes (13 ml). This solution contains 17% of propylene glycol and 2% sodium tetraborate decahydrate as stabilizing agents.

In both cases 0.2% of Proxel LV was added as preservative in detergent before mixing it with other components. The pH values of the samples prepared in Liquid Base detergent using recipe A, were approx. 8.2, and using recipe B approx. 7. pHs of the samples prepared in Ecolabel Reference Detergent were approx. 7.2 and 6.5 and in Commercial liquid detergent approx. 8.2 and 7.5, respectively. Test tubes were incubated at 37°C and the protease activity was measured at certain intervals according to the method described in Example 1a.

Results of detergent stability tests are shown in Fig. 11 - 13. In Liquid Base detergent (Fig. 11) the stability of mutants, except m8 and m21 was similar or slightly improved compared to the wild type protease. The stability of m-series mutants, except m8 and m21, was considerably improved in Ecolabel (Fig. 12) and Commercial liquid detergents (Fig. 13). The results show that mutant proteases were constructed that have improved stability compared and also have similar or even better stain removal performance compared to the wild type protease.


Based on thermal stability tests (Example 11) and preliminary stain removal tests at 30°C made with shake flask supernatants of mutant proteases of D-series produced in *Trichoderma*, as described in Examples 10, the transformants producing the best mutant protease candidates were cultivated in laboratory scale bioreactors. Fermentation culture supernatants containing the mutant proteases were stabilized with 50% propylen glycol and were then tested for their ability to remove blood/milk/ink standard stain at 30°C and 50°C in the presence of liquid detergent at concentration of 5 g/l. Wild type and mutant m26 used as background molecule for D-series mutants, were used for comparison. The testing method and dosing was similar to Example 12, except that the Commercial liquid detergent, described in Example 13, was used. Also the colour of the swatches after treatment was measured with Minolta CM 2500.
spectrophotometer using L*a*b* colour space coordinates and stain removal effect calculated as AL* as described in Example 12.

The results are shown in Figures 14 and 15. In stain removal tests at 30°C (Figs 14A, 14B and 15A) all mutant proteases, except D19, had better performance than wild type and similar or better performance compared to m26. D19 had similar performance compared to wild type. At 50°C (Figs. 14C, 14D and 15B) only mutant D7 had weaker performance than wild type. Mutants D17 and D19 were slightly better than the wild type and mutant proteases D2, D3, D5, D6, D8, D13, D16, D22 and D23 had relatively similar stain removal performance compared to m26.

EXAMPLE 15. Stability of mutants of D-series in liquid detergents

Stability of D-series mutant proteases in fermentation culture supernatants (containing 50 % propylene glycol) was tested at 37°C for 20 hours in Commercial liquid detergent and using similar test systems as described Example 13. Mutant m26 used as background molecule for mutations in D-series and having improved stability and stain removal effect compared to wild type was used as reference. The stability of the best performing mutants from Example 14 and most stable mutants in Commercial liquid detergent were tested also with Ecolabel Reference Detergent, light duty (wfk Testgewebe GmbH)

Results of detergent stability tests in Commercial liquid detergent are shown in Figures 16 - 17. Stability of mutants D2, D3, D6, D19 and D22 was better compared to m26. The mutants D5, D8, D13 and D23 had similar stability as m26 in Commercial liquid detergent containing no stabilizers (Figs 16A and B). The stability of mutants D16, D17 and especially D7 was lower compared to m26 (Fig. 16A). However, D16 and D17 were more stable than the wild type (Fig. 12B) in the previous test.

In detergent test system which contains sodium tetraborate decahydrate and propylene glycol, the stability of mutants D2, D3, D6, D13, D22 and especially D19, was better compared to m26 (Fig. 17). The stability of mutants D8, D16, D17, D23 and especially D7 was lower
compared to m26. However all the mutants having lower stability compared to m26, except D7, were more stable than the wild type (Fig. 13B) in previous tests. All the mutant proteases tested showed considerably improved stability in Ecolabel Reference detergent compared to wild type (Fig 18). Mutant proteases D2, D3, D6 and especially D19 had also better stability than m26.

The results show that mutant proteases were constructed that have improved stability compared and also have similar or even better stain removal performance compared to the wild type protease.

