



(86) Date de dépôt PCT/PCT Filing Date: 2012/10/17
(87) Date publication PCT/PCT Publication Date: 2013/04/25
(45) Date de délivrance/Issue Date: 2023/05/23
(85) Entrée phase nationale/National Entry: 2014/04/16
(86) N° demande PCT/PCT Application No.: EP 2012/070575
(87) N° publication PCT/PCT Publication No.: 2013/057141
(30) Priorité/Priority: 2011/10/17 (EP11185478.2)

(51) Cl.Int./Int.Cl. *C07K 14/435* (2006.01)
(72) Inventeurs/Inventors:
ANDERSEN, CARSTEN, DK;
LARSEN, SIGNE ESKILDSEN, DK;
FRIIS, ESBEN PETER, DK;
HANSEN, PETER KAMP, DK;
FRIIS-MADSEN, HENRIK, DK;
VIKSOE-NIELSEN, ANDERS, DK;
MICHEELSEN, PERNILLE OLLENDORFF, DK
(73) Propriétaire/Owner:
NOVOZYMES A/S, DK
(74) Agent: WILSON LUE LLP

(54) Titre : VARIANTS D'ALPHA-AMYLASE ET POLYNUCLEOTIDES LES CODANT
(54) Title: ALPHA-AMYLASE VARIANTS AND POLYNUCLEOTIDES ENCODING SAME

(57) Abrégé/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.



Abstract

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

ALPHA-AMYLASE VARIANTS AND POLYNUCLEOTIDES ENCODING SAME

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form.

5

Background of the Invention

Field of the Invention

The present invention relates to alpha-amylase variants, polynucleotides encoding the variants, methods of producing the variants, and methods of using the variants.

10

Description of the Related Art

Alpha-amylases (E.C. 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch, glycogen and related polysaccharides and oligosaccharides.

Alpha-amylases are used commercially for a variety of purposes such as in the initial stages of starch processing (e.g., liquefaction); in wet milling processes; and in alcohol production from carbohydrate sources. They are also used as cleaning agents or adjuncts in detergent matrices; in the textile industry for starch desizing; in baking applications; in the beverage industry; in oil fields in drilling processes; in recycling processes, e.g., for de-inking paper; and in animal feed.

Some commercial alpha-amylases for, e.g., starch liquefaction originate from *Bacillus licheniformis* or *Bacillus stearothermophilus*. Protein engineered variants of wild type enzymes have been developed to overcome process issues. There is still a need, though, for novel alpha-amylases with improved properties, such as higher stability at low pH, low calcium and high temperature. Such enzymes will allow the starch liquefaction process to be run at reduced pH which has a positive influence on chemical savings.

It is an object of the present invention to provide novel alpha-amylase variants having an increased stability at low pH and/or at high temperature, in particular at low calcium concentrations.

Summary of the Invention

The present inventors have found that alpha-amylase variants comprising a substitution with proline at a position corresponding to position 185 combined with a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID

NO: 1 have an increased stability at low pH and/or at high temperature, in particular at low calcium concentrations.

The present invention therefore relates to alpha-amylase variants comprising a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and
 5 further comprising a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1, wherein the variant has at least 60% and less than 100% sequence identity to (i) the mature polypeptide of any of SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of
 10 SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12,
 15 amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14, and wherein the variant has alpha-amylase activity.

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

20 The present invention also relates to uses of the variants of the invention and to a method of producing liquefied starch and a method of producing a fermentation product.

Brief Description of the Figures

Figure 1 shows an alignment of alpha-amylases with the amino acid sequences of:
 25 SEQ ID NO: 1 is a *Bacillus licheniformis* alpha-amylase.
 SEQ ID NO: 2 is a *Bacillus stearothermophilus* alpha-amylase.
 SEQ ID NO: 3 is the *Bacillus* alpha-amylase TS-23 described in J. Appl. Microbiology, 1997, 82: 325-334 (SWALL:q59222).
 SEQ ID NO: 4 is *Bacillus flavothermus* alpha-amylase AMY1048 described in WO
 30 2005/001064.
 SEQ ID NO: 5 is *Bacillus* alpha-amylase TS-22 described as SEQ ID NO: 21 in WO 04/113511.
 SEQ ID NO: 6 is a *Bacillus amyloliquefaciens* alpha-amylase.

SEQ ID NO: 7 is *Bacillus* alkaline sp. SP690 amylase described as SEQ ID NO 1 in WO 95/26397.

SEQ ID NO: 8 is *Bacillus halmapalus* alpha-amylase described as SEQ ID NO 2 in WO 95/26397.

5 SEQ ID NO: 9 is *Bacillus* alkaline sp. AA560 amylase described as SEQ ID NO 4 in WO 00/60060.

SEQ ID NO: 10 is *Bacillus* alkaline sp. A 7-7 amylase described as SEQ ID NO 2 in WO200210356.

10 SEQ ID NO: 11 is *Bacillus* alkaline sp. SP707 amylase described in Tsukamoto et al., 1988, *Biochem. Biophys. Res. Commun.* 151: 25-33).

SEQ ID NO: 12 is *Bacillus* alkaline sp. K-38 amylase described as SEQ ID NO 2 in EP 1022334.

SEQ ID NO: 13 is a *Bacillus licheniformis* alpha-amylase described in Lee et al, 2006, *J. Biochem.*, 139: 997-1005.

15 SEQ ID NO: 14 is a variant alpha-amylase LE399 previously disclosed in, e.g., WO 2002/010355.

Definitions

20 **Allelic variant:** The term "allelic variant" means any of two or more 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.

25 **Alpha-amylase:** Alpha-amylases (E.C. 3.2.1.1) are a group of enzymes which catalyze the hydrolysis of starch and other linear and branched 1,4 glucosidic oligo- and polysaccharides. The skilled person will know how to determine alpha-amylase activity. It may be determined according to the procedure described in the Examples, e.g., by the PNP-G7 assay. In one aspect, the variants of the present invention have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the
30 mature polypeptide of SEQ ID NO: 1. In another aspect, a variant of the present application has 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 its parent.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell.

cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

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

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

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

Fragment: The term "fragment" means a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has alpha-amylase activity. In one aspect, a fragment contains at least 300 amino acid residues, at least 350 amino acid residues, at least 400 amino acid residues, at least 450 amino acid residues, at least 470 amino acid residues, or at least 480 amino acid residues.

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

Isolated: The term "isolated" means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally

occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. The mature form of some alpha-amylases, e.g., some bacterial alpha-amylases, comprises a catalytic domain containing the active site for substrate hydrolysis and one or more carbohydrate-binding modules (CBM) for binding to the carbohydrate substrate (starch) and optionally a polypeptide linking the CBM(s) with the catalytic domain, a region of the latter type usually being denoted a "linker".

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

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

Parent or parent alpha-amylase: The term "parent" or "parent alpha-amylase" means 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 purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.*

16: 276-277), preferably version 5.0.0 or later. The parameters used 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:

5
$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

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. 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 one or more (*e.g.*, several) amino acids, *e.g.*, 1-5 amino acids, adjacent to the amino acid occupying a position. In one aspect, the variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the mature polypeptide of SEQ ID NO: 1. In another aspect, a variant of the present application has 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 its parent. The alpha-amylase activity may be determined by the PNP-G7 assay described in the Examples.

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 mature polypeptide disclosed in SEQ ID NO: 1 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 mature polypeptide disclosed in SEQ ID NO: 1, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO: 1 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

Identification of the corresponding amino acid residue in another alpha-amylase can be determined by an alignment of multiple polypeptide sequences using several computer programs

including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later; Edgar, 2004, *Nucleic Acids Research* 32: 1792-1797), MAFFT (version 6.857 or later; Katoh and Kuma, 2002, *Nucleic Acids Research* 30: 3059-3066; Katoh *et al.*, 2005, *Nucleic Acids Research* 33: 511-518; Katoh and Toh, 2007, *Bioinformatics* 23: 372-374; Katoh *et al.*, 2009, *Methods in Molecular Biology* 537: 39-64; Katoh and Toh, 2010, *Bioinformatics* 26: 1899-1900), and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson *et al.*, 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

When the other enzyme has diverged from the mature polypeptide of SEQ ID NO: 1 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. In the Examples of the present application, multiple mutations are separated by a space, e.g., G205R S411F representing G205R + S411F.

Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), e.g., "Gly195* + Ser411*" or "G195* + S411*".

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

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

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

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

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

"Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167Ala+Arg170Ala".

Detailed Description of the Invention

5 The present invention relates to alpha-amylase variants comprising a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and further comprising a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1, wherein the variant has at least 60% and less than 100%
 10 sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to
 15 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14, and wherein the variant has alpha-amylase activity.

Preferably, the variants are isolated.

20 Variants

The present invention provides alpha-amylase variants comprising a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1.

The variants further comprise a substitution at one or more (e.g., several) positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197,
 25 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1.

The variant comprises a substitution at a position corresponding to position 185 with Pro.

In one embodiment, the variant comprises a substitution at a position corresponding to position 15 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Leu, Ser or Thr.

30 In one embodiment, the variant comprises a substitution at a position corresponding to position 48 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ala.

In one embodiment, the variant comprises a substitution at a position corresponding to position 49 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val, in particular with Gly, His, Ile or Leu.

5 In one embodiment, the variant comprises a substitution at a position corresponding to position 50 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Thr.

In one embodiment, the variant comprises a substitution at a position corresponding to position 107 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ala.

10 In one embodiment, the variant comprises a substitution at a position corresponding to position 116 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val, in particular with Gly.

15 In one embodiment, the variant comprises a substitution at a position corresponding to position 133 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr.

In one embodiment, the variant comprises a substitution at a position corresponding to position 138 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr or Val, in particular with Phe or Tyr.

20 In one embodiment, the variant comprises a substitution at a position corresponding to position 156 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr.

In one embodiment, the variant further comprises a substitution at a position corresponding to position 176 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Leu.

25 In one embodiment, the variant comprises a substitution at a position corresponding to position 181 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Asp, Glu or Thr.

30 In one embodiment, the variant comprises a substitution at a position corresponding to position 187 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Asp.

In one embodiment, the variant comprises a substitution at a position corresponding to position 188 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser or Thr.

