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(54) Title: ALICYCLOBACILLUS VARIANTS AND POLYNUCLEOTIDES ENCODING SAME

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

ALICYCLOBACILLUS VARIANTS AND POLYNUCLEOTIDES ENCODING SAME**REFERENCE TO A SEQUENCE LISTING**

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

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

10 The present invention relates to Alicyclobacillus variants, polynucleotides encoding the variants, methods of producing the variants, and methods of using the variants.

Description of the Related Art

Alpha-amylases have for many years been used in laundry where it is well-known that alpha-amylases have a beneficial effect in removal of starch containing stains.

15 WO95/26397 discloses alkaline *Bacillus* amylases having good wash performance measured at temperatures in the range of 30-60°C.

WO00/60060 and WO00/60058 discloses further bacterial alpha-amylases having good wash performance.

20 In recent years there has been a desire to reduce the temperature of the laundry in order to reduce the energy consumption. Lowering the temperature in laundry often means that the performance of the detergent composition and the enzyme is reduced and a lower wash performance is therefore obtained at low temperature. It is therefore desired to find new alpha-amylases having good wash performance at low temperature. Accordingly, it is an object of the present invention to provide alpha-amylases which have good wash performance at low
25 temperature, such as at 15°C. It is another object to provide alpha-amylases which have improved wash performance in model detergent J (see below) compared to the above mentioned known alpha-amylases such as the alpha-amylase disclosed as SEQ ID NO: 2 in WO00/60060.

30 SUMMARY OF THE INVENTION

The present invention provides variants having alpha-amylase activity and polynucleotides encoding the polypeptides. It further provides methods of obtaining the same.

35 Accordingly, the present invention relates to an alpha-amylase variant, comprising a substitution at one or more positions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide according to SEQ ID NO:3, wherein the variant has alpha-amylase activity, and wherein said parent alpha-amylase has at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, sequence identity to the polypeptide of SEQ ID NOs: 3 or 13.

The present invention also relates to polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of producing the variants.

5 The invention also relates to compositions comprising the variants. In particular, detergent compositions are disclosed herein.

The present invention also relates to the use of a variant according to the invention in a cleaning process, such as laundry and hard surface cleaning.

The present invention also provides a method of obtaining a variant having alpha-
10 amylase activity, wherein the method comprises the steps of introducing a substitution in one or more positions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide according to SEQ ID NO:3, wherein the variant has at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%,
15 such as at least 95%, such as at least 96%, such as at least 97%, such as at least 99%, but less than 100% sequence identity with the amino acid sequence of SEQ ID NOs: 3 or 13, wherein the variant has alpha-amylase activity; and recovering said variant.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a sequence alignment of the amino acid sequence of SEQ ID NO: 3 and
20 SEQ ID NO: 11.

DEFINITIONS

Alpha-amylase: The term “alpha-amylase” as used herein, refers to an enzyme that has alpha-amylase activity (EC 3.2.1.1) that hydrolyses alpha bonds of large, alpha-linked
25 polysaccharides, such as starch and glycogen, yielding glucose and maltose. The terms “alpha-amylase” and “amylase” may be used interchangeably and constitute the same meaning and purpose within the scope of the present invention. For purposes of the present invention, alpha-amylase activity is determined according to the procedure described in the Examples. In one aspect, the variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%,
30 at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the polypeptide of SEQ ID NO: 3. The alpha-amylase activity may be determined according to a method using the Amylazyme substrate which is described in the section “Amylazyme activity assay”.

Allelic variant: The term “allelic variant” as used herein, refers to any of two or more
35 alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having

altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

cDNA: The term "cDNA" as used herein, refers to 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" as used herein, refers to 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" as used herein, refers to 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.

Expression: The term "expression" as used herein, refers to any step involved in the production of a variant including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" as used herein, refers to 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.

Fragment: The term "fragment" as used herein, refers to a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has alpha-amylase activity. In one aspect, a fragment contains at least 85% of the amino acid residues of SEQ ID NO:3, at least 90% of the amino acid residues of SEQ ID NO:3, or at least 95% of the amino acid residues of SEQ ID NO:3.

High stringency conditions: The term "high stringency conditions" as used herein, refers to for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The

carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

Very high stringency conditions: The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 70°C.

Host cell: The term "host cell" as used herein, refers to 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.

Improved property: The term "improved property" as used herein, refers to a characteristic associated with a variant that is improved compared to the parent. Such improved properties include, but are not limited to, performance, such as wash performance, stability, such as stability under storage conditions.

Isolated: The term "isolated" as used herein, refers to 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 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.

Low stringency conditions: The term "low stringency conditions" as used herein, refers to probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 50°C.

Very low stringency conditions: The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C.

Mature polypeptide: The term "mature polypeptide" as used herein, refers to a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. Also contemplated within the term "mature polypeptide" is that the signal peptide of the polypeptide has been cleaved off e.g. during a natural maturation process within the cell expressing the polypeptide. In one aspect, the mature polypeptide is the amino acid sequence of SEQ ID NO:3. 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.

Polynucleotide coding sequence: The term "polynucleotide coding sequence" as used herein, refers to a polynucleotide that encodes a mature polypeptide having alpha-amylase activity. In one aspect, the polynucleotide coding sequence is nucleotides of SEQ ID NO: 1.

Medium stringency conditions: The term "medium stringency conditions" as used herein, refers to probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 55°C.

Medium-high stringency conditions: The term "medium-high stringency conditions" as used herein, refers to probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

Mutant: The term "mutant" as used herein, refers to a polynucleotide encoding a variant. Thus, the terms "mutant" and "variant" may be used interchangeably and constitute the same meaning and purpose within the present invention.

Nucleic acid construct: The term "nucleic acid construct" as used herein, refers to 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.

Operably linked: The term "operably linked" as used herein, refers to 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 terms "parent" or "parent alpha-amylase" may be used interchangeably herein, both terms refer to an alpha-amylase to which an alteration is made to produce the enzyme variants of the present invention. The parent may be a naturally occurring (wild-type) polypeptide or a variant or fragment thereof.

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

For the purposes of the present invention, the sequence identity between two amino acid sequences may be determined using the program Vector NTI® which is well-known in the art.

5 Another well-known program is the ClustalW program. Thus, identification of the corresponding amino acid residue in another alpha-amylase may 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: 1899-1880), and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson *et al.*, 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

15 The sequence identity between two amino acid sequences may also be 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. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

25 The sequence identity between two deoxyribonucleotide sequences may also be determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are 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:

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

35 **Subsequence:** The term “subsequence” as used herein, refers to 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.

Enzyme Detergency benefit: The term “enzyme detergency benefit” used herein, refers to the advantageous effect an enzyme may add to a detergent compared to the same detergent without the enzyme. Important detergency benefits which can be provided by enzymes are stain removal with no or very little visible soils after washing and/or cleaning, prevention or reduction of re-deposition of soils released in the washing process (an effect that also is termed anti-redeposition), restoring fully or partly the whiteness of textiles which originally were white but after repeated use and wash have obtained a greyish or yellowish appearance (an effect that also is termed whitening). Textile care benefits, which are not directly related to catalytic stain removal or prevention of re-deposition of soils, are also important for enzyme detergency benefits. Examples of such textile care benefits are prevention or reduction of dye transfer from one fabric to another fabric or another part of the same fabric (an effect that is also termed dye transfer inhibition or anti-backstaining), removal of protruding or broken fibers from a fabric surface to decrease pilling tendencies or remove already existing pills or fuzz (an effect that also is termed anti-pilling), improvement of the fabric-softness, colour clarification of the fabric and removal of particulate soils which are trapped in the fibers of the fabric or garment. Enzymatic bleaching is a further enzyme detergency benefit where the catalytic activity generally is used to catalyze the formation of bleaching component such as hydrogen peroxide or other peroxides.

Textile care benefit: The term “textile care benefits”, as used herein, is defined as not being directly related to catalytic stain removal or prevention of re-deposition of soils, are also important for enzyme detergency benefits. Examples of such textile care benefits are prevention or reduction of dye transfer from one textile to another textile or another part of the same textile (an effect that is also termed dye transfer inhibition or anti-backstaining), removal of protruding or broken fibers from a textile surface to decrease pilling tendencies or remove already existing pills or fuzz (an effect that also is termed anti-pilling), improvement of the textile-softness, colour clarification of the textile and removal of particulate soils which are trapped in the fibers of the textile. Enzymatic bleaching is a further enzyme detergency benefit where the catalytic activity generally is used to catalyze the formation of bleaching component such as hydrogen peroxide or other peroxides or other bleaching species.”

Dish washing composition: The term “dish washing composition” as used herein, refers to all forms of compositions for cleaning hard surfaces. The present invention is not restricted to any particular type of dish wash composition or any particular detergent. Thus, in one embodiment, the dish washing composition is a liquid dish washing composition, a powder dish washing composition, wherein the composition may optionally be in the form of a unit dose.

Hard surface cleaning: The term “hard surface cleaning” as used herein, refers to cleaning of hard surfaces wherein hard surfaces may include floors, tables, walls, roofs etc. as well as surfaces of hard objects such as cars (car wash) and dishes (dish wash). Dish washing includes but are not limited to cleaning of plates, cups, glasses, bowls, cutlery such as spoons, knives, forks, serving utensils, ceramics, plastics, metals, china, glass and acrylics.

Improved wash performance: The term “improved wash performance” as used herein, refers to an improvement of the wash performance of an alpha-amylase of the present invention relative to the wash performance of the alpha-amylases known in the art. Improved wash performance may be measured by comparing of the so-called Intensity value. The improved wash performance is determined according to the section “Wash performance of alpha-amylases using Automatic Mechanical Stress Assay” and using model detergent J at 15°C. Other model detergents may be used, such as model detergent A, model detergent X or model detergent T.

Wash performance: the term “wash performance” as used herein, refers to an enzyme’s ability to remove starch or starch-containing stains present on the object to be cleaned during e.g. laundry or hard surface cleaning, such as dish wash. The term “wash performance” includes cleaning in general e.g. hard surface cleaning as in dish wash, but also wash performance on textiles such as laundry, and also industrial and institutional cleaning. The wash performance may be quantified by calculating the so-called Intensity value. Wash performance may be determined as in described in the Methods section herein.

Low temperature: “Low temperature” as used herein, refers to is a temperature of 5-40°C, such as 5-35°C, preferably 5-30°C, more preferably 5-25°C, more preferably 5-20°C, most preferably 5-15°C, and in particular 5-10°C. In a preferred embodiment, “Low temperature” is a temperature of 10-35°C, preferably 10-30°C, more preferably 10-25°C, most preferably 10-20°C, and in particular 10-15°C. Most preferred, low temperature means 15 °C.

Intensity value: The wash performance is measured as the brightness expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower, than that of a clean sample. Therefore, the intensity of the reflected light can be used to measure wash performance, where a higher intensity value correlates with higher wash performance. Color measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak) used to capture an image of the washed textile. To extract a value for the light intensity from the scanned images, 24-bit pixel values from the image are 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:

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

Variant: The term “variant” means a polypeptide having alpha-amylase activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (*e.g.*, several) positions as compared to a “parent”. 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 at least one (*e.g.* several) amino

acids, e.g. 1 to 5 amino acids, adjacent to and immediately following the amino acid occupying a position. Amino acid substitutions may exchange a native amino acid for another naturally-occurring amino acid, or for a non-naturally-occurring amino acid derivative. 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 activity of the mature polypeptide of SEQ ID NO: 3.

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 fungus found in nature.

Conventions for Designation of Variants

For purposes of the present invention, the polypeptide disclosed in SEQ ID NO: 3 is used to determine the corresponding amino acid residue in another alpha-amylase. The amino acid sequence of another alpha-amylase is aligned with the polypeptide disclosed in SEQ ID NO: 3, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the polypeptide disclosed in SEQ ID NO: 3 is determined. In particular, Figure 1 shows an alignment of SEQ ID NO:3 and SEQ ID NO:11 both included herein. Thus, unless otherwise explicitly stated, amino acid positions herein described are numbered according to SEQ ID NO: 3, and alignment to determine the corresponding amino acid position in other polypeptides may be needed.

Thus, 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: 1899-1880), 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 enzyme 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 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. Multiple substitutions may alternatively be separated by “/”, “,” or a “ ” (i.e. space), and constitute the same meaning and purpose.

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*”. Multiple deletions may alternatively be separated by “/”, “,” or a “ ” (i.e. space), and constitute the same meaning and purpose.

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:

Parent:	Variant:
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”.

Different alterations may be also be separated by “/”, “,” or a “ ” (i.e. space), and constitute the same meaning and purpose. Accordingly, the substitution of threonine at position 226 with alanine or lysine or valine, is designated as “Thr226Ala/Lys/Val” or “T226A/K/V”.

20 DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention relates to variants having improved performance, in particular improved wash performance in laundry and/or dish wash, compared to the polypeptide of the parent alpha-amylase. Thus, in one embodiment, the alpha-amylase variant has improved performance, in particular improved wash performance in laundry and/or dish wash, compared to the polypeptide of the parent alpha-amylase.

Thus, in one aspect, the present invention relates to an alpha-amylase variant, comprising a substitution at one or more positions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide according to SEQ ID NO:3, wherein the variant has alpha-amylase activity, and wherein said parent alpha-amylase has at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, sequence identity to the polypeptide of SEQ ID NOs: 3 or 13.

The term “variant” as used herein, refers to a polypeptide which has been altered in specific amino acid positions, and thus, comprises a different amino acid sequence than the parent polypeptide.

The terms "parent polypeptide" or "parent alpha-amylase" as used herein, refers to a polypeptide which has at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13. Thus, in one embodiment, the parent polypeptide has at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NO: 3. Thus, in one embodiment, the parent polypeptide has at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NO: 13. The parent polypeptide according to SEQ ID NOs: 3 or 13 may comprise alterations in positions which are not positions 51, 109, 193, 201, 269, 294, 298, and 314 when referring to SEQ ID NO: 3. *I.e.* when the parent alpha-amylase is a polypeptide having at least 89% sequence identity to SEQ ID NO: 13, and the numbering is according to SEQ ID NO: 13, the parent alpha-amylase may comprise alterations in positions which are not positions 51, 109, 195, 203, 271, 296, and 316. In one embodiment, the parent polypeptide is a polypeptide which in at least the amino acid positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 when referring to SEQ ID NO: 3 has the amino acids Q, M, N, G, T, M, Q, A, and N, respectively. Accordingly, the amino acid positions of a parent polypeptide as used in relation to the present invention at least have the following amino acids; Q51, M109, N193, G201, T269, M294, Q297, A298, and N314. In a further embodiment, the parent polypeptide has at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13 but wherein the amino acids in positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 when referring to SEQ ID NO: 3 are the amino acids Q, M, N, G, T, M, Q, A, and N, respectively.

The parent polypeptide of the present invention is not limited to the polypeptide of SEQ ID NO: 3, but may also refer to other known alpha-amylase sequences, such as the sequence set forth in SEQ ID NO: 6, 7, 8, 9, 10, 11, 12, or 13. The skilled person knows how to align any of SEQ ID NOs: 6, 7, 8, 9, 10, 11, 12, or 13 with SEQ ID NO: 3 as described herein in order to determine which position the variant according to the present invention corresponds to in the aligned sequences.

The term "alpha-amylase" means, as described herein, an enzyme that has alpha-amylase activity (EC 3.2.1.1) which facilitates the removal of starch-containing stains such as those from pasta and potato. The alpha-amylase variants of the present invention typically consist of three domains (A, B, and C) with the catalytic site between the A and B domains.

The term "polypeptide" as used herein, refers to a sequence of amino acids which are connected to one another via peptide (amide) bonds, *i.e.* a long, continuous, and unbranched peptide chain. It is within the knowledge of the skilled person to define a polypeptide.

The term "having alpha-amylase activity" as used herein, refers to a polypeptide which can be shown to be active in an assay testing for alpha-amylase activity. Such as assay may be the Amylazyme activity assay. Thus, in one embodiment, the alpha-amylase activity is determined by a method comprising the steps of; (i) incubating the polypeptide with a dyed amylose substrate, and (ii) measuring the amylase activity of the polypeptide. In another embodiment, the alpha-amylase activity is determined by a method comprising the steps of; (i) adding to a composition comprising AZCL-amylose, lactose, magnesium stearate and a blue dye covalently bound in the composition, the polypeptide to be tested; and (ii) measuring the concentration of dye released upon degradation at 590 nm. The term "polypeptide of the invention" or merely "polypeptide" are to be understood as a "polypeptide having alpha-amylase activity according to the invention" unless otherwise clearly stated.

The variants according to the present invention have an improved performance, such as wash performance, as when compared to the parent alpha-amylase. Additionally, the variants may also have an improved stability, such as storage stability, when compared to the parent alpha-amylase. In particular embodiments, the variants have similar stability as compared to the parent alpha-amylase, but an improved performance.

In one embodiment, the variant is a variant of a parent alpha-amylase selected from the group consisting of:

- a. a polypeptide having at least 89%, sequence identity to the polypeptide of SEQ ID NOs: 3 or 13;
- b. a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with (i) the mature polynucleotide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i);
- c. a polypeptide encoded by a polynucleotide having at least 89% sequence identity to the mature polynucleotide coding sequence of SEQ ID NO:1; and
- d. a fragment of the polypeptide of SEQ ID NOs: 3 or 13, which has alpha-amylase activity.

The term "sequence identity" as used herein, has the same meaning and purpose as stated elsewhere herein. Furthermore, it is within the knowledge of the skilled person to determine the sequence identity of a polypeptide as well as a polynucleotide.

The term "hybridizes" as used herein, refers to the process of establishing a non-covalent, sequence-specific interaction between two or more complementary strands of nucleic acids into a complex. The skilled person would know how to recognize hybridization of complementary sequences.

The term "low stringency conditions" as used herein, has the same meaning and purpose as elsewhere described herein.

The term "full-length" as used herein, refers to the complete sequence of sequence of a polypeptide sequence provided in any of the sequences submitted herewith in the sequence

listing. Other full-length sequences may also be contemplated to be part of such definition. It is within the knowledge of the skilled person to determine a full-length of e.g. a nucleotide sequence.

5 In one embodiment, the parent alpha-amylase has at least 67%, such as at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polynucleotide of SEQ ID NO:1.

10 In another embodiment, the parent alpha-amylase is encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the polynucleotide coding sequence of SEQ ID NO: 1 or (ii) the full-length complement of (i).

In yet another embodiment, the parent alpha-amylase is encoded by a polynucleotide having at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide coding sequence of SEQ ID NO: 3.

15 In yet another embodiment, the parent alpha-amylase is encoded by a polynucleotide having at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the polypeptide coding sequence of SEQ ID NO: 13.

20 In yet another embodiment, the parent alpha-amylase comprises or consists of the mature polynucleotide of SEQ ID NO: 1.

In one embodiment, the parent alpha-amylase is a fragment of the polypeptide of SEQ ID NO: 3, wherein the fragment has alpha-amylase activity.

25 In one embodiment, the total number of alterations in the parent alpha-amylase is between 3 and 40, preferably between 3 and 30, more preferably between 3 and 20, even more preferably between 3 and 15, most preferably between 3 and 10 alterations, such as substitutions. In one embodiment, the total number of alterations in the parent alpha-amylase is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 alterations, such as substitutions.

30 **Variants**

In one aspect of the invention, the variant according to the invention has a sequence identity of at least 67%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide according to SEQ ID NOs: 3 or 13.

35 In one embodiment, the variant has a sequence identity of at least 67%, such as at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least

97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide according to SEQ ID NOs: 3 or 13.

In one embodiment, the variant consists of 400 to 490, such as 410 to 490, such as 420 to 490, such as 440 to 486 amino acids.

5 In one embodiment, the number of substitutions in the variants of the present invention is 1 to 40, e.g. 1 to 30, e.g. 1 to 20, e.g., 1 to 10 and 1 to 5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 substitutions. In a particular embodiment, the variant according to any one of the preceding claims, wherein the number of substitutions is 1 to 20, e.g., 1 to 10 and 1 to 5,
10 such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 alterations.

In a further embodiment, the variant of the present invention comprises further alterations. Such alterations may be an insertion or a deletion. An insertion means adding one or more (e.g. several) amino acids, e.g. 1 to 5 amino acids, adjacent to the amino acid occupying a position. A deletion means deleting one or more (e.g. several) amino acids, e.g. 1
15 to 5 amino acids, in a polypeptide. The term "alteration" has the same meaning and purpose as described elsewhere herein.

In one embodiment, the alpha-amylase variant has improved performance, in particular improved wash performance in laundry and/or dish washing, compared to the polypeptide of SEQ ID NO:2 or the polypeptide of SEQ ID NOs: 3 or 13.

20 Thus, in one embodiment, the variant comprises a substitution at one position corresponding to any one of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO:3.

In one embodiment, the parent alpha-amylase is the alpha-amylase having the amino acid sequence of SEQ ID NO:2 or the amino acid sequence of SEQ ID NOs: 3 or 13.

25 In one embodiment, the variant comprises a substitution at a position corresponding to position 51 of SEQ ID NO:3. In one embodiment, the substitution is Q51X, wherein X may be any amino acid selected from the group consisting of: T, A, R, N, D, C, E, G, H, I, L, K, M, F, P, S, W, Y, and V. Thus, in one embodiment, the substitution is Q51T, Q51A, Q51R, Q51N, Q51D, Q51C, Q51E, Q51E, Q51H, Q51I, Q51L, Q51L, Q51K, Q51M, Q51F, Q51P, Q51S, Q51W,
30 Q51Y, or Q51V. In a particular embodiment, the substitution is Q51T. Thus, in one embodiment, the variant comprises or consists of the substitution Q51T of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is Q51D. Thus, in one embodiment, the variant comprises or consists of the substitution Q51D of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution Q51T of the polypeptide of
35 SEQ ID NO:13. In another particular embodiment, the substitution is Q51D. Thus, in one embodiment, the variant comprises or consists of the substitution Q51D of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to position 109 of SEQ ID NO:3. In one embodiment, the substitution is M109X, wherein X may be any amino acid selected from the group consisting of: G, A, R, N, D, C, E, Q, H, I, L, K, F, P, S, T, W, Y, and V. Thus, in one embodiment, the substitution is M109G, M109A, M109R, M109N, 5 M109D, M109C, M109E, M109Q, M109H, M109I, M109L, M109K, M109F, M109P, M109S, M109T, M109Y, or M109V. In a particular embodiment, the substitution is M109G. Thus, in one embodiment, the variant comprises or consists of the substitution M109G of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is M109A. Thus, in one embodiment, the variant comprises or consists of the substitution M109A of the polypeptide of 10 SEQ ID NO:3. In another particular embodiment, the substitution is M109G. Thus, in one embodiment, the variant comprises or consists of the substitution M109H. Thus, in one embodiment, the variant comprises or consists of the substitution M109H of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is M109L. Thus, in one embodiment, the variant comprises or consists of the substitution M109L of the polypeptide of 15 SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution M109G of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is M109A. Thus, in one embodiment, the variant comprises or consists of the substitution M109A of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is M109G. Thus, in one embodiment, the variant comprises or consists of the substitution 20 M109H. Thus, in one embodiment, the variant comprises or consists of the substitution M109H of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is M109L. Thus, in one embodiment, the variant comprises or consists of the substitution M109L of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to 25 position 193 of SEQ ID NO:3. In one embodiment, the substitution is N193X, wherein X may be any amino acid selected from the group consisting of: F, A, R, D, C, E, Q, G, H, I, L, K, M, P, S, T, W, Y, and V. Thus, in one embodiment, the substitution is N193F, N193A, N193R, N193D, N193C, N193E, N193Q, N193G, N193H, N193I, N193L, N193K, N193M, N193P, N193S, N193T, N193W, N193Y, or N193V. In a particular embodiment, the substitution is N193F. Thus, 30 in one embodiment, the variant comprises or consists of the substitution N193F of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution N193F of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to 35 position 201 of SEQ ID NO:3. In one embodiment, the substitution is G201X, wherein X may be any amino acid selected from the group consisting of: Y, A, R, D, C, E, Q, H, I, L, K, M, N, P, S, T, W, Y, and V. Thus, in one embodiment, the substitution is G201Y, G201A, G201R, G201D, G201C, G201E, G201Q, G201H, G201I, G201L, G201K, G201M, G201N, G201P, G201S, G201T, G201W, G201Y, or G201V. In a particular embodiment, the substitution is G201Y.