**EXAMPLE 16. Design of D31 - D68 mutant proteases, construction of their expression cassettes and production of mutant proteases in T. reesei**

Additional mutations were designed to improve the stability of protease. The m26 protease was chosen as a background molecule, as in Example 9.

The mutant proteases designed and the modifications made to the m26 protease cDNA sequence are listed in Table 9. For the mutations already included in m26, see Table 4. The full-length m26 nucleotide (cDNA) and protein sequences are included as SEQ ID NO:1 1 and SEQ ID NO: 12, respectively. The cDNA sequence encoding the full-length m26 protease is shown in Fig. 8. The synthetic genes were ordered from GenScript as described in Example 3. The D-series mutant genes (with the 5′- and 3′-additional cbhl promoter and terminator sequences, see Fig. 2) were isolated from the pUC57 backbone by SacII - AgeI digestion as described in Example 3. The inserts were ligated to pALK2777, digested with SacII and AgeI as described in Example 3.

**Table 9. The mutant proteases D31 - D68.** The mutant protease code, modifications made on the amino acid and nucleotide sequences (codons) of the m26 mutant protease and the codes for the expression plasmids are shown. The modified native amino acid, its position in the mature m26 protease sequence (SEQ ID NO: 12) and the amino acid replacing the native amino acid after mutation are shown. The cDNA encoding the mature m26 amino acid
sequence and m26 mature amino acid sequences are included as SEQ ID NO: 11 and SEQ ID NO: 12, respectively.

<table>
<thead>
<tr>
<th>Modification (amino acid sequence)</th>
<th>Modification (cDNA sequence)</th>
<th>Expression cassette code</th>
</tr>
</thead>
<tbody>
<tr>
<td>D31 A8V</td>
<td>GCT→GTC</td>
<td>pALK3101</td>
</tr>
<tr>
<td>D32 G34S</td>
<td>GGT→AGC</td>
<td>pALK3102</td>
</tr>
<tr>
<td>D33 G34N</td>
<td>GGT→AAC</td>
<td>pALK3103</td>
</tr>
<tr>
<td>D34 Y36D</td>
<td>TAC→GAC</td>
<td>pALK3104</td>
</tr>
<tr>
<td>D35 G52E</td>
<td>GGC→GAG</td>
<td>pALK3105</td>
</tr>
<tr>
<td>D36 A91T</td>
<td>GCC→ACC</td>
<td>pALK3106</td>
</tr>
<tr>
<td>D37 V100T</td>
<td>GTC→ACG</td>
<td>pALK3107</td>
</tr>
<tr>
<td>D38 V100D</td>
<td>GTC→GAC</td>
<td>pALK3108</td>
</tr>
<tr>
<td>D39 V100K</td>
<td>GTC→AAG</td>
<td>pALK3109</td>
</tr>
<tr>
<td>D40 T106A</td>
<td>ACC→GCC</td>
<td>pALK3110</td>
</tr>
<tr>
<td>D41 T106N</td>
<td>ACC→AAC</td>
<td>pALK3111</td>
</tr>
<tr>
<td>D42 P138D</td>
<td>CCC→GAC</td>
<td>pALK3112</td>
</tr>
<tr>
<td>D43 P138K</td>
<td>CCC→AAG</td>
<td>pALK3113</td>
</tr>
<tr>
<td>D44 A144N</td>
<td>GCT→AAC</td>
<td>pALK3114</td>
</tr>
<tr>
<td>D45 A144T</td>
<td>GCT→ACC</td>
<td>pALK3115</td>
</tr>
<tr>
<td>D46 E205N</td>
<td>GAG→AAC</td>
<td>pALK3116</td>
</tr>
<tr>
<td>D47 E205R</td>
<td>GAG→CGC</td>
<td>pALK3117</td>
</tr>
<tr>
<td>D48 L249T</td>
<td>CTC→ACC</td>
<td>pALK3118</td>
</tr>
<tr>
<td>D49 L252T</td>
<td>CTC→ACC</td>
<td>pALK3119</td>
</tr>
<tr>
<td>D50 L252A</td>
<td>CTC→GCC</td>
<td>pALK3120</td>
</tr>
<tr>
<td>D51 A256S</td>
<td>GCT→AGC</td>
<td>pALK3121</td>
</tr>
<tr>
<td>D52 Q247L, L249T</td>
<td>CAG→CTC; CTC→ACC</td>
<td>pALK3122</td>
</tr>
<tr>
<td>D53 T3C, T29C, S6R, T24D</td>
<td>ACC→TGC; ACC→TGC; ACC→GCC; ACC→GAC</td>
<td>pALK3123</td>
</tr>
<tr>
<td>D54 I185C, T259C</td>
<td>ATC→TGC; ACC→TGC</td>
<td>pALK3124</td>
</tr>
<tr>
<td>D55 G210A, I185M</td>
<td>GGT→GCC; ATC→ATG</td>
<td>pALK3125</td>
</tr>
<tr>
<td>D56 A33E, A47E, N83D</td>
<td>GCC→GAG; GCC→GAG; AAC→GAC</td>
<td>pALK3126</td>
</tr>
<tr>
<td>D57 A14T</td>
<td>GCC→ACC</td>
<td>pALK3127</td>
</tr>
<tr>
<td>D58 G22S</td>
<td>GGC→TCC</td>
<td>pALK3128</td>
</tr>
<tr>
<td>D59 G37A</td>
<td>GGT→GCC</td>
<td>pALK3129</td>
</tr>
<tr>
<td>D60 A65D</td>
<td>GCC→GAC</td>
<td>pALK3130</td>
</tr>
<tr>
<td>D61 V100Q</td>
<td>GTC→CAG</td>
<td>pALK3131</td>
</tr>
</tbody>
</table>
The NotI expression cassettes were isolated from the plasmids and transformed into *T. reesei* as described in Example 4. The screening and cultivation of the *T. reesei* transformants (in shake flasks and in fermentors) was performed as described in Example 4.

