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(54) Title: ALPHA-AMYLASE VARIANTS AND POLYNUCLEOTIDES ENCODING THE SAME

(57) Abstract: The present invention relates to polypeptide having alpha-amylase activity. The present invention also relates to polynucleotides encoding the polypeptides; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of using the polypeptides.



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ALPHA-AMYLASE VARIANTS AND POLYNUCLEOTIDES ENCODING THE SAME**Reference to a Sequence Listing**

5 This application comprises a Sequence Listing in computer readable form, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION**10 Field of the Invention**

The present invention relates to alpha-amylase variants (*i.e.* polypeptides having alpha-amylase activity), nucleic acids encoding the alpha-amylases, methods of producing the alpha-amylases, compositions comprising the alpha-amylases and methods of using the alpha-amylases.

15 Description of the Related Art

Alpha-amylases (alpha-1,4-glucan-4-glucohydrolases, E.C. 3.2.1.1) constitute a group of enzymes which catalyse hydrolysis of starch and other linear and branched 1,4-glycosidic oligo- and polysaccharides.

20 There is a long history of industrial use of alpha-amylases in several known applications such as detergent, baking, brewing, starch liquefaction and saccharification *e.g.* in preparation of high fructose syrups or as part of ethanol production from starch. These and other applications of alpha-amylases are known and utilize alpha-amylases derived from microorganisms, in particular bacterial alpha-amylases.

Bacillus alpha-amylases, such as Termamyl, AA560 (SEQ ID NO: 2; see also
25 WO 2000/060060) and SP707 (SEQ ID NO: 8; see also Tsukamoto *et al.*, 1988, *Biochem. Biophys. Res. Comm.* **151**:25-31) form a particular group of alpha-amylases that have found use in detergents. These amylases have been modified to improve the stability in detergents. For example, WO 96/23873 discloses deletion mutants of alpha-amylases SP690, SP722 (SEQ ID NO: 5) and SP707 (SEQ ID NO: 8) (see also SEQ ID NOs: 1, 2 and 7 of WO 96/23873) to improve
30 the stability of these amylases. WO 96/23873 further discloses substitution mutants to stabilize the amylases towards oxidation. Additional alpha-amylase mutants with improved properties are disclosed in WO 2006/002643 and WO 01/66712.

Thus, it is known to modify naturally-occurring amylases to improve certain properties.

35 It is an object of the present invention to provide polypeptides having alpha-amylase activity (alpha-amylases) which have improved wash performance, especially for use in automatic washing machines and/or dishwashers.

SUMMARY OF THE INVENTION

The present invention relates to polypeptides having alpha-amylase activity corresponding to variants of an alpha amylase derived from *Bacillus*. In particular, the polypeptides of the invention comprise or consist of an amino acid sequence of SEQ ID NO:1 in which one or more
5 of the amino acids are mutated (for example, by substitution or deletion), such that the polypeptide exhibits enhanced wash performance (especially in automatic washing machines and/or dishwashers).

The present invention also relates to polynucleotides encoding the variant alpha-amylase polypeptides; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and
10 methods of producing the variant alpha-amylase polypeptides.

The present invention also relates to detergent compositions comprising the variant alpha-amylase polypeptides and uses of the same in domestic and industrial cleaning processes, such as the laundry cleaning and dishwashing.

15 FIGURES

Figure 1 shows an alignment of SEQ ID NO: 1 and SEQ ID NO: 2

DEFINITIONS

Alpha-amylase: The term "alpha-amylase" (alpha-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1) constitutes a group of enzymes which catalyse hydrolysis of starch and other linear and
20 branched 1,4-glycosidic oligo- and polysaccharides. For purposes of the present invention, alpha-amylase activity is determined according to the procedure described in Example 1. In one aspect, the variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase
25 activity of the mature polypeptide of SEQ ID NO: 1.

Amino acid: The term 'amino acid' as used herein includes the standard twenty genetically-encoded amino acids and their corresponding stereoisomers in the 'D' form (as compared to the natural 'L' form), omega-amino acids other naturally-occurring amino acids, unconventional amino acids (*e.g.* α , α -disubstituted amino acids, N-alkyl amino acids, *etc.*) and
30 chemically derivatised amino acids. Chemical derivatives of one or more amino acids may be achieved by reaction with a functional side group. Such derivatised molecules include, for example, those molecules in which free amino groups have been derivatised to form amine hydrochlorides, *p*-toluene sulphonyl groups, carboxybenzoxy groups, *t*-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatised to form salts,
35 methyl and ethyl esters or other types of esters and hydrazides. Free hydroxyl groups may be derivatised to form O-acyl or O-alkyl derivatives. Also included as chemical derivatives are those peptides which contain naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be

substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine for lysine. Derivatives also include peptides containing one or more additions or deletions as long as the requisite activity is maintained. Other included modifications are amidation, amino terminal acylation (e.g. acetylation or thioglycolic acid amidation), terminal carboxylamidation (e.g. with ammonia or methylamine), and the like terminal modifications.

When an amino acid is being specifically enumerated, such as 'alanine' or 'Ala' or 'A', the term refers to both L-alanine and D-alanine unless explicitly stated otherwise. Other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded amino acid residue, where appropriate, is represented by a single letter designation, corresponding to the trivial name of the conventional amino acid. In one embodiment, the polypeptides of the invention comprise or consist of L-amino acids.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a variant. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a variant of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the variant or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a variant.

Corresponding to: The term "corresponding to" as used herein, refers to a way of determining the specific amino acid of a sequence wherein reference is made to a specific amino acid sequence. *E.g.* for the purposes of the present invention, when references are made to specific amino acid positions, the skilled person would be able to align another amino acid sequence to said amino acid sequence that reference has been made to, in order to determine

which specific amino acid may be of interest in said another amino acid sequence. Alignment of another amino acid sequence with e.g. the sequence as set forth in SEQ ID NO: 1 or 2, or any other sequence listed herein, has been described elsewhere herein. Alternative alignment methods may be used, and are well-known for the skilled person.

5 **Enhanced wash performance:** The terms “enhanced wash performance” or “improved wash performance” mean the ability of the polypeptide of the invention to provide a cleaning effect (e.g. stain removal) in a wash process, such as laundry or dishwashing, is improved compared to that of the parent alpha-amylase of SEQ ID NO:1. Wash performance may be determined using methods well known in the art, such as using an automatic mechanical stress assay (AMSA) or
10 automatic dish wash (ADW) (see Examples 2 and 3). It will be appreciated by persons skilled in the art that the enhanced wash performance may be achieved under only some or perhaps all wash conditions, for example at wash temperatures of 40°C or higher (such as at 40°C and/or at 50°C) and/or with or without the presence of bleach.

Expression: The term “expression” includes any step involved in the production of a
15 variant including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a variant and is operably linked to control sequences that provide for its expression.

20 **Fragment:** The term “fragment” means a polypeptide having one or more (e.g., several) amino acids absent from the amino and/or carboxyl terminus of the mature polypeptide of SEQ ID NO:1; wherein the fragment has alpha-amylase activity. In one aspect, a fragment contains at least 200 contiguous amino acid residues of SEQ ID NO: 1, for example at least 300 contiguous amino acid residues, or at least 350 contiguous amino acid residues, or at least 400 contiguous
25 amino acid residues, or at least 450 contiguous amino acid residues of SEQ ID NO: 1.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

30 **Isolated:** The term “isolated” means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified
35 by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., multiple copies of a gene encoding the substance; use of a stronger promoter

than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, *etc.* It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having alpha-amylase activity.

Mutation: The term "mutation", in the context of the polypeptides of the invention, means that one or more amino acids within the reference amino acid sequence (*i.e.* SEQ ID NO:1) are altered by substitution with a different amino acid or by deletion. Additionally, the mutation may correspond to an insertion of one or more extra amino acid(s) within the reference amino acid sequence.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Numbering is according to: The term "numbering is according to" as used herein, refers to the way each of the amino acid residues in a polypeptide of the present invention is numbered. *I.e.* the skilled person would know that when, *e.g.* position 202 is numbered according to SEQ ID NO: 2, he would know that by alignment of any other polypeptide with SEQ ID NO: 2, he will be able to determine the corresponding amino acid residue in the other polypeptide. Alignment of two or more amino acid sequences has been described elsewhere herein.

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Parent or parent alpha-amylase: The term "parent" or "parent alpha-amylase" means the alpha-amylase of SEQ ID NO:1 (commercialised by Novozymes A/S under the trade name "Stainzyme® Plus"; see WO 2006/002643). Alternatively, it may also mean the alpha-amylase of SEQ ID NO: 2 (also known as AA560).

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used may be gap open

penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$\text{(Identical Residues x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

5 Alternatively, the parameters used may be gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

10
$$\text{(Identical Deoxyribonucleotides x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Subsequence: The term “subsequence” means a polynucleotide having one or more (e.g., several) nucleotides absent from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having alpha-amylase activity.

15 **Variant:** The term “variant” means a polypeptide having alpha-amylase activity comprising a mutation, i.e., a substitution, insertion, and/or deletion, at one or more (e.g., several) positions relative to the ‘parent’ alpha-amylase of SEQ ID NO:1 or SEQ ID NO: 2. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position. The variants of the present invention have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%,
20 at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the mature polypeptide of SEQ ID NO: 1 or SEQ ID NO: 2.

Wild-type alpha-amylase: The term “wild-type” alpha-amylase means an alpha-amylase expressed by a naturally occurring microorganism, such as a bacterium, yeast, or filamentous
25 fungus found in nature.

Conventions for Designation of Variants

The polypeptides of the invention having alpha-amylase activity correspond to variants of an alpha-amylase derived from *Bacillus*, as shown in SEQ ID NO: 1 (which is marketed by
30 Novozymes A/S under the name *Stainzyme Plus*TM).