In one embodiment, the variant comprises a substitution at a position corresponding to position 190 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Phe.

5 In one embodiment, the variant comprises a substitution at a position corresponding to position 197 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ile, Leu, Ser, Thr or Val.

In one embodiment, the variant comprises a substitution at a position corresponding to position 201 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Phe or Tyr.

10 In one embodiment, the variant comprises a substitution at a position corresponding to position 205 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr.

In one embodiment, the variant comprises a substitution at a position corresponding to position 209 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Val.

15 In one embodiment, the variant comprises a substitution at a position corresponding to position 213 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Thr.

In one embodiment, the variant comprises a substitution at a position corresponding to position 239 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Ala, Asn, Asp, Cys, Gln, Glu or Met.

In one embodiment, the variant comprises a substitution at a position corresponding to position 241 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Asp.

25 In one embodiment, the variant comprises a substitution at a position corresponding to position 255 with Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Gly or Pro.

In one embodiment, the variant comprises a substitution at a position corresponding to position 264 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser.

30 In one embodiment, the variant comprises a substitution at a position corresponding to position 299 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg.

In one embodiment, the variant comprises a substitution at a position corresponding to position 360 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser.

In one embodiment, the variant comprises a substitution at a position
5 corresponding to position 375 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Gly or Val.

In one embodiment, the variant comprises a substitution at a position corresponding to position 416 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Val.

10 In one embodiment, the variant comprises a substitution at a position corresponding to position 437 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Trp.

In one embodiment, the variant comprises a substitution at a position corresponding to position 474 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys,
15 Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg, Gln, Glu or Lys.

In one embodiment, the variant comprises a substitution at a position corresponding to position 475 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg, Gln, Glu or Lys.

The variant has sequence identity of at least 60%, *e.g.*, at least 65%, at least 70%, at least
20 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%, to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO:
25 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

In a preferred embodiment, the variant has at least 60%, *e.g.*, at least 65%, at least 70%, at
30 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 (i) the mature polypeptide of SEQ ID NO: 1, or (ii) amino acids 1-483 of SEQ ID NO: 1.

In one embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 2, (ii) the mature polypeptide of SEQ ID NO: 2 comprising the deletions I181*+G182*, (iii) amino acids 1-483 of SEQ ID NO: 2, or (iv) amino acids 1-483 of SEQ ID NO: 2 comprising the deletions I181*+G182*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 3, (ii) the mature polypeptide of SEQ ID NO: 3 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 3, or (iv) amino acids 1-485 of SEQ ID NO: 3 comprising the deletions T183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 4, (ii) the mature polypeptide of SEQ ID NO: 4 comprising the deletions T180*+G181*, (iii) amino acids 1-482 of SEQ ID NO: 4, or (iv) amino acids 1-482 of SEQ ID NO: 4 comprising the deletions T180*+G181*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 5, (ii) the mature polypeptide of SEQ ID NO: 5 comprising the deletions T182*+G183*, (iii) amino acids 1-484 of SEQ ID NO: 5, or (iv) amino acids 1-484 of SEQ ID NO: 5 comprising the deletions T182*+G183*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 6, (ii) the mature polypeptide of SEQ ID NO: 6 comprising the deletions E178*+G179*, (iii) amino acids 1-483 of SEQ ID NO: 6, or (iv) amino acids 1-483 of SEQ ID NO: 6 comprising the deletions E178*+G179*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 7, (ii) the mature polypeptide

of SEQ ID NO: 7 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 7, or (iv) amino acids 1-485 of SEQ ID NO: 7 comprising the deletions T183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 8, (ii) the mature polypeptide of SEQ ID NO: 8 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 8, or (iv) amino acids 1-485 of SEQ ID NO: 8 comprising the deletions D183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 9, (ii) the mature polypeptide of SEQ ID NO: 9 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 9, or (iv) amino acids 1-485 of SEQ ID NO: 9 comprising the deletions D183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 10, (ii) the mature polypeptide of SEQ ID NO: 10 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 10, or (iv) amino acids 1-485 of SEQ ID NO: 10 comprising the deletions D183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 11, (ii) the mature polypeptide of SEQ ID NO: 11 comprising the deletions H183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 11, or (iv) amino acids 1-485 of SEQ ID NO: 11 comprising the deletions H183*+G184*.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 12, or (ii) amino acids 1-480 of SEQ ID NO: 12.

In another embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 13, or (ii) amino acids 1-483 of SEQ ID NO: 13.

In another preferred embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 14, or (ii) amino acids 1-481 of SEQ ID NO: 14.

A variant of the invention comprises a substitution with proline at position 185. Further, the variant comprises a substitution at one or more (*e.g.*, several) positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1. In one embodiment, the variant comprises a substitution at one position corresponding to any of positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475. In another embodiment, the variant comprises a substitution at two positions corresponding to any of positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475. In another embodiment, the variant comprises a substitution at three positions corresponding to any of positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475. In another embodiment, the variant comprises a substitution at four positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475. In another embodiment, the variant comprises a substitution at more than four, *e.g.*, five, six, seven, eight, nine or ten, positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475. In another embodiment, the variant comprises a substitution at more than 10 positions, *e.g.*, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 positions, corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475.

A variant of the invention comprises the substitution E185P. In one embodiment, the variant further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, K176L, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M,

S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

In a preferred embodiment, the variant comprises a substitution at one or more positions corresponding to positions 49, 50, 116, 176, 201, 205, 213, 241, 360, 375, 416, and 437. The variant preferably comprises one or more substitutions selected among T49H, T49G, T49L, S50T, T116G, K176L, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W.

In one embodiment, the variant comprises a substitution at one or more positions corresponding to positions 49, 50, 116, 176, 201, 205, 213, 241, 360, 375, 416, and 437 and further comprises a substitution at one or more positions corresponding to positions 15, 48, 107, 133, 138, 156, 181, 187, 188, 190, 197, 209, 239, 255, 264, 299, 474 and 475. The variant preferably comprises one or more substitutions selected among T49H, T49G, T49L, S50T, T116G, K176L, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W, and further one or more substitutions selected among M15T, M15S, M15L, G48A, G107A, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, A209V, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, E255P, E255G, Q264S, G299R, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 15. Preferably, the variant comprises the substitutions M15T+E185P; M15S+E185P or M15L+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 48. Preferably, the variant comprises the substitutions G48A+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 49. Preferably, the variant comprises the substitutions T49H+E185P; T49G+E185P; T49L+E185P or T49I+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 50. Preferably, the variant comprises the substitutions S50T+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 107. Preferably, the variant comprises the substitutions G107A+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 116. Preferably, the variant comprises the substitutions T116G+E185P.

5 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 133. Preferably, the variant comprises the substitutions H133Y+E185P.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 138. Preferably, the variant comprises the substitutions W138Y+E185P or W138F+E185P.

10 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 156. Preferably, the variant comprises the substitutions H156Y+E185P.

In a preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176. Preferably, the variant comprises the substitutions K176L+E185P.

20 In another preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176 and further in combination with a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475.

In another preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

30 In a more preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of T49H, T49G, T49L, S50T, T116G, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W.

In another preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176, wherein the variant has at least 60%, e.g., at least 65%, 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 SEQ ID NO: 1. Preferably, the variant comprises the substitutions K176L+E185P, wherein the variant has at least 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 1.

In another preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176 and further in combination with a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475, wherein the variant has at least 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 1.

In another preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q, wherein the variant has at least 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 1.

In a more preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of T49H, T49G, T49L, S50T, T116G, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W, wherein the variant has at least 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 1.

In another preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176, wherein the

variant has at least 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 14. Preferably, the variant comprises the substitutions K176L+E185P, wherein the variant has at least

5 60%, *e.g.*, at least 65%, 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 SEQ ID NO: 14.

In another preferred embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 176 and further in

10 combination with a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475, wherein the variant has at least 60%, *e.g.*, at least 65%, 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

15 than 100%, sequence identity to SEQ ID NO: 14.

In another preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L,

20 M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q, wherein the variant has at least 60%, *e.g.*, at least 65%, 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

25 least 98%, or at least 99%, but less than 100%, sequence identity to SEQ ID NO: 14.

In a more preferred embodiment, the variant comprises the substitutions K176L+E185P and further comprises one or more substitutions selected from the group consisting of T49H, T49G, T49L, S50T, T116G, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W, wherein the variant has at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at

30 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 SEQ ID NO: 14.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 181. Preferably, the variant comprises the substitutions A181T+E185P; A181E+E185P or A181D+E185P.

5 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 187. Preferably, the variant comprises the substitutions E185P+S187D.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 188. Preferably, the variant comprises the substitutions E185P+N188S or E185P+N188T.

10 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 190. Preferably, the variant comprises the substitutions E185P+N190F.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 197. Preferably, the variant
15 comprises the substitutions E185P+M197S, E185P+M197T, E185P+M197V, E185P+M197L or E185P+M197I.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 201. Preferably, the variant comprises the substitutions E185P+I201Y or E185P+I201F.

20 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 205. Preferably, the variant comprises the substitutions E185P+H205Y.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 209. Preferably, the variant
25 comprises the substitutions E185P+A209V.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 213. Preferably, the variant comprises the substitutions E185P+K213T.

In one embodiment, the variant comprises the substitution E185P in combination
30 with a substitution at a position corresponding to position 239. Preferably, the variant comprises the substitutions E185P+S239Q, E185P+S239E, E185P+S239N, E185P+S239D, E185P+S239A, E185P+S239M or E185P+S239C.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 241. Preferably, the variant comprises the substitutions E185P+L241D.

5 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 255. Preferably, the variant comprises the substitutions E185P+E255P or E185P+E255G.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 264. Preferably, the variant comprises the substitutions E185P+Q264S.

10 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 299. Preferably, the variant comprises the substitutions E185P+G299R.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 360. Preferably, the variant comprises the substitutions E185P+Q360S.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 375. Preferably, the variant comprises the substitutions E185P+R375V or E185P+R375G.

20 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 416. Preferably, the variant comprises the substitutions E185P+D416V.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 437. Preferably, the variant comprises the substitutions E185P+R437W.