The properties of the mutant proteases were analysed from the culture supernatants as described in Example 5. The mutant proteases showing the best thermal stability in these assays and good performance in the preliminary stain removal tests (not shown) were produced in larger amounts by cultivating the corresponding *T. reesei* transformants in laboratory scale bioreactors as described in Example 4. The spent culture medium obtained from the shake flask and fermentor cultivations was used for characterization of the mutant proteases, thermal stability tests, stain removal performance and detergent stability tests (Example 17).

**EXAMPLE 17. Stain removal performance and stability of mutants of DH-series (D32 - D68) in liquid detergents**

Selected DH-series mutant proteases (shake flask and fermentation cultivation supernatants), produced in *Trichoderma* as described in Example 16, were tested for their thermostability, wash performance and detergent stability. The results obtained with the mutant proteases were compared to those obtained with the wild type protease and mutant protease m26.

The thermal stability of the mutant proteases at 45 °C in pH 8.5 buffer was tested as described in Example 1 (b). Especially, D38, D41, D53 and D55 show clearly better thermal stability than m26.
The ability of proteases to remove blood/milk/ink standard stain in the presence of Commercial liquid detergent was tested at 30 °C using similar test system as described in Examples 12 and 14. The wash performance of all the mutant proteases (Commercial detergent, 30°C, 60 min), except D39, was similar or better compared to m26 protease. Especially good result was obtained with D35, D37, D38 and D44.

Fermentation culture supernatants were tested for their stability in Commercial liquid detergent and Ecolabel Reference detergent at 37 °C using test system A as described in Example 13, except that the incubation time in Ecolabel tests was 15.5 hours at 37°C. All the mutant proteases showed better stability than the wild type protease in Commercial liquid detergent (not shown) and in Ecolabel Reference Detergent. D39, D53, D55, D61, D63, D64, D65, D66 and D67 showed especially good stability in Ecolabel Reference Detergent compared to m26. The summary of results of thermostability and stability in Ecolabel Reference Detergent are shown in Table 10.

**Table 10. Summary of the stability tests of selected DII series mutant proteases.** Those marked with "+" show better stability than the wild type enzyme. The mutants with "++" have similar stability as the m26 mutant protease and those with "+++" symbol show better stability than m26. ND, not determined.