Thus, in one embodiment, the variant comprises or consists of the substitution G201Y of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is G201F. Thus, in one embodiment, the variant comprises or consists of the substitution G201F of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution G201Y of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is G201F. Thus, in one embodiment, the variant comprises or consists of the substitution G201F of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to position 269 of SEQ ID NO:3. In one embodiment, the substitution is T269X, wherein X may be any amino acid selected from the group consisting of: N, A, R, D, C, E, G, F, Q, H, I, L, K, M, P, S, W, Y, and V. Thus, in one embodiment, the substitution is T269N, T269A, T269R, T269D, T269C, T269E, T269G, T269F, T269Q, T269H, T269I, T269L, T269K, T269M, T269P, T269S, T269W, T269Y, or T269V. In a particular embodiment, the substitution is T269N. Thus, in one embodiment, the variant comprises or consists of the substitution T269N of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is T269Y. Thus, in one embodiment, the variant comprises or consists of the substitution T269Y of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is T269G. Thus, in one embodiment, the variant comprises or consists of the substitution T269G of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution T269N of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is T269Y. Thus, in one embodiment, the variant comprises or consists of the substitution T269Y of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is T269G. Thus, in one embodiment, the variant comprises or consists of the substitution T269G of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to position 294 of SEQ ID NO:3. In one embodiment, the substitution is M294X, wherein X may be any amino acid selected from the group consisting of: Y, A, R, D, C, E, Q, G, F, H, I, L, K, N, P, S, T, W, and V. Thus, in one embodiment, the substitution is M294G, M294A, M294R, M294N, M294D, M294C, M294E, M294Q, M294H, M294I, M294L, M294K, M294F, M294P, M294S, M294T, M294Y, or M294V. In a particular embodiment, the substitution is M294Y. Thus, in one embodiment, the variant comprises or consists of the substitution M294Y of the polypeptide of SEQ ID NO:3. In another particular embodiment, the substitution is M294N. Thus, in one embodiment, the variant comprises or consists of the substitution M294N of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution M294Y of the polypeptide of SEQ ID NO:13. In another particular embodiment, the substitution is M294N. Thus, in one embodiment, the variant comprises or consists of the substitution M294N of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to position 297 of SEQ ID NO:3. In one embodiment, the substitution is Q297X, wherein X may be any amino acid selected from the group consisting of: Y, A, R, N, D, C, E, G, H, I, L, K, M, F, P, S, T, W, and V. Thus, in one embodiment, the substitution is Q297T, Q297A, Q297R, Q297N,
 5 Q297D, Q297C, Q297E, Q297E, Q297H, Q297I, Q297L, Q297L, Q297K, Q297M, Q297F, Q297P, Q297S, Q297W, Q297Y, or Q297V. In a particular embodiment, the substitution is Q297Y. Thus, in one embodiment, the variant comprises or consists of the substitution Q297Y of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution Q297Y of the polypeptide of SEQ ID NO:13.

10 In one embodiment, the variant comprises a substitution at a position corresponding to position 298 of SEQ ID NO:3. In one embodiment, the substitution is A298X, wherein X may be any amino acid selected from the group consisting of: N, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, and V. Thus, in one embodiment, the substitution is A298N, A298R, A298D, A298C, A298E, A298Q, A298G, A298H, A298I, A298L, A298K, A298M, A298F, A298P, A298S, A298T,
 15 A298W, A298Y, or A298V. In a particular embodiment, the substitution is A298N. Thus, in one embodiment, the variant comprises or consists of the substitution A298N of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution A298N of the polypeptide of SEQ ID NO:13.

In one embodiment, the variant comprises a substitution at a position corresponding to position 314 of SEQ ID NO:3. In one embodiment, the substitution is N314X, wherein X may be any amino acid selected from the group consisting of: G, A, R, D, C, E, Q, H, I, L, K, M, F, P, S, T, W, Y, and V. Thus, in one embodiment, the substitution is N314F, N314A, N314R, N314D, N314C, N314E, N314Q, N314G, N314H, N314I, N314L, N314K, N314M, N314P, N314S, N314T, N314W, N314Y, or N314V. In a particular embodiment, the substitution is N314G. Thus,
 25 in one embodiment, the variant comprises or consists of the substitution N314G of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the variant comprises or consists of the substitution N314G of the polypeptide of SEQ ID NO:13.

In an embodiment, the alpha-amylase variant comprises or consists of one or more of the substitutions in Table 1, wherein each position corresponds to the corresponding position of the polypeptide of SEQ ID NO:3.
 30

Table 1: Alpha-amylase variants

Q51T
M109G
N193F
G201Y
T269N
M294Y

Q297Y
A298N
N314G

In a preferred embodiment, the variant is a polypeptide having said substitutions according to the invention and having an amino acid sequence which is at least 67% identical to SEQ ID NO: 3. Thus, in one embodiment, the alpha-amylase variant comprises or consists of the substitution Q51T, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution M109G, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution N193F, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution G201Y, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution T269N, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution M294Y, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution Q297Y, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and

wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution
5 A298N, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In one embodiment, the alpha-amylase variant comprises or consists of the substitution
10 N314G, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

Accordingly, in one embodiment, the substitution is selected from the list consisting of;

- 15 i. Q51T, Q51A, Q51R, Q51N, Q51D, Q51C, Q51E, Q51G, Q51H, Q51I, Q51L, Q51K, Q51M, Q51F, Q51P, Q51S, Q51W, Q51Y, or Q51V;
- ii. M109G, M109A, M109H, M109L, M109R, M109N, M109D, M109C, M109E, M109Q, M109I, M109K, M109F, M109P, M109S, M109T, M109W, M109Y, or M109V;
- 20 iii. N193F, N193A, N193R, N193D, N193C, N915E, N193Q, N193G, N193H, N193I, N193L, N193K, N193M, N193P, N193S, N193T, N193W, N193Y, or N193V;
- iv. G201Y, G201A, G201R, G201D, G201C, G201E, G201Q, G201H, G201I, G201L, G201K, G201M, G201N, G201P, G201S, G201T, G201W, G201Y, or G201V;
- v. T269N, T269Y, T269G, T269A, T269R, T269D, T269C, T269E, T269F, T269Q, T269H, T269I, T269L, T269K, T269M, T269P, T269S, T269W, or T269V;
- 25 vi. M294Y, M294N, M294A, M294R, M294D, M294C, M294E, M294Q, M294G, M294F, M294H, M294I, M294L, M294K, M294P, M294S, M294T, M294W, or M294V;
- vii. Q297Y, Q297A, Q297R, Q297N, Q297D, Q297C, Q297E, Q297G, Q297H, Q297I, Q297L, Q297K, Q297M, Q297F, Q297P, Q297S, Q297T, Q297W, or Q297V;
- viii. A298N, A298R, A298D, A298C, A298E, A298Q, A298G, A298H, A298I, A298L, A298K, A298M, A298F, A298P, A298S, A298T, A298W, A298Y, or A298V; or
- 30 ix. N314G, N314A, N314R, N314D, N314C, N314E, N314Q, N314H, N314I, N314L, N314K, N314M, N314F, N314P, N314S, N314T, N314W, N314Y, or N314V, wherein each position corresponds to the corresponding position in the polypeptide of SEQ ID NO:3.

In another aspect, the present invention relates to a variant which comprises a
35 substitution at two positions corresponding to any one of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO: 3. The substitution in each amino acid position is contemplated to be any of those described elsewhere herein.

Accordingly, the invention also relates to alpha-amylase variants comprising substitutions in two positions corresponding to those pairwise listed in Table 2, wherein each position corresponds to the corresponding position of the mature polypeptide of SEQ ID NO:2 or the polypeptide of SEQ ID NOs: 3 or 13.

5

Table 2: Alpha-amylase variants

Q51+M109	Q51+N193	Q51+G201
Q51+T269	Q51+M294	Q51+Q297
Q51+A298	Q51+N314	M109+N193
M109+G201	M109+T269	M109+M294
M109+Q297	M109+A298	M109+N314
N193+G201	N193+T269	N193+M294
N193+Q297	N193+A298	N193+N314
T269+M294	T269+Q297	T269+A298
T269+N314	M294+Q297	M294+A298
M294+N314	Q297+A298	Q297+N314
A298+N314		

Combining two positions may further enhance the improved performance of the variants. Thus, in one embodiment, the alpha-amylase variants of the present invention comprises the specific pairwise amino acid substitutions listed in Table 3, wherein each position corresponds to the position of the mature polypeptide of SEQ ID NO: 2 or the polypeptide of SEQ ID NO: 3.

10

Table 3: Alpha-amylase variants

Q51T+M109G	Q51T+N193F	Q51T+G201Y
Q51T+T269N	Q51T+M294Y	Q51T+Q297Y
Q51T+A298N	Q51T+N314G	M109G+N193F
M109G+G201Y	M109G+T269N	M109G+M294Y
M109G+Q297Y	M109G+A298N	M109G+N314G
N193F+G201Y	N193F+T269N	N193F+M294Y
N193F+Q297Y	N193F+A298N	N193F+N314G
T269N+M294Y	T269N+Q297Y	T269N+A298N
T269N+N314G	M294Y+Q297Y	M294Y+A298N
M294Y+N314G	Q297Y+A298N	Q297Y+N314G
A298N+N314G		

In a particular embodiment, the variant comprises two of the substitutions selected from the list consisting of Q51T, Q51D, M109G, M109G, M109H, M109L, N193F, G201Y, G201F, T269N, T269Y, T269G, M294Y, M294N, Q297Y, A298N, and N314G, wherein each position corresponds to the corresponding position of the polypeptide of SEQ ID NO:3.

5 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 109, such as those described above, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid
10 sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 193, such as those described above, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such
15 as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 269, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase
20 variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 294, such as those described above wherein the position
25 corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions
30 corresponding to positions 51 and 297, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

35 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 298, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such

as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

5 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 51 and 314, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

10 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 193, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

15 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 269, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

20 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 294, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

25 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 297, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

30 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 298, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 109 and 314, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 193 and 269, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 193 and 294, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 193 and 297, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 193 and 298, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 193 and 314, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 269 and 294, such as those described above wherein the position

corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

5 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 269 and 297, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid
10 sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 269 and 298, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO:3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such
15 as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 269 and 314, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase
20 variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 294 and 297, such as those described above wherein the position
25 corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 294 and 298, such as those described above wherein the position
30 corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

35 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 294 and 314, such as those described above wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such

as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

5 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 297 and 298, such as those described above, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

10 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 297 and 314, such as those described above, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

15 In another embodiment, the variant comprises or consists of substitutions at positions corresponding to positions 298 and 314, such as those described above, wherein the position corresponds to the corresponding positions of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

20 In another embodiment, the alpha-amylase variants comprises amino acid substitutions in three positions corresponding to those listed in Table 4, wherein each position corresponds to the corresponding positions of the polypeptide of SEQ ID NO:3. Thus, in one embodiment, the alpha-amylase variant comprises or consists of substitutions in three positions each selected from the list consisting of 51, 109, 193, 201, 269, 294, 297, 298, and 314, wherein each position corresponds to the corresponding positions of the polypeptide of SEQ ID NO:3. In one embodiment, the alpha-amylase variant comprises or consists of substitutions in three positions each selected from the list consisting of 51, 109, 193, 201, 269, 294, 297, 298, and 314, wherein each position corresponds to the corresponding positions of the polypeptide of SEQ ID NO:3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In a particular embodiment, the variant comprises a substitution at each position corresponding to positions;

- 35
- (i) 51 and 109;
 - (ii) 109 and 201;
 - (iii) 269 and 294; or
 - (iv) 294 and 297;

wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID NO: 3.

In one particular embodiment, the variant comprises the substitutions;

- 5 (i) Q51T and M109G;
(ii) M109G and G201Y;
(iii) M109G and G201F
(iv) T269N and M294Y;
(v) T269N and M294F;
10 (vi) T269Y and M294N; or
(vii) M294Y and Q297Y;

wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID NO: 3.

15 In one embodiment, the variant comprises a substitution at three positions corresponding to any of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO:3. In one embodiment, the variant comprises a substitution at three positions corresponding to any of positions 109, 51, 203, 271, 296, 299, 300, 195, and 316 of the polypeptide of SEQ ID NO:13.

Table 4: Alpha-amylase variants

Q51+M109+N193	Q51+M109+G201	Q51+M109+T269
Q51+M109+M294	Q51+M109+Q297	Q51+M109+A298
Q51+M109+N314	Q51+N193+G201	Q51+N193+T269
Q51+N193+M294	Q51+N193+Q297	Q51+N193+A298
Q51+N193+N314	Q51+G201+T269	Q51+G201+M294
Q51+G201+Q297	Q51+G201+A298	Q51+G201+N314
Q51+T269+M294	Q51+T269+Q297	Q51+T269+A298
Q51+T269+N314	Q51+M294+Q297	Q51+M294+A298
Q51+M294+N314	Q51+Q297+A298	Q51+Q297+N314
Q51+A298+N314	M109+N193+G201	M109+N193+T269
M109+N193+M294	M109+N193+Q297	M109+N193+A298
M109+N193+N314	M109+G201+T269	M109+G201+M294
M109+G201+Q297	M109+G201+A298	M109+G201+N314
M109+T269+M294	M109+T269+Q297	M109+T269+A298
M109+T269+N314	M109+M294+Q297	M109+M294+A298
M109+M294+N314	M109+Q297+A298	M109+Q297+N314
M109+A298+N314	N193+G201+T269	N193+G201+M294
N193+G201+Q297	N193+G201+A298	N193+G201+N314

N193+T269+M294	N193+T269+Q297	N193+T269+A298
N193+T269+N314	N193+M294+Q297	N193+M294+A298
N193+M294+N314	N193+Q297+A298	N193+Q297+N314
N193+A298+N314	G201+T269+M294	G201+T269+Q297
G201+T269+A298	G201+T269+N314	G201+M294+Q297
G201+M294+A298	G201+M294+N314	G201+Q297+A298
G201+Q297+N314	G201+A298+N314	T269+M294+Q297
T269+M294+A298	T269+M294+N314	T269+Q297+A298
T269+Q297+N314	T269+A298+N314	M294+Q297+A298
M294+Q297+N314	M294+A298+N314	Q297+A298+N314

In one embodiment, the alpha-amylase variants of the present invention comprise or consist of the amino acid substitutions listed in Table 5. Thus, in one embodiment, the alpha-amylase variant comprises or consists of three of the amino acid substitutions individually selected from the list consisting of Q51T, Q51D, M109G, M109G, M109H, M109L, N193F, G201Y, G201F, T269N, T269Y, T269G, M294Y, M294N, Q297Y, A298N, and N314G, wherein each position corresponds to the corresponding position of SEQ ID NO: 3. In one embodiment, the alpha-amylase variant comprises or consists of three of the amino acid substitutions individually selected from the list consisting of Q51T, Q51D, M109G, M109G, M109H, M109L, N193F, G201Y, G201F, T269N, T269Y, T269G, M294Y, M294N, Q297Y, A298N, and N314G, wherein each position corresponds to the corresponding position of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

15

Table 5: Alpha-amylase variants

Q51T+M109G+G201Y	Q51D+T269G+M294Y	M109L+G201Y+T269N
Q51T+M109G+G201F	Q51D+T269G+M294N	M109L+G201Y+T269Y
Q51T+M109G+T269N	Q51D+T269G+Q297Y	M109L+G201Y+T269G
Q51T+M109G+T269Y	Q51D+T269G+A298N	M109L+G201Y+M294Y
Q51T+M109G+T269G	Q51D+T269G+N314G	M109L+G201Y+M294N
Q51T+M109G+M294Y	Q51D+M294Y+Q297Y	M109L+G201Y+Q297Y
Q51T+M109G+M294N	Q51D+M294Y+A298N	M109L+G201Y+A298N
Q51T+M109G+Q297Y	Q51D+M294Y+N314G	M109L+G201Y+N314G
Q51T+M109G+A298N	Q51D+M294N+Q297Y	M109L+G201F+T269N
Q51T+M109G+N314G	Q51D+M294N+A298N	M109L+G201F+T269Y
Q51T+M109G+N193F	Q51D+M294N+N314G	M109L+G201F+T269G

Q51T+M109G+G201Y	Q51D+Q297Y+A298N	M109L+G201F+M294Y
Q51T+M109G+G201F	Q51D+Q297Y+N314G	M109L+G201F+M294N
Q51T+M109G+T269N	Q51D+A298N+N314G	M109L+G201F+Q297Y
Q51T+M109G+T269Y	M109G+N193F+G201Y	M109L+G201F+A298N
Q51T+M109G+T269G	M109G+N193F+G201F	M109L+G201F+N314G
Q51T+M109G+M294Y	M109G+N193F+T269N	M109L+T269N+M294Y
Q51T+M109G+M294N	M109G+N193F+T269Y	M109L+T269N+M294N
Q51T+M109G+Q297Y	M109G+N193F+T269G	M109L+T269N+Q297Y
Q51T+M109G+A298N	M109G+N193F+M294Y	M109L+T269N+A298N
Q51T+M109G+N314G	M109G+N193F+M294N	M109L+T269N+N314G
Q51T+M109H+M109L	M109G+N193F+Q297Y	M109L+T269Y+M294Y
Q51T+M109H+N193F	M109G+N193F+A298N	M109L+T269Y+M294N
Q51T+M109H+G201Y	M109G+N193F+N314G	M109L+T269Y+Q297Y
Q51T+M109H+G201F	M109G+G201Y+T269N	M109L+T269Y+A298N
Q51T+M109H+T269N	M109G+G201Y+T269Y	M109L+T269Y+N314G
Q51T+M109H+T269Y	M109G+G201Y+T269G	M109L+T269G+M294Y
Q51T+M109H+T269G	M109G+G201Y+M294Y	M109L+T269G+M294N
Q51T+M109H+M294Y	M109G+G201Y+M294N	M109L+T269G+Q297Y
Q51T+M109H+M294N	M109G+G201Y+Q297Y	M109L+T269G+A298N
Q51T+M109H+Q297Y	M109G+G201Y+A298N	M109L+T269G+N314G
Q51T+M109H+A298N	M109G+G201Y+N314G	M109L+M294Y+Q297Y
Q51T+M109H+N314G	M109G+G201F+T269N	M109L+M294Y+A298N
Q51T+M109L+N193F	M109G+G201F+T269Y	M109L+M294Y+N314G
Q51T+M109L+G201Y	M109G+G201F+T269G	M109L+M294N+Q297Y
Q51T+M109L+G201F	M109G+G201F+M294Y	M109L+M294N+A298N
Q51T+M109L+T269N	M109G+G201F+M294N	M109L+M294N+N314G
Q51T+M109L+T269Y	M109G+G201F+Q297Y	M109L+Q297Y+A298N
Q51T+M109L+T269G	M109G+G201F+A298N	M109L+Q297Y+N314G
Q51T+M109L+M294Y	M109G+G201F+N314G	M109L+A298N+N314G
Q51T+M109L+M294N	M109G+T269N+M294Y	N193F+G201Y+T269N
Q51T+M109L+Q297Y	M109G+T269N+M294N	N193F+G201Y+T269Y
Q51T+M109L+A298N	M109G+T269N+Q297Y	N193F+G201Y+T269G
Q51T+M109L+N314G	M109G+T269N+A298N	N193F+G201Y+M294Y
Q51T+N193F+G201Y	M109G+T269N+N314G	N193F+G201Y+M294N
Q51T+N193F+G201F	M109G+T269Y+M294Y	N193F+G201Y+Q297Y
Q51T+N193F+T269N	M109G+T269Y+M294N	N193F+G201Y+A298N

Q51T+N193F+T269Y	M109G+T269Y+Q297Y	N193F+G201Y+N314G
Q51T+N193F+T269G	M109G+T269Y+A298N	N193F+G201F+T269N
Q51T+N193F+M294Y	M109G+T269Y+N314G	N193F+G201F+T269Y
Q51T+N193F+M294N	M109G+T269G+M294Y	N193F+G201F+T269G
Q51T+N193F+Q297Y	M109G+T269G+M294N	N193F+G201F+M294Y
Q51T+N193F+A298N	M109G+T269G+Q297Y	N193F+G201F+M294N
Q51T+N193F+N314G	M109G+T269G+A298N	N193F+G201F+Q297Y
Q51T+G201Y+T269N	M109G+T269G+N314G	N193F+G201F+A298N
Q51T+G201Y+T269Y	M109G+M294Y+Q297Y	N193F+G201F+N314G
Q51T+G201Y+T269G	M109G+M294Y+A298N	N193F+T269N+M294Y
Q51T+G201Y+M294Y	M109G+M294Y+N314G	N193F+T269N+M294N
Q51T+G201Y+M294N	M109G+M294N+Q297Y	N193F+T269N+Q297Y
Q51T+G201Y+Q297Y	M109G+M294N+A298N	N193F+T269N+A298N
Q51T+G201Y+A298N	M109G+M294N+N314G	N193F+T269N+N314G
Q51T+G201Y+N314G	M109G+Q297Y+A298N	N193F+T269Y+M294Y
Q51T+G201F+T269N	M109G+Q297Y+N314G	N193F+T269Y+M294N
Q51T+G201F+T269Y	M109G+A298N+N314G	N193F+T269Y+Q297Y
Q51T+G201F+T269G	M109G+N193F+G201Y	N193F+T269Y+A298N
Q51T+G201F+M294Y	M109G+N193F+G201F	N193F+T269Y+N314G
Q51T+G201F+M294N	M109G+N193F+T269N	N193F+T269G+M294Y
Q51T+G201F+Q297Y	M109G+N193F+T269Y	N193F+T269G+M294N
Q51T+G201F+A298N	M109G+N193F+T269G	N193F+T269G+Q297Y
Q51T+G201F+N314G	M109G+N193F+M294Y	N193F+T269G+A298N
Q51T+T269N+M294Y	M109G+N193F+M294N	N193F+T269G+N314G
Q51T+T269N+M294N	M109G+N193F+Q297Y	N193F+M294Y+Q297Y
Q51T+T269N+Q297Y	M109G+N193F+A298N	N193F+M294Y+A298N
Q51T+T269N+A298N	M109G+N193F+N314G	N193F+M294Y+N314G
Q51T+T269N+N314G	M109G+G201Y+T269N	N193F+M294N+Q297Y
Q51T+T269Y+M294Y	M109G+G201Y+T269Y	N193F+M294N+A298N
Q51T+T269Y+M294N	M109G+G201Y+T269G	N193F+M294N+N314G
Q51T+T269Y+Q297Y	M109G+G201Y+M294Y	N193F+Q297Y+A298N
Q51T+T269Y+A298N	M109G+G201Y+M294N	N193F+Q297Y+N314G
Q51T+T269Y+N314G	M109G+G201Y+Q297Y	N193F+A298N+N314G
Q51T+T269G+M294Y	M109G+G201Y+A298N	G201Y+T269N+M294Y
Q51T+T269G+M294N	M109G+G201Y+N314G	G201Y+T269N+M294N
Q51T+T269G+Q297Y	M109G+G201F+T269N	G201Y+T269N+Q297Y