25 In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 474. Preferably, the variant comprises the substitutions E185P+474K, E185P+474R, E185P+474E or E185P+474Q.

In one embodiment, the variant comprises the substitution E185P in combination with a substitution at a position corresponding to position 475. Preferably, the variant comprises the substitutions E185P+475K, E185P+475R, E185P+475E or E185P+475Q.

30 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 15. Preferably, the variant comprises the substitutions M15T+K176L+E185P; M15S+K176L+E185P or M15L+K176L+E185P.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 48. Preferably, the variant comprises the substitutions G48A+K176L+E185P.

5 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 49. Preferably, the variant comprises the substitutions T49H+K176L+E185P; T49G+K176L+E185P; T49L+K176L+E185P or T49I+K176L+E185P.

10 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 50. Preferably, the variant comprises the substitutions S50T+K176L+E185P.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 107. Preferably, the variant comprises the substitutions G107A+K176L+E185P.

15 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 116. Preferably, the variant comprises the substitutions T116G+K176L+E185P.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 133. Preferably, the variant comprises the substitutions H133Y+K176L+E185P.

20 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 138. Preferably, the variant comprises the substitutions W138Y+K176L+E185P or W138F+K176L+E185P.

25 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 156. Preferably, the variant comprises the substitutions H156Y+K176L+E185P.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 181. Preferably, the variant comprises the substitutions K176L+A181T+E185P; K176L+A181E+E185P or K176L+A181D+E185P.

30 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 187. Preferably, the variant comprises the substitutions K176L+E185P+S187D.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 188. Preferably, the variant comprises the substitutions K176L+E185P+N188S or K176L+E185P+N188T.

5 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 190. Preferably, the variant comprises the substitutions K176L+E185P+N190F.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 197. Preferably, the variant comprises the substitutions K176L+E185P+M197S, K176L+E185P+M197T, K176L+E185P+M197V,
10 K176L+E185P+M197L or K176L+E185P+M197I.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 201. Preferably, the variant comprises the substitutions K176L+E185P+I201Y or K176L+E185P+I201F.

15 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 205. Preferably, the variant comprises the substitutions K176L+E185P+H205Y.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 209. Preferably, the variant comprises the substitutions K176L+E185P+A209V.

20 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 213. Preferably, the variant comprises the substitutions K176L+E185P+K213T.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 239. Preferably, the variant comprises the
25 substitutions K176L+E185P+S239Q, K176L+E185P+S239E, K176L+E185P+S239N, K176L+E185P+S239D, K176L+E185P+S239A, K176L+E185P+S239M or K176L+E185P+S239C.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 241. Preferably, the variant comprises the substitutions K176L+E185P+L241D.

30 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 255. Preferably, the variant comprises the substitutions K176L+E185P+E255P or K176L+E185P+E255G.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 264. Preferably, the variant comprises the substitutions K176L+E185P+Q264S.

5 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 299. Preferably, the variant comprises the substitutions K176L+E185P+G299R.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 360. Preferably, the variant comprises the substitutions K176L+E185P+Q360S.

10 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 375. Preferably, the variant comprises the substitutions K176L+E185P+R375V or K176L+E185P+R375G.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 416. Preferably, the variant comprises the substitutions K176L+E185P+D416V.

15 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 437. Preferably, the variant comprises the substitutions K176L+E185P+R437W.

20 In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 474. Preferably, the variant comprises the substitutions K176L+E185P+G474K, K176L+E185P+G474R, K176L+E185P+G474E or K176L+E185P+G474Q.

In one embodiment, the variant comprises the substitutions E185P+K176L in combination with a substitution at a position corresponding to position 475. Preferably, the variant comprises the substitutions K176L+E185P+G475K, K176L+E185P+G475R, K176L+E185P+G475E or K176L+E185P+G475Q.

In a preferred embodiment, the variant comprises a set of substitutions selected from the group consisting of:

30 T49H+K176L+E185P,
T49G+K176L+E185P,
T49L+S50T+K176L+E185P,
T116G+K176L+E185P,
K176L+E185P,
K176L+E185P+I201Y+H205Y+K213T+Q360S+D416V+R437W,

K176L+E185P+L241D,
 K176L+E185P+R375V, and
 K176L+E185P+R375G.

In another preferred embodiment, the variant comprises a set of substitutions selected from
 5 the group consisting of:

- G48A+T49H+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
- G48A+T49G+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
- G48A+T49L+S50T+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
- G48A+T49I+G107A+T116G+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
- 10 G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
- G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201Y+H205Y+A209V+K213T+Q264S+Q360S+D416V+R437W;
- G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+L241D+A209V+Q264S;
- G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S+R375V;
- 15 G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S+R375G; and
- G48A+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S.

In one embodiment, the variant further comprises a deletion at both of the two positions immediately before the position corresponding to position 180 of SEQ ID NO: 1. I.e., a deletion of the two amino acids corresponding to positions 181 and 182 of SEQ ID NO: 2.

20 In another embodiment, the variant further comprises a deletion of two amino acids after the position corresponding to position 177 of SEQ ID NO: 1 and before the position corresponding to position 180 of SEQ ID NO: 1. I.e., a deletion of two amino acids in the R179-G180-I181-G182 peptide of SEQ ID NO: 2, or homologous amino acids in any of SEQ ID NO: 3 to 11.

The variants may further comprise one or more (e.g., several) additional alterations, e.g.,
 25 one or more (e.g., several) additional substitutions.

The additional 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
 30 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.

The variants may consist of 300 to 700, e.g., 350 to 650, 400 to 600, 450 to 500 or 470 to 490, amino acids.

In a preferred embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 at high temperature, low calcium and low pH.

In another preferred embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 at high temperature, low calcium and low pH.

In another preferred embodiment, the variant has increased thermostability compared to its parent at high temperature, low calcium and low pH.

In the context of the present invention "high temperature" means temperatures from 70-120°C, preferably 80-100°C, more preferably 85-95°C.

In the context of the present invention the term "low pH" means a pH in the range from 4-6, preferably 4.2-5.5, more preferably 4.5-5.

In the context of the present invention the term “low calcium” means free calcium levels lower than 60 ppm, preferably 40 ppm, more preferably 25 ppm, even more preferably 5 ppm calcium. 0.125 mM CaCl_2 provides 5 ppm calcium.

In one embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.5.

In one embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5. In another embodiment, the variant has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.5.

In one embodiment, the variant has increased thermostability compared to its parent when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to its parent when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8. In another embodiment, the variant has increased thermostability compared to its parent when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5. In another embodiment, the variant has increased thermostability compared to its parent when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.5.

The skilled person will know how to determine thermostability of the enzymes. It may be done by determining the residual activity half-life as shown in the Examples of the present application. The skilled person will know how to choose the relevant conditions for the assay, such as the incubation time.

In a preferred embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 at high temperature, low calcium and low pH. In one embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8. In another

embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8. In another embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5. In another embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.5.

In a preferred embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 at high temperature, low calcium and low pH. In one embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8. In another embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8. In another embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5. In another embodiment, the variant has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.5.

In a preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8, which is at least 5 minutes, e.g., at least 10 minutes or at least 20 minutes. In a more preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8, which is at least 50 minutes, e.g., at least 60 minutes or at least 70 minutes. In one embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.8, which is at least 100 minutes.

In a preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5, which is at least 2 minute, e.g., at least 3 minutes or at least 5 minutes. In a more preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5, which is at least 10 minutes, e.g., at least 15 minutes or at least 20 minutes. In one embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 90°C and pH 4.5, which is at least 30 minutes.

In a preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8, which is at least 1 minute, e.g., at least 2 minutes or at least 5 minutes. In a more preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8, which is at least 10 minutes, e.g., at least 15 minutes or at least 20 minutes. In one embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl_2 at 95°C and pH 4.8, which is at least 30 minutes.

In a preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.5, which is at least 1 minute, e.g., at least 2 minutes or at least 3 minutes. In a more preferred embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.5, which is at least 5 minutes, e.g., at least 7 minutes or at least 10 minutes. In one embodiment, the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.8, which is at least 15 minutes.

In one embodiment, the variant has increased thermostability, e.g., an increased residual activity half-life, compared to the parent enzyme when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.8. In another embodiment, the variant has increased thermostability, e.g., an increased residual activity half-life, compared to the parent enzyme when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.8. In another embodiment, the variant has increased thermostability, e.g., an increased residual activity half-life, compared to the parent enzyme when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.5. In another embodiment, the variant has increased thermostability, e.g., an increased residual activity half-life, compared to the parent enzyme when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.5.

In other embodiments, the variant has improved catalytic efficiency, improved catalytic rate, improved chemical stability, improved oxidation stability, improved specific activity, improved stability under storage conditions, improved substrate binding, improved substrate cleavage, improved substrate specificity, improved substrate stability, improved surface properties, improved thermal activity, or improved thermostability compared to the parent enzyme.

Parent alpha-amylase

The variant is preferably a variant of a parent alpha-amylase selected from the group consisting of:

- (a) a polypeptide having at least 60% sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14; or
- (b) a fragment of the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, which has alpha-amylase activity.

In one embodiment, the parent alpha-amylase has at least 65%, *e.g.*, 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 (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

In one embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 1, or (ii) amino acids 1-483 of SEQ ID NO: 1.

In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 2, (ii) the mature polypeptide of SEQ ID NO: 2 comprising the deletions I181*+G182*, (iii) amino acids 1-483 of SEQ ID NO: 2, or (iv) amino acids 1-483 of SEQ ID NO: 2 comprising the deletions I181*+G182*.

In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 3, (ii) the mature polypeptide of SEQ ID NO: 3 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 3, or (iv) amino acids 1-485 of SEQ ID NO: 3 comprising the deletions T183*+G184*.

In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 4, (ii) the mature polypeptide of SEQ ID NO: 4 comprising the deletions T180*+G181*, (iii) amino acids 1-482 of SEQ ID NO: 4, or (iv) amino acids 1-482 of SEQ ID NO: 4 comprising the deletions T180*+G181*.

In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 5, (ii) the mature polypeptide of SEQ ID NO: 5 comprising the deletions T182*+G183*, (iii) amino acids 1-484 of SEQ ID NO: 5, or (iv) amino acids 1-484 of SEQ ID NO: 5 comprising the deletions T182*+G183*.