<table>
<thead>
<tr>
<th>Mutant protease</th>
<th>Thermal stability</th>
<th>Stability in Ecolabel (residual activity at 37°C, 15.5 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D32</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>D33</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>D35</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>D36</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>D37</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>D38</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>D39</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>D41</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>D42</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D44</td>
<td>++</td>
<td>ND</td>
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<tr>
<td>D51</td>
<td>++</td>
<td>ND</td>
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<tr>
<td>D53</td>
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<td>+++</td>
</tr>
<tr>
<td>D67</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>
REFERENCES


CLAIRS

1. A serine protease variant of the *Fusarium equiseti* Fe_RF6318 serine protease, characterized in that the variant has serine protease activity and comprises an amino acid sequence having a substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid other than valine, wherein the position of the substitution corresponds to the amino acid sequence of the mature Fe_RF6318 enzyme as defined in SEQ ID NO:2.

2. The serine protease variant according to claim 1, characterized in that the variant has improved thermal stability and/or stability in the presence of detergents compared to the parent mature Fe_RF6318 serine protease having the amino acid sequence of SEQ ID NO:2.

3. The serine protease variant according to claims 1 and 2, characterized in that the substitution is valine at position 208 with isoleucine (V208I), leucine (V208L) or methionine (V208M).

4. The serine protease variant according to any of claims 1 to 3, characterized in that the substitution is V208I.

5. The serine protease variant according to any of claims 1 to 4, characterized in that the variant further comprises one or more amino acid changes, which are selected from the group consisting of substitution, insertion or deletion.

6. The serine protease variant according to any of claims 1 to 5, characterized in that the variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284,
286, 287 or 288 of the parent Fe_RF6318 serine protease, a deletion of asparagine at position 167 of the parent Fe_RF6318 serine protease, a deletion of alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

7. The serine protease variant according to any of claims 1 to 6, characterized in that the variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 14, 24, 29, 33, 34, 47, 52, 61, 63, 65, 83, 91, 100, 103, 106, 111, 121, 144, 153, 157, 158, 164, 175, 176, 185, 210, 234, 236, 256, 268 or 281, and an amino acid insertion at position 104, said substitution or insertion resulting in increase in thermostability.

8. The serine protease variant according to any of claims 1 to 7, characterized in that the variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from a group consisting of an amino acid substitution at position 3, 6, 24, 29, 33, 47, 100, 103, 106, 111, 185, 210, 268 or 281, and an amino acid insertion at position 104, said substitution or insertion resulting in increase in thermostability.

10. The serine protease variant according to any of claims 1 to 6, characterized in that the variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 37, 47, 61, 63, 65, 83, 100, 111, 123, 157, 175, 176, 185, 210, 234, 236, 247, 268 or 281 of the parent Fe_RF6318 serine protease, said substitution resulting in increased stability in detergents.

11. The serine protease variant according to any of claims 1 to 6 and claim 10, characterized in that the variant comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid substitutions at position 3, 6, 24, 29, 33, 47, 63, 100, 111, 123, 157, 175, 176, 185, 210, 236, 268 or 281, said substitution resulting in increased stability in detergents.


13. The serine protease variant according to any of claims 1 to 12, characterized in that the variant is encoded by an isolated polynucleotide sequence, which encodes a polypeptide comprising the amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28,
14. An isolated nucleic acid molecule comprising a nucleotide sequence which encodes the Fe_RF6318 variant according to any of claims 1 to 13, characterized in that the Fe_RF6318 variant has serine protease activity and comprises an amino acid sequence having substitution of valine at position 208 of the parent Fe_RF6318 serine protease with an amino acid other than valine, wherein the amino acid position corresponds to the amino acid position of the mature Fe_RF6318 serine protease defined in SEQ ID NO:2.

15. The isolated nucleic acid molecule according to claim 14, characterized in that said nucleic acid molecule comprises a nucleotide sequence encoding a Fe_RF6318 variant which comprises, in addition to the substitution of valine at position 208 of the parent Fe_RF6318 serine protease, one or more amino acid changes selected from the group consisting of an amino acid substitution at position 3, 6, 7, 8, 14, 17, 18, 22, 24, 25, 28, 29, 33, 34, 36, 37, 46, 47, 52, 56, 61, 63, 65, 69, 76, 77, 83, 88, 91, 100, 103, 106, 111, 113, 114, 121, 123, 138, 144, 151, 153, 155, 157, 158, 164, 167, 169, 173, 174, 175, 176, 185, 196, 205, 206, 210, 214, 216, 230, 234, 236, 239, 247, 248, 249, 252, 256, 260, 268, 281, 282, 283, 284, 286, 287 or 288 of the parent Fe_RF6318 serine protease, a deletion of asparagine at position 167 of the parent Fe_RF6318 serine protease, a deletion of alanine at position 65 and histidine at position 66 of the parent Fe_RF6318 serine protease, and an amino acid insertion at position 104 of the parent Fe_RF6318 serine protease, wherein the amino acid positions correspond to the amino acid sequence of the mature Fe_RF6318 serine protease of SEQ ID NO:2.