Q51T+T269G+A298N	M109G+G201F+T269Y	G201Y+T269N+A298N
Q51T+T269G+N314G	M109G+G201F+T269G	G201Y+T269N+N314G
Q51T+M294Y+Q297Y	M109G+G201F+M294Y	G201Y+T269Y+M294Y
Q51T+M294Y+A298N	M109G+G201F+M294N	G201Y+T269Y+M294N
Q51T+M294Y+N314G	M109G+G201F+Q297Y	G201Y+T269Y+Q297Y
Q51T+M294N+Q297Y	M109G+G201F+A298N	G201Y+T269Y+A298N
Q51T+M294N+A298N	M109G+G201F+N314G	G201Y+T269Y+N314G
Q51T+M294N+N314G	M109G+T269N+M294Y	G201Y+T269G+M294Y
Q51T+Q297Y+A298N	M109G+T269N+M294N	G201Y+T269G+M294N
Q51T+Q297Y+N314G	M109G+T269N+Q297Y	G201Y+T269G+Q297Y
Q51T+A298N+N314G	M109G+T269N+A298N	G201Y+T269G+A298N
Q51D+M109G+N193F	M109G+T269N+N314G	G201Y+T269G+N314G
Q51D+M109G+G201Y	M109G+T269Y+T269G	G201Y+M294Y+Q297Y
Q51D+M109G+G201F	M109G+T269Y+M294Y	G201Y+M294Y+A298N
Q51D+M109G+T269N	M109G+T269Y+M294N	G201Y+M294Y+N314G
Q51D+M109G+T269Y	M109G+T269Y+Q297Y	G201Y+M294N+Q297Y
Q51D+M109G+T269G	M109G+T269Y+A298N	G201Y+M294N+A298N
Q51D+M109G+M294Y	M109G+T269Y+N314G	G201Y+M294N+N314G
Q51D+M109G+M294N	M109G+T269G+M294Y	G201Y+Q297Y+A298N
Q51D+M109G+Q297Y	M109G+T269G+M294N	G201Y+Q297Y+N314G
Q51D+M109G+A298N	M109G+T269G+Q297Y	G201Y+A298N+N314G
Q51D+M109G+N314G	M109G+T269G+A298N	G201F+T269N+M294Y
Q51D+M109G+N193F	M109G+T269G+N314G	G201F+T269N+M294N
Q51D+M109G+G201Y	M109G+M294Y+Q297Y	G201F+T269N+Q297Y
Q51D+M109G+G201F	M109G+M294Y+A298N	G201F+T269N+A298N
Q51D+M109G+T269N	M109G+M294Y+N314G	G201F+T269N+N314G
Q51D+M109G+T269Y	M109G+M294N+Q297Y	G201F+T269Y+M294Y
Q51D+M109G+T269G	M109G+M294N+A298N	G201F+T269Y+M294N
Q51D+M109G+M294Y	M109G+M294N+N314G	G201F+T269Y+Q297Y
Q51D+M109G+M294N	M109G+Q297Y+A298N	G201F+T269Y+A298N
Q51D+M109G+Q297Y	M109G+Q297Y+N314G	G201F+T269Y+N314G
Q51D+M109G+A298N	M109G+A298N+N314G	G201F+T269G+M294Y
Q51D+M109G+N314G	M109H+N193F+G201Y	G201F+T269G+M294N
Q51D+M109H+M109L	M109H+N193F+G201F	G201F+T269G+Q297Y
Q51D+M109H+N193F	M109H+N193F+T269N	G201F+T269G+A298N
Q51D+M109H+G201Y	M109H+N193F+T269Y	G201F+T269G+N314G

Q51D+M109H+G201F	M109H+N193F+T269G	G201F+M294Y+Q297Y
Q51D+M109H+T269N	M109H+N193F+M294Y	G201F+M294Y+A298N
Q51D+M109H+T269Y	M109H+N193F+M294N	G201F+M294Y+N314G
Q51D+M109H+T269G	M109H+N193F+Q297Y	G201F+M294N+Q297Y
Q51D+M109H+M294Y	M109H+N193F+A298N	G201F+M294N+A298N
Q51D+M109H+M294N	M109H+N193F+N314G	G201F+M294N+N314G
Q51D+M109H+Q297Y	M109H+G201Y+T269N	G201F+Q297Y+A298N
Q51D+M109H+A298N	M109H+G201Y+T269Y	G201F+Q297Y+N314G
Q51D+M109H+N314G	M109H+G201Y+T269G	G201F+A298N+N314G
Q51D+M109L+N193F	M109H+G201Y+M294Y	T269N+M294Y+Q297Y
Q51D+M109L+G201Y	M109H+G201Y+M294N	T269N+M294Y+A298N
Q51D+M109L+G201F	M109H+G201Y+Q297Y	T269N+M294Y+N314G
Q51D+M109L+T269N	M109H+G201Y+A298N	T269N+M294N+Q297Y
Q51D+M109L+T269Y	M109H+G201Y+N314G	T269N+M294N+A298N
Q51D+M109L+T269G	M109H+G201F+T269N	T269N+M294N+N314G
Q51D+M109L+M294Y	M109H+G201F+T269Y	T269N+Q297Y+A298N
Q51D+M109L+M294N	M109H+G201F+T269G	T269N+Q297Y+N314G
Q51D+M109L+Q297Y	M109H+G201F+M294Y	T269N+A298N+N314G
Q51D+M109L+A298N	M109H+G201F+M294N	T269Y+M294Y+Q297Y
Q51D+M109L+N314G	M109H+G201F+Q297Y	T269Y+M294Y+A298N
Q51D+N193F+G201Y	M109H+G201F+A298N	T269Y+M294Y+N314G
Q51D+N193F+G201F	M109H+G201F+N314G	T269Y+M294N+Q297Y
Q51D+N193F+T269N	M109H+T269N+M294Y	T269Y+M294N+A298N
Q51D+N193F+T269Y	M109H+T269N+M294N	T269Y+M294N+N314G
Q51D+N193F+T269G	M109H+T269N+Q297Y	T269Y+Q297Y+A298N
Q51D+N193F+M294Y	M109H+T269N+A298N	T269Y+Q297Y+N314G
Q51D+N193F+M294N	M109H+T269N+N314G	T269Y+A298N+N314G
Q51D+N193F+Q297Y	M109H+T269Y+M294Y	T269G+M294Y+Q297Y
Q51D+N193F+A298N	M109H+T269Y+M294N	T269G+M294Y+A298N
Q51D+N193F+N314G	M109H+T269Y+Q297Y	T269G+M294Y+N314G
Q51D+G201Y+T269N	M109H+T269Y+A298N	T269G+M294N+Q297Y
Q51D+G201Y+T269Y	M109H+T269Y+N314G	T269G+M294N+A298N
Q51D+G201Y+T269G	M109H+T269G+M294Y	T269G+M294N+N314G
Q51D+G201Y+M294Y	M109H+T269G+M294N	T269G+Q297Y+A298N
Q51D+G201Y+M294N	M109H+T269G+Q297Y	T269G+Q297Y+N314G
Q51D+G201Y+Q297Y	M109H+T269G+A298N	T269G+A298N+N314G

Q51D+G201Y+A298N	M109H+T269G+N314G	M294Y+Q297Y+A298N
Q51D+G201Y+N314G	M109H+M294Y+Q297Y	M294Y+Q297Y+N314G
Q51D+G201F+T269N	M109H+M294Y+A298N	M294Y+A298N+N314G
Q51D+G201F+T269Y	M109H+M294Y+N314G	M294N+Q297Y+A298N
Q51D+G201F+T269G	M109H+M294N+Q297Y	M294N+Q297Y+N314G
Q51D+G201F+M294Y	M109H+M294N+A298N	M294N+A298N+N314G
Q51D+G201F+M294N	M109H+M294N+N314G	Q297Y+A298N+N314G
Q51D+G201F+Q297Y	M109H+Q297Y+A298N	M109L+N193F+M294N
Q51D+G201F+A298N	M109H+Q297Y+N314G	M109L+N193F+Q297Y
Q51D+G201F+N314G	M109H+A298N+N314G	M109L+N193F+A298N
Q51D+T269N+M294Y	M109L+N193F+G201Y	M109L+N193F+N314G
Q51D+T269N+M294N	M109L+N193F+G201F	Q51D+T269Y+M294N
Q51D+T269N+Q297Y	M109L+N193F+T269N	Q51D+T269Y+Q297Y
Q51D+T269N+A298N	M109L+N193F+T269Y	Q51D+T269Y+A298N
Q51D+T269N+N314G	M109L+N193F+T269G	Q51D+T269Y+N314G
Q51D+T269Y+M294Y	M109L+N193F+M294Y	

In another embodiment, the alpha-amylase variant of the invention comprises amino acid substitutions in four or five or six or seven or eight of the positions selected from the list consisting of Q51T, M109G, N193F, G201Y, T269N, M294Y, Q297Y, A298N, and N314G, wherein each position corresponds to the corresponding position of SEQ ID NO: 3. In one embodiment, the alpha-amylase variant comprises or consists of amino acid substitutions in four or five or six or seven or eight of the positions individually selected from the list consisting of Q51T, M109G, N193F, G201Y, T269N, M294Y, Q297Y, A298N, and N314G, wherein each position corresponds to the corresponding position of SEQ ID NO: 3, and wherein the alpha-amylase variant is a polypeptide having at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NOs: 3 or 13.

In a particular embodiment, the variant comprises a substitution at each positions corresponding to positions;

- (i) 51, 109, 193, and 201;
(ii) 109, 201, 269, and 294;
(iii) 201, 269, 294 and 297; or
(iv) 51, 109, 193, 201, 269, 294, 297, 298, and 314 ;

wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID NO: 3.

In a particular embodiment, the variant comprises a substitution at each positions corresponding to positions;

- (i) Q51T, M109G, N193F, and G201Y;
 (ii) M109G, G201Y, T269N, and M294Y;
 5 (iii) G201Y, T269N, M294Y, and Q297Y; or
 (ii) Q51T, M109G, N193F, G201Y, T269N, M294Y, Q297Y, A298N, and N314G;

wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID NO: 3.

It is contemplated that each alpha-amylase variant herein described may further has an improved performance, such as an improved wash performance in laundry or in automated dish washing, compared to the parent polypeptide of SEQ ID NOs: 3 or 13.

The variants may further comprise one or more additional substitutions at one or more (e.g., several) other positions.

Thus, in one embodiment, the variant further comprises an alteration at positions corresponding to positions:

105L,I,F+206Y; 105L,I+206Y+217I; 105F+206Y+208Y+217V+246V; 105L+206F;
 105I+206Y+208Y+217I+246V; 195F+213S+214T; 195F+206Y+208Y+213T+214T+217M,V;
 195F+206Y+208F,L+213T+214T+217V; 195F+206Y+213S+214T;
 195F+206Y+208Y+213S+214T+217M; 195F+206Y+208F+213T+214T+217M;
 20 195F+206Y+208Y+213T+214T+217Q; 195F+206Y+213G+214T; 195F+206Y+213S;
 195F+206Y+208Y+213T+214T+217M; 195F+213S; 195F+206Y+208L+213T+214T+217M;
 195F+213G+214T; 206Y,F+208Y+217Q; 206Y+208Y+217I; 206F+208Y+217M; 206Y+208Y;
 206Y+217M; 206Y+208Y+213A+217M; 206Y+208Y+217V+246V; 206Y+213G;
 206Y+208F+217V; 206N+208Y+217M; 206F+208Y+217V; 206Y+246V; 206Y+217I,V;
 25 206F+208F+217I; 206Y+208L+213S; 206F+217I; 206Y+217I+246I; 206L+217V;
 206Y+208F+217H; 206L+208F+217I; 206L+217V+246L; 206F+246V; 208Y+213S+217M;
 208Y+213A+217Q; 63I+206Y; 63I+206Y+241V; 63V+206Y; 63V+105L+206Y; 63V+206Y+217I;
 63V+105F+206Y+208F+217I; 63V+206Y+246V; 63V+206F; 63V+206L+217V;
 63V+105F+206Y; 63V+206Y+241V+246L; 195F+206Y+208Y+214T+217V; 186E+195F+206Y;
 30 195F+206Y+208Y+213T+217V; 186E+195F+202T+206Y+209S; 63I+195F+206Y+210S;
 195F+206Y+213P+214T; 195F+206Y+208Y+213T+214T+217I; 186E+195F+206Y+210S;
 195F+213P; 186E+195F+202T+206Y+210S; 195F+206H; 195F+208Y+213T+214T+217V;
 206Y+208Y+213T+214T+217V; 195F+206Y+217V; 195F+206Y+208Y+213S+214T;
 195F+206Y+208Y; 195F+213I+214P; 195F+206Y+208Y+213T+214T; 195F+206Y;
 35 206Y+213S; 182P+186E; 182S+186E; 182V+186K; 179L+186H+190P; 179L+186K,R,S+190P;
 179L+190P; 179L+182C+186K+190P; 179L+182P+186S,V+190P; 179L+182S+186Q+190P;
 173F+174Q; 173Y+174S; 172K+173Y+174E; 193A,D,N,S+195F; 213A+214Q; 213P+214L;

213S+214R; 48V+60V; 213G+214T; 213I+214P; 213N+214I; 213N+214Q; and 213P,S+214T; wherein numbering is according to SEQ ID NO:11.

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 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 can 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 variant comprises a pairwise deletion selected from the list consisting of: 181 and 182; 181 and 183; 181 and 184; 182 and 183; 182 and 184; and 183 and 184; wherein the positions correspond to the positions of SEQ ID NO: 13.

The term "pairwise deletion" as used herein, refers to a double deletion of two amino acids in close proximity to one another. In close proximity to one another may be two amino

acids positioned within five amino acids, such as within four, such as within three, and such as two, distance from one another in an amino acid sequence. The amino acids may also be positioned adjacent to one another.

In a preferred embodiment, the polypeptide has the alterations according to the invention and has an amino acid sequence which is at least 67% identical to SEQ ID NO: 13. Thus, in one embodiment, the polypeptide comprises or consists of the pairwise deletion of amino acids selected from the group consisting of: (a) 181 and 183; (b) 181 and 184; (c) 182 and 183; (d) 182 and 184; and (e) 183 and 184, wherein the positions correspond to the corresponding positions of SEQ ID NO: 13, and wherein the polypeptide has at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 90%, such as at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 13.

In one embodiment, the variant of the invention has an improved stability in detergent compositions relative to the parent alpha-amylase of SEQ ID NOs: 3 or 13.

The term "improved stability" as used herein, refers to when the stability, such as the storage stability, of a polypeptide has been enhanced. In particular, it is well-known within the art that polypeptides degrade over time. Thus, an improvement of the stability means that the time before a polypeptide is degraded, in particular the time until the activity of the polypeptide is lost due to degradation, is extended. Accordingly, it is contemplated that an improved stability of the polypeptides of the present invention maintains the activity longer than for, e.g., a parent polypeptide.

The term "detergent composition" as used herein, refers to the definition elsewhere described herein.

In a further embodiment, the improved stability is improved storage stability.

In an embodiment, the improved stability is determined by a method comprising the steps of;

- a) incubating an alpha-amylase variant sample and a parent alpha-amylase sample, respectively, in a model detergent composition, such as Model A, Model J, Model T, or Model X, for a period of time;
- b) measuring the activity of the variant alpha-amylase and the parent alpha-amylase, respectively; and
- c) calculating the residual activity of the samples.

In a further embodiment, the improved stability is determined by a method comprising the steps of;

- a) incubating an alpha-amylase variant sample and a parent alpha-amylase sample, respectively, in a model detergent composition, such as Model A, Model J, Model T, or Model X, at 40°C to 60°C for 2 to 168hrs;
- b) measuring the activity of the variant alpha-amylase and the parent alpha-amylase, respectively; and

- c) calculating the residual activity of the samples as the average of activity in the samples relative to the average of the activity to frozen control samples.

In another embodiment, the variant of the invention has an improved performance in detergent compositions relative to the parent alpha-amylase of SEQ ID NOs: 3 or 13.

- 5 In a further embodiment, the improved performance is improved wash performance.

The term "improved performance" as used herein, refers to when the performance, such as the wash performance, has been enhanced. Performance may be measured by intensity of light reflected from a sample illuminated with white light, i.e. the ability of the polypeptide to remove or reduce stains on, e.g., fabrics. The performance may then be quantified by an
10 "Improvement Factor", which is well-known within the knowledge of the skilled person.

In one embodiment, the improved performance is determined according to an AMSA as described in the method section.

Thus, in one embodiment, the improved performance is determined by a method comprising the steps of;

- 15 a) washing a fabric stained with starch with an alpha-amylase variant and a parent alpha-amylase sample added, respectively, to a model detergent composition, such as Model A, Model J, Model T, or Model X;
- b) measuring the intensity of light reflected from the sample when illuminated with white light; and
- 20 c) optionally, calculating the improvement factor (IF) as the ration of delta intensity of the alpha-amylase sample over the delta intensity of the parent alpha-amylase sample.

In one embodiment, the improved performance is determined by a method comprising the steps of;

- 25 a) washing a fabric stained with starch with an alpha-amylase variant and a parent alpha-amylase sample added, respectively, to a model detergent composition, such as Model A, Model J, Model T, or Model X, for 20 minutes at 15°C and 30°C;
- b) measuring the intensity of light reflected from the sample when illuminated with white light; and
- 30 c) optionally, calculating the improvement factor (IF) as the ration of delta intensity of the alpha-amylase sample over the delta intensity of the parent alpha-amylase sample.

In a particular embodiment, the variant of the invention has both an improved stability and improved performance in detergent compositions relative to the parent alpha-amylase of
35 SEQ ID NOs: 3 or 13.

Parent alpha-amylases

The parent alpha-amylase may be (a) a polypeptide having at least 67% sequence identity to the mature polypeptide of SEQ ID NO: 2, or the polypeptide of SEQ ID NOs: 3 or 13; (b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with (i) the mature polynucleotide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i); or (c) a polynucleotide encoded by a polynucleotide having at least 67% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1.

In one embodiment, the parent alpha-amylase has a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 67%, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one embodiment, the amino acid sequence of the parent differs by up to 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the polypeptide of SEQ ID NOs: 3 or 13.

In another embodiment, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another embodiment, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another embodiment, the parent alpha-amylase comprises or consists of the polypeptide of SEQ ID NO:3. In another embodiment, the parent alpha-amylase comprises or consists of the polypeptide of SEQ ID NO: 13.

In another embodiment, the parent alpha-amylase is a polypeptide which in at least the amino acid positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 when referring to SEQ ID NO: 3 has the amino acids Q, M, N, G, T, M, Q, A, and N, respectively. Accordingly, the amino acid positions of a parent polypeptide as used in relation to the present invention at least have the following amino acids; Q51, M109, N193, G201, T269, M294, Q297, A298, and N314. In a further embodiment, the parent polypeptide has at least 67%, such as at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13 but wherein the amino acids in positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 when referring to SEQ ID NO: 3 are the amino acids Q, M, N, G, T, M, Q, A, and N, respectively.

In another embodiment, the parent is a fragment of the polypeptide of SEQ ID NOs: 3 or 13 containing at least 430 amino acid residues, *e.g.*, at least 440, at least 450, at least 460, at least 470, at least 480 amino acid residues.

In another embodiment, the parent is an allelic variant of the polypeptide of SEQ ID NOs: 3 or 13.

In another embodiment, the parent is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, or (ii) the full-

length complement of (i) (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the polypeptide of SEQ ID NOs: 3 or 13, or a fragment thereof may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of a cell of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, *e.g.*, at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, *e.g.*, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ³²P, ³H, ³⁵S, biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that hybridizes with SEQ ID NO: 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to (i) SEQ ID NO: 1; (ii) the full-length complement thereof; or (iii) a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film or any other detection means known in the art.

In one embodiment, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another embodiment, the nucleic acid probe comprises at least 50% of the nucleotides of SEQ ID NO: 1. In another embodiment, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2; the mature polypeptide thereof; or a fragment thereof.

In another embodiment, the parent alpha-amylase is encoded by a polynucleotide having a sequence identity to the mature polynucleotide coding sequence of SEQ ID NO: 1 of at least 60%, *e.g.*, at least 67%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

The parent may be a fusion polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. Thus, the polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide.

5 A fusion polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s) and terminator. Fusion polypeptides may also be constructed
10 using intein technology in which fusion polypeptides are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*,
15 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug*
20 *Discovery World* 4: 35-48.

The parent may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the parent encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the parent is
25 secreted extracellularly. The parent polypeptide of the present invention has been obtained from an Alicyclobacillus species found in humus from a Danish spruce and beech forest.

The parent may be a bacterial alpha-amylase. For example, the parent may be a Gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces*
30 alpha-amylase, or a Gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, *cytophaga*, or *Ureaplasma* alpha-amylase.

In one aspect, the parent is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*,
35 *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* alpha-amylase.

In another aspect, the parent is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* alpha-amylase.

In another aspect, the parent is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* alpha-amylase.

The parent may be a fungal alpha-amylase. For example, the parent may be a yeast
 5 alpha-amylase such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*,
Schizosaccharomyces, or *Yarrowia* alpha-amylase; or a filamentous fungal alpha-amylase such
 as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*,
Ceriporiopsis, *Chaetomidium*, *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*,
 10 *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*,
Fusarium, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*, *Lentinula*, *Leptosphaeria*,
Magnaporthe, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*,
Paecilomyces, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*, *Pseudoplectania*,
Pseudotriconympha, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*, *Thermoascus*,
Thielavia, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or *Xylaria* alpha-
 15 amylase.

In another aspect, the parent is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* alpha-amylase.

In another aspect, the parent is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*,
 20 *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*,
Aspergillus nidulans, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*,
Chrysosporium keratinophilum, *Chrysosporium lucknowense*, *Chrysosporium merdarium*,
Chrysosporium pannicola, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*,
Chrysosporium zonatum, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*,
 25 *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*,
Fusarium negundi, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*,
Fusarium sarcochromum, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
Fusarium torulosum, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*,
Humicola insolens, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora*
 30 *thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*,
Phanerochaete chrysosporium, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*,
Thielavia australeinsis, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*,
Thielavia peruviana, *Thielavia setosa*, *Thielavia spededonium*, *Thielavia subthermophila*,
Thielavia terrestris, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma*
 35 *longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* alpha-amylase.

In another aspect, the parent is an *Alicyclobacillus* alpha-amylase, e.g., the alpha-amylase of SEQ ID NO: 2 or the mature polypeptide thereof.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, *e.g.*, anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

5 Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

10 The parent alpha-amylase may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) or DNA samples obtained directly from natural materials (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding a parent may then be obtained by similarly
15 screening a genomic DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a parent has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

20 **Preparation of Variants**

The present invention also relates to a method of producing an alpha-amylase of the invention, comprising (a) cultivating a host cell under conditions suitable for expression of the variant of the invention, and (b) recovering the variant.

25 The present invention also relates to methods for obtaining an alpha-amylase variant, comprising introducing into a parent alpha-amylase having at least 67% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13 a substitution at one or more positions said substitutions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298 and 314 of SEQ ID NO: 3, wherein the variant has alpha-amylase activity; and recovering said variant.

30 In a particular embodiment, the present invention relates to methods for obtaining a variant having alpha-amylase activity, comprising: (a) introducing into a parent alpha-amylase having at least 67% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13 a substitution at one or more positions said substitutions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of SEQ ID NO:3, wherein the variant has at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%,
35 such as at least 95%, such as at least 96%, such as at least 97%, such as at least 99%, but less than 100% sequence identity with the amino acid sequence of SEQ ID NOs: 3 or 13, wherein the variant has alpha-amylase activity; and (b) recovering the variant.