35 HHNGTNGTLM QYFEWYLPND GNHWNRLRSD ASNLKDKGIS AVWIPPAWKG
ASQNDVGYGA YDLYDLGEFN QKGTIRTKYG TRNQLQAAVN ALKSNGIQVY
GDVVMNHKGG ADATEMVKAV EVNPNNRNQE VSGEYTIKAW TKFDFPGRAN
THSNFKWRWY HFDGVDWDQS RKLNNRIYKF RTKAWDWEVD TEFGNIDYLL
YADIDMDHPE VVNELRNWGV WYTNTLGLDG FRIDAVKHIK YSFTRDWINH
VRS AIGKNMF AVAEFWKNDL GAIENYLNKT NWNHVSFVDP LHFNLVYASK
SGGNVDMRQI FNGTVVQKHP THAVTFVDNH DSQPEESLES FVREWFKPLA
YALTLTREQG YPSVFGDYD GIPTHGVPAM KSKIDPILEA RQKYAYGRQN
40 DYLDHHNIIG WTREGNTAHP NSGLATIMSD GAGGNKWMFV GRNKAGQVWT

DITGNKAGTV TINADGWGNF SVNGGSVSIW VNK

[SEQ ID NO:1]

The variant, *i.e.* mutated, amino acids in the polypeptides of the invention are defined by reference to the amino acid numbering of SEQ ID NO: 2 (which corresponds to the mature protein AA560 of *B. subtilis*). The amino acid sequence differences relative to SEQ ID NO:1 are shown below in bold, underlined.

10 HHNGTNGT**M**MQYFEWYLPNDGNHWNRLRSDASNLKDKGISAVWI PPAWKGASQNDVGYGAYD
 LYDLGEFNQKGTIRTKYGTRNQLQAAVNALKSNGIQVYGDVVMNHKGGADATEM**R**AVEVNP
 NNRNQEVSGEYTI EAWTKFDFPGR**G**NTHSNFKWRWYHFDGVDWDQSRKLNRIYK**F****R****G****D****G****K****G**
 WDWEVDTE**N**GNYDYL**M**YADIDMDHPEVVNELRNWGVWYTNLTGLDGFRIDAVKHIKYSFTRD
 WINHVRSA**T**GKNMFVAEAEFWKNDLGAIENYLKTNWNHVSFVDVPLH**Y**NLY**N**ASKSGGNYDMR
 15 QIFNGTVV**Q****R**H**P****M**HAVTFVDNHDSQPEE**A**LESFV**E**EWFKPLAYALTLTREQGYPSVFYGDYY
 GIPTHGVPAMKSKIDPILEARQKYAYGRQNDYLDHHNIIGWTREGNTAHPNSGLATIMSDGA
 GGNKWMFVGRNKAGQVWTDITGN**R**AGTVTINADGWGNFSVNGGSVSIWVNK

[SEQ ID NO:2]

Consequently, amino acid positions 1 to 182 in SEQ ID NO:2 correspond to the same numbering in SEQ ID NO:1, amino acid positions 183 and 184 of SEQ ID NO: 2 are absent in SEQ ID NO:1 and amino acid positions 185 to 485 of SEQ ID NO:2 correspond to positions 183 to 483 of SEQ ID NO:1, respectively. The alignment of SEQ ID NO: 1 and SEQ ID NO: 2 can be seen in Figure 1.

Thus, for purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO: 2 is used to determine the corresponding amino acid residue in another alpha-amylase polypeptide. The amino acid sequence of another alpha-amylase is aligned with the mature polypeptide disclosed in SEQ ID NO: 2, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO: 2 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

Identification of the corresponding amino acid residue in another alpha-amylase can be determined by an alignment of multiple polypeptide sequences using several computer programs including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later; Edgar, 2004, *Nucleic Acids Research* 32: 1792-1797), MAFFT (version 6.857 or later; Katoh and Kuma, 2002, *Nucleic Acids Research* 30: 3059-3066; Katoh *et al.*, 2005, *Nucleic Acids Research* 33: 511-518; Katoh and Toh, 2007, *Bioinformatics* 23: 372-374; Katoh *et al.*, 2009, *Methods in Molecular Biology* 537: 39-64; Katoh and Toh, 2010, *Bioinformatics* 26: 1899-1900),

and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson *et al.*, 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

When the other alpha-amylase has diverged from the mature polypeptide of SEQ ID NO: 2 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* 295: 613-615), other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Atschul *et al.*, 1997, *Nucleic Acids Res.* 25: 3389-3402). Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such as GenTHREADER (Jones, 1999, *J. Mol. Biol.* 287: 797-815; McGuffin and Jones, 2003, *Bioinformatics* 19: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough *et al.*, 2000, *J. Mol. Biol.* 313: 903-919, can be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* 33: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* 11: 739-747), and implementation of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs (*e.g.*, Holm and Park, 2000, *Bioinformatics* 16: 566-567).

In describing the alpha-amylase variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

Substitutions: For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), *e.g.*, "Gly205Arg + Ser411Phe" or "G205R + S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

Deletions: For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or

“G195*”. Multiple deletions are separated by addition marks (“+”), e.g., “Gly195* + Ser411*” or “G195* + S411*”.

Insertions: For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated “Gly195GlyLys” or “G195GK”. An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as “Gly195GlyLysAla” or “G195GKA”.

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

<u>Parent:</u>	<u>Variant:</u>
195	195 195a 195b
G	G - K - A

Multiple alterations: Variants comprising multiple alterations are separated by addition marks (“+”), e.g., “Arg170Tyr+Gly195Glu” or “R170Y+G195E” representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively.

Different alterations: Where different alterations can be introduced at a position, the different alterations are separated by a comma, e.g., “Arg170Tyr,Glu” represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Thus, “Tyr167Gly,Ala + Arg170Gly,Ala” designates the following variants:

“Tyr167Gly+Arg170Gly”, “Tyr167Gly+Arg170Ala”, “Tyr167Ala+Arg170Gly”, and “Tyr167Ala+Arg170Ala”.

DETAILED DESCRIPTION OF THE INVENTION

Polypeptides having alpha-amylase activity

The present invention relates to variant alpha-amylase polypeptides, comprising a mutation at one or more (e.g., several) positions within the amino acid sequence of the mature polypeptide of SEQ ID NO: 1, wherein the variant exhibits an enhanced wash performance compared to the polypeptide of SEQ ID NO: 1; said variant amino acid sequence comprises a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2; and has at least 80%, such as at least 85%, at least 90%, or at least 95% sequence identity to SEQ ID NOs: 1 or 2.

The amino acid sequence of the parent alpha amylase is shown below:

5
 HHNGTNGTLM QYFEWYLPND GNHWNRLRSD ASNLKDKGIS AVWIPPAWKG
 ASQNDVGYGA YDLYDLGEFN QKGTIRTKYG TRNQLQAAVN ALKSNIGIQVY
 GDVVMNHKGG ADATEMVKAV EVNPNRNQEQ VSGEYTIKAW TKFDFPGRAN
 THSNFKWRWY HFDGVDWDQS RKLNNRIYKF RTKAWDWEVD TEFGNIDYLL
 YADIDMDHPE VVNELRNWGV WYTNTLGLDG FRIDAVKHIK YSFTRDWINH
 VRSAIGKNMF AVAEFWKNDL GAIENYLNKT NWNHVSFVDP LHFNLYYASK
 10 SGGNYDMRQI FNGTVVQKHP THAVTFVDNH DSQPEESLES FVREWFKPLA
 YALTLTREQG YPSVFGDYD GIPTHGVPAM KSKIDPILEA RQKYAYGRQN
 DYLDHHNIIG WTREGNTAHP NSGLATIMSD GAGGNKWMFV GRNKAGQVWT
 DITGNKAGTV TINADGWGNF SVNGGSVSIW VNK

[SEQ ID NO:1]

15
 In one embodiment, the polypeptide is a variant amino acid sequence of SEQ ID NO: 1, wherein the polypeptide has alpha-amylase activity and exhibits an improved wash performance compared to the parent polypeptide of SEQ ID NO: 1, the variant amino acid sequence comprises a mutation at amino acid position corresponding to positions 202 and/or 186 wherein numbering
 20 is according to SEQ ID NO: 2, with the proviso that the mutation in position 186 is not A186G, and wherein the variant has at least 80% sequence identity to SEQ ID Nos: 1 or 2.

In one embodiment, the polypeptide is a variant amino acid sequence comprising a substitution at amino acid position 202 wherein numbering is according to SEQ ID NO: 2, such as L202M. In one embodiment, the polypeptide is a variant amino acid sequence consisting of
 25 the substitution L202M wherein numbering is according to SEQ ID NO: 2. In another embodiment, the polypeptide variant does not comprise a mutation in position 202 but in at least position 186, which is not the substitution A186D, and potentially other positions, wherein numbering is according to SEQ ID NO: 2.

In another embodiment, the polypeptide is a variant amino acid sequence comprising a
 30 substitution at amino acid position 186 wherein numbering is according to SEQ ID NO: 2, such as A186D.

The polypeptides of the invention represent variants of the parent alpha amylase of SEQ ID NO: 1, which variants exhibit enhanced wash performance in domestic and/or industrial cleaning processes, such as the laundry cleaning and dishwashing. Wash performance may be
 35 determined using methods well known in the art, such as using an automatic dish wash (ADW) (see Example 5) or an automatic mechanical stress assay (AMSA) (see Example 6).

In one embodiment, the polypeptide exhibits enhanced wash performance during high temperature washes, for example wherein the temperature is at least 40°C, such as at least 45°C, and such as at least 50°C.

40 In one embodiment, enhanced wash performance is assessed in an automatic dish wash

(ADW) assay using melamine tiles stained with starch. Exemplary wash conditions for determining such improved wash performance of the polypeptides of the invention include:

- (a) ADW detergent at 40°C and a wash cycle of 10 min; and
- (b) ADW detergent at 50°C and a wash cycle of 20 min;

5 wherein the polypeptide is present at a concentration of 0.03 or 0.24 mg/L and wherein said ADW detergent comprises a bleach catalyst, non-ionic surfactant(s), and a sulfonated polymer.