5 In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 6, (ii) the mature polypeptide of SEQ ID NO: 6 comprising the deletions E178*+G179*, (iii) amino acids 1-483 of SEQ ID NO: 6, or (iv) amino acids 1-483 of SEQ ID NO: 6 comprising the deletions E178*+G179*.

10 In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 7, (ii) the mature polypeptide of SEQ ID NO: 7 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 7, or (iv) amino acids 1-485 of SEQ ID NO: 7 comprising the deletions T183*+G184*.

15 In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 8, (ii) the mature polypeptide of SEQ ID NO: 8 comprising the deletions D183*+G184*, (iii) amino acids 1-486 of SEQ ID NO: 8, or (iv) amino acids 1-486 of SEQ ID NO: 8 comprising the deletions D183*+G184*.

20 In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 9, (ii) the mature polypeptide of SEQ ID NO: 9 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 9, or (iv) amino acids 1-485 of SEQ ID NO: 9 comprising the deletions D183*+G184*.

25 In another embodiment, the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 10, (ii) the mature polypeptide of SEQ ID NO: 10 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 10, or (iv) amino acids 1-485 of SEQ ID NO: 10 comprising the deletions D183*+G184*.

In another embodiment, the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 11, (ii) the mature polypeptide of SEQ ID NO: 11 comprising the deletions H183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 11, or (iv) amino acids 1-485 of SEQ ID NO: 11 comprising the deletions H183*+G184*.

In another embodiment, the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 12, or (ii) amino acids 1-480 of SEQ ID NO: 12.

In another embodiment, the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 13, or (ii) amino acids 1-483 of SEQ ID NO: 13.

In another embodiment, the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 14, or (ii) amino acids 1-481 of SEQ ID NO: 14.

In one embodiment, the parent alpha-amylase comprises or consists of the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14.

In one embodiment, the parent alpha-amylase comprises or consists of amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

In another embodiment, the parent alpha-amylase is a fragment of the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, wherein the fragment has alpha-amylase activity.

In one embodiment, the variant has at least 60%, *e.g.*, at least 65%, 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%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the parent alpha-amylase.

5 In one embodiment, the total number of alterations in the variants of the present invention is 2-20, *e.g.*, 2-10 and 2-5, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 alterations.

The parent 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.

10 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. 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
15 terminator. Fusion polypeptides may also be constructed 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
20 of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 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 Discovery World* 4: 35-48.
25

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 secreted
30 extracellularly.

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* alpha-amylase, or a Gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*,

Flavobacterium, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, 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*,
 5 *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus*
stearothermophilus, *Bacillus subtilis*, or *Bacillus thuringiensis* alpha-amylase.

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
 10 identity of appropriate equivalents.

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
 15 (NRRL).

The parent 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
 20 polynucleotide encoding a parent may then be obtained by similarly 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*).

25

Preparation of Variants

The present invention also relates to methods for obtaining a variant having alpha-amylase activity, comprising: (a) introducing into a parent alpha-amylase a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and further a substitution at one or more
 30 positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1; 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.

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 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 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.

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.

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

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells

(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.

5 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 be synthesized *de novo*, while other regions may be amplified using site-specific mutagenic primers,
10 while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

15 The present invention also relates to isolated polynucleotides encoding a variant of the present invention.

Nucleic Acid Constructs

20 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.

25 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.

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

 Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*),

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

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

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: 423-488.

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

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

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* TKA amylase, and *Fusarium oxysporum* trypsin-like protease.

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

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

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

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

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

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 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, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

5 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 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
10 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
15 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*
20 *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
25 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*
30 *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
 5 *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
 10 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
 15 alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the variant would be operably linked with the
 20 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
 25 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. In creating the
 30 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 recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with

the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The 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.

5 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*.

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

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

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

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

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

30 **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 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 depend upon the gene encoding the variant and its source.

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

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

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

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

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

 The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation
25 (see, *e.g.*, Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, *e.g.*, Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, *e.g.*,
30 Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (see, *e.g.*, Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, *e.g.*, Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, *e.g.*, Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by

electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397), or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

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

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

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

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

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

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*,
 5 *Ceriporiopsis subvermispore*, *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*,
 10 *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium 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*,
 15 *Trichoderma harzianum*, *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 in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic
 20 Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a variant, comprising: (a)
 30 cultivating a host cell of the present invention under conditions suitable for expression of the variant; 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 place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the variant is secreted into the nutrient medium, the variant can be recovered directly from the medium. If the variant is not secreted, it can be recovered from cell lysates.

The variant may be detected using methods known in the art that are specific for the variants. 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.

Compositions

The present invention also relates to compositions comprising an alpha-amylase variant and at least one additional enzyme. The additional enzyme(s) may be selected from the group consisting of beta-amylase, cellulase (beta-glucosidase, cellobiohydrolase and endoglucanase), glucoamylase, hemicellulase (e.g., xylanase), isoamylase, isomerase, lipase, phytase, protease, pullulanase, and/or other enzymes useful in a commercial process in conjunction with an alpha-amylase. The additional enzyme may also be a second alpha-amylase. Such enzymes are known in the art in starch processing, sugar conversion, fermentations for alcohol and other useful end-products, commercial detergents and cleaning aids, stain removal, fabric treatment or desizing, and the like.

Uses

The variants of the present invention possess valuable properties allowing for a variety of industrial applications. In particular, the variants may be used in detergents, in particular laundry

detergent compositions and dishwashing detergent compositions, hard surface cleaning compositions, and for desizing textiles, fabrics or garments, production of pulp and paper, beer making, ethanol production, and starch conversion processes.

5 The alpha-amylase variants may be used for starch processes, in particular starch conversion, especially liquefaction of starch (see, e.g., U.S. Patent No. 3,912,590, EP 252730 and EP 063909, WO 99/19467, and WO 96/28567). Also contemplated are compositions for starch conversion purposes, which may beside the variant of the invention also comprise an AMG, pullulanase, and other alpha-amylases.

10 Further, the variants are particularly useful in the production of sweeteners and ethanol (see, e.g., U.S. Patent No. 5,231,017), such as fuel, drinking and industrial ethanol, from starch or whole grains.

The variants may also be used for desizing of textiles, fabrics, and garments (see, e.g., WO 95/21247, U.S. Patent No. 4,643,736, and EP 119920), beer making or brewing, and in pulp and paper production or related processes.

15

Starch Processing

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

Conventional starch-conversion processes, such as liquefaction and saccharification processes are described, e.g., in U.S. Patent No. 3,912,590, EP 252730 and EP 063909.

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

In the case of converting starch into a sugar, the starch is depolymerized. Such a depolymerization process consists of, e.g., a pre-treatment step and two or three consecutive process steps, i.e., a liquefaction process, a saccharification process, and depending on the desired end-product, an optional isomerization process.

30

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

Production of Fermentation Products

In general, alcohol production (ethanol) from whole grain can be separated into 4 main steps: milling, liquefaction, saccharification, and fermentation.

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

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

15 During a typical enzymatic liquefaction, the long-chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. Enzymatic liquefaction is generally carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C (e.g., 77-86°C, 80-85°C, or 83-85°C) and the enzyme(s) is (are) added. The liquefaction process is carried out at 85°C for 1-2 hours. The pH is generally between 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, 1 mM of calcium is added (to provide about 40 ppm free calcium ions). After such treatment, the liquefied starch will have a "dextrose equivalent" (DE) of 20 10-15.

25 The slurry is subsequently jet-cooked at between 95-140°C, e.g., 105-125°C, cooled to 60-95°C and more enzyme(s) is (are) added to obtain the final hydrolysis. The liquefaction process is carried out at pH 4.5-6.5, typically at a pH between 5 and 6. Milled and liquefied grain is also known as mash.

Liquefied starch-containing material is saccharified in the presence of saccharifying enzymes such as glucoamylases. The saccharification process may last for 12 hours to 120 hours (e.g., 12 to 90 hours, 12 to 60 hours and 12 to 48 hours).

30 However, it is common to perform a pre-saccharification step for about 30 minutes to 2 hours (e.g., 30 to 90 minutes) at a temperature of 30 to 65°C, typically around 60°C which is followed by a complete saccharification during fermentation referred to as simultaneous saccharification and fermentation (SSF). The pH is usually between 4.2-4.8, e.g., pH 4.5. In a simultaneous saccharification and fermentation (SSF) process, there is no holding stage for saccharification, rather, the yeast and enzymes are added together.

In a typical saccharification process, maltodextrins produced during liquefaction are converted into dextrose by adding a glucoamylase and a debranching enzyme, such as an isoamylase (U.S. Patent No. 4,335,208) or a pullulanase. The temperature is lowered to 60°C, prior to the addition of the glucoamylase and debranching enzyme. The saccharification process
5 proceeds for 24-72 hours.

Prior to addition of the saccharifying enzymes, the pH is reduced to below 4.5, while maintaining a high temperature (above 95°C), to inactivate the liquefying alpha-amylase. This process reduces the formation of short oligosaccharide called "panose precursors," which cannot be hydrolyzed properly by the debranching enzyme. Normally, about 0.2-0.5% of the
10 saccharification product is the branched trisaccharide panose (Glc α 1-6Glc α 1-4Glc), which cannot be degraded by a pullulanase. If active amylase from the liquefaction remains present during saccharification (*i.e.*, no denaturing), the amount of panose can be as high as 1-2%, which is highly undesirable since it lowers the saccharification yield significantly.

Fermentable sugars (*e.g.*, dextrins, monosaccharides, particularly glucose) are produced
15 from enzymatic saccharification. These fermentable sugars may be further purified and/or converted to useful sugar products. In addition, the sugars may be used as a fermentation feedstock in a microbial fermentation process for producing end-products, such as alcohol (*e.g.*, ethanol and butanol), organic acids (*e.g.*, succinic acid and lactic acid), sugar alcohols (*e.g.*, glycerol), ascorbic acid intermediates (*e.g.*, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate,
20 and 2-keto-L-gulonic acid), amino acids (*e.g.*, lysine), proteins (*e.g.*, antibodies and fragment thereof).