The variants 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.

5 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.

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
10 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.

Site-directed mutagenesis can also be accomplished *in vivo* by methods known in the
15 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.

20 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.

25 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,
30 phage 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
35 cells (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 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
5 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 amplification. Polynucleotide subsequences may then be shuffled.

The present invention furthermore relates to methods of producing an alpha-amylase variant, comprising: (a) cultivating a host cell of the present invention under conditions suitable
10 for expression of the alpha-amylase variant; and (b) recovering the alpha-amylase variant. Thus, in one aspect, the present invention relates to a method of producing an alpha-amylase variant, comprising a) cultivating a host cell as described herein under conditions suitable for expression of the variant, and b) recovering the variant. In a particular embodiment, the method of producing an alpha-amylase variant comprises the steps of a) cultivating a host cell
15 comprising a polynucleotide, a nucleic acid construct or an expression vector as described herein; b) recovering the variant. In one embodiment, the method of producing an alpha-amylase variant comprises the steps of a) cultivating a host cell comprises a polynucleotide encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3, a
20 nucleic acid construct encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3, or an expression vector encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294,
25 297, 298, and 314 of SEQ ID NO:3; and b) recovering the variant.

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
30 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
35 lysates.

The variant may be detected using methods known in the art that are specific for the variants having alpha-amylase activity. These 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 activity of the variant.

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.

In one aspect, the present invention relates to a method of improving the stability, in particular the detergent stability, preferably liquid detergent stability, of a parent alpha-amylase having the amino acid sequence of SEQ ID NO:3, or having at least 67% sequence identity thereto, wherein the method comprises the steps of:

a) a substitution at one or more positions said substitutions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3 when using the mature polypeptide of SEQ ID NO:3 for numbering, wherein the resulting variant has at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 99%, but less than 100% sequence identity with the amino acid sequence of SEQ ID NOs: 3 or 13, where the variant has alpha-amylase activity; and

b) introducing into the parent alpha-amylase one or more of the following substitutions;
 105L,I,F+206Y; 105L,I+206Y+217I; 105F+206Y+208Y+217V+246V; 105L+206F;
 105I+206Y+208Y+217I+246V; 195F+213S+214T; 195F+206Y+208Y+213T+214T+217M,V;
 195F+206Y+208F,L+213T+214T+217V; 195F+206Y+213S+214T;
 195F+206Y+208Y+213S+214T+217M; 195F+206Y+208F+213T+214T+217M;
 195F+206Y+208Y+213T+214T+217Q; 195F+206Y+213G+214T; 195F+206Y+213S;
 195F+206Y+208Y+213T+214T+217M; 195F+213S; 195F+206Y+208L+213T+214T+217M;
 195F+213G+214T; 206Y,F+208Y+217Q; 206Y+208Y+217I; 206F+208Y+217M; 206Y+208Y;
 206Y+217M; 206Y+208Y+213A+217M; 206Y+208Y+217V+246V; 206Y+213G;
 206Y+208F+217V; 206N+208Y+217M; 206F+208Y+217V; 206Y+246V; 206Y+217I,V;
 206F+208F+217I; 206Y+208L+213S; 206F+217I; 206Y+217I+246I; 206L+217V;
 206Y+208F+217H; 206L+208F+217I; 206L+217V+246L; 206F+246V; 208Y+213S+217M;
 208Y+213A+217Q; 63I+206Y; 63I+206Y+241V; 63V+206Y; 63V+105L+206Y; 63V+206Y+217I;

WO 2016/079305

PCT/EP2015/077236

63V+105F+206Y+208F+217I; 63V+206Y+246V; 63V+206F; 63V+206L+217V;
 63V+105F+206Y; 63V+206Y+241V+246L; 195F+206Y+208Y+214T+217V; 186E+195F+206Y;
 195F+206Y+208Y+213T+217V; 186E+195F+202T+206Y+209S; 63I+195F+206Y+210S;
 195F+206Y+213P+214T; 195F+206Y+208Y+213T+214T+217I; 186E+195F+206Y+210S;
 5 195F+213P; 186E+195F+202T+206Y+210S; 195F+206H; 195F+208Y+213T+214T+217V;
 206Y+208Y+213T+214T+217V; 195F+206Y+217V; 195F+206Y+208Y+213S+214T;
 195F+206Y+208Y; 195F+213I+214P; 195F+206Y+208Y+213T+214T; 195F+206Y;
 206Y+213S; 182P+186E; 182S+186E; 182V+186K; 179L+186H+190P; 179L+186K,R,S+190P;
 179L+190P; 179L+182C+186K+190P; 179L+182P+186S,V+190P; 179L+182S+186Q+190P;
 10 173F+174Q; 173Y+174S; 172K+173Y+174E; 193A,D,N,S+195F; 213A+214Q; 213P+214L;
 213S+214R; 48V+60V; 213G+214T; 213I+214P; 213N+214I; 213N+214Q, and 213P,S+214T,
 wherein numbering is according to SEQ ID NO:11, and wherein the resulting variant has at least
 67%, such as at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%,
 at least 97%, at least 98%, at least 99%, but less than 100% sequence identity with the
 15 polypeptide of SEQ ID NOs: 3 or 13, and wherein the variant has alpha-amylase activity and
 improved detergent stability and/or improved performance compared to the parent alpha-
 amylase.

In a further embodiment, the variant has at least 50%, such as at least 60%, or at least
 70%, or at least 80%, or at least 90%, or at least 100% of the activity of the parent alpha-
 20 amylase having the amino acid sequence of SEQ ID NOs: 3 or 13.

In a further embodiment, the activity is determined according to the Phadebas assay as
 described herein. Thus, in one embodiment, the activity is determined by a method comprising
 the steps of;

- a) incubating an alpha-amylase variant according to the invention with a dyed amylose
 25 substrate for 15 minute at 37°C; and
- b) measuring the absorption at OD 620 nm.

In a further embodiment, the activity is determined by a method comprising the steps of;

- a) incubating an alpha-amylase variant according to the invention with a dyed amylose
 substrate for 15 minute at 37°C; and
- 30 b) centrifuging the sample;
- c) transferring the supernatant to reader plate, and
- d) measuring the absorption at OD 620 nm.

Polynucleotides

35 The present invention also relates to polynucleotides encoding a variant of the present
 invention. Thus, in one aspect, the present invention relates to a polynucleotide encoding a
 variant as described herein. In one embodiment, the polynucleotide is an isolated
 polynucleotide.

In a particular embodiment, the polynucleotide encodes an alpha-amylase variant comprising a substitution at one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3.

5 Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present 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. Thus, in one embodiment, the invention relates to a nucleic acid construct comprising a polynucleotide as described herein. In a particular
10 embodiment, the nucleic acid construct comprises a polynucleotide encoding an alpha-amylase variant as described herein. Thus, the nucleic acid construct comprises a polynucleotide encoding a variant comprising a substitution at one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3.

15 The term "nucleic acid construct" as used herein, refers to a DNA construct which is an artificially constructed segment of nucleic acids to be transplanted into target tissue or cell, such as a host cell. A nucleic acid construct comprises at least a gene sequence encoding the polypeptide. It is within the skills of the skilled person to understand the meaning and purpose of a nucleic acid construct.

20 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
25 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.

30 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
35 *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.

5 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),
 10 *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, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III,
 15 *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 untranslated leader has been replaced by an untranslated leader from an
 20 *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.

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. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8:
 25 423-488.
 30

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 be used.

35 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 *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

5 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 cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be an mRNA stabilizer region downstream of a promoter
10 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).

The control sequence may also be a leader, a nontranslated region of an mRNA that is
15 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.

20 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).

The control sequence may also be a polyadenylation sequence, a sequence operably
25 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.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase,
30 *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.

The control sequence may also be a signal peptide coding region that encodes a signal
35 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 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.

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
5 would be operably linked with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant of the present invention, a promoter, and transcriptional and
10 translational stop signals. 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.
15 In creating the expression 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 term "expression vector" as used herein, refers to any vector which may comprise an origin of replication, a selectable marker, and a suitable site for the insertion of a gene such as the multiple cloning site. The cloned gene may be transferred from a specialized cloning
20 vector to an expression vector, although it is possible to clone directly into an expression vector. It is within the skills of the skilled person to determine the meaning and purpose of an expression vector.

Accordingly, in one aspect, the present invention relates to an expression vector comprising a polynucleotide as described herein. Thus, in a particular embodiment, the
25 expression vector comprises a polynucleotide encoding an alpha-amylase variant comprising a substitution at one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3.

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
30 the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector 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,
35 *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The 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.

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* 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), *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 hygroscopicus 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*.

5 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
10 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
15 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
20 (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences
25 that direct the production of a variant of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the 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
30 depend upon the gene encoding the variant and its source.

Accordingly, in one aspect the present invention relates to a host cell comprising a polynucleotide, a nucleic acid construct or an expression vector as described herein. Thus, in one embodiment, the host cell comprises a polynucleotide encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201,
35 269, 294, 297, 298, and 314 of SEQ ID NO:3, a nucleic acid construct encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3, or an expression vector encoding

an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3.

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

5 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*. Gram-negative bacteria include, but are not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*,
10 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 megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and
15 *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.

The bacterial host cell may also be any *Streptomyces* cell, including, but not limited to,
20 *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
25 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
30 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.*
35 *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.

5 The host cell may also be a eukaryote, such as a mammalian, insect, 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).

10 The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporegenous yeast (Endomycetales), basidiosporegenous 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.*
15 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*,
20 *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
25 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.

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

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*,
35 *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *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*
 5 *roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *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*,
 10 *Trichoderma koningii*, *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
 15 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*,
 20 Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Fermentation Broth Formulations or Cell Compositions

The present invention also relates to a fermentation broth formulation or a cell
 25 composition comprising a polypeptide of the present invention. Thus, in one embodiment, the fermentation broth formulation or the cell composition comprises a polynucleotide encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3, a nucleic acid construct encoding an alpha-amylase variant comprising a substitution in one or more positions
 30 corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3, or an expression vector encoding an alpha-amylase variant comprising a substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3. The fermentation broth product may further comprise additional ingredients used in the fermentation process, such as, for example, cells (including, the host cells containing the
 35 gene encoding the polypeptide of the present invention which are used to produce the polypeptide of interest), cell debris, biomass, fermentation media and/or fermentation products. In some embodiments, the composition is a cell-killed whole broth containing organic acid(s), killed cells and/or cell debris, and culture medium.

The term "fermentation broth" as used herein refers to a preparation produced by cellular fermentation that undergoes no or minimal recovery and/or purification. For example, fermentation broths are produced when microbial cultures are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (e.g., expression of enzymes by host
5 cells) and secretion into cell culture medium. The fermentation broth can contain unfractionated or fractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the fermentation broth is unfractionated and comprises the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are removed, e.g., by centrifugation. In some embodiments, the fermentation broth contains spent cell culture
10 medium, extracellular enzymes, and viable and/or nonviable microbial cells.

In an embodiment, the fermentation broth formulation and cell compositions comprise a first organic acid component comprising at least one 1-5 carbon organic acid and/or a salt thereof and a second organic acid component comprising at least one 6 or more carbon organic acid and/or a salt thereof. In a particular embodiment, the first organic acid component is acetic
15 acid, formic acid, propionic acid, a salt thereof, or a mixture of two or more of the foregoing and the second organic acid component is benzoic acid, cyclohexanecarboxylic acid, 4-methylvaleric acid, phenylacetic acid, a salt thereof, or a mixture of two or more of the foregoing.

In one embodiment, the composition contains an organic acid(s), and optionally further
20 contains killed cells and/or cell debris. In one embodiment, the killed cells and/or cell debris are removed from a cell-killed whole broth to provide a composition that is free of these components.

The fermentation broth formulations or cell compositions may further comprise a preservative and/or anti-microbial (e.g., bacteriostatic) agent, including, but not limited to,
25 sorbitol, sodium chloride, potassium sorbate, and others known in the art.

The cell-killed whole broth or composition may comprise the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the cell-killed whole broth or composition comprises the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are grown to saturation, incubated under carbon-
30 limiting conditions to allow protein synthesis. In some embodiments, the cell-killed whole broth or composition comprises the spent cell culture medium, extracellular enzymes, and killed filamentous fungal cells. In some embodiments, the microbial cells present in the cell-killed whole broth or composition may be permeabilized and/or lysed using methods known in the art.

A whole broth or cell composition as described herein is typically a liquid, but may
35 comprise insoluble components, such as killed cells, cell debris, culture media components, and/or insoluble enzyme(s). In some embodiments, insoluble components may be removed to provide a clarified liquid composition.

The whole broth formulations and cell compositions of the present invention may be produced by a method described in WO 90/15861 or WO 2010/096673.

Enzyme Compositions

5 The present invention also relates to compositions comprising an alpha-amylase variant of the present invention. Thus, in one aspect the present invention relates to a composition comprising an alpha-amylase variant as described herein. In a particular embodiment, the composition comprises a variant which comprises a substitution in one or more positions corresponding to position 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3.
10 Preferably, the compositions are enriched in such a variant. The term "enriched" indicates that the alpha-amylase activity of the composition has been increased, e.g., with an enrichment factor of at least 1.1.

The compositions may comprise a variant of the present invention as the major enzymatic component, e.g., a mono-component composition. In another embodiment, the
15 composition comprises at least one further active component.

The term "active component" as used herein, refers to any biological or non-biological molecule which in itself is active. For example, an active component is an enzyme.

Thus, in one embodiment, the further active component is an enzyme, such as a protease, lipase, cellulose, pectate lyase, and mannanase. Thus, the compositions may
20 comprise multiple enzymatic activities, such as one or more (e.g., several) enzymes selected from the group consisting of hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, e.g., an alpha-galactosidase, alpha-glucosidase, aminopeptidase, amylase, beta-galactosidase, beta-glucosidase, beta-xylosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase,
25 deoxyribonuclease, endoglucanase, esterase, glucoamylase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase.

In a particular embodiment, the further active component is an enzyme, which is selected from the group consisting of;

- 30 i. a protease comprising one or more modifications in the following positions: 32, 33, 48-54, 58-62, 94-107, 116, 123-133, 150, 152-156, 158-161, 164, 169, 175-186, 197, 198, 203-216 as compared with the protease in SEQ ID NO:4;
- ii. a lipase comprising one or more modifications in the following positions: 1-5, 27, 33, 38, 57, 91, 94, 96, 97, 111, 163, 210, 225, 231, 233, 249, and 254-256 as
35 compared with the lipase in SEQ ID NO:5;
- iii. an alpha-amylase comprising one or more modifications in the following positions: 9, 118, 149, 182, 186, 195, 202, 257, 295, 299, 320, 323, 339, 345, and 458 as compared with the alpha-amylase in SEQ ID NO:6;

- iv. an alpha-amylase comprising one or more modifications in the following positions: 140, 195, and 206, 243, 260, and 476 as compared with the alpha-amylase in SEQ ID NO:7;
- 5 v. an alpha-amylase comprising one or more modifications in the following positions: 180, 181, 243, and 475 as compared with the alpha-amylase in SEQ ID NO:8;
- vi. an alpha-amylase comprising one or more modifications in the following positions: 178, 179, 187, 203, 458, 459, 460, and 476 as compared with the alpha-amylase in SEQ ID NO:9;
- 10 vii. an alpha-amylase comprising a modifications in the following position: 202 as compared with the alpha-amylase in SEQ ID NO:10;
- viii. an alpha-amylase comprising one or more modifications in the following positions: 405, 421, 422, and 428 as compared with the alpha-amylase in SEQ ID NO:11; and/or
- 15 ix. an alpha-amylase according to SEQ ID NO:12.

The detergent composition may comprise one or more additional enzymes, herein also termed "further active components", such as a protease, lipase, cutinase, an amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, oxidase, e.g., a laccase, and/or peroxidase.

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

Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera
25 *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 color care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372,
30 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.

Example of cellulases exhibiting endo-beta-1,4-glucanase activity (EC 3.2.1.4) are those having described in WO02/099091.

35 Other examples of cellulases include the family 45 cellulases described in WO96/29397, and especially variants thereof having substitution, insertion and/or deletion at one or more of the positions corresponding to the following positions in SEQ ID NO: 8 of WO 02/099091: 2, 4, 7, 8, 10, 13, 15, 19, 20, 21, 25, 26, 29, 32, 33, 34, 35, 37, 40, 42, 42a, 43, 44, 48, 53, 54, 55,

58, 59, 63, 64, 65, 66, 67, 70, 72, 76, 79, 80, 82, 84, 86, 88, 90, 91, 93, 95, 95d, 95h, 95j, 97, 100, 101, 102, 103, 113, 114, 117, 119, 121, 133, 136, 137, 138, 139, 140a, 141, 143a, 145, 146, 147, 150e, 150j, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160c, 160e, 160k, 161, 162, 164, 165, 168, 170, 171, 172, 173, 175, 176, 178, 181, 183, 184, 185, 186, 188, 191, 192, 195, 5 196, 200, and/or 20, preferably selected among P19A, G20K, Q44K, N48E, Q119H or Q146 R.

Commercially available cellulases include Celluzyme, and Carezyme (Novozymes A/S), Clazinase, and Puradax HA (Genencor International Inc.), and KAC-500(B) (Kao Corporation).

The additional enzyme (or further active component) may be a protease or protease variant. The protease may be of animal, vegetable or microbial origin, including chemically or 10 genetically modified mutants. Microbial origin is preferred. It may be an alkaline protease, such as a serine protease or a metalloprotease. A serine protease may for example be of the S1 family, such as trypsin, or the S8 family such as subtilisin. A metalloproteases protease may for example be a thermolysin from e.g. family M4, M5, M7 or M8.

The term "subtilases" refers to a sub-group of serine protease according to Siezen et al., 15 Protein Engng. 4 (1991) 719-737 and Siezen et al. Protein Science 6 (1997) 501-523. Serine proteases are a subgroup of proteases characterized by having a serine in the active site, which forms a covalent adduct with the substrate. The subtilases may be divided into 6 sub-divisions, i.e. the Subtilisin family, the Thermitase family, the Proteinase K family, the Lantibiotic peptidase family, the Kexin family and the Pyrolysin family. In one aspect of the invention the protease 20 may be a subtilase, such as a subtilisin or a variant hereof. Further the subtilases (and the serine proteases) are characterised by having two active site amino acid residues apart from the serine, namely a histidine and an aspartic acid residue.

Examples of subtilisins are those derived from *Bacillus* such as subtilisin lentus, *Bacillus* 25 lentus, subtilisin Novo, subtilisin Carlsberg, *Bacillus licheniformis*, subtilisin BPN', subtilisin 309, subtilisin 147 and subtilisin 168 described in WO 89/06279 and protease PD138 (WO 93/18140). Additional serine protease examples are described in WO 98/020115, WO 01/44452, WO 01/58275, WO 01/58276, WO 03/006602 and WO 04/099401. An example of a subtilase variants may be those having mutations in any of the positions: 3, 4, 9, 15, 27, 36, 68, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 118, 120, 123, 128, 129, 130, 160, 30 167, 170, 194, 195, 199, 205, 217, 218, 222, 232, 235, 236, 245, 248, 252 and 274 using the BPN' numbering. More preferred the subtilase variants may comprise the mutations: S3T, V4I, S9R, A15T, K27R, *36D, V68A, N76D, N87S,R, *97E, A98S, S99G,D,A, S99AD, S101G,M,R S103A, V104I,Y,N, S106A, G118V,R, H120D,N, N123S, S128L, P129Q, S130A, G160D, Y167A, R170S, A194P, G195E, V199M, V205I, L217D, N218D, M222S, A232V, K235L, 35 Q236H, Q245R, N252K, T274A (using BPN' numbering). A further preferred protease is the alkaline protease from *Bacillus lentus* DSM 5483, as described for example in WO 95/23221, and variants thereof which are described in WO 92/21760, WO 95/23221, EP 1921147 and EP 1921148.

Examples of trypsin-like proteases 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, 5 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235, and 274.

Examples of metalloproteases are the neutral metalloprotease as described in WO 07/044993.

Preferred commercially available protease enzymes include AlcalaseTM, CoronaseTM, DuralaseTM, DurazymTM, EsperaseTM, EverlaseTM, KannaseTM, LiquanaseTM, Liquanase 10 UltraTM, OvozymeTM, PolarzymeTM, PrimaseTM, RelaseTM, SavinaseTM and Savinase UltraTM, (Novozymes A/S), AxapemTM (Gist-Brocades N.V.), BLAP and BLAP X (Henkel AG & Co. KGaA), ExcellaseTM, FN2TM, FN3TM, FN4TM, MaxacaTM, MaxapemTM, MaxataseTM, ProperaseTM, PurafastTM, PurafectTM, Purafect OxPTM, Purafect PrimeTM and PuramaxTM (Genencor int.).

15 Suitable lipases and cutinases include those of bacterial or fungal origin. Chemically modified or protein engineered mutant enzymes are included. Examples include lipase from *Thermomyces*, e.g. from *T. lanuginosus* (previously named *Humicola lanuginosa*) as described in EP258068 and EP305216, cutinase from *Humicola*, e.g. *H. insolens* (WO96/13580), lipase from strains of *Pseudomonas* (some of these now renamed to *Burkholderia*), e.g. *P. alcaligenes* or *P. pseudoalcaligenes* (EP218272), *P. cepacia* (EP331376), *P. sp.* strain SD705 20 (WO95/06720 & WO96/27002), *P. wisconsinensis* (WO96/12012), GDSL-type *Streptomyces* lipases (WO10/065455), cutinase from *Magnaporthe grisea* (WO10/107560), cutinase from *Pseudomonas mendocina* (US5,389,536), lipase from *Thermobifida fusca* (WO11/084412), *Geobacillus stearothermophilus* lipase (WO11/084417), lipase from *Bacillus subtilis* 25 (WO11/084599), and lipase from *Streptomyces griseus* (WO11/150157) and *S. pristinaespiralis* (WO12/137147).

Further examples are lipases sometimes referred to as acyltransferases or perhydrolases, e.g. acyltransferases with homology to *Candida antarctica* lipase A (WO10/111143), acyltransferase from *Mycobacterium smegmatis* (WO05/56782), 30 perhydrolases from the CE 7 family (WO09/67279), and variants of the *M. smegmatis* perhydrolase in particular the S54V variant used in the commercial product Gentle Power Bleach from Huntsman Textile Effects Pte Ltd (WO10/100028).

Other examples are lipase variants such as those described in EP407225, WO92/05249, WO94/01541, WO94/25578, WO95/14783, WO95/30744, WO95/35381, WO95/22615, 35 WO96/00292, WO97/04079, WO97/07202, WO00/34450, WO00/60063, WO01/92502, WO07/87508 and WO09/109500.

Preferred commercial lipase products include include Lipolase™, Lipex™; Lipolex™ and Lipoclean™ (Novozymes A/S), Lumafast (originally from Genencor) and Lipomax (originally from Gist-Brocades).

5 The further active component is not limited to other categories of enzymes. Thus, the further active component may be an additional amylase such as an alpha-amylase or a glucoamylase and may be 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 *Bacillus licheniformis*, described in more detail in GB 1,296,839.

10 Examples of amylases are those described in WO 95/10603 or variants thereof. Preferred variants are described in WO 94/02597, WO 94/18314, WO 97/43424 and SEQ ID NO: 4 of WO 99/019467, such as variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 178, 179, 181, 188, 190, 197, 201, 202, 207, 208, 209, 211, 243, 264, 304, 305, 391, 408, and 444 of SEQ ID NO: 3 in WO 95/10603.