In addition to enhanced wash performance, it will be appreciated by persons skilled in the art that the polypeptides of the invention may also exhibit improvements in one or more of the
10 following properties relative to the parent alpha-amylase of SEQ ID NO:1:

- (i) Substrate specificity;
- (ii) Substrate binding;
- (iii) Specific activity;
- 15 (iv) Thermal stability
- (v) pH stability profile;
- (vi) Ca²⁺ dependency;
- (vii) Oxidation stability;
- (viii) Increased/decreased pI; and/or
- 20 (ix) Sensitivity to surfactants.

Assays for determining the above properties of a protein are described in WO 2006/002643, WO 2001/066712 and EP 2 264 460 A.

The polypeptides of the invention may be longer or shorter than the parent alpha-amylase
25 of SEQ ID NO:1. Thus, the polypeptide may be 1000 or fewer amino acids in length, for example 900, 800, 700, 600, 500, 400, 300, 200, 175, 150, 125, 100 or fewer amino acids. In one embodiment, the polypeptide is between 400 and 600 amino acids in length, for example between 450 and 500, between 460 and 500, or between 470 and 490 amino acids in length.

The variant polypeptides of the invention comprise a mutation (*i.e.*, a substitution,
30 insertion, and/or deletion) at one or more amino acid positions relative to the 'parent' alpha-amylase of SEQ ID NO:1.

The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such
35 as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a polypeptide may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for alpha-amylase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

In one embodiment, the polypeptide of the invention is a variant of the alpha-amylase of SEQ ID NO:1 having a mutation (such as a substitution, deletion, and/or insertion) at one or more positions corresponding to positions 51, 186, 202, 246 and 334, wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2. Thus, the variant of the present invention comprises a mutation in at least one of the positions corresponding to positions 202 and/or 186 wherein numbering is according to SEQ ID NO: 2 and may further comprise a mutation in one or more of positions 51, 246 and 334, and in the case wherein the variant comprises a mutation in position 202, then it may also comprise a mutation in position 186 and potentially also any other position, and vice versa. *I.e.* when the variant comprises a mutation in position 186, then it may also comprise a mutation in position 202 and potentially also any other position. It is to be understood that the variant according to the present invention does not necessarily comprise a mutation in both position 202 and position 186 when it comprises a mutation in other positions.

In one particular embodiment, the polypeptide of the invention has at least 80%, such as at least 85%, such as at least 90%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, but less than 100% sequence identity to the amino acid sequence of SEQ ID NO: 1 or 2.

Thus, the polypeptide may comprise one or more of the following substitutions relative to the amino acid sequence of SEQ ID NO:2; A51T, A186D, L202M, T246(I/L/V) and S334T.

In one embodiment, the polypeptide exhibits an enhanced wash performance corresponding to an Improvement Factor (IF) of at least 1.2 when the polypeptide is evaluated in an AMSA with a detergent comprising bleach and at 40°C for 10 min (for example, see Example 6). Suitable polypeptides may be a variant of SEQ ID NO:1 comprising a mutation at one or more positions corresponding to positions 51, 186, 202, 246 and 334, wherein numbering of amino acid positions is according to the amino acid sequence set forth in SEQ ID NO: 2. For example, the polypeptide may be selected from the group consisting of polypeptides of the amino acid sequence of SEQ ID NO:1 with the following mutations; A51T, A186D, L202M, T246(I/L/V) and S334T, and fragments thereof having alpha amylase activity, wherein numbering of amino acid positions is according to the amino acid sequence set forth in SEQ ID NO: 2.

It will be appreciated by persons skilled in the art that different examples of the polypeptides of the invention will possess a different degree of amino acid sequence identity with the sequence of SEQ ID NO:1. Thus, the polypeptide may comprise or consist of an amino acid sequence which shares at least 85% sequence identity with SEQ ID NO: 1, for example at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 1. In one embodiment, the number of mutations within the polypeptide relative to the amino acid sequence of SEQ ID NO:1 is between 1 and 20, *e.g.*, between 1 and 10 mutations or between 1 and 5 mutations, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mutations.

In one particular subset of the polypeptides of the invention, the polypeptide consists of the amino acid sequence of SEQ ID NO:1 with mutations selected from the group consisting of; A51T+A186D, A51T+L202M, A51T+T246I, A51T+S334T, A186D+L202M, A186D+T246I, A186D+S334T, L202M+T246I, L202M+S334T, T246I+S334T, A51T+A186D+L202M, A51T+A186D+T246I, A51T+A186D+S334T, A51T+L202M+T246I, A51T+L202M+S334T, A51T+T246I+S334T, A186D+L202M+T246I, A186D+L202M+S334T, A186D+T246I+S334T, L202M+T246I+S334T, A51T+A186D+L202M+T246I, A51T+A186D+L202M+S334T, A51T+A186D+T246I+S334T, A51T+L202M+T246I+S334T, A186D+L202M+T246I+S334T, and A51T+A186D+L202M+T246I+S334T, or a fragment thereof having alpha amylase activity.

For example, the polypeptide may comprise or consist of an amino acid sequence of SEQ ID NO:1 with mutations selected from the group consisting of the following:

- (a) L202M;
 (b) A186D;
 (c) L202M+T246I+S334T;
 (d) L202M+T246L+S334T;
 5 (e) A51T+L202M+T246I+S334T;
 (f) L202M+T246V; and
 (g) L202M+T246V+S334T;

wherein numbering of amino acid positions is according to the amino acid sequence set forth in SEQ ID NO: 2.

10 One exemplary polypeptide of the invention consists of the amino acid sequence of SEQ ID NO:3 (wherein the mutated amino acids relative to SEQ ID NO:1 are underlined).

15 HHNGTNGTLM QYFEWYLPND GNHWNRLRSD ASNLKDKGIS AVWI¹PPAWKG
 ASQNDVGYGA YDLYDLGEFN QKGTIRTKYG TRNQLQAAVN ALKSNGIQVY
 GDVVMNHKGG ADATEMVKAV EVNPNNRNQE VSGEY²TIEAW TKFDFPGRAN
 THSNFKWRWY HFDGVDWDQS RKLNNRIYKF RTKA³WDWEVD TEF⁴GN⁵YDYL⁶M
 YADIDMDHPE VVNELRNWGV WYTNTLGLDG FRIDAVKHIK YSF⁷V⁸RDWINH
 VRSAIGKNMF AVAEFWKNDL GAIENYLNKT NWNH⁹SVFDVP LHF¹⁰NLYYASK
 SGGNYDMRQI FNGTVVQKHP THAVTFVDNH DSQPEESLES FVREWFKPLA
 20 YALTLTREQG YPSV¹¹FYGDYY GIP¹²THGVPAM KSKIDPILEA RQKYAYGRQN
 DYLDHHNIIG WTREGNTAHP NSGLATIMSD GAGGNKWMFV GRNKAGQVWT
 DITGNKAGTV TINADG¹³WGNF SVNGGSVSIW VNK

[SEQ ID NO:3]

25 Preparation of Polypeptides of the Invention

The polypeptides of the invention can be prepared using any mutagenesis procedure known in the art, such as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, *etc.*

30 Site-directed mutagenesis is a technique in which one or more (*e.g.*, several) mutations are introduced at one or more defined sites in a polynucleotide encoding the parent alpha-amylase.

Site-directed mutagenesis can be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed *in vitro* by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and the insert to ligate to one another. See, *e.g.*, Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* 76: 4949-4955; and Barton *et al.*, 1990, *Nucleic Acids Res.* 18: 7349-4966.

40 Site-directed mutagenesis can also be accomplished *in vivo* by methods known in the art. See, *e.g.*, U.S. Patent Application Publication No. 2004/0171154; Storici *et al.*, 2001, *Nature*

Biotechnol. 19: 773-776; Kren *et al.*, 1998, *Nat. Med.* 4: 285-290; and Calissano and Macino, 1996, *Fungal Genet. Newslett.* 43: 15-16.

Any site-directed mutagenesis procedure can be used in the present invention. There are many commercial kits available that can be used to prepare variants.

5 Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian *et al.* (2004, *Nature* 432: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

10 Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage
15 display (*e.g.*, Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204) and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells
20 (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene
25 construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized *de novo*, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR
30 amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

The present invention also relates to polynucleotides encoding a polypeptide of the present invention. Accordingly, the present invention relates to polynucleotides encoding a variant
35 polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80%, such as at least 85%, at least 90% sequence identity to SEQ ID NOs: 1 or 2.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant polypeptide of the invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences. Accordingly, the present invention relates to nucleic acid constructs comprising a polynucleotide encoding a variant polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80%, such as at least 85%, at least 90% sequence identity to SEQ ID NOs: 1 or 2, operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of a variant. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide which is recognized by a host cell for expression of the polynucleotide. The promoter contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis cryIIIA* gene (Agaisse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli lac* operon, *E. coli trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*. Examples of tandem promoters are disclosed in WO 99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate

isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, 5 *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the 10 untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

15 In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. 20 Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the variant. Any terminator that is functional in the host cell may 25 be used.

Preferred terminators for bacterial host cells are obtained from the genes for *Bacillus clausii* alkaline protease (*aprH*), *Bacillus licheniformis* alpha-amylase (*amyL*), and *Escherichia coli* ribosomal RNA (*rrnB*).

Preferred terminators for filamentous fungal host cells are obtained from the genes for 30 *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host 35 cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis cryIIIA* gene (WO 94/25612) and a *Bacillus subtilis* SP82 gene (Hue *et al.*, 1995, *Journal of Bacteriology* 177: 3465-3471).

5 The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the variant. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

10 Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

15 The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the variant-encoding sequence and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

20 Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

25 The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a variant and directs the variant into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the variant. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal
30 peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the variant. However, any signal peptide coding sequence that directs the expressed variant into the secretory pathway of a host cell may be used.

35 Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis*

prsA. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a variant. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of the variant and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the variant relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the variant would be operably linked with the regulatory sequence.

35 **Expression Vectors**

The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant polypeptide of the present invention, a promoter, and transcriptional and translational stop signals. Accordingly, the present invention relates to

recombinant expression vectors comprising a polynucleotide encoding a variant polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80% sequence identity to SEQ ID NOs: 1 or 2, a promoter, and transcriptional and translational stop signals.