16. The isolated nucleic acid molecule according to claims 14 and 15, characterized in that the nucleic acid molecule comprises a nucleotide sequence defined in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49.
17. A recombinant expression vector comprising a nucleotide sequence encoding the Fe-
RF6318 variant according to any of claims 1 to 13 operably linked to regulatory sequences
capable of directing expression of the gene encoding said Fe_RF63 18 serine protease variant
in a suitable host.

18. A host cell comprising the recombinant expression vector according to claim 17.

19. The host cell according to claim 18, characterized in that said host is a microbial host.

20. The host cell according to claims 18 and 19, characterized in that said host is a
filamentous fungus.

21. The host cell according to any of claims 18 to 20, characterized in that said host is of a
genus Trichoderma, Aspergillus, Fusarium, Humicola, Chrysosporium, Neurospora,
Rhizopus, Penicillium and Mortiriella.

22. The host cell according to claim 21, characterized in that said host is Trichoderma or
Aspergillus.

23. The host cell according to claim 22, characterized in that said host is T. reesei.

24. A process of producing a Fe_RF6318 serine protease variant according to any of claims 1
to 13, said process comprising the steps of culturing the host cell according to any of claims
18 to 23 and recovering the polypeptide.

25. A process for obtaining an enzyme preparation, which comprises the Fe_RF6318 serine
protease variant according to any of claims 1 to 13, said process comprising the steps of
culturing a host cell according to any of claims 18 to 23 and either recovering the polypeptide
from the cells or separating the cells from the culture medium and obtaining the supernatant.
26. An enzyme preparation comprising the serine protease variant according to any of claims 1 to 13.

27. The enzyme preparation according to claim 26, **characterized** in that said preparation comprises other enzymes selected from the group of protease, amylase, cellulase, lipase, xylanase, mannanase, cutinase, pectinase or oxidase with or without a mediator.

28. The enzyme preparation according to claims 26 and 27, **characterized** in that said preparation comprises a suitable additive selected from the group of stabilizers, buffers, surfactants, builders, bleaching agents, mediators, anti-corrosion agents, antiredeposition agents, caustics, abrasives, optical brighteners, dyes, pigments, and preservatives.

29. The enzyme preparation according to any one of claims 26 to 28, **characterized** in that said enzyme preparation is in the form of liquid, powder or granulate.

30. The Fe_RF6318 serine protease variant according to any of claims 1 to 13 or the enzyme preparation according to any of claims 26 to 29 for use in detergents, in treating fibers, in treating wool, in treating hair, in treating leather, in treating food or feed, or in any application involving modification, degradation or removal of proteinaceous material.

31. The Fe_RF6318 serine protease variant according to any of claims 1 to 13 or the enzyme preparation according to any of claims 26 to 29 for use as a detergent additive.

32. The Fe_RF6318 serine protease variant according to any of claims 1 to 13 or the enzyme preparation according to any of claims 26 to 29 for use in detergent liquids.

33. The Fe_RF6318 serine protease variant according to any of claims 1 to 13 or the enzyme preparation according to any of claims 26 to 29 for use in detergent powders.

34. A detergent composition comprising the serine protease variant of any of claims 1 to 13 or the enzyme preparation according to any of claims 26 to 29.
FIG. 1B