In an embodiment, the fermentable sugars obtained during the liquefaction process steps are used to produce alcohol and particularly ethanol. In ethanol production, an SSF process is commonly used wherein the saccharifying enzymes and fermenting organisms (*e.g.*, yeast) are
25 added together and then carried out at a temperature of 30-40°C.

The organism used in fermentation will depend on the desired end-product. Typically, if ethanol is the desired end product yeast will be used as the fermenting organism. In some preferred embodiments, the ethanol-producing microorganism is a yeast and specifically *Saccharomyces* such as strains of *S. cerevisiae* (U.S. Patent No. 4,316,956). A variety of *S.*
30 *cerevisiae* are commercially available and these include but are not limited to FALI (Fleischmann's Yeast), SUPERSTART™ (Alltech), FERMIOL™ (DSM Specialties), RED STAR (Lesaffre) and Angel alcohol yeast (Angel Yeast Company, China). The amount of starter yeast employed in the methods is an amount effective to produce a commercially significant amount of ethanol in a suitable amount of time, (*e.g.*, to produce at least 10% ethanol from a substrate having between 25-

40% DS in less than 72 hours). Yeast cells are generally supplied in amounts of about 10^4 to about 10^{12} , and preferably from about 10^7 to about 10^{10} viable yeast count per mL of fermentation broth. After yeast is added to the mash, it is typically subjected to fermentation for about 24-96 hours, e.g., 35-60 hours. The temperature is between about 26-34°C, typically at about 32°C, and the pH is from pH 3-6, e.g., around pH 4-5.

The fermentation may include, in addition to a fermenting microorganisms (e.g., yeast), nutrients, and additional enzymes, including phytases. The use of yeast in fermentation is well known in the art.

In further embodiments, use of appropriate fermenting microorganisms, as is known in the art, can result in fermentation end product including, e.g., glycerol, 1,3-propanediol, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, 2-keto-L-gulonic acid, succinic acid, lactic acid, amino acids, and derivatives thereof. More specifically when lactic acid is the desired end product, a *Lactobacillus* sp. (*L. casei*) may be used; when glycerol or 1,3-propanediol are the desired end-products *E. coli* may be used; and when 2-keto-D-gluconate, 2,5-diketo-D-gluconate, and 2-keto-L-gulonic acid are the desired end products, *Pantoea citrea* may be used as the fermenting microorganism. The above enumerated list is only examples and one skilled in the art will be aware of a number of fermenting microorganisms that may be used to obtain a desired end product.

Processes for producing fermentation products from ungelatinized starch-containing material

The invention relates to processes for producing fermentation products from starch-containing material without gelatinization (*i.e.*, without cooking) of the starch-containing material. The fermentation product, such as ethanol, can be produced without liquefying the aqueous slurry containing the starch-containing material and water. In one embodiment a process of the invention includes saccharifying (e.g., milled) starch-containing material, e.g., granular starch, below the initial gelatinization temperature, preferably in the presence of alpha-amylase and/or carbohydrate-source generating enzyme(s) to produce sugars that can be fermented into the fermentation product by a suitable fermenting organism. In this embodiment the desired fermentation product, e.g., ethanol, is produced from ungelatinized (*i.e.*, uncooked), preferably milled, cereal grains, such as corn. Accordingly, in the first aspect the invention relates to processes for producing fermentation products from starch-containing material comprising simultaneously saccharifying and fermenting starch-containing material using a carbohydrate-source generating enzyme and a fermenting organism at a temperature below the initial gelatinization temperature of said starch-containing material. In an embodiment a protease is also present. The protease may be any acid fungal protease or metalloprotease. The fermentation product, e.g., ethanol, may optionally be

recovered after fermentation, e.g., by distillation. Typically amylase(s), such as glucoamylase(s) and/or other carbohydrate-source generating enzymes, and/or alpha-amylase(s), is(are) present during fermentation. Examples of glucoamylases and other carbohydrate-source generating enzymes include raw starch hydrolyzing glucoamylases. Examples of alpha-amylase(s) include acid alpha-amylases such as acid fungal alpha-amylases. Examples of fermenting organisms include yeast e.g., a strain of *Saccharomyces cerevisiae*. The term "initial gelatinization temperature" means the lowest temperature at which starch gelatinization commences. In general, starch heated in water begins to gelatinize between about 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch and can readily be determined by the skilled artisan.

Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch-containing material may be determined as the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein and Lii, 1992, *Starch/Stärke* 44(12): 461-466. Before initiating the process a slurry of starch-containing material, such as granular starch, having 10-55 w/w % dry solids (DS), preferably 25-45 w/w % dry solids, more preferably 30-40 w/w % dry solids of starch-containing material may be prepared. The slurry may include water and/or process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side-stripper water from distillation, or process water from other fermentation product plants. Because the process of the invention is carried out below the initial gelatinization temperature, and thus no significant viscosity increase takes place, high levels of stillage may be used if desired. In an embodiment the aqueous slurry contains from about 1 to about 70 vol. %, preferably 15-60 vol. %, especially from about 30 to 50 vol. % water and/or process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side-stripper water from distillation, or process water from other fermentation product plants, or combinations thereof, or the like. The starch-containing material may be prepared by reducing the particle size, preferably by dry or wet milling, to 0.05 to 3.0 mm, preferably 0.1-0.5 mm. After being subjected to a process of the invention at least 85%, at least 86%, at least 87%, at least 88%, 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%, or preferably at least 99% of the dry solids in the starch-containing material are converted into a soluble starch hydrolyzate. A process in this aspect of the invention is conducted at a temperature below the initial gelatinization temperature, which means that the temperature typically lies in the range between 30-75°C, preferably between 45-60°C. In a preferred embodiment the process carried at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around 32°C. In an embodiment the

process is carried out so that the sugar level, such as glucose level, is kept at a low level, such as below 6 w/w %, such as below about 3 w/w %, such as below about 2 w/w %, such as below about 1 w/w %, such as below about 0.5 w/w %, or below 0.25 w/w %, such as below about 0.1 w/w %. Such low levels of sugar can be accomplished by simply employing adjusted quantities of enzyme and fermenting organism. A skilled person in the art can easily determine which doses/quantities of enzyme and fermenting organism to use. The employed quantities of enzyme and fermenting organism may also be selected to maintain low concentrations of maltose in the fermentation broth. For instance, the maltose level may be kept below about 0.5 w/w %, such as below about 0.2 w/w %. The process of the invention may be carried out at a pH from about 3 and 7, preferably from pH 3.5 to 6, or more preferably from pH 4 to 5. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

Processes for producing fermentation products from gelatinized starch-containing material

In this aspect the invention relates to processes for producing fermentation products, especially ethanol, from starch-containing material, which process includes a liquefaction step and sequentially or simultaneously performed saccharification and fermentation steps. Consequently, the invention relates to processes for producing fermentation products from starch-containing material comprising the steps of:

- (a) liquefying starch-containing material in the presence of an alpha-amylase variant, or;
- (b) saccharifying the liquefied material obtained in step (a) using a carbohydrate-source generating enzyme;
- (c) fermenting using a fermenting organism.

In an aspect, a pullulanase such as a family GH57 pullulanase is also used in the liquefaction step. In an embodiment a protease, such as an acid fungal protease or a metalloprotease is added before, during and/or after liquefaction. In an embodiment the metalloprotease is derived from a strain of *Thermoascus*, e.g., a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670. In an embodiment the carbohydrate-source generating enzyme is a glucoamylase derived from a strain of *Aspergillus*, e.g., *Aspergillus niger* or *Aspergillus awamori*, a strain of *Talaromyces*, especially *Talaromyces emersonii*; or a strain of *Athelia*, especially *Athelia rolfsii*; a strain of *Trametes*, e.g., *Trametes cingulata*; a strain of the genus *Pachykytospora*, e.g., a strain of *Pachykytospora papyracea*; or a strain of the genus *Leucopaxillus*, e.g., *Leucopaxillus giganteus*; or a strain of the genus *Peniophora*, e.g., a strain of the species *Peniophora rufomarginata*; or a mixture thereof. Saccharification step (b) and fermentation step (c) may be carried out either sequentially or simultaneously. A pullulanase and/or

metalloprotease may be added during saccharification and/or fermentation when the process is carried out as a sequential saccharification and fermentation process and before or during fermentation when steps (b) and (c) are carried out simultaneously (SSF process). The pullulanase and/or metalloprotease may also advantageously be added before liquefaction (pre-liquefaction treatment), *i.e.*, before or during step (a), and/or after liquefaction (post liquefaction treatment), *i.e.*, after step (a). The pullulanase is most advantageously added before or during liquefaction, *i.e.*, before or during step (a). The fermentation product, such as especially ethanol, may optionally be recovered after fermentation, *e.g.*, by distillation. The fermenting organism is preferably yeast, preferably a strain of *Saccharomyces cerevisiae*. In a particular embodiment, the process of the invention further comprises, prior to step (a), the steps of:

x) reducing the particle size of the starch-containing material, preferably by milling (*e.g.*, using a hammer mill);

y) forming a slurry comprising the starch-containing material and water.

In an embodiment the particle size is smaller than a # 7 screen, *e.g.*, a # 6 screen. A # 7 screen is usually used in conventional prior art processes. The aqueous slurry may contain from 10-55, *e.g.*, 25-45 and 30-40, w/w % dry solids (DS) of starch-containing material. The slurry is heated to above the gelatinization temperature and an alpha-amylase variant may be added to initiate liquefaction (thinning). The slurry may in an embodiment be jet-cooked to further gelatinize the slurry before being subjected to alpha-amylase in step (a). Liquefaction may in an embodiment be carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C, preferably between 70-90°C, such as preferably between 80-85°C at pH 4-6, preferably 4.5-5.5, and alpha-amylase variant, optionally together with a pullulanase and/or protease, preferably metalloprotease, are added to initiate liquefaction (thinning). In an embodiment the slurry may then be jet-cooked at a temperature between 95-140°C, preferably 100-135°C, such as 105-125°C, for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes. The slurry is cooled to 60-95°C and more alpha-amylase variant and optionally pullulanase variant and/or protease, preferably metalloprotease, is(are) added to finalize hydrolysis (secondary liquefaction). The liquefaction process is usually carried out at pH 4.0-6, in particular at a pH from 4.5 to 5.5. Saccharification step (b) may be carried out using conditions well known in the art. For instance, a full saccharification process may last up to from about 24 to about 72 hours, however, it is common only to do a pre-saccharification of typically 40-90 minutes at a temperature between 30-65°C, typically about 60°C, followed by complete saccharification during fermentation in a simultaneous saccharification and fermentation process (SSF process). Saccharification is typically carried out at temperatures from 20-75°C, preferably from 40-70°C, typically around 60°C, and at a

pH between 4 and 5, normally at about pH 4.5. The most widely used process to produce a fermentation product, especially ethanol, is a simultaneous saccharification and fermentation (SSF) process, in which there is no holding stage for the saccharification, meaning that a fermenting organism, such as yeast, and enzyme(s), may be added together. SSF may typically be carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

Beer Making

10 The alpha-amylase variants may also be used in a beer-making process and similar fermentations; the alpha-amylases will typically be added during the mashing process. The process is substantially similar to the milling, liquefaction, saccharification, and fermentation processes described above.