15 Other amylases which can be used are amylases having SEQ ID NO: 6 in WO 02/010355 or variants thereof as well as hybrid alpha-amylase comprising residues 1-33 of the alpha-amylase derived from *Bacillus amyloliquefaciens* shown in SEQ ID NO: 6 of WO 2006/066594 and residues 36-483 of the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4 of WO 2006/066594.

20 Further amylase examples are amylases having SEQ ID NO: 6 in WO 99/019467 or variants thereof and amylases having SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 7 of WO 96/023873 or variants thereof. Other amylases which can be used are amylases having SEQ ID NO: 2 of WO 08/153815, SEQ ID NO: 10 in WO 01/66712 or variants thereof.

25 Additional amylases which can be used are amylases having SEQ ID NO: 2 of WO 09/061380 or variants thereof and alpha-amylases having SEQ ID NO: 12 in WO01/66712 or a variant thereof.

30 Commercially available amylases are Duramyl, Termamyl, Fungamyl, Stainzyme, Stainzyme Plus, Natalase, BAN, Everest and Amplify (Novozymes A/S), Powerase, Preferenz S100, Preferenz S110, Preferenz S1000, Preferenz S2000, Excellenz S1000 (from Genencor International Inc.).

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.

35 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, for example, as a granulate, liquid, 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
5 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 nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and
10 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.

15 The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. The compositions may be stabilized in accordance with methods known in the art.

Detergent compositions

20 In one aspect, the invention relates to detergent compositions comprising an alpha-amylase variant as described herein. Thus, in one aspect the invention relates to a composition which is a detergent composition. In a particular embodiment, the detergent composition is a liquid or powder composition. In one embodiment, the composition comprises an alpha-amylase variant which comprises a substitution in one or more positions corresponding to positions 51,
25 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO: 3. In a further embodiment, the composition comprises a detergent composition of the present invention in combination with one or more additional cleaning composition components. In an embodiment, the detergent is a liquid detergent composition. In another embodiment the detergent composition is a powder detergent composition.

30 The detergent composition may be a laundry detergent composition or a dish wash detergent composition. A dish wash detergent composition may be both for use in manual dish wash as well as for use in automated dish wash.

The choice of additional components is within the skill of the artisan and includes conventional ingredients, including the exemplary non-limiting components set forth below. The
35 choice of components may include, for fabric care, the consideration of the type of fabric to be cleaned, the type and/or degree of soiling, the temperature at which cleaning is to take place, and the formulation of the detergent product. Although components mentioned below are categorized by general header according to a particular functionality, this is not to be construed

as a limitation, as a component may comprise additional functionalities as will be appreciated by the skilled artisan.

In one embodiment of the present invention, the a polypeptide of the present invention may be added to a detergent composition in an amount corresponding to 0.001-100 mg of protein, such as 0.01-100 mg of protein, preferably 0.005-50 mg of protein, more preferably 0.01-25 mg of protein, even more preferably 0.05-10 mg of protein, most preferably 0.05-5 mg of protein, and even most preferably 0.01-1 mg of protein per liter of wash liquor. The term "protein" in this context is contemplated to be understood to include an alpha-amylase variant according to the present invention.

A composition for use in automatic dish wash (ADW), for example, may include 0.0001%-50%, such as 0.001%-20%, such as 0.01%-10%, such as 0.05-5% of enzyme protein by weight of the composition.

A composition for use in laundry granulation, for example, may include 0.0001%-50%, such as 0.001%-20%, such as 0.01%-10%, such as 0.05%-5% of enzyme protein by weight of the composition.

A composition for use in laundry liquid, for example, may include 0.0001%-10%, such as 0.001-7%, such as 0.1%-5% of enzyme protein by weight of the composition.

The alpha-amylase variants of the invention as well as the further active components, such as additional enzymes, 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, for example, WO92/19709 and WO92/19708.

In certain markets different wash conditions and, as such, different types of detergents are used. This is disclosed in e.g. EP 1 025 240. For example, In Asia (Japan) a low detergent concentration system is used, while the United States uses a medium detergent concentration system, and Europe uses a high detergent concentration system.

A low detergent concentration system includes detergents where less than about 800 ppm of detergent components are present in the wash water. Japanese detergents are typically considered low detergent concentration system as they have approximately 667 ppm of detergent components present in the wash water.

A medium detergent concentration includes detergents where between about 800 ppm and about 2000ppm of detergent components are present in the wash water. North American detergents are generally considered to be medium detergent concentration systems as they have approximately 975 ppm of detergent components present in the wash water.

A high detergent concentration system includes detergents where greater than about 2000 ppm of detergent components are present in the wash water. European detergents are

generally considered to be high detergent concentration systems as they have approximately 4500-5000 ppm of detergent components in the wash water.

Latin American detergents are generally high suds phosphate builder detergents and the range of detergents used in Latin America can fall in both the medium and high detergent concentrations as they range from 1500 ppm to 6000 ppm of detergent components in the wash water. Such detergent compositions are all embodiments of the invention.

An alpha-amylase variant of the present invention may also be incorporated in the detergent formulations disclosed in WO97/07202, which is hereby incorporated by reference.

Examples are given herein of preferred uses of the compositions of the present invention. The dosage of the composition and other conditions under which the composition is used may be determined on the basis of methods known in the art.

Surfactants

The detergent composition may comprise one or more surfactants, which may be anionic and/or cationic and/or non-ionic and/or semi-polar and/or zwitterionic, or a mixture thereof. In a particular embodiment, the detergent composition includes a mixture of one or more nonionic surfactants and one or more anionic surfactants. The surfactant(s) is typically present at a level of from about 0.1% to 60% by weight, such as about 1% to about 40%, or about 3% to about 20%, or about 3% to about 10%. The surfactant(s) is chosen based on the desired cleaning application, and includes any conventional surfactant(s) known in the art. Any surfactant known in the art for use in detergents may be utilized.

When included therein the detergent will usually contain from about 1% to about 40% by weight, such as from about 5% to about 30%, including from about 5% to about 15%, or from about 20% to about 25% of an anionic surfactant. Non-limiting examples of anionic surfactants include sulfates and sulfonates, in particular, linear alkylbenzenesulfonates (LAS), isomers of LAS, branched alkylbenzenesulfonates (BABS), phenylalkanesulfonates, alpha-olefinsulfonates (AOS), olefin sulfonates, alkene sulfonates, alkane-2,3-diylbis(sulfates), hydroxyalkanesulfonates and disulfonates, alkyl sulfates (AS) such as sodium dodecyl sulfate (SDS), fatty alcohol sulfates (FAS), primary alcohol sulfates (PAS), alcohol ethersulfates (AES or AEOS or FES, also known as alcohol ethoxysulfates or fatty alcohol ether sulfates), secondary alkanesulfonates (SAS), paraffin sulfonates (PS), ester sulfonates, sulfonated fatty acid glycerol esters, alpha-sulfo fatty acid methyl esters (alpha-SFMe or SES) including methyl ester sulfonate (MES), alkyl- or alkenylsuccinic acid, dodecenyyl/tetradecenyl succinic acid (DTSA), fatty acid derivatives of amino acids, diesters and monoesters of sulfo-succinic acid or soap, and combinations thereof.

When included therein the detergent will usually contain from about 0 % to about 40% by weight of a cationic surfactant. Non-limiting examples of cationic surfactants include alkyl dimethylethanolamine quat (ADMEAQ), cetyltrimethylammonium bromide (CTAB),

dimethyldistearylammonium chloride (DSDMAC), and alkylbenzyltrimethylammonium, alkyl quaternary ammonium compounds, alkoxyated quaternary ammonium (AQA) compounds, and combinations thereof.

When included therein the detergent will usually contain from about 0.2% to about 40%
5 by weight of a non-ionic surfactant, for example from about 0.5% to about 30%, in particular from about 1% to about 20%, from about 3% to about 10%, such as from about 3% to about 5%, or from about 8% to about 12%. Non-limiting examples of non-ionic surfactants include alcohol ethoxylates (AE or AEO), alcohol propoxylates, propoxylated fatty alcohols (PFA), alkoxyated fatty acid alkyl esters, such as ethoxylated and/or propoxylated fatty acid alkyl
10 esters, alkylphenol ethoxylates (APE), nonylphenol ethoxylates (NPE), alkylpolyglycosides (APG), alkoxyated amines, fatty acid monoethanolamides (FAM), fatty acid diethanolamides (FADA), ethoxylated fatty acid monoethanolamides (EFAM), propoxylated fatty acid monoethanolamides (PFAM), polyhydroxy alkyl fatty acid amides, or *N*-acyl *N*-alkyl derivatives of glucosamine (glucamides, GA, or fatty acid glucamide, FAGA), as well as products available
15 under the trade names SPAN and TWEEN, and combinations thereof.

When included therein the detergent will usually contain from about 0 % to about 40% by weight of a semipolar surfactant. Non-limiting examples of semipolar surfactants include amine oxides (AO) such as alkyldimethylamineoxide, *N*-(coco alkyl)-*N,N*-dimethylamine oxide and *N*-(tallow-alkyl)-*N,N*-bis(2-hydroxyethyl)amine oxide, fatty acid alkanolamides and ethoxylated fatty
20 acid alkanolamides, and combinations thereof.

When included therein the detergent will usually contain from about 0% to about 40% by weight of a zwitterionic surfactant. Non-limiting examples of zwitterionic surfactants include betaine, alkyldimethylbetaine, sulfobetaine, and combinations thereof.

The detergent composition may also comprise one or more isoprenoid surfactants as
25 disclosed in US 20130072416 or US 20130072415.

Hydrotropes

A hydrotrope is a compound that solubilises hydrophobic compounds in aqueous solutions (or oppositely, polar substances in a non-polar environment). Typically, hydrotropes
30 have both hydrophilic and a hydrophobic character (so-called amphiphilic properties as known from surfactants); however the molecular structure of hydrotropes generally do not favor spontaneous self-aggregation, see e.g. review by Hodgdon and Kaler (2007), Current Opinion in Colloid & Interface Science 12: 121-128. Hydrotropes do not display a critical concentration above which self-aggregation occurs as found for surfactants and lipids forming micellar,
35 lamellar or other well defined meso-phases. Instead, many hydrotropes show a continuous-type aggregation process where the sizes of aggregates grow as concentration increases. However, many hydrotropes alter the phase behavior, stability, and colloidal properties of systems containing substances of polar and non-polar character, including mixtures of water, oil,

surfactants, and polymers. Hydrotropes are classically used across industries from pharma, personal care, food, to technical applications. Use of hydrotropes in detergent compositions allow for example more concentrated formulations of surfactants (as in the process of compacting liquid detergents by removing water) without inducing undesired phenomena such as phase separation or high viscosity.

The detergent may contain 0-5% by weight, such as about 0.5 to about 5%, or about 3% to about 5%, of a hydrotrope. Any hydrotrope known in the art for use in detergents may be utilized. Non-limiting examples of hydrotropes include sodium benzene sulfonate, sodium p-toluene sulfonate (STS), sodium xylene sulfonate (SXS), sodium cumene sulfonate (SCS), sodium cymene sulfonate, amine oxides, alcohols and polyglycoethers, sodium hydroxynaphthoate, sodium hydroxynaphthalene sulfonate, sodium ethylhexyl sulfate, and combinations thereof.

Builders and Co-Builders

The detergent composition may contain about 0-65% by weight, such as about 10% to about 40% of a detergent builder or co-builder, or a mixture thereof. In a dish wash detergent, the level of builder is typically 40-65%, particularly 50-65%. The builder and/or co-builder may particularly be a chelating agent (ie. a chelator) that forms water-soluble complexes with Ca and Mg. Any builder and/or co-builder known in the art for use in laundry detergents may be utilized. Non-limiting examples of builders include zeolites, diphosphates (pyrophosphates), triphosphates such as sodium triphosphate (STP or STPP), carbonates such as sodium carbonate, soluble silicates such as sodium metasilicate, layered silicates (e.g., SKS-6 from Hoechst), ethanolamines such as 2-aminoethan-1-ol (MEA), diethanolamine (DEA, also known as iminodiethanol), triethanolamine (TEA, also known as 2,2',2''-nitrilotriethanol), and carboxymethyl inulin (CMI), and combinations thereof.

The detergent composition may also contain 0-50% by weight, such as about 10% to about 40%, of a detergent co-builder, or a mixture thereof. The detergent composition may include a co-builder alone, or in combination with a builder, for example a zeolite builder. Non-limiting examples of co-builders include homopolymers of polyacrylates or copolymers thereof, such as poly(acrylic acid) (PAA) or copoly(acrylic acid/maleic acid) (PAA/PMA). Further non-limiting examples include citrate, chelators such as aminocarboxylates, aminopolycarboxylates and phosphonates, and alkyl- or alkenylsuccinic acid. Additional specific examples include 2,2',2''-nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), iminodisuccinic acid (IDS), ethylenediamine-*N,N'*-disuccinic acid (EDDS), methylglycinediacetic acid (MGDA), glutamic acid-*N,N*-diacetic acid (GLDA), 1-hydroxyethane-1,1-diphosphonic acid (HEDP), ethylenediaminetetra(methylenephosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylenephosphonic acid) (DTMPA or DTPMPA), *N*-(2-

hydroxyethyl)iminodiacetic acid (EDG), aspartic acid-*N*-monoacetic acid (ASMA), aspartic acid-*N,N*-diacetic acid (ASDA), aspartic acid-*N*-monopropionic acid (ASMP), iminodisuccinic acid (IDA), *N*-(2-sulfomethyl)-aspartic acid (SMAS), *N*-(2-sulfoethyl)-aspartic acid (SEAS), *N*-(2-sulfomethyl)-glutamic acid (SMGL), *N*-(2-sulfoethyl)-glutamic acid (SEGL), *N*-methyliminodiacetic acid (MIDA), α -alanine-*N,N*-diacetic acid (α -ALDA), serine-*N,N*-diacetic acid (SEDA), isoserine-*N,N*-diacetic acid (ISDA), phenylalanine-*N,N*-diacetic acid (PHDA), anthranilic acid-*N,N*-diacetic acid (ANDA), sulfanilic acid-*N,N*-diacetic acid (SLDA), taurine-*N,N*-diacetic acid (TUDA) and sulfomethyl-*N,N*-diacetic acid (SMDA), *N*-(2-hydroxyethyl)-ethylidenediamine-*N,N',N'*-triacetate (HEDTA), diethanolglycine (DEG), diethylenetriamine penta(methylenephosphonic acid) (DTPMP), aminotris(methylenephosphonic acid) (ATMP), and combinations and salts thereof. Further exemplary builders and/or co-builders are described in, e.g., WO 09/102854, US 5977053.

Chelating agents or chelators are chemicals which form molecules with certain metal ions, inactivating the ions so that they cannot react with other elements thus a binding agent that suppresses chemical activity by forming chelates. Chelation is the formation or presence of two or more separate bindings between a ligand and a single central atom. The ligand may be any organic compound, a silicate or a phosphate. In the present context the term "chelating agents" comprises chelants, chelating agent, chelating agents, complexing agents, or sequestering agents that forms water-soluble complexes with metal ions such as calcium and magnesium. The chelate effect describes the enhanced affinity of chelating ligands for a metal ion compared to the affinity of a collection of similar nonchelating ligands for the same metal. Chelating agents having binding capacity with metal ions, in particular calcium (Ca^{2+}) ions, and has been used widely in detergents and compositions in general for wash, such as laundry or dish wash. Chelating agents have however shown themselves to inhibit enzymatic activity. The term chelating agent is used in the present application interchangeably with "complexing agent" or "chelating agent" or "chelant".

Since most alpha-amylases are calcium sensitive the presence of chelating agents these may impair the enzyme activity. The calcium sensitivity of alpha-amylases can be determined by incubating a given alpha-amylase in the presence of a strong chelating agent and analyze the impact of this incubation on the activity of the alpha-amylase in question. A calcium sensitive alpha-amylase will lose a major part or all of its activity during the incubation. Chelating agent may be present in the composition in an amount from 0.0001 wt% to 20wt%, preferably from 0.01 to 10 wt%, more preferably from 0.1 to 5wt%.

Strong chelating agents may be but are not limited to the following: ethylene-diamine-tetraacetic acid (EDTA), diethylene triamine penta methylene phosphonic acid (DTMPA, DTPMP), hydroxy-ethane diphosphonic acid (HEDP), ethylenediamine *N,N'*-disuccinic acid (EDDS),

methyl glycine di-acetic acid (MGDA), diethylene triamine penta acetic acid (DTPA), propylene diamine tetraacetic acid (PDTA), 2-hydroxypyridine-N-oxide (HPNO), methyl glycine diacetic acid (MGDA), glutamic acid N,N-diacetic acid (N,N-dicarboxymethyl glutamic acid tetrasodium salt (GLDA) and nitrilotriacetic acid (NTA) or mixtures thereof. The chelating agents may be present in their acid form or a salt, preferably the chelating agents may be present as a sodium, ammonium or potassium salt.

Characterizing chelating agents: As mentioned the chelate effect or the chelating effect describes the enhanced affinity of chelating ligands for a metal ion compared to the affinity of a collection of similar nonchelating ligands for the same metal. However, the strength of this chelate effect can be determined by various types of assays or measure methods thereby differentiating or ranking the chelating agents according to their chelating effect (or strength).

In an assay the chelating agents may be characterized by their ability to reduce the concentration of free calcium ions (Ca^{2+}) from 2.0 mM to 0.10 mM or less at pH 8.0, e.g. by using a test based on the method described by M.K.Nagarajan et al., JAOCS, Vol. 61, no. 9 (September 1984), pp. 1475-1478.

For reference, a chelator having the same ability to reduce the concentration of free calcium ions (Ca^{2+}) from 2.0 mM to 0.10 mM at pH as EDTA at equal concentrations of the chelator are said to be strong chelators.

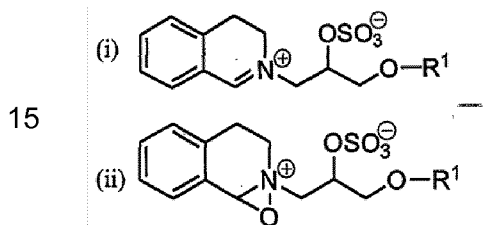
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Bleaching Systems

The detergent may contain 0-20% by weight, such as about 0 % to about 10%, of a bleaching system. Any bleaching system known in the art for use in laundry + dish wash + I&I detergents may be utilized. Suitable bleaching system components include bleaching catalysts, photobleaches, bleach activators, sources of hydrogen peroxide such as sodium percarbonate and sodium perborates, preformed peracids and mixtures thereof. Suitable preformed peracids include, but are not limited to, peroxy-carboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxy-monosulfuric acids and salts, for example, Oxone (R), and mixtures thereof. Non-limiting examples of bleaching systems include peroxide-based bleaching systems, which may comprise, for example, an inorganic salt, including alkali metal salts such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, persulfate, perphosphate, persilicate salts, in combination with a peracid-forming bleach activator. The term bleach activator is meant herein as a compound which reacts with peroxygen bleach like hydrogen peroxide to form a peracid. The peracid thus formed constitutes the activated bleach. Suitable bleach activators to be used herein include those belonging to the class of esters amides, imides or anhydrides. Suitable examples are tetracetylene diamine (TAED), sodium 4-[(3,5,5-trimethylhexanoyl)oxy]benzene sulfonate (ISONOBS), diperoxy dodecanoic acid, 4-(dodecanoyloxy)benzenesulfonate (LOBS), 4-(decanoyloxy)benzenesulfonate, 4-

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(decanoyloxy)benzoate (DOBS), 4-(nonanoyloxy)-benzenesulfonate (NOBS), and/or those disclosed in WO98/17767. A particular family of bleach activators of interest was disclosed in EP624154 and particularly preferred in that family is acetyl triethyl citrate (ATC). ATC or a short chain triglyceride like triacetin has the advantage that it is environmental friendly as it eventually
 5 degrades into citric acid and alcohol. Furthermore acetyl triethyl citrate and triacetin has a good hydrolytical stability in the product upon storage and it is an efficient bleach activator. Finally ATC provides a good building capacity to the laundry additive. Alternatively, the bleaching system may comprise peroxyacids of, for example, the amide, imide, or sulfone type. The bleaching system may also comprise peracids such as 6-(phthalimido)peroxyhexanoic acid
 10 (PAP). The bleaching system may also include a bleach catalyst. In some embodiments the bleach component may be an organic catalyst selected from the group consisting of organic catalysts having the following formulae:



(iii) and mixtures thereof; wherein each R¹ is independently a branched alkyl group containing from 9 to 24 carbons or linear alkyl group containing from 11 to 24 carbons, preferably each R¹
 20 is independently a branched alkyl group containing from 9 to 18 carbons or linear alkyl group containing from 11 to 18 carbons, more preferably each R¹ is independently selected from the group consisting of 2-propylheptyl, 2-butyloctyl, 2-pentylonyl, 2-hexyldecyl, n-dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, iso-nonyl, iso-decyl, iso-tridecyl and iso-pentadecyl. Other exemplary bleaching systems are described, e.g. in WO2007/087258, WO2007/087244,
 25 WO2007/087259 and WO2007/087242. Suitable photobleaches may for example be sulfonated zinc phthalocyanine.

Polymers

The detergent may contain 0-10% by weight, such as 0.5-5%, 2-5%, 0.5-2% or 0.2-1%
 30 of a polymer. Any polymer known in the art for use in detergents may be utilized. The polymer may function as a co-builder as mentioned above, or may provide antiredeposition, fiber protection, soil release, dye transfer inhibition, grease cleaning and/or anti-foaming properties. Some polymers may have more than one of the above-mentioned properties and/or more than one of the below-mentioned motifs. Exemplary polymers include (carboxymethyl)cellulose
 35 (CMC), poly(vinyl alcohol) (PVA), poly(vinylpyrrolidone) (PVP), poly(ethyleneglycol) or poly(ethylene oxide) (PEG), ethoxylated poly(ethyleneimine), carboxymethyl inulin (CMI), and polycarboxylates such as PAA, PAA/PMA, poly-aspartic acid, and lauryl methacrylate/acrylic acid copolymers, hydrophobically modified CMC (HM-CMC) and silicones, copolymers of

terephthalic acid and oligomeric glycols, copolymers of poly(ethylene terephthalate) and poly(oxyethene terephthalate) (PET-POET), PVP, poly(vinylimidazole) (PVI), poly(vinylpyridine-*N*-oxide) (PVPO or PVPNO) and polyvinylpyrrolidone-vinylimidazole (PVPVI). Further exemplary polymers include sulfonated polycarboxylates, polyethylene oxide and polypropylene oxide (PEO-PPO) and diquaternium ethoxy sulfate. Other exemplary polymers are disclosed in, e.g., WO 2006/130575. Salts of the above-mentioned polymers are also contemplated.

Fabric hueing agents

The detergent compositions of the present invention may also include fabric hueing agents such as dyes or pigments, which when formulated in detergent compositions can deposit onto a fabric when said fabric is contacted with a wash liquor comprising said detergent compositions and thus altering the tint of said fabric through absorption/reflection of visible light. Fluorescent whitening agents emit at least some visible light. In contrast, fabric hueing agents alter the tint of a surface as they absorb at least a portion of the visible light spectrum. Suitable fabric hueing agents include dyes and dye-clay conjugates, and may also include pigments. Suitable dyes include small molecule dyes and polymeric dyes. Suitable small molecule dyes include small molecule dyes selected from the group consisting of dyes falling into the Colour Index (C.I.) classifications of Direct Blue, Direct Red, Direct Violet, Acid Blue, Acid Red, Acid Violet, Basic Blue, Basic Violet and Basic Red, or mixtures thereof, for example as described in WO2005/03274, WO2005/03275, WO2005/03276 and EP1876226 (hereby incorporated by reference). The detergent composition preferably comprises from about 0.00003 wt% to about 0.2 wt%, from about 0.00008 wt% to about 0.05 wt%, or even from about 0.0001 wt% to about 0.04 wt% fabric hueing agent. The composition may comprise from 0.0001 wt% to 0.2 wt% fabric hueing agent, this may be especially preferred when the composition is in the form of a unit dose pouch. Suitable hueing agents are also disclosed in, e.g. WO 2007/087257 and WO2007/087243.