961 TCTTACCCA ACTACGGTCC TGAGGTCGAT GTCTTCGGTC CTGGTGTAAC CATCCAGTCC
321 S F T N Y G P E V D V F G P G V N I Q S

1021 ACCTGGTACA CCTCAACAG CGCTACCAAAC ACCATCAGCG GTACCTCCAT GGCTTGCCCT
341 T W Y T S N S A T N T I S G T S M A C P

1081 CACGTTGCTG GTCTTGCTCT CTACCTCCAG GCTCTCGAGA ACCCTATAC CCCTGCTGCC
361 H V A G L A L Y L Q A L E N L N T P A A

1141 GTCACCAACC GCATCAAGTC TCTTGCCACT ACCGGCGCCA TCACTGCGAG CCTCAGCGGC
381 V T N R I K S L A T T G I T G S L S G

1201 AGCCCCAACC CCATGGCTTT CAACGCGCGT ACTGCTTAA
401 S P N A M A F N G A T A *
FIG. 2A

SacII

1  cggcggactg gcacatcatga ctagcttcgg cggatatcgtc ctggcctttg cagctctgct

>>...\pcbh1...>>

>>..................Pe_Prt8A_cDNA.......................>

61  gcgcgcagtc ctgcgccgctc ccacccgagaa gcgcagagag ctcactgccg gcgcctgacaa

>.........................Pe_Prt8A_cDNA........................>

121  gtacatcatac acccctcaacgc cggagctgctg tgagcggcttg gcacgagctgc acatggcctg

>..........................Pe_Prt8A_cDNA........................>

181  ggttacgagg cgcagctccgg gcagccggttc gcagctgctgg tggagagagag

>..........................Pe_Prt8A_cDNA........................>

241  gttcaacatcg agcgagctgaga cgcctactc tggcgggtttc gacgactgta cccattgcga

>..........................Pe_Prt8A_cDNA........................>

301  gatcagagag agccccggagg tgtcctctcttg gcagcggagg tacattgctca cccctgacta

>..........................Pe_Prt8A_cDNA........................>

361  caaggttgag cctctctctctg acgcgtctctg gaccactcag agcaacgctc cttggggtct

>..........................Pe_Prt8A_cDNA........................>

421  tgctgccactc tcgccggcagg cccccgggtgg gcacacactc acctctgcac ccacggccgg

>..........................Pe_Prt8A_cDNA........................>

481  tgccggctactc taggtgtcttc tggtatcaac cccgcggcaac ctcagtttgg

>..........................Pe_Prt8A_cDNA........................>

541  cgggccggtctc ttctctggttt acaacagctgc tggcgccgcc cacactgata cccctggcga

>..........................Pe_Prt8A_cDNA........................>

601  cgggtacccaag tgcgttggta cccatgtcttc ccaacccttc ggtgttgcca agcggtccaa

>..........................Pe_Prt8A_cDNA........................>

661  cgctcagctct tctcaaggtttcgctcgttacttt ccaagctctctgccctacttg

>..........................Pe_Prt8A_cDNA........................>

721  tttacactgggtcgtcaacag acatcaacctc caagacacgtg tgcagccgct cagtcatcaa

>..........................Pe_Prt8A_cDNA........................>

781  catgtctactc ctgggtccctc tctctcagac gctgggtactg gccatcaacag cgtctacaag

>..........................Pe_Prt8A_cDNA........................>

841  ccaaggtgatc cttccgggttg tgcgtccggcg taacggtgat tccacgggtc gtcctctccc

>..........................Pe_Prt8A_cDNA........................>
FIG. 2B

cgcctctggc cagtcacctg ccaacgttcc caacgtatct acggttgctg cgcggcgactc
>..........................Fe_Prt8A_cDNA..............................>

cagctggcga actgcctcttt tcaccaacta cggctctgag gtgcgtgtct tcgggtctggt
>..........................Fe_Prt8A_cDNA..............................>

tgtaacacatc cagtccacct ggtacacctc caacagcgtc acacaacacca tcacggtac
>..........................Fe_Prt8A_cDNA..............................>

cgccatggct tcgcct cacg ttgcgtct tgcgtactac ctccaggtctc tcgagaacct
>..........................Fe_Prt8A_cDNA..............................>

caacccctg gctgccgtca ccaacgcat caagtctttt gccactaccg gccgcatac
>..........................Fe_Prt8A_cDNA..............................>

tggcagccctc aggccgagcc ccaacgccct ggctttcaac ggccgtacty cttaagctc
>..........................Fe_Prt8A_cDNA..............................>

AgeI

tcbhl >>..>

cgtggcgaaa gcctgacgca cgcgt
>............tcbhl'............>>
FIG. 5B

pALK2888
9686 bps
FIG. 6

![Graph showing residual activity over time for different samples.]