5 The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the variant at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression
10 vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector
15 with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The
20 vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

25 The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* *dal*
30 genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase),
35 *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae* *amdS* and *pyrG* genes and a *Streptomyces hygrosopicus* *bar* gene.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the variant or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a variant. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

5 Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide encoding a variant polypeptide of the present invention operably linked to one or more control sequences that direct the production of the variant polypeptide of the present invention. Accordingly, the present invention relates to recombinant host cells, comprising a polynucleotide
10 encoding a variant polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80% sequence identity to SEQ ID NOs: 1 or 2, operably linked to one or more control sequences that direct the production of the variant polypeptide.

A construct or vector comprising a polynucleotide is introduced into a host cell so that the
15 construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the variant and its source.

20 The host cell may be any cell useful in the recombinant production of a variant, e.g., a prokaryote or a eukaryote.

The prokaryotic host cell may be any Gram-positive or Gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*.
25 Gram-negative bacteria include, but are not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus*
30 *megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

35 The bacterial host cell may also be any *Streptomyces* cell, including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397), or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, In, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, editors, *Soc. App. Bacteriol. Symposium Series No.* 9, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of

chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

5 The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

10 For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispota*, *Chrysosporium inops*, *Chrysosporium keratinophilum*,
15 *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
20 *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*,
25 *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163;
35 and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a variant polypeptide, comprising: (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant polypeptide; and (b) recovering the variant polypeptide. Accordingly, the present invention relates to methods of producing a variant polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80% sequence identity to SEQ ID NOs: 1 or 2, the methods comprising (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant polypeptide; and (b) recovering the variant polypeptide.

The host cells are cultivated in a nutrient medium suitable for production of the variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the variant to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the variant is secreted into the nutrient medium, the variant can be recovered directly from the medium. If the variant is not secreted, it can be recovered from cell lysates.

The variant may be detected using methods known in the art. Suitable detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the alpha-amylase activity of the variant (see Examples).

The variant may be recovered using methods known in the art. For example, the variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The variant may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure variants.

In an alternative aspect, the variant is not recovered, but rather a host cell of the present invention expressing the variant is used as a source of the variant.

Compositions

The alpha-amylase polypeptides of the invention may be added to and thus become a component of a detergent composition. Accordingly, the present invention relates to a

composition comprising a polypeptide having alpha-amylase activity and exhibits an improved wash performance compared to the parent polypeptide of SEQ ID NO: 1, the variant polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2 and has at least 80% sequence identity to SEQ ID Nos: 1 or 2.

5 Thus, a further aspect of the invention provides a detergent composition comprising a polypeptide of the invention and a surfactant (such as an anionic surfactant, a cationic surfactant, a nonionic surfactant and/or an amphoteric surfactant). Also provided is a concentrate or additive for making such detergent compositions, which concentrate or additive comprises a polypeptide of the invention and, optionally, a surfactant.

10 As discussed in detail below, the detergent composition may further comprise one or more additional components selected from the group consisting of oxidizing agents, bleach activators, bulking agents, builders, buffering agents, structurants, sequestrants, optical brighteners, antifoaming agents, enzymes, fragrances, anti-redeposition agents, skin conditioning agents, softness extenders, emulsifiers, and colorants.

15 In one embodiment, the composition is a liquid or powder laundry detergent composition. In a further embodiment, the composition is a liquid or powder automatic dishwashing (ADW) detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment
20 of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent concentrate/additive comprising the alpha-amylase polypeptide of the invention. The detergent concentrate/additive, as well as the detergent
25 composition, may comprise one or more other enzymes such as a protease, a lipase, a peroxidase, another amylolytic enzyme, e.g., another alpha-amylase, glucoamylase, maltogenic amylase, CGTase and/or a cellulase, mannanase (such as MANNAWAY™ from Novozymes, Denmark)), pectinase, pectine lyase, cutinase, and/or laccase.

In general the properties of the chosen enzyme(s) should be compatible with the selected
30 detergent, (i.e., pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The
35 protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like pro-teases are trypsin

(e.g., of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274. Preferred commercially available protease enzymes include ALCALASE®, SAVINASE® (SEQ ID NO: 3), PRIMASE®, DURALASE®, ESPERASE®, and KANNASE® (from Novozymes A/S), MAXATASE®, MAXACAL, MAXAPEM®, PROPERASE®, PURAFECT®, PURAFECT OXP®, FN2®, FN3®, FN4® (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g., from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580 , a *Pseudomonas* lipase, e.g., from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g., from *B. subtilis* (Dartois *et al.* (1993), *Biochemica et Biophysica Acta*, **1131**:253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422). Other examples are lipase variants such as those described in WO 92/05249 , WO 94/01541 , EP 407 225 , EP 260 105 , WO 95/35381 , WO 96/00292 , WO 95/30744 , WO 94/25578 , WO 95/14783, WO 95/22615 , WO 97/04079 and WO 97/07202 .

Preferred commercially available lipase enzymes include LIPOLASE<™> and LIPOLASE ULTRA<™> (Novozymes A/S).

Amylases: Suitable amylases (alpha and/or beta) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus*, e.g., a special strain of *B. licheniformis*, described in more detail in GB 1,296,839 . Examples of useful alpha-amylases are the variants described in WO 94/02597 , WO 94/18314 , WO 96/23873 , and WO 97/43424 , especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444. Commercially available alpha-amylases are DURAMYL<™>, LIQUEZYME™, TERMAMYL<™>, NATALASE<™>, FUNGAMYL<™> and BAN<™> (Novozymes A/S), Preferenz S100, Preferenz S110, Preferenz S1000 (SEQ ID NO: 11), Excellenz S110, Excellenz S1000, Excellenz S2000, RAPIDASE<™> and PURASTAR<™> (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*, *Acremonium*, *e.g.*, the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307 , US 5,648,263 , US 5,691,178 , US 5,776,757 and WO 89/09259. Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998 , EP 0 531 315 , US 5,457,046 , US 5,686,593 , US 5,763,254 , WO 95/24471 , WO 98/12307 and PCT/DK98/00299 .

Commercially available cellulases include CELLUZYME®, and CAREZYME® (Novozymes A/S), CLAZINASE®, and PURADAX HA® (Genencor International Inc.), and KAC-500(B)® (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, *e.g.*, from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257. Commercially available peroxidases include GUARDZYME® (Novozymes A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, *i.e.*, a separate additive or a combined additive, can be formulated, *e.g.*, granulate, a liquid, a slurry, *etc.* Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, *e.g.*, as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

The detergent composition of the invention may be in any convenient form, *e.g.*, a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

5 The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight.

When included therein the detergent will usually comprise from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid 10 methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually comprise from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanol-amide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine 15 ("glucamides").

The detergent may comprise 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetri-aminepentaacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (*e.g.* SKS-6 from Hoechst).

20 The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly(ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

25 The detergent may contain a bleaching system, which may comprise a H₂O₂ source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of, *e.g.*, the amide, imide, or sulfone type.

30 The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, *e.g.*, a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, *e.g.*, an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in, *e.g.*, WO 92/19709 and WO 92/19708.

35 The detergent may also contain other conventional detergent ingredients such as *e.g.* fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil re-deposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the alpha amylase polypeptides of the invention, may be added in an amount corresponding to

0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.05 - 5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The alpha amylase polypeptides of the invention may additionally be incorporated in the detergent formulations disclosed in WO 2006/002643, which is hereby incorporated as reference.

5

Examples of Dishwash Detergent Compositions of the Invention

The alpha-amylase polypeptide of the invention may also be used in dish wash detergent compositions, including the following:

10 (a) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	0.4 - 2.5%
	Sodium metasilicate	0 - 20%
	Sodium disilicate	3 - 20%
15	Sodium triphosphate	20 - 40%
	Sodium carbonate	0 - 20%
	Sodium perborate	2 - 9%
	Tetraacetyl ethylene diamine (TAED)	1 - 4%
	Sodium sulphate	5 - 33%
20	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%

(b) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1 - 2%
25	Sodium disilicate	2 - 30%
	Sodium carbonate	10 - 50%
	Sodium phosphonate	0 - 5%
	Trisodium citrate dihydrate	9 - 30%
	Nitritotrisodium acetate (NTA)	0 - 20%
30	Sodium perborate monohydrate	5 - 10%
	Tetraacetyl ethylene diamine (TAED)	1 - 2%
	Polyacrylate polymer (e.g. maleic acid/acrylic acid copolymer)	6 - 25%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
	Perfume	0.1 - 0.5%
35	Water	5 - 10%

(c) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	0.5 - 2.0%
	Sodium disilicate	25 - 40%
	Sodium citrate	30 - 55%
	Sodium carbonate	0 - 29%
5	Sodium bicarbonate	0 - 20%
	Sodium perborate monohydrate	0 - 15%
	Tetraacetyl ethylene diamine (TAED)	0 - 6%
	Maleic acid/acrylic acid copolymer	0 - 5%
	Clay	1 - 3%
10	Polyamino acids	0 - 20%
	Sodium polyacrylate	0 - 8%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%

(d) POWDER AUTOMATIC DISHWASHING COMPOSITION

15	Nonionic surfactant	1 - 2%
	Zeolite MAP	15 - 42%
	Sodium disilicate	30 - 34%
	Sodium citrate	0 - 12%
20	Sodium carbonate	0 - 20%
	Sodium perborate monohydrate	7 - 15%
	Tetraacetyl ethylene diamine (TAED)	0 - 3%
	Polymer	0 - 4%
	Maleic acid/acrylic acid copolymer	0 - 5%
25	Organic phosphonate	0 - 4%
	Clay	1 - 2%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
	Sodium sulphate	Balance

30 (e) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1 - 7%
	Sodium disilicate	18 - 30%
	Trisodium citrate	10 - 24%
35	Sodium carbonate	12 - 20%
	Monopersulphate (2 KHSO ₅ .KHSO ₄ .K ₂ SO ₄)	15 - 21%
	Bleach stabilizer	0.1 - 2%

Maleic acid/acrylic acid copolymer	0 - 6%
Diethylene triamine pentaacetate, pentasodium salt	0 - 2.5%
Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
Sodium sulphate, water	Balance