15 Starch Slurry Processing with Stillage

Milled starch-containing material is combined with water and recycled thin-stillage resulting in an aqueous slurry. The slurry can comprise between 15 to 55% ds w/w (e.g., 20 to 50%, 25 to 50%, 25 to 45%, 25 to 40%, 20 to 35% and 30-36% ds). In some embodiments, the recycled thin-stillage (backset) is in the range of about 10 to 70% v/v (e.g., 10 to 60%, 10 to 50%, 10 to 40%, 10 to 30%, 10 to 20%, 20 to 60%, 20 to 50%, 20 to 40% and also 20 to 30%).

Once the milled starch-containing material is combined with water and backset, the pH is not adjusted in the slurry. Further the pH is not adjusted after the addition of a phytase and optionally an alpha-amylase to the slurry. In an embodiment, the pH of the slurry will be in the range of about pH 4.5 to less than about 6.0 (e.g., pH 4.5 to 5.8, pH 4.5 to 5.6, pH 4.8 to 5.8, pH 25 5.0 to 5.8, pH 5.0 to 5.4, pH 5.2 to 5.5 and pH 5.2 to 5.9). The pH of the slurry may be between about pH 4.5 and 5.2 depending on the amount of thin stillage added to the slurry and the type of material comprising the thin stillage. For example, the pH of the thin stillage may be between pH 3.8 and pH 4.5.

During ethanol production, acids can be added to lower the pH in the beer well, to reduce 30 the risk of microbial contamination prior to distillation.

In some embodiments, a phytase is added to the slurry. In other embodiments, in addition to phytase, an alpha-amylase is added to the slurry. In some embodiments, a phytase and alpha-amylase are added to the slurry sequentially. In other embodiments, a phytase and alpha-amylase are added simultaneously. In some embodiments, the slurry comprising a phytase and optionally,

an alpha-amylase, are incubated (pretreated) for a period of about 5 minutes to about 8 hours (e.g., 5 minutes to 6 hours, 5 minutes to 4 hours, 5 minutes to 2 hours, and 15 minutes to 4 hours). In other embodiments, the slurry is incubated at a temperature in the range of about 40 to 115°C (e.g., 45 to 80°C, 50 to 70°C, 50 to 75°C, 60 to 110°C, 60 to 95°C, 70 to 110°C, 70 to 85°C and 77 to 86°C).

In other embodiments, the slurry is incubated at a temperature of about 0 to about 30°C (e.g., 0 to 25°C, 0 to 20°C, 0 to 15°C, 0 to 10°C and 0 to 5°C) below the starch gelatinization temperature of the starch-containing material. In some embodiments, the temperature is below about 68°C, below about 65°C, below about 62°C, below about 60°C and below about 55°C. In some embodiments, the temperature is above about 45°C, above about 50°C, above about 55°C and above about 60°C. In some embodiments, the incubation of the slurry comprising a phytase and an alpha-amylase at a temperature below the starch gelatinization temperature is referred to as a primary (1°) liquefaction.

In one embodiment, the milled starch-containing material is corn or milo. The slurry comprises 25 to 40% DS, the pH is in the range of 4.8 to 5.2, and the slurry is incubated with a phytase and optionally an alpha-amylase for 5 minutes to 2 hours, at a temperature range of 60 to 75°C.

Currently, it is believed that commercially available microbial alpha-amylases used in the liquefaction process are generally not stable enough to produce liquefied starch substrate from a dry mill process using whole ground grain at a temperature above about 80°C at a pH level that is less than pH 5.6. The stability of many commercially available alpha-amylases is reduced at low pH.

In a further liquefaction step, the incubated or pretreated starch-containing material is exposed to an increase in temperature such as about 0 to about 45°C above the starch gelatinization temperature of the starch-containing material (e.g., 70°C to 120°C, 70°C to 110°C, and 70°C to 90°C) for a period of time of about 2 minutes to about 6 hours (e.g., 2 minutes to 4 hrs, 90 minutes, 140 minutes and 90 to 140 minutes) at a pH of about 4.0 to 5.5 more preferably between 1 hour to 2 hours. The temperature can be increased by a conventional high temperature jet cooking system for a short period of time, for example, for 1 to 15 minutes. Then the starch maybe further hydrolyzed at a temperature ranging from about 75°C to 95°C (e.g., 80°C to 90°C and 80°C to 85°C) for a period of about 15 to 150 minutes (e.g., 30 to 120 minutes). In a preferred embodiment, the pH is not adjusted during these process steps and the pH of the liquefied mash is in the range of about pH 4.0 to pH 5.8 (e.g., pH 4.5 to 5.8, pH 4.8 to 5.4, and pH 5.0 to 5.2). In

some embodiments, a second dose of thermostable alpha-amylase is added to the secondary liquefaction step, but in other embodiments there is no additional dosage of alpha-amylase.

The incubation and liquefaction steps may be followed by saccharification and fermentation steps well known in the art.

5

Distillation

Optionally, following fermentation, an alcohol (e.g., ethanol) may be extracted by, for example, distillation and optionally followed by one or more process steps.

In some embodiments, the yield of ethanol produced by the methods provided herein is at least 8%, at least 10%, at least 12%, at least 14%, at least 15%, at least 16%, at least 17% and at least 18% (v/v) and at least 23% v/v. The ethanol obtained according to the process provided herein may be used as, for example, fuel ethanol, drinking ethanol, *i.e.*, potable neutral spirits, or industrial ethanol.

15 By-Products

Left over from the fermentation is the grain, which is typically used for animal feed either in liquid or dried form. In further embodiments, the end product may include the fermentation co-products such as distiller's dried grains (DDG) and distiller's dried grain plus solubles (DDGS), which may be used, for example, as an animal feed.

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

According to the process provided herein, the saccharification and fermentation may be carried out simultaneously or separately.

25 Pulp and Paper Production

The alpha-amylase variants may also be used in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste paper and cardboard, especially where re-pulping occurs at pH above 7 and where amylases facilitate the disintegration of the waste material through degradation of the reinforcing starch. The alpha-amylase variants are especially useful in a process for producing a papermaking pulp from starch-coated printed-paper. 30 The process may be performed as described in WO 95/14807, comprising the following steps:

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

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

5

Desizing of Textiles, Fabrics and Garments

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

Desizing and bleaching processes are well known in the art. For instance, such processes are described in e.g., WO 95/21247, U.S. Patent No. 4,643,736, EP 119920.

Cleaning Processes and Detergent Compositions

The alpha-amylase variants may be added as a component of a detergent composition for various cleaning or washing processes, including laundry and dishwashing. For example, the variants may be used in the detergent compositions described in WO 96/23874 and WO 97/07202.

The alpha-amylase variants may be incorporated in detergents at conventionally employed concentrations. For example, a variant of the invention may be incorporated in an amount corresponding to 0.00001-10 mg (calculated as pure, active enzyme protein) of alpha-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

The detergent composition may for example be formulated as a hand or machine laundry detergent composition, including a laundry additive composition suitable for pretreatment of stained

fabrics and a rinse added fabric softener composition or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

5 The detergent composition may further comprise one or more other enzymes, such as a lipase, peroxidase, protease, another amylolytic enzyme, *e.g.*, another alpha-amylase, glucoamylase, maltogenic amylase, CGTase, cellulase, mannanase (such as Mannaway™ from Novozymes, Denmark)), pectinase, pectin lyase, cutinase, and/or laccase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent (*i.e.*, pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, 10 etc.), and the enzyme(s) should be present in effective amounts.

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, *e.g.*, a separate additive or a combined additive, can be formulated, *e.g.*, granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are 15 granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, *e.g.*, as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; 20 ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols, fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric 25 acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238216.

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

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

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty

alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

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

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

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

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

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

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

The detergent compositions may comprise any enzyme in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably 0.055 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

One or more of the variant enzymes described herein may additionally be incorporated in the detergent formulations disclosed in WO 97/07202.

The invention is further defined by the following embodiments:

Embodiment 1. An alpha-amylase variant comprising a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and further comprising a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1, wherein the variant has at least 60% and less than 100% sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14, and wherein the variant has alpha-amylase activity.