- Wild type
- m18
- m8
- m21
- m22

Residual activity (%) vs. Minutes
FIG. 7

![Graph showing residual activity over minutes for wild type, m24, m25, and m26 samples.]
FIG. 8A

1  atgactagct tccgccgtat cgctcttgcc ctggcagctc tgctgccccg agtctctgcc
2  mts frr rial glaal all pav la
61  gctcccaccg agaagcgaca ggagctcact gcgccgccttg acaagtacat catcaccctc
21  aptekr qelt aap dky iiil
121  aagcccgagg cgctcgagggc caggtctgag gtcacatgg cctgggttac cagctctcacc
41  kpe aae aek ve ahma wtvdhv
181  cgccgcaaggc tcgccaagcc tcgacacttc ggtgtgtaga agaagttcga cttcagcagctc
61  rsslgkrdtsgv ekkfniss
241  tggagccgctt actctgagcgat gttgagctgt ctgagctcaga gaagagcccc
81  wnasgeffddaitaeiikksp
301  gaggttgcct tgcctcagcc cgactacatt gtcacccctg actacaaggt tgagcctctc
101  evafvdpy lvdty k v ep l
361  tcgcagcgct CTCGACCAC CCTGCCC GTTTTGTGC CATCCCGCC
121  sdraltqsnapw glaal aISR
421  CGAACCCCGGG TGGCGCGCCAC TACACCTCTG GACGCGACTG CCGTGGCGCC TCTTACTGGT
141  RTPPGGSTY TD TAAGAGTY G
481  TACGCGGCTG ACTCTGGTTAT CAACACCGCC CACACTGACT TGGCGGGCGG TCGCTCTCTC
161  YVVDSGINATACHTDF GGRASL
541  GGTACACCGC CTGCTGTTGCG CGGCCACACT GATACCCCTT GACCAGTCTA CCACAGTTGCT
181  GNYGAGGD TDTLGHTHVA
601  GGTACCCATTG CCGCCCAACCG CTCGCGGCTT GCCAGCGGCGCCAAGCAGCAT CTACTGCAAG
201  GTIASNTYGV WA KRANV I SVK
661  GTTTTCTGCGT GGAACAAAGC TTCTACCTCT GTTATCTTGG AGGTTCCTAA CTGGGCGGCTC
221  VFVGNQASTSVILDFGFNWAV
721  AAGCAGATCA CCTCCAAGAA CGCGCTAGGC CGCTCCTGCA TCAACATGTC TCTGCTGTTGCT
241  NDI T RARS RSV INMSLGG
781  CCTCCTTCTC AAGCTCTGCGC TACCTGACATC AAGCTGCTTCACGCAGCGGCTGCTCTCC
261  PSSQTWATAYNAASQGVLG
841  GGTGTGCGTG CGCGGAAACGG TGATTCAAC CGGTCGCTTCT GTCGCGCCTT GAGGACAGCTC
281  VVAAANGDSN GRNLPAASGQS
FIG. 8B

901  CCTGCCAAACG TTCCCAACGC TATCACCGTT GCTGCCGCGC ACTCCAGCTG GCGRACTGCC
1001 P A N V P M A I T V A A A D S S W R T A

961  TCTTTCAACCA ACTACGGTCC TGAGGTCGAT ATCTTTGCGTC CTGGTGTCAA CATCCAGTCC
1161 S F T N Y G P E V D F G P G V N I Q S

1021  ACCTGGTACA CCTCCACAG CGCTACCAAC ACCATCAGCG GTACCTCCAT GGTCTGCCCT
1221 T W Y T S N S A T N T I S G T S M A C P

1081  CACGTTGCTG GTCTTGGCTCT CTACCTCCAG GCTCCTGAGA ACCTCAATAC CCCTGCTGCC
1281 H V A G L A L Y L Q A L E N L N T P A A

1141  GTCACCCAACC GCATCAAGTC TCTTGGCACT ACCGGCGCGA TCACCTGCCAG CCCTACGGGC
1341 V T N R I K S L A T T G I T G S L S G

1201  AGCCCAACC TAATGGGCTTT CAACGCGCGT ACTGCTTAA
1401 S P N M A F N G A T A *
FIG. 10A
Wash performance, blood/milk/ink (Art. 117, EMPA), Liquid Base Detergent at 5 g/l (60 min, 30°C, pH approx. 7.4)