Adjunct materials

Any detergent components known in the art for use in laundry detergents may also be utilized. Other optional detergent components include anti-corrosion agents, anti-shrink agents, anti-soil redeposition agents, anti-wrinkling agents, bactericides, binders, corrosion inhibitors, disintegrants/disintegration agents, dyes, enzyme stabilizers (including boric acid, borates, CMC, and/or polyols such as propylene glycol), fabric conditioners including clays, fillers/processing aids, fluorescent whitening agents/optical brighteners, foam boosters, foam (suds) regulators, perfumes, soil-suspending agents, softeners, suds suppressors, tarnish inhibitors, and wicking agents, either alone or in combination. Any ingredient known in the art for use in laundry detergents may be utilized. The choice of such ingredients is well within the skill of the artisan.

The detergent compositions of the present invention may also comprise dispersants. In particular powdered detergents may comprise dispersants. Suitable water-soluble organic materials include the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated from each other by not more than two carbon atoms. Suitable dispersants are for example described in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc.

The detergent compositions of the present invention may also include one or more dye transfer inhibiting agents. Suitable polymeric dye transfer inhibiting agents include, but are not limited to, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinylloxazolidones and polyvinylimidazoles or mixtures thereof. When present in a subject composition, the dye transfer inhibiting agents may be present at levels from about 0.0001 % to about 10%, from about 0.01% to about 5% or even from about 0.1% to about 3% by weight of the composition.

The detergent compositions of the present invention may preferably also comprise additional components that may tint articles being cleaned, such as fluorescent whitening agent or optical brighteners. Where present the brightener is preferably at a level of about 0.01% to about 0.5%. Any fluorescent whitening agent suitable for use in a laundry detergent composition may be used in the composition of the present invention. The most commonly used fluorescent whitening agents are those belonging to the classes of diaminostilbene-sulphonic acid derivatives, diarylpyrazoline derivatives and bisphenyl-distyryl derivatives. Examples of the diaminostilbene-sulphonic acid derivative type of fluorescent whitening agents include the sodium salts of: 4,4'-bis-(2-diethanolamino-4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate; 4,4'-bis-(2,4-dianilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate; 4,4'-bis-(2-anilino-4(N-methyl-N-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate, 4,4'-bis-(4-phenyl-2,1,3-triazol-2-yl)stilbene-2,2'-disulphonate; 4,4'-bis-(2-anilino-4(1-methyl-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate and 2-(stilbyl-4"-naphtho-1.,2':4,5)-1,2,3-triazole-2"-sulphonate. Preferred fluorescent whitening agents are Tinopal DMS and Tinopal CBS available from Ciba-Geigy AG, Basel, Switzerland. Tinopal DMS is the disodium salt of 4,4'-bis-(2-morpholino-4 anilino-s-triazin-6-ylamino) stilbene disulphonate. Tinopal CBS is the disodium salt of 2,2'-bis-(phenyl-styryl) disulphonate. Also preferred are fluorescent whitening agents is the commercially available Parawhite KX, supplied by Paramount Minerals and Chemicals, Mumbai, India. Other fluorescers suitable for use in the invention include the 1-3-diaryl pyrazolines and the 7-alkylaminocoumarins. Suitable fluorescent brightener levels include lower levels of from about 0.01, from 0.05, from about 0.1 or even from about 0.2 wt % to upper levels of 0.5 or even 0.75 wt%.

The detergent compositions of the present invention may also comprise one or more soil release polymers which aid the removal of soils from fabrics such as cotton and polyester based fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil

release polymers may for example be nonionic or anionic terephthalate based polymers, polyvinyl caprolactam and related copolymers, vinyl graft copolymers, polyester polyamides see for example Chapter 7 in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc. Another type of soil release polymers are amphiphilic alkoxyated grease cleaning
5 polymers comprising a core structure and a plurality of alkoxyate groups attached to that core structure. The core structure may comprise a polyalkylenimine structure or a polyalkanolamine structure as described in detail in WO 2009/087523 (hereby incorporated by reference). Furthermore random graft co-polymers are suitable soil release polymers Suitable graft co-polymers are described in more detail in WO 2007/138054, WO 2006/108856 and WO
10 2006/113314 (hereby incorporated by reference). Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose derivatives such as those described in EP 1867808 or WO 2003/040279 (both are hereby incorporated by reference). Suitable cellulosic polymers include cellulose, cellulose ethers, cellulose esters, cellulose amides and mixtures thereof. Suitable cellulosic polymers include
15 anionically modified cellulose, nonionically modified cellulose, cationically modified cellulose, zwitterionically modified cellulose, and mixtures thereof. Suitable cellulosic polymers include methyl cellulose, carboxy methyl cellulose, ethyl cellulose, hydroxyl ethyl cellulose, hydroxyl propyl methyl cellulose, ester carboxy methyl cellulose, and mixtures thereof.

The detergent compositions of the present invention may also comprise one or more
20 anti-redeposition agents such as carboxymethylcellulose (CMC), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyoxyethylene and/or polyethyleneglycol (PEG), homopolymers of acrylic acid, copolymers of acrylic acid and maleic acid, and ethoxylated polyethyleneimines. The cellulose based polymers described under soil release polymers above may also function as anti-redeposition agents.

25 Other suitable adjunct materials include, but are not limited to, anti-shrink agents, anti-wrinkling agents, bactericides, binders, carriers, dyes, enzyme stabilizers, fabric softeners, fillers, foam regulators, hydrotropes, perfumes, pigments, sod suppressors, solvents, and structurants for liquid detergents and/or structure elasticizing agents.

30 **Formulation of Detergent Products**

The detergent composition of the invention may be in any convenient form, e.g., a bar, a homogenous tablet, a tablet having two or more layers, a pouch having one or more compartments, a regular or compact powder, a granule, a paste, a gel, or a regular, compact or concentrated liquid. There are a number of detergent formulation forms such as layers (same or
35 different phases), pouches, as well as forms for machine dosing unit.

Pouches can be configured as single or multicompartments. It can be of any form, shape and material which is suitable for hold the composition, e.g. without allowing the release of the composition from the pouch prior to water contact. The pouch is made from water soluble film

which encloses an inner volume. Said inner volume can be divided into compartments of the pouch. Preferred films are polymeric materials preferably polymers which are formed into a film or sheet. Preferred polymers, copolymers or derivatives thereof are selected polyacrylates, and water soluble acrylate copolymers, methyl cellulose, carboxy methyl cellulose, sodium dextrin, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, malto dextrin, poly methacrylates, most preferably polyvinyl alcohol copolymers and, hydroxyprpyl methyl cellulose (HPMC). Preferably the level of polymer in the film for example PVA is at least about 60%. Preferred average molecular weight will typically be about 20,000 to about 150,000. Films can also be of blend compositions comprising hydrolytically degradable and water soluble polymer blends such as polyactide and polyvinyl alcohol (known under the Trade reference M8630 as sold by Chris Craft In. Prod. Of Gary, Ind., US) plus plasticisers like glycerol, ethylene glycerol, Propylene glycol, sorbitol and mixtures thereof. The pouches can comprise a solid laundry cleaning composition or part components and/or a liquid cleaning composition or part components separated by the water soluble film. The compartment for liquid components can be different in composition than compartments containing solids. Ref: (US2009/0011970 A1).

Detergent ingredients may be separated physically from each other by compartments in water dissolvable pouches or in different layers of tablets. Thereby negative storage interaction between components may be avoided. Different dissolution profiles of each of the compartments can also give rise to delayed dissolution of selected components in the wash solution.

A liquid or gel detergent, which is not unit dosed, may be aqueous, typically containing at least 20% by weight and up to 95% water, such as up to about 70% water, up to about 65% water, up to about 55% water, up to about 45% water, up to about 35% water. Other types of liquids, including without limitation, alkanols, amines, diols, ethers and polyols may be included in an aqueous liquid or gel. An aqueous liquid or gel detergent may contain from 0-30% organic solvent. A liquid or gel detergent may be non-aqueous.

Laundry Soap Bars

The enzymes of the invention may be added to laundry soap bars and used for hand washing laundry, fabrics and/or textiles. The term laundry soap bar includes laundry bars, soap bars, combo bars, syndet bars and detergent bars. The types of bar usually differ in the type of surfactant they contain, and the term laundry soap bar includes those containing soaps from fatty acids and/or synthetic soaps. The laundry soap bar has a physical form which is solid and not a liquid, gel or a powder at room temperature. The term solid is defined as a physical form which does not significantly change over time, i.e. if a solid object (e.g. laundry soap bar) is placed inside a container, the solid object does not change to fill the container it is placed in. The bar is a solid typically in bar form but can be in other solid shapes such as round or oval.

The laundry soap bar may comprise one or more additional enzymes, protease inhibitors such as peptide aldehydes (or hydrosulfite adduct or hemiacetal adduct), boric acid, borate, borax and/or phenylboronic acid derivatives such as 4-formylphenylboronic acid, one or more soaps or synthetic surfactants, polyols such as glycerine, pH controlling compounds such as
 5 fatty acids, citric acid, acetic acid and/or formic acid, and/or a salt of a monovalent cation and an organic anion wherein the monovalent cation may be e.g. Na⁺, K⁺ or NH₄⁺ and the organic anion may be for example formate, acetate, citrate or lactate such that the salt of a monovalent cation and an organic anion may be, for example, sodium formate.

The laundry soap bar may also comprise complexing agents like EDTA and HEDP,
 10 perfumes and/or different type of fillers, surfactants e.g. anionic synthetic surfactants, builders, polymeric soil release agents, detergent chelators, stabilizing agents, fillers, dyes, colorants, dye transfer inhibitors, alkoxyated polycarbonates, suds suppressers, structurants, binders, leaching agents, bleaching activators, clay soil removal agents, anti-redeposition agents, polymeric dispersing agents, brighteners, fabric softeners, perfumes and/or other compounds
 15 known in the art.

The laundry soap bar may be processed in conventional laundry soap bar making equipment such as but not limited to: mixers, plodders, e.g. a two stage vacuum plodder, extruders, cutters, logo-stampers, cooling tunnels and wrappers. The invention is not limited to preparing the laundry soap bars by any single method. The premix of the invention may be
 20 added to the soap at different stages of the process. For example, the premix comprising a soap, an enzyme, optionally one or more additional enzymes, a protease inhibitor, and a salt of a monovalent cation and an organic anion may be prepared and the mixture may then plodded. The enzyme and optional additional enzymes may be added at the same time as an enzyme inhibitor, e.g. a protease inhibitor, for example in liquid form. Besides the mixing step and the
 25 plodding step, the process may further comprise the steps of milling, extruding, cutting, stamping, cooling and/or wrapping.

Granular detergent formulations

A granular detergent may be formulated as described in WO09/092699, EP1705241,
 30 EP1382668, WO07/001262, US6472364, WO04/074419 or WO09/102854. Other useful detergent formulations are described in WO09/124162, WO09/124163, WO09/117340, WO09/117341, WO09/117342, WO09/072069, WO09/063355, WO09/132870, WO09/121757, WO09/112296, WO09/112298, WO09/103822, WO09/087033, WO09/050026, WO09/047125, WO09/047126, WO09/047127, WO09/047128, WO09/021784, WO09/010375, WO09/000605,
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 10 WO2010142539, WO2010118959, WO2010115813, WO2010105942, WO2010105961,
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 WO2010069905, WO2010076165, WO2010072603, WO2010066486, WO2010066631,
 WO2010066632, WO2010063689, WO2010060821, WO2010049187, WO2010031607, and
 WO2010000636.

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Method of producing the composition

The present invention also relates to methods of producing the composition. The method may be relevant for the (storage) stability of the detergent composition: e.g. soap bar premix method WO2009155557.

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Uses

The present invention is directed to methods for using the alpha-amylase variants, or compositions thereof, in a cleaning process such as laundry or hard surface cleaning including automated dish wash. The soils and stains that are important for cleaning are composed of
 25 many different substances, and a range of different enzymes, all with different substrate specificities, have been developed for use in detergents both in relation to laundry and hard surface cleaning, such as dishwashing. These enzymes are considered to provide an enzyme detergency benefit, since they specifically improve stain removal in the cleaning process that they are used in, compared to the same process without enzymes. Stain removing enzymes
 30 that are known in the art include enzymes such as proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases and mannanases.

In one aspect, the invention relates the use of alpha-amylases variants of the present invention in detergent compositions, for use in cleaning hard-surfaces, such as dish wash, or in
 35 laundering or for stain removal. In another aspect, the invention relates to the use of an alpha-amylase variant according to the invention in a cleaning process such as laundry or hard surface cleaning including, but not limited to, dish wash and industrial cleaning. Thus, in one embodiment, the invention relates to the use of an alpha-amylase variant comprising a

substitution in one or more positions corresponding to positions 51, 109, 193, 201, 269, 294, 297, 298, and 314 of SEQ ID NO:3 in a cleaning process such as laundry or hard surface cleaning including dish wash and industrial cleaning.

5 In one embodiment of the invention relates the use of the detergent composition comprising an alpha-amylase variant of the present invention together with one or more surfactants and optionally one or more detergent components, selected from the list comprising of hydrotropes, builders and co-builders, bleaching systems, polymers, fabric hueing agents and adjunct materials, or any mixture thereof in detergent compositions and in detergent applications.

10 A further embodiment is the use of the detergent composition comprising an alpha-amylase of the present invention together with one or more surfactants, and one or more additional enzymes selected from the group comprising of proteases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases and mannanases, or any mixture thereof in
15 detergent compositions and in detergent applications.

In another aspect, the invention relates to a laundering process which may be for household laundering as well as industrial laundering. Furthermore, the invention relates to a process for the laundering of textiles (e.g. fabrics, garments, cloths etc.) where the process comprises treating the textile with a washing solution containing a detergent composition and an
20 alpha-amylase of the present invention. The laundering can for example be carried out using a household or an industrial washing machine or be carried out by hand using a detergent composition containing a glucoamylase of the invention.

In another aspect, the invention relates to a dish wash process which may be for household dish wash as well as industrial dish wash. The term "dish wash" as used herein,
25 refers to both manual dish wash and automated dish wash. Furthermore, the invention relates to a process for the washing of hard surfaces (e.g. cutlery such as knives, forks, spoons; crockery such as plates, glasses, bowls; and pans) where the process comprises treating the hard surface with a washing solution containing a detergent composition and an alpha-amylase variant of the present invention. The hard surface washing can for example be carried out using
30 a household or an industrial dishwasher or be carried out by hand using a detergent composition containing an alpha-amylase of the invention, optionally together with one or more further enzymes selected from the group comprising of proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases, mannanases, or any mixture thereof.

35 In a further aspect, the invention relates to a method for removing a stain from a surface comprising contacting the surface with a composition comprising an alpha-amylase of the present invention together with one or more surfactants and optionally one or more detergent components selected from the list comprising of hydrotropes, builders and co-builders,

bleaching systems, polymers, fabric hueing agents and adjunct materials, or any mixture thereof in detergent compositions and in detergent applications. A further aspect is a method for removing a stain from a surface comprising contacting the surface with a composition comprising an alpha-amylase variant of the present invention together with one or more
 5 surfactants, one or more additional enzymes selected from the group comprising of proteases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidases, haloperoxygenases, catalases and mannanases, or any mixture thereof in detergent compositions and in detergent applications.

10 **pNP-G7 assay – alpha-amylase activity**

The alpha-amylase activity may be determined by a method employing the G7-pNP substrate. G7-pNP which is an abbreviation for 4,6-ethylidene(G₇)-p-nitrophenyl(G₁)-α,D-maltoheptaoside, a blocked oligosaccharide which can be cleaved by an endo-amylase, such as an alpha-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the
 15 hydrolysed substrate further to liberate a free PNP molecule which has a yellow color and thus can be measured by visible spectrophotometry at λ=405nm (400-420 nm.). Kits containing G7-pNP substrate and alpha-Glucosidase is manufactured by Roche/Hitachi (cat. No.11876473).

REAGENTS:

20 The G7-pNP substrate from this kit contains 22 mM 4,6-ethylidene- G7-pNP and 52.4 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), pH 7.0) .

The alpha-Glucosidase reagent contains 52.4 mM HEPES, 87 mM NaCl, 12.6 mM MgCl₂, 0.075 mM CaCl₂, ≥ 4 kU/L alpha-glucosidase).

The substrate working solution is made by mixing 1 mL of the alpha-Glucosidase
 25 reagent with 0.2 mL of the G7-pNP substrate. This substrate working solution is made immediately before use.

Dilution buffer: 50 mM MOPS, 0.05% (w/v) Triton X100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (C₁₄H₂₂O(C₂H₄O)_n (n = 9-10))), 1mM CaCl₂, pH8.0.

30 PROCEDURE:

The amylase sample to be analyzed is diluted in dilution buffer to ensure the pH in the diluted sample is 7. The assay is performed by transferring 20µl diluted enzyme samples to 96 well microtiter plate and adding 80µl substrate working solution. The solution is mixed and pre-incubated 1 minute at room temperature and absorption is measured every 20 sec. over 5
 35 minutes at OD 405 nm.

The slope (absorbance per minute) of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question

under the given set of conditions. The amylase sample should be diluted to a level where the slope is below 0.4 absorbance units per minute.

Phadebas activity assay

5 The alpha-amylase activity can also be determined by a method using the Phadebas substrate (from for example Magle Life Sciences, Lund, Sweden). A Phadebas tablet includes interlinked starch polymers that are in the form of globular microspheres that are insoluble in water. A blue dye is covalently bound to these microspheres. The interlinked starch polymers in the microsphere are degraded at a speed that is proportional to the alpha-amylase activity.
10 When the alpha-amylase degrades the starch polymers, the released blue dye is water soluble and concentration of dye can be determined by measuring absorbance at 620nm. The concentration of blue is proportional to the alpha-amylase activity in the sample.

 The alpha-amylase sample to be analyzed is diluted in activity buffer with the desired pH. Two substrate tablets are suspended in 5mL activity buffer and mixed on magnetic stirrer.
15 During mixing of substrate transfer 150µl to microtiter plate (MTP) or PCR-MTP. Add 30µl diluted amylase sample to 150µl substrate and mix. Incubate for 15 minutes at 37°C. The reaction is stopped by adding 30µl 1M NaOH and mix. Centrifuge MTP for 5 minutes at 4000xg. Transfer 100µl to new MTP and measure absorbance at 620nm.

 The alpha-amylase sample should be diluted so that the absorbance at 620nm is
20 between 0 and 2.2, and is within the linear range of the activity assay.

Amylzyme activity assay

 The alpha-amylase activity may also be determined by a method using the Amylzyme substrate (Megazyme® Amylzyme Test, supplied by Megazyme for the assay of cereal and
25 bacterial amylases) comprising AZCL-amylose, which has been mixed with lactose and magnesium stearate and tabletted. A blue dye is covalently bound to these microspheres. The interlinked amylose polymers in the microsphere are degraded at a speed that is proportional to the alpha-amylase activity. When the alpha-amylase degrades the starch polymers, the released blue dye is water soluble and concentration of dye may be determined by measuring
30 absorbance at 590 nm. The concentration of blue is proportional to the alpha-amylase activity in the sample.

 The alpha-amylase sample to be analyzed is diluted in activity buffer with the desired pH. Two substrate tablets are suspended in 5 mL activity buffer and mixed on magnetic stirrer. During mixing of substrate 150 µl is transferred to a microtiter plate (MTP) or PCR-MTP. Next,
35 25 µl diluted amylase sample is added to 150 µl substrate and mixed. The mixture is incubated for 10 minutes at 37°C. The reaction is stopped by adding 25 µl 1M NaOH and mixed. MTP is centrifuged for 5 minutes at 4000xg, followed by transferring 100 µl to a new MTP and absorbance is measured at 590 nm.

The alpha-amylase sample may be diluted so that the absorbance at 590 nm is between 0 and 2.2, and is within the linear range of the activity assay.

Reducing sugar activity assay

5 The alpha-amylase activity can also be determined by reducing sugar assay with for example corn starch substrate. The number of reducing ends formed by the alpha-amylase hydrolysing the alpha-1,4-glycosidic linkages in starch is determined by reaction with p-Hydroxybenzoic acid hydrazide (PHBAH). After reaction with PHBAH the number of reducing ends can be measured by absorbance at 405nm and the concentration of reducing ends is
10 proportional to the alpha-amylase activity in the sample.

 The corns starch substrate (3mg/ml) is solubilised by cooking for 5 minutes in milliQ water and cooled down before assay. For the stop solution prepare a Ka-Na-tartrate/NaOH solution (K-Na-tartrate (Merck 8087) 50g/l, NaOH 20g/l) and prepare freshly the stop solution by adding p-Hydroxybenzoic acid hydrazide (PHBAH, Sigma H9882) to Ka-Na-tartrate/NaOH
15 solution to 15mg/ml.

 In PCR-MTP 50µl activity buffer is mixed with 50µl substrate. Add 50µl diluted enzyme and mix. Incubate at the desired temperature in PCR machine for 5 minutes. Reaction is stopped by adding 75µl stop solution (Ka-Na-tartrate/NaOH/PHBAH). Incubate in PCR machine for 10 minutes at 95°C. Transfer 150µl to new MTP and measure absorbance at 405nm.

20 The alpha-amylase sample should be diluted so that the absorbance at 405nm is between 0 and 2.2, and is within the linear range of the activity assay.

EnzChek® assay

 For the determination of residual amylase activity an EnzChek® Ultra Amylase Assay Kit
25 (E33651, Invitrogen, La Jolla, CA, USA) may be used.

 The substrate is a corn starch derivative, DQ™ starch, which is corn starch labeled with BODIPY® FL dye to such a degree that fluorescence is quenched. One vial containing approx. 1 mg lyophilized substrate is dissolved in 100 microliters of 50 mM sodium acetate (pH 4.0). The vial is vortexed for 20 seconds and left at room temperature, in the dark, with occasional mixing
30 until dissolved. Then 900 microliters of 100 mM acetate, 0.01% (w/v) TRITON® X100, 0.125 mM CaCl₂, pH 5.5 is added, vortexed thoroughly and stored at room temperature, in the dark until ready to use. The stock substrate working solution is prepared by diluting 10-fold in residual activity buffer (100 mM acetate, 0.01% (w/v) TRITON® X100, 0.125 mM CaCl₂, pH 5.5). Immediately after incubation the enzyme is diluted to a concentration of 10-20 ng enzyme
35 protein/ml in 100 mM acetate, 0.01% (W/v) TRITON® X100, 0.125 mM CaCl₂, pH 5.5.

 For the assay, 25 microliters of the substrate working solution is mixed for 10 second with 25 microliters of the diluted enzyme in a black 384 well microtiter plate. The fluorescence intensity is measured (excitation: 485 nm, emission: 555 nm) once every minute for 15 minutes

in each well at 25°C and the V_{\max} is calculated as the slope of the plot of fluorescence intensity against time. The plot should be linear and the residual activity assay has been adjusted so that the diluted reference enzyme solution is within the linear range of the activity assay.

5 **Wash performance of alpha-amylases using Automatic Mechanical Stress Assay (AMSA)**

In order to assess the wash performance of the polypeptides 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 to be washed against all the slot openings. During the washing time, the plate, test solutions, textile and lid are vigorously shaken to bring the test solution in contact with the textile 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.