15

Embodiment 2. The variant of Embodiment 1, which comprises:

a substitution at a position corresponding to position 15 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Leu, Ser or Thr;

20 a substitution at a position corresponding to position 48 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ala;

25 a substitution at a position corresponding to position 49 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val, in particular with Gly, His, Ile or Leu;

a substitution at a position corresponding to position 50 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Thr;

30 a substitution at a position corresponding to position 107 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ala;

a substitution at a position corresponding to position 116 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val, in particular with Gly;

a substitution at a position corresponding to position 133 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr;

a substitution at a position corresponding to position 138 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr or Val, in particular with Phe or Tyr;

5 a substitution at a position corresponding to position 156 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr;

a substitution at a position corresponding to position 176 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Leu;

10 a substitution at a position corresponding to position 181 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Asp, Glu or Thr;

a substitution at a position corresponding to position 187 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Asp;

15 a substitution at a position corresponding to position 188 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser or Thr;

a substitution at a position corresponding to position 190 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Phe;

20 a substitution at a position corresponding to position 197 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ile, Leu, Ser, Thr or Val;

a substitution at a position corresponding to position 201 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Phe or Tyr;

a substitution at a position corresponding to position 205 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Tyr;

25 a substitution at a position corresponding to position 209 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Val;

a substitution at a position corresponding to position 213 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Thr;

30 a substitution at a position corresponding to position 239 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val, in particular with Ala, Asn, Asp, Cys, Gln, Glu or Met;

a substitution at a position corresponding to position 241 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Asp;

a substitution at a position corresponding to position 255 with Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Gly or Pro;

5 a substitution at a position corresponding to position 264 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser;

a substitution at a position corresponding to position 299 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg;

10 a substitution at a position corresponding to position 360 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Ser;

15 a substitution at a position corresponding to position 375 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Gly or Val;

a substitution at a position corresponding to position 416 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Val;

20 a substitution at a position corresponding to position 437 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Trp;

a substitution at a position corresponding to position 474 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg, Gln, Glu or Lys; or

25 a substitution at a position corresponding to position 475 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val, in particular with Arg, Gln, Glu or Lys.

Embodiment 3. The variant of any of Embodiments 1-2, wherein the variant has at least 65%,
30 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% or at least 99%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID

NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

Embodiment 4. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 1, or (ii) amino acids 1-483 of SEQ ID NO: 1.

Embodiment 5. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 2, (ii) the mature polypeptide of SEQ ID NO: 2 comprising the deletions I181*+G182*, (iii) amino acids 1-483 of SEQ ID NO: 2, or (iv) amino acids 1-483 of SEQ ID NO: 2 comprising the deletions I181*+G182*.

Embodiment 6. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 3, (ii) the mature polypeptide of SEQ ID NO: 3 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 3, or (iv) amino acids 1-485 of SEQ ID NO: 3 comprising the deletions T183*+G184*.

Embodiment 7. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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% sequence identity, but less than 100%, to (i) the mature polypeptide of SEQ ID NO: 4, (ii) the mature polypeptide of SEQ ID NO: 4 comprising the deletions T180*+G181*, (iii) amino acids 1-482 of SEQ ID NO: 4, or (iv) amino acids 1-482 of SEQ ID NO: 4 comprising the deletions T180*+G181*.

Embodiment 8. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 5, (ii) the mature polypeptide of SEQ ID NO: 5 comprising the deletions T182*+G183*, (iii) amino acids 1-484 of SEQ ID NO: 5, or (iv) amino acids 1-484 of SEQ ID NO: 5 comprising the deletions T182*+G183*.

Embodiment 9. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 6, (ii) the mature polypeptide of SEQ ID NO: 6 comprising the deletions E178*+G179*, (iii) amino acids 1-483 of SEQ ID NO: 6, or (iv) amino acids 1-483 of SEQ ID NO: 6 comprising the deletions E178*+G180*.

Embodiment 10. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 7, (ii) the mature polypeptide of SEQ ID NO: 7 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 7, or (iv) amino acids 1-485 of SEQ ID NO: 7 comprising the deletions T183*+G184*.

Embodiment 11. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 8, (ii) the mature polypeptide of SEQ ID NO: 8 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 8, or (iv) amino acids 1-485 of SEQ ID NO: 8 comprising the deletions D183*+G184*.

Embodiment 12. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 9, (ii) the mature polypeptide of SEQ ID NO: 9 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 9, or (iv) amino acids 1-485 of SEQ ID NO: 9 comprising the deletions D183*+G184*.

Embodiment 13. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 10, (ii) the mature polypeptide of SEQ ID NO: 10 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 10, or (iv) amino acids 1-485 of SEQ ID NO: 10 comprising the deletions D183*+G184*.

Embodiment 14. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 11, (ii) the mature polypeptide of SEQ ID NO: 11 comprising the deletions H183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 11, or (iv) amino acids 1-485 of SEQ ID NO: 11 comprising the deletions H183*+G184*.

Embodiment 15. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 12, or (ii) amino acids 1-480 of SEQ ID NO: 12.

Embodiment 16. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 13, or (ii) amino acids 1-483 of SEQ ID NO: 13.

5 Embodiment 17. The variant of any of Embodiments 1-2, wherein the variant has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 14, or (ii) amino acids 1-481 of SEQ ID NO: 14.

10 Embodiment 18. The variant of any of Embodiments 1-17, which is a variant of a parent alpha-amylase selected from the group consisting of:

- (a) a polypeptide having at least 60% sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14; or
- 15 20 (b) a fragment of the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, which has alpha-amylase activity.

Embodiment 19. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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 (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

25 30

Embodiment 20. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 1, or (ii) amino acids 1-483 of SEQ ID NO: 1.

5

Embodiment 21. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 2, (ii) the mature polypeptide of SEQ ID NO: 2 comprising the deletions I181*+G182*, (iii) amino acids 1-483 of SEQ ID NO: 2, or (iv) amino acids 1-483 of SEQ ID NO: 2 comprising the deletions I181*+G182*.

10

Embodiment 22. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 3, (ii) the mature polypeptide of SEQ ID NO: 3 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 3, or (iv) amino acids 1-485 of SEQ ID NO: 3 comprising the deletions T183*+G184*.

15

Embodiment 23. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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 (i) the mature polypeptide of SEQ ID NO: 4, (ii) the mature polypeptide of SEQ ID NO: 4 comprising the deletions T180*+G181*, (iii) amino acids 1-482 of SEQ ID NO: 4, or (iv) amino acids 1-482 of SEQ ID NO: 4 comprising the deletions T180*+G181*.

20

25

Embodiment 24. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, *e.g.*, at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 5, (ii) the mature polypeptide of SEQ ID NO: 5 comprising the deletions T182*+G183*, (iii) amino acids 1-484 of SEQ ID NO: 5, or (iv) amino acids 1-484 of SEQ ID NO: 5 comprising the deletions T182*+G183*.

30

Embodiment 25. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 6, (ii) the mature polypeptide of SEQ ID NO: 6 comprising the deletions E178*+G179*, (iii) amino acids 1-483 of SEQ ID NO: 6, or (iv) amino acids 1-483 of SEQ ID NO: 6 comprising the deletions E178*+G179*.

Embodiment 26. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 7, (ii) the mature polypeptide of SEQ ID NO: 7 comprising the deletions T183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 7, or (iv) amino acids 1-485 of SEQ ID NO: 7 comprising the deletions T183*+G184*.

Embodiment 27. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 8, (ii) the mature polypeptide of SEQ ID NO: 8 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 8, or (iv) amino acids 1-485 of SEQ ID NO: 8 comprising the deletions D183*+G184*.

Embodiment 28. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 9, (ii) the mature polypeptide of SEQ ID NO: 9 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 9, or (iv) amino acids 1-485 of SEQ ID NO: 9 comprising the deletions D183*+G184*.

Embodiment 29. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 10, (ii) the mature polypeptide of SEQ ID NO: 10 comprising the deletions D183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 10, or (iv) amino acids 1-485 of SEQ ID NO: 10 comprising the deletions D183*+G184*.

Embodiment 30. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 11, (ii) the mature polypeptide of SEQ ID NO: 11 comprising the deletions H183*+G184*, (iii) amino acids 1-485 of SEQ ID NO: 11, or (iv) amino acids 1-485 of SEQ ID NO: 11 comprising the deletions H183*+G184*.

Embodiment 31. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 12, or (ii) amino acids 1-480 of SEQ ID NO: 12.

Embodiment 32. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 13, or (ii) amino acids 1-483 of SEQ ID NO: 13.

Embodiment 33. The variant of Embodiment 18, wherein the parent alpha-amylase has at least 60%, e.g., at least 65%, 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% sequence identity to (i) the mature polypeptide of SEQ ID NO: 14, or (ii) amino acids 1-481 of SEQ ID NO: 14.

Embodiment 34. The variant of Embodiment 18, wherein the parent alpha-amylase comprises or consists of (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2,

amino acids 1 to 485 of SEQ ID NO: 3, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

Embodiment 35. The variant of Embodiment 18, wherein the parent alpha-amylase is a fragment of the mature polypeptide of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, wherein the fragment has alpha-amylase activity.

10

Embodiment 36. The variant of any of Embodiments 1-35, wherein the variant consists of 300 to 700, e.g., 350 to 650, 400 to 600, 450 to 500 or 470 to 490, amino acids.

Embodiment 37. The variant of any of Embodiments 1-36, which is an isolated alpha-amylase variant.

15

Embodiment 38. The variant of any of Embodiments 1 to 37, which comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, K176L, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

20

Embodiment 39. The variant of any of Embodiments 1 to 38, which comprises a substitution at a position corresponding to position 176.

25

Embodiment 40. The variant of any of Embodiments 1 to 39, which comprises the substitution K176L.

30

Embodiment 41. The variant of any of Embodiments 39-40, which further comprises a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264, 299, 360, 375, 416, 437, 474 and 475.

35

Embodiment 42. The variant of Embodiment 40, which further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V,
 5 K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

Embodiment 43. The variant of any of Embodiments 1 to 42, which comprises a substitution at
 10 a position corresponding to position 15.

Embodiment 44. The variant of any of Embodiments 1 to 42, which comprises the substitution M15T.

15 Embodiment 45. The variant of any of Embodiments 1 to 42, which comprises the substitution M15S.

Embodiment 46. The variant of any of Embodiments 1 to 42, which comprises the substitution M15L.

20 Embodiment 47. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 48.

Embodiment 48. The variant of any of Embodiments 1 to 42, which comprises the substitution
 25 G48A.

Embodiment 49. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 49.

30 Embodiment 50. The variant of any of Embodiments 1 to 42, which comprises the substitution T49H.

Embodiment 51. The variant of any of Embodiments 1 to 42, which comprises the substitution
 35 T49G.