5

(f) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

	Nonionic surfactant	0 - 1.5%
10	Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
	80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
15	70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%
	C ₁₃ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of	3 0 - 10%
20	C ₁₂ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of	3 0 - 5%
	C ₁₃ -C ₁₅ ethoxylated alcohol with an average degree of ethoxylation of	12 0 - 5%
	A blend of C ₁₂ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of	9 0 - 6.5%
25	A blend of C ₁₃ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of	30 0 - 4%
	Sodium disilicate	0 - 33%
	Sodium tripolyphosphate	0 - 46%
	Sodium citrate	0 - 28%
30	Citric acid	0 - 29%
	Sodium carbonate	0 - 20%
	Sodium perborate monohydrate	0 - 11.5%
	Tetraacetyl ethylene diamine (TAED)	0 - 4%
	Maleic acid/acrylic acid copolymer	0 - 7.5%
35	Sodium sulphate	0 - 12.5%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%

(g) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
	Alkali metal silicate	3.0 - 15.0%
	Alkali metal phosphate	20.0 - 40.0%
5	Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycoethers	25.0 - 45.0%
	Stabilizer (e.g. a partial ester of phosphoric acid and a C ₁₆ -C ₁₈ alkanol)	0.5 - 7.0%
	Foam suppressor (e.g. silicone)	0 - 1.5%
10	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%

(h) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
15	Sodium silicate	3.0 - 15.0%
	Alkali metal carbonate	7.0 - 20.0%
	Sodium citrate	0.0 - 1.5%
	Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
20	Low molecule weight polyacrylate polymer	5.0 - 15.0%
	Clay gel thickener (e.g. bentonite)	0.0 - 10.0%
	Hydroxypropyl cellulose polymer	0.0 - 0.6%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
25	Liquid carrier selected from higher glycols, polyglycols, polyoxides and glycol ethers	Balance

(i) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

30	C ₁₂ -C ₁₄ fatty acid	0 - 0.5%
	Block co-polymer surfactant	1.5 - 15.0%
	Sodium citrate	0 - 12%
	Sodium tripolyphosphate	0 - 15%
	Sodium carbonate	0 - 8%
35	Aluminium tristearate	0 - 0.1%
	Sodium cumene sulphonate	0 - 1.7%
	Polyacrylate thickener	1.32 - 2.5%
	Sodium polyacrylate	2.4 - 6.0%

	Boric acid	0 - 4.0%
	Sodium formate	0 - 0.45%
	Calcium formate	0 - 0.2%
	Sodium n-decydiphenyl oxide disulphonate	0 - 4.0%
5	Monoethanol amine (MEA)	0 - 1.86%
	Sodium hydroxide (50%)	1.9 - 9.3%
	1,2-Propanediol	0 - 9.4%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
	Suds suppressor, dye, perfumes, water	Balance

10

(j) LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Alcohol ethoxylate	0 - 20%
	Fatty acid ester sulphonate	0 - 30%
15	Sodium dodecyl sulphate	0 - 20%
	Alkyl polyglycoside	0 - 21%
	Oleic acid	0 - 10%
	Sodium disilicate monohydrate	18 - 33%
	Sodium citrate dihydrate	18 - 33%
20	Sodium stearate	0 - 2.5%
	Sodium perborate monohydrate	0 - 13%
	Tetraacetyl ethylene diamine (TAED)	0 - 8%
	Maleic acid/acrylic acid copolymer	4 - 8%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%

25

(k) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

30

	Sodium silicate	5 - 10%
	Tetrapotassium pyrophosphate	15 - 25%
	Sodium triphosphate	0 - 2%
	Potassium carbonate	4 - 8%
35	Protected bleach particles, e.g. chlorine	5 - 10%
	Polymeric thickener	0.7 - 1.5%
	Potassium hydroxide	0 - 2%
	Enzymes (e.g. alpha-amylase polypeptide)	0.0001 - 0.1%
	Water	Balance

(l) Automatic dishwashing compositions as described in (a), (b), (c), (d), (f) and (j), wherein perborate is replaced by percarbonate.

5 (m) Automatic dishwashing compositions as described in (a) to (f) which additionally contain a manganese catalyst. The manganese catalyst may, *e.g.*, be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", *Nature* 369, 1994, pp. 637-639.

10 Uses

The present invention is also directed to methods for using an alpha-amylase polypeptide of the invention in detergents, in particular laundry detergent compositions and dishwashing detergent compositions. Accordingly, the present invention relates to methods of using a polypeptide having alpha-amylase activity and exhibits an improved wash performance compared
15 to the parent polypeptide of SEQ ID NO: 1, the polypeptide comprising a mutation at amino acid position 202 and/or 186 wherein numbering is according to SEQ ID NO: 2 and has at least 80% sequence identity to SEQ ID NOs: 1 or 2.

Thus, the invention provides the use of an alpha-amylase polypeptide or composition of the invention, in a domestic or industrial cleaning process.

20 In one embodiment, the use is cleaning of fabric, for example laundry.

In another embodiment, the use is cleaning of ceramic, plastic or glass material, for example dishwashing.

Accordingly, the alpha-amylase polypeptides of the invention are applicable as a component in washing, dishwashing, and hard surface cleaning detergent compositions (in either
25 a domestic or industrial setting).

The alpha-amylase variants of this invention possess valuable properties allowing for a variety of other industrial applications. For example, alpha-amylase polypeptides of the invention may be used for starch processes, in particular starch conversion, especially liquefaction of starch (see, *e.g.*, US 3,912,590, EP patent application nos. 252 730 and 63 909, WO 99/19467, and
30 WO 96/28567 all references hereby incorporated by reference). Also contemplated are compositions for starch conversion purposes, which may beside the variant of the invention also comprise a glucoamylase, pullulanase, and other alpha-amylases.

Furthermore, alpha-amylase variants of this invention are also particularly useful in the production of sweeteners and ethanol (see, *e.g.*, US patent no. 5,231,017 hereby incorporated
35 by reference), such as fuel, drinking and industrial ethanol, from starch or whole grains.

Alpha-amylase variants of the invention may also be useful for desizing of textiles, fabrics and garments (see, *e.g.*, WO 95/21247, US patent 4,643,736, EP 119,920 hereby in corporate by reference), beer making or brewing, in pulp and paper production.

Starch Conversion

Conventional starch-conversion processes, such as liquefaction and saccharification processes are described, *e.g.*, in US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909, hereby incorporated by reference.

5 In an embodiment the starch conversion process degrading starch to lower molecular weight carbohydrate components such as sugars or fat replacers includes a debranching step.

In the case of converting starch into a sugar, the starch is depolymerized. Such depolymerization processes may consist of a pre-treatment step and two or three consecutive process steps, viz. a liquefaction process, a saccharification process and dependent on the
10 desired end product optionally an isomerization process.

(i) Pre-treatment of native starch

Native starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst,
15 dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typically industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation.

20 (ii) Liquefaction

During the liquefaction step, the long chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. The liquefaction process is carried out at 105-110°C for 5 to 10 minutes followed by 1-2 hours at 95°C. The pH lies between 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, 1 mM of calcium is added (40
25 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

(iii) Saccharification

After the liquefaction process the maltodextrins are converted into dextrose by addition of
30 a glucoamylase (*e.g.*, AMG) and a debranching enzyme, such as an isoamylase (US patent no. 4,335,208) or a pullulanase (*e.g.*, Promozyme™) (US patent no. 4,560,651). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C) to inactivate the liquefying alpha-amylase to reduce the formation of short oligosaccharide called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

35 The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

Normally, when denaturing the α -amylase after the liquefaction step about 0.2-0.5% of the saccharification product is the branched trisaccharide α -glucosyl maltose (panose)

which cannot be degraded by a pullulanase. If active amylase from the liquefaction step is present during saccharification (i.e., no denaturing), this level can be as high as 1-2%, which is highly undesirable as it lowers the saccharification yield significantly.

5 (iv) Isomerization

When the desired final sugar product is, e.g., high fructose syrup the dextrose syrup may be converted into fructose. After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucose isomerase (such
10 as Sweetzyme[™] IT).

Ethanol production

In general alcohol production (ethanol) from whole grain can be separated into 4 main steps

- Milling
- 15 - Liquefaction
- Saccharification
- Fermentation

(i) Milling

20 The grain is milled in order to open up the structure and allowing for further processing. Two processes are used wet or dry milling. In dry milling the whole kernel is milled and used in the remaining part of the process. Wet milling gives a very good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where there is a parallel production of syrups.

25

(ii) Liquefaction

In the liquefaction process the starch granules are solubilized by hydrolysis to maltodextrins mostly of a DP higher than 4. The hydrolysis may be carried out by acid treatment or enzymatically by alpha-amylase. Acid hydrolysis is used on a limited basis. The raw material
30 can be milled whole grain or a side stream from starch processing.

Enzymatic liquefaction is typically carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably 80-85°C, and the enzyme(s) is (are) added. Then the slurry is jet-cooked at between 95-140°C, preferably 105-125°C, cooled to 60-95°C and more enzyme(s) is (are) added to obtain the final hydrolysis. The liquefaction process is carried out at
35 pH 4.5-6.5, typically at a pH between 5 and 6. Milled and liquefied grain is also known as mash.

(iii) Saccharification

To produce low molecular sugars DP1-3 that can be metabolized by yeast, the maltodextrin from the liquefaction must be further hydrolyzed. The hydrolysis is typically done enzymatically by glucoamylases, alternatively alpha-glucosidases or acid alpha-amylases can be used. A full saccharification step may last up to 72 hours, however, it is common only to do a pre-saccharification of typically 40-90 minutes and then complete saccharification during fermentation (SSF). Saccharification is typically carried out at temperatures from 30-65°C, typically around 60°C, and at pH 4.5.

(iv) Fermentation

Yeast typically from *Saccharomyces* spp. is added to the mash and the fermentation is ongoing for 24-96 hours, such as typically 35-60 hours. The temperature is between 26-34°C, typically at about 32°C, and the pH is from pH 3-6, preferably around pH 4-5.