15

General laundry wash performance description

A test solution comprising water (6°dH), 0.79 g/L detergent, e.g. model detergent J as described below, and the polypeptide, i.e. enzyme, of the invention at concentration of 0 or 0.6 mg enzyme protein/L, is prepared. Fabrics stained with starch (CS-28 from Center For Test materials BV, P.O. Box 120, 3133 KT, Vlaardingen, The Netherlands) is added and washed for 20 minutes at 15°C and/or 30°C, or alternatively 20 minutes at 15°C and/or 40°C as specified in the examples. After thorough rinse under running tap water and drying in the dark, the light intensity values of the stained fabrics are subsequently measured as a measure for wash performance. The test with 0 mg enzyme protein/L is used as a blank and corresponds to the contribution from the detergent. Preferably mechanical action is applied during the wash step, e.g. in the form of shaking, rotating or stirring the wash solution with the fabrics. The AMSA wash performance experiments were conducted under the experimental conditions specified below:

30 Table A: Experimental condition

Detergent	Liquid Model detergent J (see Table B)
Detergent dosage	0.79 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C or 30°C
Water hardness	6°dH

Enzyme concentration in test	0.6 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

Table B: Model detergent J

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	5.15	5.00
AS	5.00	4.50
AEOS	14.18	10.00
Coco fatty acid	1.00	1.00
AEO	5.00	5.00
MEA	0.30	0.30
MPG	3.00	3.00
Ethanol	1.50	1.35
DTPA (as Na5 salt)	0.25	0.10
Sodium citrate	4.00	4.00
Sodium formate	1.00	1.00
Sodium hydroxide	0.66	0.66
H ₂ O, ion exchanged	58.95	58.95

Water hardness was adjusted to 6°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the textiles were flushed in tap water and dried.

5

Table C: Experimental condition

Detergent	Liquid Model detergent A (see Table D)
Detergent dosage	3.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C, 30°C, or 40°C
Water hardness	15°dH
Enzyme concentration in test	0.6 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

Table D: Model detergent A

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	12.00	11.60
AEOS, SLES	17.63	4.90
Soy fatty acid	2.75	2.48
Coco fatty acid	2.75	2.80
AEO	11.00	11.00
Sodium hydroxide	1.75	1.80
Ethanol / Propan-2-ol	3.00	2.70/0.30
MPG	6.00	6.00
Glycerol	1.71	1.70
TEA	3.33	3.30
Sodium formate	1.00	1.00
Sodium citrate	2.00	2.00
DTMPA	0.48	0.20
PCA	0.46	0.18
Phenoxy ethanol	0.50	0.50
H ₂ O, ion exchanged	33.64	33.64

Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 4:1:7.5) to the test system. After washing the textiles were flushed in tap water and dried.

5

Table E: Experimental condition

Detergent	Powder Model detergent X (see Table F)
Detergent dosage	1.75 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C, 30°C, or 40°C
Water hardness	12°dH
Enzyme concentration in test	0.6 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

Table F: Model detergent X

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	16.50	15.00
AEO*	2.00	2.00
Sodium carbonate	20.00	20.00
Sodium (di)silicate	12.00	9.90
Zeolite A	15.00	12.00
Sodium sulfate	33.50	33.50
PCA	1.00	1.00

*Model detergent X is mixed without AEO. AEO is added separately before wash.

5 Water hardness was adjusted to 12°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the textiles were flushed in tap water and dried.

Table G: Experimental condition

Detergent	Model detergent T (see Table H)
Detergent dosage	5.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C, 30°C, or 40°C
Water hardness	15°dH
Enzyme concentration in test	0.6 mg/L
Test material	CS-28 (Rice starch cotton)

10 Table H: Model detergent T

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS, sodium salt	11.00	10.00
AS, sodium salt	2.00	1.80
Soap	2.00	2.00
AEO*	3.00	3.00
Sodium carbonate	15.15	14.90
Sodium (di)silicate	3.00	2.50
Zeolite A	18.75	15.00
HEDP-Na ₄	0.15	0.13

Sodium citrate	2.00	2.00
PCA	1.65	1.50
CMC	2.50	1.60
SRP	0.50	0.50
SPC	22.20	20.00
TAED	3.25	3.00
Sodium sulfate	10.85	10.70
Silicone	2.00	2.00

*Model detergent T is mixed without AEO. AEO is added separately before wash.

Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 4:1:7.5) to the test system. After washing the textiles were flushed in tap water and dried.

General AMSA automatic dish wash performance description

A test solution comprising water (6°dH), 4.53 g/L detergent, e.g. Liquid model detergent containing phosphate, as described below, and the polypeptide of the invention at concentration of 0 or 0.5 mg enzyme protein/L, is prepared. Melamine plates stained with mixed starch (DM-177 from Center For Test materials BV, P.O. Box 120, 3133 KT, Vlaardingen, The Netherlands) was added and washed for 20 minutes at 15°C. After short rinse under running tap water and drying in the dark, the light intensity values of the stained plates are subsequently measured as a measure for wash performance. The test with 0 mg enzyme protein/L is used as a blank and corresponds to the contribution from the detergent. Preferably mechanical action is applied during the wash step, e.g. in the form of shaking, rotating or stirring the wash solution with the plates. The AMSA automatic dish wash performance experiments were conducted under the experimental conditions specified below:

20 Table I: Experimental condition

Detergent	Liquid model detergent containing phosphate (see Table J)
Detergent dosage	4.53 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C, 30°C, or 40°C
Water hardness	6°dH
Enzyme concentration in test	0.5 mg/L
Test material	Melamine plates (Mixed Starch)

Table J: Liquid model automatic dish wash detergent containing phosphate

Compound	Content of compound (% w/w)
STPP	50.0
Sodium carbonate	20.0
Sodium percarbonate	10.0
Sodium disilicate	5.0
TAED	2.0
Sokalan CP5 (39,5%)	5.0
Surfac 23-6.5 (100%)	2.0
Sodium Sulfate	6.0

Water hardness was adjusted to 6°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the plates were flushed in tap water and dried.

5

Table K: Experimental condition

Detergent	Powder model detergent containing phosphate (see Table L)
Detergent dosage	3.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C, 30°C, or 40°C
Water hardness	21°dH
Enzyme concentration in test	0.5 mg/L
Test material	Melamine plates (Mixed Starch)

Table L: Powder automatic dish wash model detergent containing phosphate

Compound	Content of compound (% w/w)
Na ₅ P ₃ O ₁₀	23.0
Pluronic PE 6800	1.0
Sokalan PA 30	2.0
ACUSOL 805S	2.0
Xantan gum	1.0
Water	74.0

Water hardness was adjusted to 21°dH by addition of CaCl₂, MgCl₂, and NaHCO₃

10 (Ca²⁺:Mg²⁺:HCO₃⁻ = 4:1:10) to the test system. After washing the plates were flushed in tap water and dried.

Evaluation of wash performance

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

Color measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak) used to capture an image of the washed textile and with a controlled digital imaging system (DigiEye) for capture an image of the washed melamine plates.

To extract a value for the light intensity from the scanned images, 24-bit pixel values from the image are 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:

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

Textile/Melamine:

Textile sample CS-28 (rice starch on cotton) and melamine plates stained with mixed starch (DM-177) are obtained from Center For Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands.

Reference alpha-amylase

The reference alpha-amylase may be the alpha-amylase of SEQ ID NO: 3.

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

EXAMPLES**Example 1 Amylase activity using Amylazyme assay**

The amylase activity of the variants of the present invention was determined by the Amylazyme assay as described herein.

The amylases were expressed from *B.subtilis* host strains by fermenting in a deep well micro titer plate with 1 ml fermentation media in each well. They were incubated at 37°C for 3 days under vigorous shaking at 600 rpm.

Fermentation media

	Chemical name	Supplier	Lot no	For 1 liter	
di-Ammonium hydrogen	(NH ₄) ₂ HPO ₄	Merck		6	g

phosphate		1207			
Potato protein (7.5%)		(Avebe TAK)		26	ml
Magnesium sulfate	MgSO ₄ .7H ₂ O	Merck 5886		1,2	g
Potassium di-hydrogen phosphate	KH ₂ PO ₄	Merck 4873		12	g
di-Sodium hydrogen phosphate	Na ₂ HPO ₄ .2H ₂ O	Merck 6580		5	g
Micro PM Low MSA-SUB-FS-0516				18	ml
Potassium sulfate	K ₂ SO ₄	Merck 5153		1,8	g
Calcium chloride 2H ₂ O	CaCl ₂ .2H ₂ O	Merck 2382		0,1	g
Soluble starch		Merck 1253		33	g
SB 2121			101- 8523	0,5	ml
Water add to				1000	ml

The supernatants from the fermentations were diluted 100 fold in assay buffer (100mM B&R buffer pH 7,3 with 0,12mM CaCl₂ and 0,01% brij) prior to measuring the activity using the Amylazyme assay. The Amylazyme tablets were also dissolved in the assay buffer.

5 Table 1: Activity of the variants of the invention

Amylase	Activity (average)
SEQ ID NO:3	1,9
T269N	1,4
T269Y	1,8
T269G	1,8
M294Y	1,4
M109A	0,4
M109G	1,3
M109H	1,3
M109L	1,5

As can be seen from Table 1, all tested variants maintain the amylase activity. Average activity values above 0 indicate that the variant show alpha-amylase activity. The reference used herein is the polypeptide according to SEQ ID NO:3. Activity of the supernatant does not reflect the wash performance of the isolated, *i.e.* purified, enzymes. Thus, these data does not indicate the wash performance. Furthermore, the data has not been normalized according to protein concentration used in the experiment. Accordingly, the data shows that all tested variants maintain the amylase activity.

15 **Example 2 Amylase activity using Phadebas assay**

The amylase activity of the variants of the present invention was determined by the Phadebas assay as described herein.

The amylases were expressed from *B.subtilis* host strains by fermenting in a deep well micro titer plate with 1 ml fermentation media from Example 1 in each well. They were incubated at 37°C for 3 days under vigorous shaking at 600 rpm.

The supernatants were diluted 100x in assay buffer (100mM B&R buffer pH 7,3 with 0,12mM CaCl₂ and 0,01% brij) prior to measuring the activity using the Phadebas assay. The Phadebas tablets were also dissolved in the assay buffer.

25 Table 2: Activity of the variants of the invention

Amylase	Activity (average)
SEQ ID NO: 3	1,7

Q51T+ M109G+N193F+G201Y	0,5
Q51T+M109G+N193F+G201Y+ T269N+M294Y+Q297Y+A298N+N314G	0,4
M109G+G201Y	0,6
M109A+G201F	0,3
M294N	1,7
Q51D+M109H	0,3
M109L+M200L	0,9

As can be seen from Table 2, all tested variants maintain the amylase activity. Average activity values above 0 indicate that the variant show alpha-amylase activity. The reference used herein is the polypeptide according to SEQ ID NO:3. Activity of the supernatant does not reflect the wash performance of the isolated, *i.e.* purified, enzymes. Thus, these data does not indicate the wash performance.

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 disclosure including definitions will control.

Sequences – **bold** and underlined parts of the sequences indicate the predicted signal sequence of the polypeptide.

SEQ ID NO:	Sequence
1	ATGAAAATCCGCAACGGTTGGAAAAAACCTTGACGCTGTTATTTGCGTCATCTTC TTGCTGCCTCATTCTGCAGCCGCGACCTTTGCCGGGACAACGGCACGATGATG CAATACTTTGAATGGTATCTGCCAACGACGGGACGCTTTGGACCAAGATGGGCA GCGACGCGTCGCACCTGAAGTCGATCGGGATCACCGGCGTCTGGTTCCCGCCG GCGTACAAAGGCCAATCGCAGTCGGACGTCGGCTACGGCGTATACGACATGTAC GACCTCGGCGAATTCAACCAAAAAGGAACCGTCCGCACCAAGTACGGCACCAAAA GCCCAGCTCCAATCGGCGATCACCTCCCTGCACAACAACGGCATCCAAGCCTAC GGGGACGTCGTCCTCAACCACCGCATGGGCGCCGATGCGACGGAGACGATCTC CGCCGTGGAAGTCAACCCGTCCAACCGCAACCAAGTCACCTCCGGGGCTTACAA CATCTCCGCTTGGACCGACTTCGAATTCGCGGGCCGCGGCAACACCTACTCCTC GTTTAAGTGGCACTCCTACTACTTTGACGGCGTGGACTGGGACCAATCCCGCCA GCTGAGCGGCAAGATCTACCAGATCCAAGGCACCGGCAAAGCGTGGGACTGGG AAGTCGATTCCGAAAACGGCAACTACGACTACCTGATGGGCGCGGACATCGACT ACGACCACCCGGACGTGCAAACGGAAGTGAAGAACTGGGGCAAGTGGTTTCGTCA ACACCCTCAACCTCGACGGCGTGCACCTCGACGCGGTCAAGCACATCAAGTTCCG ACTACATGTCTTCCCTGGCTGTCCAGCGTCAAATCCACGACCGGCAAGTCCAACCT GTTCGCCGTGCGCGAATACTGGAACACCTCGCTCGGAGCGCTGGAGAACTACGA GAACAAAACCAACTGGAGCATGTGCTGTTTCGACGTGCCGCTGCACATGAACCTC CAAGCGGCAGCGAACGGCGGGCTACTATGATATGCGCAACCTGCTCAACAAC

	<p>ACGATGATGAAAAATCACCCGATCCAAGCGGTCACCTTCGTCGACAACCACGACA CCGAGCCG GGCCAAGCCCTGCAATCGTGGGTATCCGACTGGTTCAAACCGCTGGCCTACGCG ACGATCCTGACCCGTCAAGAAGGCTACCCGTGCGTGTTCTACGGCGACTACTAC GGCATCCCGTCGCAAAGCGTCTCCGCGAAATCCACCTGGTTGGACAAGCAGCTT TCCGCACGCAAATCCTACGCGTACGGCACCCAGCAGACTACTTGGACAACCAA GACGTGATCGGCTGGACGCGCGAAGGCGATTCCGCGCACGCGGGCTCGGGTCT TGCCACCGTCATGTCGGACGGCCCTGGCGGCTCCAAGACGATGTACGTCGGCAC CGCCCATGCCGGCCAAGTCTTCAAGGACATCACCGGCAACCGCACCCGACACCGT CACGATCAACTCCGACGCAACGGCACCTTCCCCTGCAACGGCGGGCTCCGTCTC GATCTGGGTCAAACAA</p>
2	<p><u>MKIRNGWKTLTLLFALIFLLPHSAAAT</u>TFAGDNGTMMQYFEWYLPNDGTLWTKMGS DASHLKSIGITGVWFPPAYKQSQSDVGYGVYDMYDLGEFNQKGTVRTKYGTKAQL QSAITSLHNNGIQAYGDVVLNHRMGADATETISAVEVNPSNRNQVTSGAYNISAWTD FEFPRGNTYSSFKWHSYFDGVDWDQSRQLSGKIYQIQGKAWDWEVDSENGNYD YLMGADIDYDHPDVQTEVKNWGWKWFVNTLNLGDVRLDAVKHIKFDYMSSWLSSVKS TTGKSNLFAVGEYWNTSLGALENYENKTNWSMSLFDVPLHMNFQAAANGGGYYDM RNLLNNTMMKNHPIQAVTFVDNHDTEPGQALQSWVSDWFKPLAYATILTRQEGYPC VFYGDYYGIPSQSVSAKSTWLDKQLSARKSYAYGTQHDYLDNQDVIWGTREGDSA HAGSGLATVMSDGPGGSKTMYVGTAHAGQVFKDITGNRTDTVTINSAGNGTFPCNGG SVSIWVKQ</p>
3	<p>TFAGDNGTMMQYFEWYLPNDGTLWTKMGS DASHLKSIGITGVWFPPAYKQSQSD VGYGVYDMYDLGEFNQKGTVRTKYGTKAQLQSAITSLHNNGIQAYGDVVLNHRMG ADATETISAVEVNPSNRNQVTSGAYNISAWTDFEFPRGNTYSSFKWHSYFDGVDW DQSRQLSGKIYQIQGKAWDWEVDSENGNYDYLMGADIDYDHPDVQTEVKNWGWK WFVNTLNLGDVRLDAVKHIKFDYMSSWLSSVKS TTGKSNLFAVGEYWNTSLGALE NYENKTNWSMSLFDVPLHMNFQAAANGGGYYDMRNLLNNTMMKNHPIQAVTFVDN HDTEPGQALQSWVSDWFKPLAYATILTRQEGYPCVFYGDYYGIPSQSVSAKSTWLD KQLSARKSYAYGTQHDYLDNQDVIWGTREGDSA HAGSGLATVMSDGPGGSKTMYV GTAHAGQVFKDITGNRTDTVTINSAGNGTFPCNGG SVSIWVKQ</p>
4	<p>AQSPVWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPT QDGNHGHTHVAGTIAALNNSIGVLGVAPSAEL YAVKVLGASGSGSVSSIAQGLEWA GNNGMHVANLSLGSPPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANA MAVGATDQNNNRASFQYAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAG AAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLVYGSGLVNAEAATR</p>
5	<p>EVSQDLFNQFNLFAQYSAAA YCGKNNDAPAGTNICTGNACPEVEKADATFLYSFED SGVGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEINDICSGCRGHDGFTS SWRSVADTLRQKVEDAVREHPDYRVVFTGHSLGALATVAGADLRNGYDIDVFSY GAPRVGNRAFAEFLTVQTGGTLYRITHNDIVPRLPPREFGYSHSSPEYWIKSGTLVP VTRNDIVKIEGIDATGGNNQPNIPDIPAH LWYFGLIGTCL</p>
6	<p>HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLKDKGISAVWIPPAWKGASQN DVGYGAYDLYDLGEFNQKGTIRTKYGTRNQLQAAVNALKSNGIQVYGDVVMNHKGG ADATEMVRAVEVNPNNRNQEVS GEYTIEAWTKFDFPGRGNTHSNFKWRWYHFDGV DWQSRKLNRIYKFRGDGKGDWEVDTENGNYDYLMYADIDMDHPEVVNELRN WGWYTNLGLDGFRI DAVKHIKYSFTRDWINHVR SATGKNMFAVAEFWKNDLGAIE NYLNKTNWNH SVFDVPLHYNLYNASKSGGNYDMRQIFNGTVVQRHPMHA VTFVDN HDSQPEEALESFVEEFKPLAYALTLTREQQYPSVFYGDYYGIP THGVPAMKSKIDP ILEARQKYAYGRQNDYLDH HNIIGWTREGNTAHPNSGLATIMSDGAGGNKWMFVGR NKAGQVWTDITGNRAGTVTINADGWGNF SVNGG SVSIWVKN</p>
7	<p>HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDASNLNRN RGITAIWIPPAWKGTSQND VGYGAYDLYDLGEFNQKGTVRTKYGTRS QLESIAIHALKNNGVQVYGDVVMNHKGG DATENVLAVEVNPNNRNQEISGDY TIEAWTKFDFPGRGNTYSDFKWRWYHFDGVD WDQSRQFQNR IYKFRGDGKAWDWEVDSENGNYDYLMYADVDMDHPEVVNELRRW GEWYTNLNLGDGFRI DAVKHIKYSFTRDWLTHVRNATGKEMFAVAEFWKNDLGALE N YLNKTNWNH SVFDVPLHYNLYNASNSGGNYDMAKLLNGTVVQKHPMHA VTFVDN DSQPGESLESFVQEWFKPLAYALTLTREQQYPSVFYGDYYGIP THSVPAMKAKIDPIL EARQNFAYGTQHDYFDH HNIIGWTREGNTTHPNSGLATIMSDGPGGEKWMYVQGN KAGQVWHDITGNKPGTVTINADGWANF SVNGG SVSIWVKR</p>
8	<p>NTAPINETMMQYFEWDL PNDGTLWTKVKNEAANLSSLGITALWLPPAYKGT SQSDV GYGVYDLYDLGEFNQKGTIRTKYGT KTQYIQAIQAAKAAGMQVYADVFNHKAGADG TEFVDAVEVDPSNRNQETS GTYQIQAWTKFDFPGRGNTYSSFKWRWYHFDGTDWD</p>

	ESRKLNRITYKFRSTGKAWDWEVDTEGNNDYLMFADLMDHPEVVTELKNWGTWY VN TTNIDGFRLDAVKHIKYTFFPDWLTYYVRNQTGKNLFAVGEFWSYDVNKLHNYITKTNG SMSLFDAPLHNNFYTASKSSGYFDMRYLLNNTLMKDQPSLAVTLVDNHDTQPGQSL QSWVEPWFKPLAYAFILTRQEGYPCVFYGDYYGIPKYNIPGLKSKIDPLLIARRDYAYG TQRDYIDHQDIIGWTREGIDTKPNSGLAALITDGPGGSKWMYVGGKHAGKVFYDLTG NRSDTVTINADGWGEFKVNGGSSVSIWVAK
9	AATNGTMMQYFEWYVPNDGQQWNRLRTDAPYLSSVGITAVWTPPAYKGTQSQADVG YGPYDLYDLGEFNQKGTVRTKYGTGKELKSAVNTLHSNGIQVYGDVVMNHKAGADY TENVTAVEVNPSNRNQETSGEYNIQAWTGFNFPGRGTTYSNFKWQWFHFDGTDWD QSRSLSRIFKFRGTGKAWDWEVSSENGNYDLYMYADIDYDHPDVVNEMKKGWVWY ANEVGLDGYRLDAVKHIKFSFLKDWVDNARAATGKEMFTVGEYWQNDLGALNNYLA KVNYNQSLFDAPLHNFYAASTGGGYDMRNILNNTLVASNPTKAVTLVENHDTQPG QSLESTVQPWFKPLAYAFILTRSGGYPSVFYGDYMYGTGKTTTREPALKSKIEPLKA RKDYAYGTQRDYIDNPDVIGWTREGDSTKAKSGLATVITDGPGGSKRMYVGTSNAG EIWYDLTGNRTDKITIGSDGYATFPVNGGSSVSVWVQQ
10	HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLKSKGITAVWIPPAWKGASQND VGYGAYDLYDLGEFNQKGTVRTKYGTRSQAQAVTSLKNNGIQVYGDVVMNHKGGGA DATEMVRAVEVNPNRNQEVTEGYTIEAWTRDFPGRGNTSSFKWRWYHFDGVD WDQSRRLNRIYKFRGHGKAWDWEVDTEGNNDYLYMYADIDMDHPEVVNELRNW GVWYTNLGLDGFRIIDAVKHIKYSFTRDWINHVRSATGKNMFVAFAEFWKNDLGAIEN YLQKTNNHNSVFDVPLHYNLYNASKSGGNYDMRNIFNGTVVQRHPHAVTFVDNHD SQPEEALESFVEEWFKPLAYALTLTREQGYPSVFYGDYYPGIPHTGVPAMRSKIDPILE ARQKYAYGKQNDYLDHNNIIGWTREGNTAHPNSGLATIMSDGAGGSKWFMVGRNK AGQVWSDITGNRTGTVTINADGWGNFSVNGGSSVSIWVVK
11	HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLKSKGITAVWIPPAWKGASQND VGYGAYDLYDLGEFNQKGTVRTKYGTRSQAQAVTSLKNNGIQVYGDVVMNHKGGGA DATEMVRAVEVNPNRNQEVTEGYTIEAWTRDFPGRGNTSSFKWRWYHFDGVD WDQSRRLNRIYKFRGHGKAWDWEVDTEGNNDYLYMYADIDMDHPEVVNELRNWGV WYTNLGLDGFRIIDAVKHIKYSFTRDWINHVRSATGKNMFVAFAEFWKNDLGAIENYL QKTNNHNSVFDVPLHYNLYNASKSGGNYDMRNIFNGTVVQRHPHAVTFVDNHD SQPEEALESFVEEWFKPLAYALTLTREQGYPSVFYGDYYPGIPHTGVPAMRSKIDPILEA RQKYAYGKQNDYLDHNNIIGWTREGDSSHPKSGLATLITDGPGGSKRMYAGLKNAG ETWYDITGNRSDTVKIGSDGWGEFHVNDGSSVSIWVQK
12	HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLKDKGITAVWIPPAWKGASQND VGYGAYDLYDLGEFNQKGTVRTKYGTRNQLQAAVTALKSNGIQVYGDVVMNHKGGGA DATEWVRAVEVNPSNRNQEVSGDYTIEAWTKDFPGRGNTSSFKWRWYHFDGVD WDQSRQLQNRRIYKFRGDGKGDWEVDTEGNNDYLYMYADIDMDHPEVVNELRNW GVWYTNLGLDGFRIIDAVKHIKYSFTRDWLTHVRNTTGKNMFVAFAEFWKNDIGAIEN YLSKTNWNHNSVFDVPLHYNLYNASRSGGNYDMRQIFNGTVVQRHPHPTHAVTFVDNHD SQPEEALESFVEEWFKPLACALTLTRDQGYPSVFYGDYYPGIPHTGVPAMKSKIDPILE ARQKYAYGKQNDYLDHNNMIGWTREGNTAHPNSGLATIMSDGPGGKWMYVGRN KAGQVWRDITGNRSGTVTINADGWGNFSVNGGSSVSIWVNN
13	TFAGDNGTMMQYFEWYLPNDGLWTKMGSDASHLKSIGITGVWFPAYKQGSQSD VGYGVYDLYDLGEFNQKGTVRTKYGTQAQLQSAITSLHNNIQAYGDVVLNHRMGA DATETISAVEVNPSNRNQVTSGAYNISAWTDFEFPGRGNTYSSFKWHSYFYFDGVDW DQSRQLSGKIYQIQGTGKAWDWEVDSENGNYDLYMGADIDYDHPDVQTEVKNWVK WVNTLNLGDGVRILDAVKHIKFDYMSSWLSSVKSTTGKSNLFAVGEYWNTSLGALEN YENKTNWMSLFDVPLHMNFQAAANGGYYDMRNLLNNTMMKNHPIQAVTFVDNH DTEPGQALQSWVSDWFKPLAYATILTRQEGYPCVFYGDYYPGIPHTGVPAMKSKIDPILE QLSARKSYAYGTQHDYLDNQDVIGWTREGDSAHAGSGLATVMSDGPGGSKTMYVG TAHAGQVFKDITGNRTDTVTINSAGNGTFPCNGGSSVSIWVKQ