FIG. 10B
Wash performance, blood/milk/ink (Art. 117, EMPA), Liquid Base Detergent at 5 g/l (60 min, 45°C, pH approx. 7.4)
FIG. 11A
Stability in Liquid Base Detergent for colored fabrics (37°C, pH approx. 8.2)

FIG. 11B
Stability in Liquid Base Detergent for colored fabrics with 2% sodium tetraborate decahydrate and 17% propylene glycol (37°C, pH approx. 7)
FIG. 12A
Stability in Ecolabel Reference Detergent (wfk Testgewebe GmbH) (18 hrs, 37°C, pH approx. 7.2)

FIG. 12B
Stability in Commercial liquid detergent (37°C, pH approx. 8.2)
FIG. 13A
Stability in Ecolabel Reference Detergent (wfk Testgewebe GmbH) with 2% sodium tetraborate decahydrate and 17% propylene glycol (37°C, pH approx. 6.5)

FIG. 13B
Stability in Commercial liquid Detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol (37°C, pH approx. 7.5)
FIG. 14A
Wash performance, blood/milk/ink (Art.117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 30°C, pH approx. 7.8)

FIG. 14B
Wash performance, blood/milk/ink (Art. 117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 30°C, pH approx. 7.8)
FIG. 14C
Wash performance, blood/milk/ink (Art.117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 50°C, pH approx. 7.8)

FIG. 14D
Wash performance, blood/milk/ink (Art.117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 50°C, pH approx. 7.8)
FIG. 15A
Wash performance, blood/milk/ink (Art.117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 30°C, pH approx. 7.8)

FIG 15B
Wash performance, blood/milk/ink (Art.117, EMPA), Commercial liquid detergent at 5 g/l (60 min, 50°C, pH approx. 7.8)
FIG. 16A
Stability in Commercial liquid detergent (37°C, pH approx. 8.2)

FIG. 16B
Stability in Commercial liquid detergent (37°C, pH approx. 8.2)
FIG. 17A
Stability in Commercial liquid detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol (37°C, pH approx. 7.5)

FIG. 17B
Stability in Commercial liquid detergent with 2% sodium tetraborate decahydrate and 17% propylene glycol (37°C, pH approx. 7.5)
FIG. 18
Stability in Ecolabel Reference Detergent (20 hrs, 37°C, pH approx. 7.2)
# INTERNATIONAL SEARCH REPORT

**International application No**

PCT/EP2011/068837

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/58 C11D3/386

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, EMBASE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>SI EZEN R J ET AL: &quot;SUBTI LASES: THE SUPERFAMILY OF SUBTILISIN-LIKE SERINE PROTEASES&quot;, PROTEIN SCIENCE, CAMBRIDGE UNIVERSITY PRESS, vol. 6, no. 3, 1 March 1997 (1997-03-01), pages 501-523, XP0008596203, ISSN: 0961-8368 figure 2</td>
<td>1-12, 14, 15</td>
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<td>X,P</td>
<td>WO 2010/125175 AI (AB ENZYMES 0Y [FI]; JUN'TUNEN KARI [FI]; VAL'TAKARI LEENA [FI]; MA'EKINEN) 4 November 2010 (2010-11-04) cited in the application on sequence 18</td>
<td>1-12, 14, 15, 17-34</td>
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Date of the actual completion of the international search

2 December 2011

Date of mailing of the international search report

15/12/2011

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer

Schmitz, Till
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<td>DATABASE Uni Prot [Online] 11 January 2011 (2011-01-11), &quot;SubName: Full1=Subtilase; &quot;, XP002665009, retrieved from EBI access no. UNI PROT:E3Q3S5 Database access no. E3Q3S5 the whole document</td>
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| X        | DATABASE Uni Prot [Online]  
13 September 2004 (2004-09-13)  
"SubName: Full=Subtilisin-like protease; ",  
XP002665012  
retrieved from EBI accession no.  
UNIPROT:Q69I7F7  
Database accession no. Q69I7F7 sequence | 1-3, 5-12, 14, 15, 17-34 |
<p>| A, P     | Wo 2010/125174 AI (AB ENZYMES 0Y [FI]; JUNTUNEN KARI [FI]; VALTAKARI LEENA [FI]; MAEKINEN) 4 November 2010 (2010-11-04) sequence 15 | 1-15 |</p>
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