Note that the most widely used process is a simultaneous saccharification and fermentation (SSF) process where there is no holding stage for the saccharification, meaning that yeast and enzyme is added together. When doing SSF it is common to introduce a pre-saccharification step at a temperature above 50°C, just prior to the fermentation.

(v) Distillation

Following the fermentation the mash is distilled to extract the ethanol.

The ethanol obtained according to the process of the invention may be used as, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol.

(vi) By-products

Left over from the fermentation is the grain, which is typically used for animal feed either in liquid form or dried.

Further details on how to carry out liquefaction, saccharification, fermentation, distillation, and recovering of ethanol are well known to the skilled person.

According to the process of the invention the saccharification and fermentation may be carried out simultaneously or separately.

Pulp and Paper Production

Alkaline alpha-amylase polypeptides of the invention may also be used in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste paper and cardboard, especially where re-pulping occurs at pH above 7 and where amylases facilitate the disintegration of the waste material through degradation of the reinforcing starch. The alpha-amylase of the invention is especially useful in a process for producing a papermaking

pulp from starch-coated printed-paper. The process may be performed as described in WO 95/14807, comprising the following steps:

- a) disintegrating the paper to produce a pulp,
- 5 b) treating with a starch-degrading enzyme before, during or after step a), and
- c) separating ink particles from the pulp after steps a) and b).

The alpha-amylases of the invention may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such as calcium carbonate, kaolin and clays. With the alkaline alpha-amylases of the invention it becomes possible
10 to modify the starch in the presence of the filler thus allowing for a simpler integrated process.

Desizing of Textiles, Fabrics and Garments

An alpha-amylase of the invention may also be very useful in textile, fabric or garment
15 desizing. In the textile processing industry, alpha-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size, which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch breakdown is preferred because it does not
20 involve any harmful effect on the fiber material. In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional alpha-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead
25 to some fiber damage because of the rather aggressive chemicals used. Accordingly, it would be desirable to use the alpha-amylases of the invention as they have an improved performance in alkaline solutions. The alpha-amylases may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

Desizing and bleaching processes are well known in the art. For instance, such processes
30 are described in WO 95/21247, US patent 4,643,736, EP 119,920 hereby incorporated by reference.

Commercially available products for desizing include AQUAZYME® and AQUAZYME® ULTRA from Novozymes A/S.

35 *Beer making*

The alpha-amylases of the invention may also be very useful in a beer-making process; the alpha-amylases will typically be added during the mashing process.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

5 The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present
10 disclosure including definitions will control.

EXAMPLES

Example 1: Construction of the parent polypeptide as set forth in SEQ ID NO: 1

15 Construction of a polypeptide having the amino acid sequence set forth SEQ ID NO: 1 (the parent polypeptide) has been described in (WO 2006/002643). The polypeptide is a modified alpha-amylase (EC 3.2.1.1) belonging to the glycoside hydrolase GH13 Family, Subfamily 5.

Example 2: Generation of variants according to the invention

20 The variants of the present invention have been generated by site-directed mutagenesis. Genomic DNA prepared from the organism containing amylase gene at the Pel locus was used as template for generating the site-directed mutants.

Mutagenic forward primer and LBei1303 (5'-CAATCCAAGAGAACCCTGATACGGATG-3'- SEQ ID NO: 4) reverse primer was used to generate a ~2.4 - 3.8 kb fragment. This fragment
25 was used as a megaprimer along with LBei1302 (gatgtatacgtcttagctcagcagcatgac - SEQ ID NO: 5) forward primer to get 6.1 kb insertion cassette. To enable integration in the Pel locus by double cross-over upon transformation, along with the amylase and cat genes, the cassette contained upstream and downstream Pel sequences at the ends. Selection was done on LB Agar containing chloramphenicol and the mutation was confirmed by DNA sequencing of amylase gene.

30 A library of variants were generated where different combinations of alterations in the following positions were made; A51, A186, L202, T246, and S334 of SEQ ID NO: 2.

The generated variants consist of an amino acid sequence of SEQ ID NO:1 with mutations selected from the group consisting of the following:

- (a) L202M;
- 35 (b) A186D;
- (c) L202M+T246I+S334T;
- (d) L202M+T246L+S334T;

(e) A51T+L202M+T246I+S334T;

(f) L202M+T246V; and

(g) L202M+T246V+S334T;

5 wherein numbering of amino acid positions is according to the amino acid sequence set forth in SEQ ID NO: 2.

Example 3: Assay performance of generated variants using *EnzChek*[®]

In order to assess amylase activities of variants in buffer, assay experiments were conducted using *EnzChek*[®] (LifeTechnologies, Cat# E33651) in the supplied assay buffer. Variant-expressing transformants of *B. subtilis* were inoculated in 1 mL suitable medium and grown at 37°C for 3 days. The cultures were centrifuged at 1013 g for 10 min and the supernatants were isolated and stored at -18°C.

The variant enzymes were normalized using polyclonal antibodies (Ab83) raised against Stainzyme Plus in rabbits. Ab83 were precipitated in ammonium sulphate and redissolved in PBST buffer (10 mM phosphate buffer pH 7.4; 2.7 mM KCl, 140 mM NaCl, 0.05% Tween[®] 20) + 0.02% Thiomersal at 31.57 mg/mL.

Ab83 were diluted ~2000 fold in PBS buffer (10 mM phosphate buffer pH 7.4; 2.7 mM KCl, 140 mM NaCl) to approximately 15 µg/ml. MaxiSorp microtiter plates (MTP) (NUNC, Cat. No. 44-2404-21) were coated for 1h with 100 µL of the diluted antibody solution at room temperature and 800 rpm. The antibody solution was discarded and the MTPs were washed with 3x200 µL PBST buffer. Supernatant of each transformant was diluted ten-fold in PBST buffer and 100 µL was added in four replicates to the MTP.

Stainzyme Plus was used as reference and followed the same procedure as the variants tested.

25 The obtained relative activities of the variants to the reference obtained with *EnzChek*[®] in *EnzChek*[®] assay buffer are listed in **Table 1**.

Table 1

Amylase activity of exemplary amylase polypeptides of the invention

Amylase	Relative activity
Reference (SEQ ID NO: 1)	1.00
SEQ ID NO: 1 + L202M	1.03
SEQ ID NO: 1 + A186D	1.02
SEQ ID NO: 1 + L202M+T246I+S334T	1.09
SEQ ID NO: 1 + L202M+T264L+S334T	1.14
SEQ ID NO: 1 + A51T+L202M+T246I+S334T	1.17
SEQ ID NO: 1 + L202M+T246V	1.09

As shown in Table 1, the amylases clearly demonstrate alpha-amylase activity at least on the same level as the parent polypeptide.

5

Example 4: Alternative assays for Alpha-Amylase Activity

1. Phadebas assay

Alpha-amylase activity may be determined by a method employing *Phadebas*[®] tablets as substrate. Phadebas tablets (*Phadebas*[®] Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance and tableted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl₂, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

2. PNP-G7 assay

Alpha-amylase activity is determined by a method employing the PNP-G7 substrate. PNP-G7 which is an abbreviation for *p*-nitrophenyl-alpha,D-maltoheptaoside is a blocked oligo-saccharide which can be cleaved by an endo-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectrophometry at $\lambda=405\text{nm}$ (400-420 nm). Kits containing PNP-G7 substrate and alpha-Glucosidase is manufactured by Boehringer-Mannheim (cat. No.1054635).

To prepare the reagent solution 10 ml of substrate/buffer solution is added to 50 ml enzyme/buffer solution as recommended by the manufacturer. The assay is performed by transferring 20 micro l sample to a 96 well microtitre plate and incubating at 25°C. 200 micro l reagent solution pre-equilibrated to 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 30 sec. over 4 minutes at OD 405 nm in an ELISA reader.

The slope of the time dependent absorption-curve is directly proportional to the activity of the alpha-amylase in question under the given set of conditions.

10 **Example 5: Assessment of wash performance of alpha-amylase polypeptides of the invention using Automatic Dish Wash (ADW)**

In order to assess the wash performance of the polypeptides of the present invention in a detergent base composition, washing experiments may be performed using full scale Automatic Dish Wash (ADW). The full scale ADW setup is used for testing the wash performance of polypeptides in test conditions mimicking a regular consumer setup.

In the present study, test conditions were a regular 50°C wash program and a short 40°C program using a Miele Dishwasher Miele G4300 SCU machine.

General wash performance description.

Melamine tiles stained with starch (CS-28 from Center For Test materials BV, P.O. Box 120, 3133 KT, Vlaardingen, The Netherlands) were used as test material and washed at set programs at 40°C and 50°C using water with 7.68 °dH, as specified below. Three minutes after the machine program has begun, 15.53 g/wash detergent, P&G Hybrid detergent as described below, and the amylase polypeptide of the invention at concentration of 2.55 mg enzyme/wash or 5.12 mg enzyme/wash are added. After a thorough rinse under running tap water and drying in the dark, the light remission values of the soiled tiles were subsequently assessed as a measure of wash performance. The test with 0 mg enzyme protein/L was used as a blank and corresponded to the contribution from the detergent alone. The full scale wash performance experiments were conducted under the experimental conditions specified below:

Table 2

Experimental condition

Detergent	Powder ADW detergent P&G hybrid (see Table 3)
Detergent dosage	15.53 g/wash
pH	As is
Wash time	Set program.

Temperature	40°C or 50°C
Water hardness	7.68°dH
Amylase concentration in test	2.55 mg polypeptide/wash or 5.12 mg polypeptide /wash
Test material	DM-77 and DM-177 at 40°C or DM-277 and DM-377 at 50°C. All mixed starch melamine tiles.