CLAIMS

1. An alpha-amylase variant of a parent alpha-amylase, comprising a substitution at one or more positions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide according to SEQ ID NO:3, wherein said variant has alpha-amylase activity, and wherein said parent alpha-amylase has at least 89%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, sequence identity to the polypeptide of SEQ ID NOs: 3 or 13.
2. The variant according to claim 1, which is a variant of a parent alpha-amylase selected from the group consisting of:
- a polypeptide having at least 89%, sequence identity to the polypeptide of SEQ ID NOs: 3 or 13;
 - a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions with (i) the mature polynucleotide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i);
 - a polypeptide encoded by a polynucleotide having at least 89% sequence identity to the mature polynucleotide coding sequence of SEQ ID NO:1; and
 - a fragment of the polypeptide of SEQ ID NOs: 3 or 13, which has alpha-amylase activity.
3. The variant according to any one of the preceding claims, which has at least 67%, such as at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide according to SEQ ID NOs: 3 or 13.
4. The variant according to any one of the preceding claims, wherein the number of substitutions is 1 to 20, e.g., 1 to 10 and 1 to 5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 alterations.
5. The variant according to any one of the preceding claims, which comprises a substitution at one position corresponding to any one of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO: 3.
6. The variant according to claim 5, wherein the substitution is selected from the list consisting of;
- Q51T, Q51A, Q51R, Q51N, Q51D, Q51C, Q51E, Q51G, Q51H, Q51I, Q51L, Q51K, Q51M, Q51F, Q51P, Q51S, Q51W, Q51Y, or Q51V;

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- ii. M109G, M109A, M109H, M109L, M109R, M109N, M109D, M109C, M109E, M109Q, M109I, M109K, M109F, M109P, M109S, M109T, M109W, M109Y, or M109V;
- iii. N193F, N193A, N193R, N193D, N193C, N915E, N193Q, N193G, N193H, N193I, 5 N193L, N193K, N193M, N193P, N193S, N193T, N193W, N193Y, or N193V;
- iv. G201Y, G201A, G201R, G201D, G201C, G201E, G201Q, G201H, G201I, G201L, G201K, G201M, G201N, G201P, G201S, G201T, G201W, G201Y, or G201V;
- v. T269N, T269Y, T269G, T269A, T269R, T269D, T269C, T269E, T269F, T269Q, 10 T269H, T269I, T269L, T269K, T269M, T269P, T269S, T269W, or T269V;
- vi. M294Y, M294N, M294A, M294R, M294D, M294C, M294E, M294Q, M294G, M294F, M294H, M294I, M294L, M294K, M294P, M294S, M294T, M294W, or M294V;
- vii. Q297Y, Q297A, Q297R, Q297N, Q297D, Q297C, Q297E, Q297G, Q297H, 15 Q297I, Q297L, Q297K, Q297M, Q297F, Q297P, Q297S, Q297T, Q297W, or Q297V;
- viii. A298N, A298R, A298D, A298C, A298E, A298Q, A298G, A298H, A298I, A298L, A298K, A298M, A298F, A298P, A298S, A298T, A298W, A298Y, or A298V; or
- ix. N314G, N314A, N314R, N314D, N314C, N314E, N314Q, N314H, N314I, N314L, 20 N314K, N314M, N314F, N314P, N314S, N314T, N314W, N314Y, or N314V,
- wherein each position corresponds to the corresponding position in the polypeptide of SEQ ID NO: 3.

7. The variant according to any one of the preceding claims, which comprises a 25 substitution at two positions corresponding to any one of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO:3.

8. The variant according to claim 7, wherein said variant comprises a substitution at each position corresponding to positions;
- 30 (i) 51 and 109;
- (ii) 109 and 201;
- (iii) 269 and 294; or
- (iv) 294 and 297;
- wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID 35 NO: 3.

9. The variant according to any one of the preceding claims, which comprises a substitution at three positions corresponding to any of positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of the polypeptide of SEQ ID NO:3.

5 10. The variant according to any one of claims 1 to 7 and 9, wherein the variant comprises a substitution at each positions corresponding to positions;

(i) 51, 109, 193, and 201;

(ii) 109, 201, 269, and 294;

(iii) 201, 269, 294 and 297; or

10 (iv) 51, 109, 193, 201, 269, 294, 297, 298, and 314 ;

wherein each position corresponds to the corresponding positions in the polypeptide of SEQ ID NO: 3.

11. The variant according to any one of the preceding claims, which further comprises an alteration at positions corresponding to positions;

105L,I,F+206Y; 105L,I+206Y+217I; 105F+206Y+208Y+217V+246V; 105L+206F;
 105I+206Y+208Y+217I+246V; 195F+213S+214T; 195F+206Y+208Y+213T+214T+217M,V;
 195F+206Y+208F,L+213T+214T+217V; 195F+206Y+213S+214T;
 195F+206Y+208Y+213S+214T+217M; 195F+206Y+208F+213T+214T+217M;
 20 195F+206Y+208Y+213T+214T+217Q; 195F+206Y+213G+214T; 195F+206Y+213S;
 195F+206Y+208Y+213T+214T+217M; 195F+213S; 195F+206Y+208L+213T+214T+217M;
 195F+213G+214T; 206Y,F+208Y+217Q; 206Y+208Y+217I; 206F+208Y+217M; 206Y+208Y;
 206Y+217M; 206Y+208Y+213A+217M; 206Y+208Y+217V+246V; 206Y+213G;
 206Y+208F+217V; 206N+208Y+217M; 206F+208Y+217V; 206Y+246V; 206Y+217I,V;
 25 206F+208F+217I; 206Y+208L+213S; 206F+217I; 206Y+217I+246I; 206L+217V;
 206Y+208F+217H; 206L+208F+217I; 206L+217V+246L; 206F+246V; 208Y+213S+217M;
 208Y+213A+217Q; 63I+206Y; 63I+206Y+241V; 63V+206Y; 63V+105L+206Y; 63V+206Y+217I;
 63V+105F+206Y+208F+217I; 63V+206Y+246V; 63V+206F; 63V+206L+217V;
 63V+105F+206Y; 63V+206Y+241V+246L; 195F+206Y+208Y+214T+217V; 186E+195F+206Y;
 30 195F+206Y+208Y+213T+217V; 186E+195F+202T+206Y+209S; 63I+195F+206Y+210S;
 195F+206Y+213P+214T; 195F+206Y+208Y+213T+214T+217I; 186E+195F+206Y+210S;
 195F+213P; 186E+195F+202T+206Y+210S; 195F+206H; 195F+208Y+213T+214T+217V;
 206Y+208Y+213T+214T+217V; 195F+206Y+217V; 195F+206Y+208Y+213S+214T;
 195F+206Y+208Y; 195F+213I+214P; 195F+206Y+208Y+213T+214T; 195F+206Y;
 35 206Y+213S; 182P+186E; 182S+186E; 182V+186K; 179L+186H+190P; 179L+186K,R,S+190P;
 179L+190P; 179L+182C+186K+190P; 179L+182P+186S,V+190P; 179L+182S+186Q+190P;
 173F+174Q; 173Y+174S; 172K+173Y+174E; 193A,D,N,S+195F; 213A+214Q; 213P+214L;

213S+214R; 48V+60V; 213G+214T; 213I+214P; 213N+214I; 213N+214Q, and 213P,S+214T, wherein numbering is according to SEQ ID NO:11.

5 12. The variant according to any one of the preceding claims, wherein said variant comprises a pairwise deletion selected from the list consisting of:

181 and 182;

181 and 183;

181 and 184;

182 and 183;

10 182 and 184; and

183 and 184;

wherein the positions correspond to the positions of SEQ ID NO: 13.

13. The variant according to any one of the preceding claims, which has an improved
15 stability in detergent compositions relative to the parent alpha-amylase of SEQ ID NOs: 3 or 13.

14. The variant according to any one of the preceding claims, which has an improved
performance in detergent compositions relative to the parent alpha-amylase of SEQ ID NOs: 3
or 13.

20 15. The variant according to claim 14, wherein the improved performance is determined according to an AMSA as described in the method section.

16. The variant according to any one of claims 13 to 15, wherein the detergent composition
25 is a liquid detergent composition, a powder detergent composition, a unit dose detergent composition, or a soap bar detergent composition.

17. A composition comprising an alpha-amylase variant according to any one of the
preceding claims.

30 18. The composition according to claim 17, further comprising at least one further active component.

19. The composition according to any one of claims 16 and 17, wherein said further active
35 component is an enzyme, such as a protease, lipase, cellulose, pectate lyase and mannanase.

20. The composition according to claim 19, wherein the enzyme is selected from the group consisting of:

- (i) a protease comprising one or more modifications in the following positions: 32, 33, 48-54, 58-62, 94-107, 116, 123-133, 150, 152-156, 158-161, 164, 169, 175-186, 197, 198, 203-216 as compared with the protease in SEQ ID NO:4;
- 5 (ii) a lipase comprising one or more modifications in the following positions: 1-5, 27, 33, 38, 57, 91, 94, 96, 97, 111, 163, 210, 225, 231, 233, 249, and 254-256 as compared with the lipase in SEQ ID NO:5;
- (iii) an alpha-amylase comprising one or more modifications in the following positions: 9, 118, 149, 182, 186, 195, 202, 257, 295, 299, 320, 323, A339, 345, and 458 as compared with the alpha-amylase in SEQ ID NO:6;
- 10 (iv) an alpha-amylase comprising one or more modifications in the following positions: 140, 195, 206, 243, 260, and 476 as compared with the alpha-amylase in SEQ ID NO:7;
- (v) an alpha-amylase comprising one or more modifications in the following positions: 180, 181, 243, and 475 as compared with the alpha-amylase in SEQ ID NO:8;
- 15 (vi) an alpha-amylase comprising one or more modifications in the following positions: 178, 179, 187, 203, 458, 459, 460, and 476 as compared with the alpha-amylase in SEQ ID NO:9;
- (vii) an alpha-amylase comprising a modifications in the following position: 202 as compared with the alpha-amylase in SEQ ID NO:10;
- 20 (viii) an alpha-amylase comprising one or more modifications in the following positions: 405, 421, 422, and 428 as compared with the alpha-amylase in SEQ ID NO:11; and/or
- (ix) an alpha-amylase according to SEQ ID NO:12.
- 25

21. The composition according to any one of claims 15 to 20, which is a detergent composition, such as a liquid or powder detergent composition.

22. The composition according to any one of claims 17 to 21, which is a liquid laundry or liquid dish wash composition, such as an Automatic Dish Wash (ADW) liquid detergent composition, or a powder laundry, such as a soap bar, or powder dish wash composition, such as an ADW unit dose detergent composition.

30

23. A polynucleotide encoding the variant according to any one of claims 1 to 16.

35

24. A nucleic acid construct comprising the polynucleotide according to claim 23.

25. An expression vector comprising the polynucleotide according to claim 24.

26. A host cell comprising the polynucleotide according to claim 23, the nucleic acid construct according to claim 24, or the expression vector according to claim 25.

5 27. A method of producing an alpha-amylase variant, comprising:

- a. cultivating the host cell of claim 26 under conditions suitable for expression of the variant; and
- b. recovering said variant.

10 28. A method for obtaining an alpha-amylase variant, comprising introducing into a parent alpha-amylase having at least 89% sequence identity to the polypeptide of SEQ ID NOs: 3 or 13 a substitution at one or more positions said substitutions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of SEQ ID NO:3, wherein the variant has at least 67%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such
 15 as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 99%, but less than 100% sequence identity with the amino acid sequence of SEQ ID NOs: 3 or 13, wherein the variant has alpha-amylase activity; and recovering said variant.

29. A method of improving the stability, in particular the detergent stability, preferably liquid
 20 detergent stability, of a parent alpha-amylase having the amino acid sequence of SEQ ID NOs: 3 or 13, or having at least 89% sequence identity thereto, said method comprising the steps of:

a) a substitution at one or more positions said substitutions corresponding to positions 109, 51, 201, 269, 294, 297, 298, 193, and 314 of SEQ ID NO:3, wherein the variant has at least 67%, such as at least 70%, such as at least 75%, such as at least
 25 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 99%, but less than 100% sequence identity with the amino acid sequence of SEQ ID NOs: 3 or 13, wherein the variant has alpha-amylase activity; and

b) introducing into the parent alpha-amylase one or more of the following
 30 substitutions; 105L,I,F+206Y; 105L,I+206Y+217I; 105F+206Y+208Y+217V+246V; 105L+206F; 105I+206Y+208Y+217I+246V; 195F+213S+214T; 195F+206Y+208Y+213T+214T+217M,V; 195F+206Y+208F,L+213T+214T+217V; 195F+206Y+213S+214T; 195F+206Y+208Y+213S+214T+217M; 195F+206Y+208F+213T+214T+217M; 195F+206Y+208Y+213T+214T+217Q;
 35 195F+206Y+213G+214T; 195F+206Y+213S; 195F+206Y+208Y+213T+214T+217M; 195F+213S; 195F+206Y+208L+213T+214T+217M; 195F+213G+214T; 206Y,F+208Y+217Q; 206Y+208Y+217I; 206F+208Y+217M; 206Y+208Y; 206Y+217M; 206Y+208Y+213A+217M; 206Y+208Y+217V+246V; 206Y+213G; 206Y+208F+217V;

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206N+208Y+217M; 206F+208Y+217V; 206Y+246V; 206Y+217I,V; 206F+208F+217I;
 206Y+208L+213S; 206F+217I; 206Y+217I+246I; 206L+217V; 206Y+208F+217H;
 206L+208F+217I; 206L+217V+246L; 206F+246V; 208Y+213S+217M; 208Y+213A+217Q;
 63I+206Y; 63I+206Y+241V; 63V+206Y; 63V+105L+206Y; 63V+206Y+217I;
 5 63V+105F+206Y+208F+217I; 63V+206Y+246V; 63V+206F; 63V+206L+217V;
 63V+105F+206Y; 63V+206Y+241V+246L; 195F+206Y+208Y+214T+217V; 186E+195F+206Y;
 195F+206Y+208Y+213T+217V; 186E+195F+202T+206Y+209S; 63I+195F+206Y+210S;
 195F+206Y+213P+214T; 195F+206Y+208Y+213T+214T+217I; 186E+195F+206Y+210S;
 195F+213P; 186E+195F+202T+206Y+210S; 195F+206H; 195F+208Y+213T+214T+217V;
 10 206Y+208Y+213T+214T+217V; 195F+206Y+217V; 195F+206Y+208Y+213S+214T;
 195F+206Y+208Y; 195F+213I+214P; 195F+206Y+208Y+213T+214T; 195F+206Y;
 206Y+213S; 182P+186E; 182S+186E; 182V+186K; 179L+186H+190P; 179L+186K,R,S+190P;
 179L+190P; 179L+182C+186K+190P; 179L+182P+186S,V+190P; 179L+182S+186Q+190P;
 173F+174Q; 173Y+174S; 172K+173Y+174E; 193A,D,N,S+195F; 213A+214Q; 213P+214L;
 15 213S+214R; 48V+60V; 213G+214T; 213I+214P; 213N+214I; 213N+214Q, and 213P,S+214T,
 wherein numbering is according to SEQ ID NO:11,

wherein the variant has at least 67%, such as at least 70%, at least 75%, at least 80%, at least
 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, but less than 100%
 sequence identity with the polypeptide of SEQ ID NOs: 3 or 13, and wherein the variant has
 20 alpha-amylase activity and improved detergent stability and/or improved performance compared
 to the parent alpha-amylase.

30. The method according to any one of claims 27 to 29, wherein the variant has at least
 50%, such as at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 100% of
 25 the activity of the parent alpha-amylase having the amino acid sequence of SEQ ID NOs: 3 or
 13.

31. The method according to any one of claims 27 to 30, wherein the activity is determined
 according to a Phadebas assay as described in the method section.

30 32. Use of a variant according to any one of claims 1 to 16 in a cleaning process such as
 laundry or hard surface cleaning including dish wash and industrial cleaning.

	1	50
SEQ ID NO: 3	(1) TFAGDNGTMMQYFEWYLPNDGTLWTKMGSDASHLKSIGITGVWFPPAYKG	
SEQ ID NO: 11	(1) HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLKSKGITAVWIPPAWKG	
	51	100
SEQ ID NO: 3	(51) QSQSDVGYGVYDMYDLGFEFNQKGTVRTKYGTAKQLQSAITSLHNNGIQAY	
SEQ ID NO: 11	(51) ASQNDVGYGAYDLYDLGFEFNQKGTVRTKYGTRSQQAAVTSLKNNGIQVY	
	101	150
SEQ ID NO: 3	(101) GDVVLNHRMGADATETISAVEVNPSNRNQVTSGAYNISAWTDFEFPGRGN	
SEQ ID NO: 11	(101) GDVVMNHKGGADATEMVRAVEVNPNNRNQEVTEGYTIEAWTRFDFFPGRGN	
	151	200
SEQ ID NO: 3	(151) TYSSFKWHSYYFDGVDWDQSRQLSGKIYQIQKAWDWEVDSSENGNYDYLM	
SEQ ID NO: 11	(151) THSSFKWRWYHFDGVDWDQSRRLNNRIYKFRGKAWDWEVDTSENGNYDYLM	
	201	250
SEQ ID NO: 3	(201) GADIDYDHPDVQTEVKNWGWVNTLNLDGVRLDAVKHIKFDYMSSWLSS	
SEQ ID NO: 11	(199) YADIDMDHPEVVNELRNWGVWYTNLGLDGFRIIDAVKHIKYSFTRDWINH	
	251	300
SEQ ID NO: 3	(251) VKSTTGKSNLFAVGEYWNTSLGALENYENKTNWSMSLFDVPLHMNFQAAA	
SEQ ID NO: 11	(249) VRSATGK-NMFAVAEFWKNDLGAIENYLQKTNWNHVSFVPLHYNLYNAS	

Figure 1

301 350
SEQ ID NO: 3 (301) NGGGYDMRNLLNNTMMKNHPIQAVTFVDNHDTEPGQALQSWVSDWFKPL
SEQ ID NO:11 (298) KSGGNYDMRNIFNGTVVQRHPHAVTFVDNHDSQPEEALESFVEEWFKPL

351 400
SEQ ID NO: 3 (351) AYATILTRQEGYPCVFYGDYYGIPSQSVSAKSTWLDKQLSARKSYAYGTQ
SEQ ID NO:11 (348) AYALTLTREQGYPSVFYGDYYGIPTHGVPAMRSKIDPILEARQKYAYGPQ

401 450
SEQ ID NO: 3 (401) HDYLDNQDVIGWTREGDSAHAGSGLATVMSDGPGGSKTMYVGTAAHAGQVF
SEQ ID NO:11 (398) HDYLDHPDVIGWTREGDSSHPKSGLATLITDGPGGSKRMYAGLKNAGETW

451 484
SEQ ID NO: 3 (451) KDITGNRTDTVTINSAGNGTFPCNGGSVSIWVKQ
SEQ ID NO:11 (448) YDITGNRSDTVKIGSDGWGEFHVNDGSVSIYVQK

Figure 1 cont.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/077236

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12N9/26 C11D3/386 C12N15/56 C12N1/21 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)
 C12N C11D
 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 EPO-Internal, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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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 10 February 2016	Date of mailing of the international search report 23/02/2016
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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 Le Cornec, Nadine
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/077236

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>-& WANG QINGYAN ET AL: "Tumebacillus flagellatus sp nov., an alpha-amylase/pullulanase-producing bacterium isolated from cassava wastewater", INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY, vol. 63, no. Part 9, September 2013 (2013-09), pages 3138-3142, XP002739816, ----- DATABASE Geneseq [Online] 8 April 1997 (1997-04-08), "Alpha-amylase variant R124P + delta183 + delta184.", XP002739817, retrieved from EBI accession no. GSP:AAW12133 Database accession no. AAW12133 sequence -& WO 96/23873 A1 (NOVO NORDISK AS [DK]; BISGAARD FRANTZEN HENRIK [DK]; SVENDSEN ALLAN [D] 8 August 1996 (1996-08-08) the whole document</p>	1-3,7,8, 12-16, 20-32
X	<p>WO 2011/076897 A1 (NOVOZYMES AS [DK]; ANDERSEN CARSTEN; KAASGAARD SVEND [DK]; BEIER LARS) 30 June 2011 (2011-06-30) page 97 - page 99; examples ----- DATABASE Geneseq [Online] 8 April 1997 (1997-04-08), "Alpha-amylase variant delta181 + delta182.", XP002754079, retrieved from EBI accession no. GSP:AAW12115 Database accession no. AAW12115 sequence</p>	1-3,7,8, 11-16, 20-32
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X	<p>WO 2013/001078 A1 (NOVOZYMES AS [DK]; KAASGAARD SVEND [DK]; OEBRO JENS [DK]; LARSEN SIGNE) 3 January 2013 (2013-01-03) page 43 - page 45; claims ----- WO 2014/183921 A1 (NOVOZYMES AS [DK]) 20 November 2014 (2014-11-20) page 88 - page 93; examples; sequence 4 ----- -/--</p>	1-31
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International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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INTERNATIONAL SEARCH REPORT

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