Table 3

ADW detergent

5

Component	Grams of active material
Solid ingredients	
Sodium carbonate	4.00
MGDA	6.00
Sodium percarbonate	2.00
Sulfonated polymer	1.20
Bleach catalyst	0.009
Miscellaneous	Balance to 15.53
Liquid ingredients	
Lutensol TO7	1.165
LF224	0.5826
Miscellaneous	Balance to 1.8

MGDA Three-sodium Methyl glycine diacetate supplied by BASF

Bleach catalyst Pentaamineacetatocobalt (III) nitrate

LF224 Nonionic surfactant supplied by BASF

10 Sulfonated polymer Acusol 588 supplied by Dow Chemicals

Lutensol TO7 Nonionic surfactant supplied by BASF

Water hardness was adjusted to 7.68°dH by mixing tap water and deionized water to the test system. After washing the melamine tiles were flushed in tap water and dried. The wash
15 performance was measured as difference in remission. The remission measurements were made with a Color-Eye 7000 (CE7000) used for taking spectra and performing calculations of remission and/or colour difference. The remission was measured at 460 nm with no UV light in the illuminant.

The term "improved wash performance" of the present experiment is defined as displaying an alteration of the wash performance of a variant of the present invention relative to the wash
20 performance of the polypeptide having an amino acid sequence as set forth in SEQ ID NO: 1,

The alteration may e.g. be seen as increased stain removal. Improved wash performance was determined as described above. The wash performance was considered to be improved if the Improvement Factor (IF) is at least 1.0, preferably at least 1.2 in one or more of the conditions listed above; *i.e.* either at 40°C or 50°C.

- 5 The wash performance of the variants according to the invention obtained by full scale wash are the following;

Table 4

ADW improvement factors of exemplary polypeptides of the invention

10

Amylase poly-peptide (SEQ ID NO: 1 + mutations)	40°C DM177		40°C DM277		40°C DM77	
	0.47mg/L	0.94mg/L	0.47mg/L	0.94mg/L	0.47mg/L	0.94mg/L
L202M			1.19	1.56	1.00	1.09
A186D	0.95	1.00			1.01	0.98
L202M+T246V	1.42	1.16			1.14	1.05
L202M+T246I+S334T	1.24	1.13			1.10	1.01
L202M+T246V+S334T	1.94	1.38			1.49	1.16
L202M+T246L+S334T	1.38	1.22			1.13	0.99
A51T+L202M+T246I+S334T	1.14	1.14			1.07	1.01

* wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

Mutations in SEQ ID NO: 1	50°C DM277		50°C DM377	
	0.47mg/L	0.94mg/L	0.47mg/L	0.94mg/L
L202M	1.27	1.08		
A186D	1.39	1.13	1.28	1.05
L202M T246V	1.45	1.14	1.25	1.05
L202M T246I S334T	1.78	1.36		
L202M T246V S334T	1.82	1.16	1.55	1.16
L202M T246L S334T	1.82	1.28		
A51T L202M T246I S334T	1.78	1.33		

* wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

Example 6: Assessment of wash performance of alpha-amylase polypeptides of the invention using Automatic Mechanical Stress Assay (AMSA)

In order to assess the wash performance of the variants of the present invention in a detergent base composition, washing experiments may be performed using Automatic Mechanical Stress Assay (AMSA). With the AMSA test the wash performance of a large quantity of small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid firmly squeezing the textile swatch or melamine tiles to be washed against all the slot openings. During the washing time, the plate, test solutions, textile/melamine tiles and lid were vigorously shaken to bring the test solution in contact with the textile/melamine tiles and apply mechanical stress in a regular, periodic oscillating manner. For further description see WO 02/42740, especially the paragraph "Special method embodiments" at page 23-24.

General wash performance description

A test solution comprising water (7.68° dH), 2.88 g/L detergent, P&G Hybrid detergent as described above, and the enzyme of the invention at concentration as listed in Tables 8, 9, and 10 prepared. Fabrics or melamine tiles stained with starch (CS-28 and DM-277 from Center For Test materials BV, P.O. Box 120, 3133 KT, Vlaardingen, The Netherlands) was added and washed for 10 or 20 minutes at 40°C and 50°C, as specified below. After thorough rinse under running tap water and drying in the dark, the light intensity values of the stained fabrics were subsequently measured as a measure for wash performance. The test with 0 mg enzyme protein/L was used as a blank and corresponded to the contribution from the detergent. Preferably mechanical action was applied during the wash step, e.g. in the form of shaking, rotating or stirring the wash solution with the fabrics and tiles. The AMSA wash performance experiments were conducted under the experimental conditions specified below:

25

Table 5
Experimental condition

Detergent	Powder ADW detergent P&G hybrid (see Table B)
Detergent dosage	2.88 g/L
Test solution volume	160 micro L
pH	As is
Wash time	10 or 20 minutes
Temperature	40°C or 50°C
Water hardness	7.68°dH
Enzyme concentration in test	See Tables 8, 9, and 10
Test material	CS-28 (Rice starch cotton) or DM-277 (Mixed starch melamine tiles)

5

Table 6
ADW detergent

Component	Grams of active material
Solid ingredients	
Sodium carbonate	4.00
MGDA	6.00
Sodium percarbonate	2.00
Sulfonated polymer	1.20
Bleach catalyst	0.009
Miscellaneous	Balance to 15.53
Liquid ingredients	
Lutensol TO7	1.165
LF224	0.5826
Miscellaneous	Balance to 1.8

10	MGDA	Three-sodium Methyl glycine diacetate supplied by BASF
	Bleach catalyst	Pentaamineacetatocobalt (III) nitrate
	LF224	Nonionic surfactant supplied by BASF
	Sulfonated polymer	Acusol 588 supplied by Dow Chemicals
	Lutensol TO7	Nonionic surfactant supplied by BASF

15

Water hardness was adjusted to 7.68°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the textiles and melamine tiles were flushed in tap water and dried.

The wash performance was measured as the brightness expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample was stained the intensity of the reflected light was lower, than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance.

Color measurements were made with a professional flatbed scanner (EPSON Expression 10000XL, EPSON) used to capture an image of the washed textile.

To extract a value for the light intensity from the scanned images, 48×24 Bit Color pixel values from the image were converted into values for red, green and blue (RGB). The intensity value (Int) is calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

$$Int = \sqrt{r^2 + g^2 + b^2}$$

The wash performance of the variants according to the invention obtained by AMSA are the following;

For each condition below, the value is an average of performance Improvement factor measured with 4 different enzyme dosages.

Table 7
Average AMSA Performance Improvement Factor (IF)

	40°C CS28	50°C CS28	40°C DM277
Mutations in SEQ ID NO:1*	Average IF	Average IF	Average IF
L202M	0.9	1.1	0.9
A186D	0.9	0.8	0.8
L202M+T246V	1.4	1.9	1.8
L202M+T246I+S334T	1.2	1.4	0.8
L202M+T246V+S334T	1.2	1.3	0.9
L202M+T246L+S334T	1.3	1.4	1.0
A51T+L202M+T246I+S334T	1.3	1.6	0.8

* wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

Detergent: P&G ADW hybrid 1

Temp = As indicated

CS28: Textile

DM277: Tiles with a mixture of rice and corn starch

Table 8

AMSA dataset I (40°C - 10 minutes - CS28)

Mutations in SEQ ID NO:1*	0.03mg/L			0.06 mg/L		
	Mean Δ Int	Std dev	IF	Mean Δ Int	Std dev	IF
L202M	11.5	5.8	0.9			
A186D	13.2	3.1	0.8	23.5	1.1	0.9
L202M T246V	19.8	3.9	1.7	23.9	4.0	1.3
L202M T246I S334T	11.3	5.8	1.5	19.9	7.2	1.3
L202M T246V S334T	14.1	2.5	1.9	19.1	3.6	1.2
L202M T246L S334T	13.2	3.0	1.8	19.6	3.3	1.2
A51T L202M T246I S334T	12.5	8.1	1.7	18.5	3.9	1.2
Mutations in SEQ ID NO:1*	0.12mg/L			0.23 mg/L		
	Mean Δ Int	Std dev	IF	Mean Δ Int	Std dev	IF
L202M						
A186D	29.9	3.4	0.9	34.3	6.9	0.9
L202M T246V	32.7	4.4	1.4	37.2	4.7	1.3
L202M T246I S334T	24.7	4.5	1.1	27.1	3.9	0.9
L202M T246V S334T	20.6	2.6	0.9	27.2	8.1	0.9
L202M T246L S334T	26.8	5.4	1.2	30.7	6.3	1.1
A51T L202M T246I S334T	29.5	2.4	1.3	30.5	4.0	1.1

5 * wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

Table 9

AMSA dataset II (50°C - 20 minutes - CS28)

Mutations in SEQ ID NO:1*	0.01mg/L			0.03 mg/L		
	Mean Δ Int	Std dev	IF	Mean Δ Int	Std dev	IF
L202M				36.6	5.2	1.1
A186D	5.5	3.8	0.6	14.1	3.9	0.8
L202M T246V	43.3	4.5	3.9	49.2	3.3	1.6
L202M T246I S334T	19.9	1.6	2.1	30.4	3.9	1.1
L202M T246V S334T	19.1	3.3	2.0	30.4	4.5	1.1
L202M T246L S334T	24.8	4.1	2.6	31.1	2.8	1.1
A51T L202M T246I S334T	25.6	1.0	2.7	37.4	4.3	1.3
Mutations in SEQ ID NO:1*	0.06mg/L			0.12 mg/L		
	Mean Δ Int	Std dev	IF	Mean Δ Int	Std dev	IF
L202M						
A186D	31.7	5.1	1.0	43.3	3.8	1.0

L202M T246V	50.7	2.7	1.2	49.1	6.4	1.1
L202M T246I S334T	41.6	2.6	1.2	46.4	3.3	1.0
L202M T246V S334T	37.0	5.9	1.0	45.8	2.5	1.0
L202M T246L S334T	37.7	6.1	1.1	41.0	8.0	0.9
A51T L202M T246I S334T	41.9	3.8	1.2	47.1	3.2	1.1

* wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

5

Table 10
AMSA dataset III (40°C - 10 minutes - DM277)

Mutations in SEQ ID NO:1*	0.47mg/L			0.94 mg/L		
	Mean ΔInt	Std dev	IF	Mean ΔInt	Std dev	IF
L202M	26.3	7.7	0.9	50.3	6.5	0.9
A186D	23.1	1.6	0.8	46.1	10.6	0.9
L202M T246V	55.0	10.0	2.1	80.1	14.1	1.4
L202M T246I S334T	28.6	9.7	0.9	50.0	8.6	0.8
L202M T246V S334T	28.4	10.9	0.9	50.7	8.4	0.8
L202M T246L S334T	34.9	8.6	1.1	56.5	9.8	0.9
A51T L202M T246I S334T	25.6	6.0	0.8	45.9	9.9	0.8

* wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2

CLAIMS

1. A polypeptide comprising a variant amino acid sequence of SEQ ID NO: 1, wherein the polypeptide has alpha-amylase activity and exhibits an improved wash performance compared to the parent polypeptide of SEQ ID NO: 1, said variant amino acid sequence comprises a mutation at amino acid position corresponding to positions 202 and/or 186 wherein numbering is according to SEQ ID NO: 2, and has at least 80% sequence identity to SEQ ID NOs: 1 or 2.
2. A polypeptide according to claim 1 wherein the variant amino acid sequence comprises a substitution at amino acid position 202 wherein numbering is according to SEQ ID NO: 2, such as L202M.
3. A polypeptide according to claim 1 wherein the variant amino acid sequence comprises a mutation at amino acid position 186 wherein numbering is according to SEQ ID NO: 2, such as A186D.
4. A polypeptide according to any one of the preceding claims wherein the improved wash performance is assessed using an Automatic Mechanical Stress Assay (AMSA) and/or a full scale automatic dishwasher (ADW) assay.
5. A polypeptide according to any one of the preceding claims wherein the improved wash performance is exhibited during high temperature washes.
6. A polypeptide according to claim 5, wherein said high temperature is at least 40°C, such as at least 45°C, and such as at least 50°C.
7. A polypeptide according to any one of claims 5 to 6, wherein said polypeptide exhibits an improved wash performance in an AMSA assay under one or more of the conditions selected from the group consisting of:
- (a) ADW detergent at 40°C and a wash cycle of 10 min; and
 - (b) ADW detergent at 50°C and a wash cycle of 20 min;
- wherein the polypeptide is present at a concentration of 0.03 or 0.24 mg/L and wherein said ADW detergent comprises a bleach catalys, non-ionic surfactant(s), and a sulfonated polymer.
8. A polypeptide according to any one of the preceding claims wherein the polypeptide is 1000 or fewer amino acids in length, for example 900, 800, 700, 600, 500, 400, 300, 200, 175, 150, 125, 100 or fewer amino acids.

9. A polypeptide according to claim 8 wherein the polypeptide is between 400 and 600 amino acids in length, for example between 450 and 500, between 460 and 500, or between 470 and 490 amino acids in length.
- 5 10. A polypeptide according to any one of the preceding claims, wherein said polypeptide comprises a mutation at one or more positions corresponding to positions 51, 186, 202, 246 and 334, wherein numbering is according to the amino acid sequence set forth in SEQ ID NO: 2.
11. A polypeptide according to any one of the preceding claims, wherein said mutation at one
10 or more positions is a substitution, deletion, and/or insertion.
12. A polypeptide according to any one of the preceding claims, wherein said polypeptide comprises one or more of the following substitutions relative to the amino acid sequence of SEQ ID NO:2; A51T, A186D, L202M, T246(I/L/V) and S334T.
- 15 13. A polypeptide according to any one of the preceding claims, wherein said polypeptide comprises or consists of an amino acid sequence which shares at least 95% sequence identity with SEQ ID NO: 1, for example at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 1.
- 20 14. A polypeptide according to any one of the preceding claims, wherein the number of mutations relative to SEQ ID NO:1 is 1-20, e.g., 1-10 and 1-5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mutations.
- 25 15. A polypeptide according to any one of the preceding claims, wherein said polypeptide consists of the amino acid sequence of SEQ ID NO:2 with mutations selected from the group consisting of; A51T+A186D, A51T+L202M, A51T+T246I, A51T+S334T, A186D+L202M, A186D+T246I, A186D+S334T, L202M+T246I, L202M+S334T, T246I+S334T, A51T+A186D+L202M, A51T+A186D+T246I, A51T+A186D+S334T, A51T+L202M+T246I,
30 A51T+L202M+S334T, A51T+T246I+S334T, A186D+L202M+T246I, A186D+L202M+S334T, A186D+T246I+S334T, L202M+T246I+S334T, A51T+A186D+L202M+T246I, A51T+A186D+L202M+S334T, A51T+A186D+T246I+S334T, A51T+L202M+T246I+S334T, A186D+L202M+T246I+S334T, and A51T+A186D+L202M+T246I+S334T, or a fragment thereof having alpha amylase activity.
- 35 16. A polypeptide according to any one of claims 1 to 14, wherein said polypeptide comprises or consists the amino acid sequence of SEQ ID NO:2 with mutations selected from the group consisting of:

- (a) L202M;
(b) A186D;
(c) L202M+T246I+S334T;
(d) L202M+T246T+S334T;
5 (e) A51T+L202M+T246I+S334T;
(f) L202M+T246V; and
(g) L202M+T246V+S334T.
17. A polypeptide according to any one of the preceding claims, wherein said polypeptide
10 consists of the amino acid sequence of SEQ ID NO: 3.
18. A polynucleotide encoding said polypeptide according to any one of claims 1 to 17.
19. A nucleic acid construct comprising said polynucleotide according to claim 18.
15
20. An expression vector comprising said polynucleotide according to claim 18.
21. A host cell comprising said polynucleotide according to claim 18.
- 20 22. A method of producing an alpha-amylase polypeptide, comprising:
- (a) cultivating said host cell according to claim 21 under conditions suitable for
expression of said polypeptide; and
(b) recovering said polypeptide.
25

SEQ:1 HHNGTNGTLMQYFEWYLPNDGNHWNRLRSDASNLKDKGISA VWI PPAWK GASQNDVGYGA
 SEQ:2 HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLKDKGISA VWI PPAWK GASQNDVGYGA
 *****:*****

SEQ:1 YDLYDLGEFNGKGTIRTKYGTRNQLQA AVNALKSNGIQVYGDVVMNHKGGADATEMVKAV
 SEQ:2 YDLYDLGEFNGKGTIRTKYGTRNQLQA AVNALKSNGIQVYGDVVMNHKGGADATEMVRAV
 *****. **

SEQ:1 EVNPNNRNQEVSGEYTI EA WTKFDFPGRANTHSNFKWRWYHFDGVDWDQSRKLNRIYKF
 SEQ:2 EVNPNNRNQEVSGEYTI EA WTKFDFPGRANTHSNFKWRWYHFDGVDWDQSRKLNRIYKF
 *****. *****

SEQ:1 RT--KAWDWEVDTEFGNYDYLLYADIDMDHPEVVNELRNWGVWYTN T LGLDGFRIDAVKH
 SEQ:2 RGDGKGDWEVDTEFGNYDYLLYADIDMDHPEVVNELRNWGVWYTN T LGLDGFRIDAVKH
 * * .***** *****:*****

SEQ:1 IKYSFTRDWINHVRSAIGKNMFAVAEFWKNDLGAIENYLNKTNWNH SVFDVPLHFNLYYA
 SEQ:2 IKYSFTRDWINHVRSAIGKNMFAVAEFWKNDLGAIENYLNKTNWNH SVFDVPLHYNLYNA
 ***** *****:*** *

SEQ:1 SKSGGNYDMRQIFNGTVVQKHPH AVTFVDNHDSQPEESLESFVREWFKPLAYALTLTRE
 SEQ:2 SKSGGNYDMRQIFNGTVVQRHPH AVTFVDNHDSQPEEALLESFVEEWFKPLAYALTLTRE
 *****. ** *****:***** *****

SEQ:1 QGYPSVFGDYGGIPTHGVPAMKSKIDPILEARQKYAYGRQNDYLDH HNIIGWTREGNTA
 SEQ:2 QGYPSVFGDYGGIPTHGVPAMKSKIDPILEARQKYAYGRQNDYLDH HNIIGWTREGNTA

SEQ:1 HPNSGLATIMSDGAGGNKWMFVGRNKAGQVWTDITGNKAGTVTINADGWGNF SVNGGSVS
 SEQ:2 HPNSGLATIMSDGAGGNKWMFVGRNKAGQVWTDITGNRAGTVTINADGWGNF SVNGGSVS
 *****. *****

SEQ:1 IWV NK
 SEQ:2 IWV NK

Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/060473

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435 C12N9/28
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)
C07K
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 practicable, search terms used)
EPO-Internal, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE Geneseq [Online] 18 August 2011 (2011-08-18), "Bacillus sp. alpha-amylase mutein (D188N-D209S).", XP002759475, retrieved from EBI accession no. GSP:AZJ61660 Database accession no. AZJ61660 sequence & WO 2011/080353 A1 (NOVOZYMES AS [DK]; ANDERSEN CARSTEN [DK]) 7 July 2011 (2011-07-07) ----- -/--</p>	1,2,14, 18-22

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search 6 June 2017	Date of mailing of the international search report 16/06/2017
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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 Gómez Ortiz, Mariola
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/060473

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE Geneseq [Online]</p> <p>1 August 2013 (2013-08-01), "Bacillus sp. wild type alpha amylase (AA560), SEQ ID 4.", XP002759582, retrieved from EBI accession no. GSP:BAP28635 Database accession no. BAP28635 sequence & DE 10 2011 088751 A1 (HENKEL AG & CO KGAA [DE]) 20 June 2013 (2013-06-20)</p> <p>-----</p>	1,2,14, 18-22
X	<p>WO 2015/144780 A2 (NOVOZYMES AS [DK]) 1 October 2015 (2015-10-01) Example 4; SEQ ID NO 3; Claims and page 29. -& DATABASE Geneseq [Online]</p> <p>19 November 2015 (2015-11-19), "Automatic dishwashing detergent composition related amylase, SEQ ID 3.", XP002759476, retrieved from EBI accession no. GSP:BCE94625 Database accession no. BCE94625 sequence</p> <p>-----</p>	4-11,13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/060473

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO 2015144780	A2	01-10-2015	EP 3122860 A2	01-02-2017
			US 2017073619 A1	16-03-2017
			WO 2015144780 A2	01-10-2015