- Embodiment 52. The variant of any of Embodiments 1 to 42, which comprises the substitution T49L.
- 5 Embodiment 53. The variant of any of Embodiments 1 to 42, which comprises the substitution T49I.
- Embodiment 54. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 50.
- 10 Embodiment 55. The variant of any of Embodiments 1 to 42, which comprises the substitution S50T.
- Embodiment 56. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 107.
- 15 Embodiment 57. The variant of any of Embodiments 1 to 42, which comprises the substitution G107A.
- Embodiment 58. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position T116.
- 20 Embodiment 59. The variant of any of Embodiments 1 to 42, which comprises the substitution T116G.
- Embodiment 60. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 133.
- 25 Embodiment 61. The variant of any of Embodiments 1 to 42, which comprises the substitution H133Y.
- 30 Embodiment 62. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 138.
- Embodiment 63. The variant of any of Embodiments 1 to 42, which comprises the substitution W138Y.
- 35

- Embodiment 64. The variant of any of Embodiments 1 to 42, which comprises the substitution W138F.
- Embodiment 65. The variant of any of Embodiments 1 to 42, which comprises a substitution at
5 a position corresponding to position 156.
- Embodiment 66. The variant of any of Embodiments 1 to 42, which comprises the substitution H156Y.
- 10 Embodiment 67. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 181.
- Embodiment 68. The variant of any of Embodiments 1 to 42, which comprises the substitution A181T.
- 15 Embodiment 69. The variant of any of Embodiments 1 to 42, which comprises the substitution A181E.
- Embodiment 70. The variant of any of Embodiments 1 to 42, which comprises the substitution A181D.
- 20 Embodiment 71. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 187.
- Embodiment 72. The variant of any of Embodiments 1 to 42, which comprises the substitution S187D.
- 25 Embodiment 73. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 188.
- 30 Embodiment 74. The variant of any of Embodiments 1 to 42, which comprises the substitution N188S.
- Embodiment 75. The variant of any of Embodiments 1 to 42, which comprises the substitution N188T.
- 35

- Embodiment 76. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 190.
- Embodiment 77. The variant of any of Embodiments 1 to 42, which comprises the substitution
5 N190F.
- Embodiment 78. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 197.
- Embodiment 79. The variant of any of Embodiments 1 to 42, which comprises the substitution
10 M197S.
- Embodiment 80. The variant of any of Embodiments 1 to 42, which comprises the substitution
M197T.
- Embodiment 81. The variant of any of Embodiments 1 to 42, which comprises the substitution
15 M197V.
- Embodiment 82. The variant of any of Embodiments 1 to 42, which comprises the substitution
20 M197L.
- Embodiment 83. The variant of any of Embodiments 1 to 42, which comprises the substitution
M197I.
- Embodiment 84. The variant of any of Embodiments 1 to 42, which comprises a substitution at
25 a position corresponding to position 201.
- Embodiment 85. The variant of any of Embodiments 1 to 42, which comprises the substitution
I201Y.
- Embodiment 86. The variant of any of Embodiments 1 to 42, which comprises the substitution
30 I201F.
- Embodiment 87. The variant of any of Embodiments 1 to 42, which comprises a substitution at
35 a position corresponding to position 205.

- Embodiment 88. The variant of any of Embodiments 1 to 42, which comprises the substitution H205Y.
- Embodiment 89. The variant of any of Embodiments 1 to 42, which comprises a substitution at
5 a position corresponding to position 209.
- Embodiment 90. The variant of any of Embodiments 1 to 42, which comprises the substitution A209V.
- 10 Embodiment 91. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 213.
- Embodiment 92. The variant of any of Embodiments 1 to 42, which comprises the substitution K213T.
- 15 Embodiment 93. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 239.
- Embodiment 94. The variant of any of Embodiments 1 to 42, which comprises the substitution S239Q.
- 20 Embodiment 95. The variant of any of Embodiments 1 to 42, which comprises the substitution S239E.
- 25 Embodiment 96. The variant of any of Embodiments 1 to 42, which comprises the substitution S239N.
- Embodiment 97. The variant of any of Embodiments 1 to 42, which comprises the substitution S239D.
- 30 Embodiment 98. The variant of any of Embodiments 1 to 42, which comprises the substitution S239A.
- Embodiment 99. The variant of any of Embodiments 1 to 42, which comprises the substitution S239M.
- 35

- Embodiment 100. The variant of any Embodiments 1 to 42, which comprises the substitution S239C.
- 5 Embodiment 101. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 241.
- Embodiment 102. The variant of any of Embodiments 1 to 42, which comprises the substitution L241D.
- 10 Embodiment 103. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 255.
- Embodiment 104. The variant of any of Embodiments 1 to 42, which comprises the substitution E255P.
- 15 Embodiment 105. The variant of any of Embodiments 1 to 42, which comprises the substitution E255G.
- Embodiment 106. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 264.
- 20 Embodiment 107. The variant of any of Embodiments 1 to 42, which comprises the substitution Q264S.
- Embodiment 108. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 299.
- 25 Embodiment 109. The variant of any of Embodiments 1 to 42, which comprises the substitution G299R.
- 30 Embodiment 110. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 360.
- Embodiment 111. The variant of any of Embodiments 1 to 42, which comprises the substitution Q360S.
- 35

Embodiment 112. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 375.

Embodiment 113. The variant of any of Embodiments 1 to 42, which comprises the substitution
5 R375V.

Embodiment 114. The variant of any of Embodiments 1 to 42, which comprises the substitution R375G.

10 Embodiment 115. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 416.

Embodiment 116. The variant of any of Embodiments 1 to 42, which comprises the substitution D416V.

15 Embodiment 117. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 437.

Embodiment 118. The variant of any of Embodiments 1 to 42, which comprises the substitution
20 R437W.

Embodiment 119. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 474.

25 Embodiment 120. The variant of any of Embodiments 1 to 42, which comprises the substitution G474K.

Embodiment 121. The variant of any of Embodiments 1 to 42, which comprises the substitution G474R.

30 Embodiment 122. The variant of any of Embodiments 1 to 42, which comprises the substitution G474E.

Embodiment 123. The variant of any of Embodiments 1 to 42, which comprises the substitution
35 G474Q.

Embodiment 124. The variant of any of Embodiments 1 to 42, which comprises a substitution at a position corresponding to position 475.

5 Embodiment 125. The variant of any of Embodiments 1 to 42, which comprises the substitution G475K.

Embodiment 126. The variant of any of Embodiments 1 to 42, which comprises the substitution G475R.

10 Embodiment 127. The variant of any of Embodiments 1 to 42, which comprises the substitution G475E.

Embodiment 128. The variant of any of Embodiments 1 to 42, which comprises the substitution G475Q.

15 Embodiment 129. The variant of any of Embodiments 1 to 42, which comprises a set of substitutions selected from the group consisting of:

T49H+K176L+E185P,
 T49G+K176L+E185P,
 20 T49L+S50T+K176L+E185P,
 T116G+K176L+E185P,
 K176L+E185P,
 K176L+E185P+I201Y+H205Y+K213T+Q360S+D416V+R437W,
 K176L+E185P+L241D,
 25 K176L+E185P+R375V, and
 K176L+E185P+R375G.

Embodiment 130. The variant of any of Embodiments 1 to 42, which comprises a set of substitutions selected from the group consisting of:

30 G48A+T49H+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
 G48A+T49G+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
 G48A+T49L+S50T+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
 G48A+T49I+G107A+T116G+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
 G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S;
 35 G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201Y+H205Y+A209V+K213T

+Q264S+Q360S+D416V+R437W;

G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+L241D+A209V+Q264S;

G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S+R375V;

G48A+T49I+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S+R375G; and

5 G48A+G107A+H156Y+K176L+A181T+E185P+N190F+I201F+A209V+Q264S.

Embodiment 131. The variant of any of Embodiments 1 to 42, which comprises a substitution at one or more positions corresponding to positions 49, 50, 116, 176, 201, 205, 213, 241, 360, 375, 416, and 437.

10

Embodiment 132. The variant of Embodiment 131, which comprises one or more substitutions selected among T49H, T49G, T49L, S50T, T116G, K176L, I201Y, H205Y, K213T, L241D, Q360S, R375V, R375G, D416V and R437W.

15 Embodiment 133. The variant of any of Embodiments 131 to 132, which further comprises a substitution at one or more positions corresponding to positions 15, 48, 107, 133, 138, 156, 181, 187, 188, 190, 197, 209, 239, 255, 264, 299, 474 and 475.

20 Embodiment 134. The variant of Embodiment 133, which comprises one or more substitutions selected among M15T, M15S, M15L, G48A, G107A, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, A209V, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, E255P, E255G, Q264S, G299R, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

25 Embodiment 135. The variant of any of Embodiments 1-134 which further comprises a deletion at both of the two positions immediately before position 180 of SEQ ID NO: 1.

Embodiment 136. The variant of any of Embodiments 1-135, wherein the total number of substitutions is 2-20, *e.g.*, 2-10 or 2-5, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 substitutions.

30

Embodiment 137. The variant of any of Embodiments 1-136, which has increased thermostability compared to the mature polypeptide of SEQ ID NO: 1 when incubated at high temperature, low calcium and low pH.

Embodiment 138. The variant of any of Embodiments 1-136, which has increased thermostability compared to the mature polypeptide of SEQ ID NO: 14 when incubated at high temperature, low calcium and low pH

- 5 Embodiment 139. The variant of any of Embodiments 1-136, which has increased thermostability compared to its parent when incubated at high temperature, low calcium and low pH.

- Embodiment 140. The variant of any of Embodiments 1-136, which has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 1 when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.5.
- 10

- Embodiment 141. The variant of any of Embodiments 1-136, which has an increased residual activity half-life compared to the mature polypeptide of SEQ ID NO: 14 when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.5.
- 15

Embodiment 142. The variant of any of Embodiments 1-136, which has an increased residual activity half-life compared to its parent when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.5.

- 20 Embodiment 143. A detergent composition comprising the variant of any of Embodiments 1-142 and a surfactant.

- Embodiment 144. A composition comprising the variant of any of Embodiments 1-142 and one or more enzymes selected from the group consisting of beta-amylase, cellulase (beta-glucosidase, cellobiohydrolase, and endoglucanase) glucoamylase, hemicellulase (e.g., xylanase), isoamylase, isomerase, lipase, phytase, protease and pullulanase.
- 25

- Embodiment 145. Use of the variant of any of Embodiments 1 to 142 for washing and/or dishwashing.
- 30

Embodiment 146. Use of the variant of any of Embodiments 1 to 142 for desizing a textile.

- Embodiment 147. Use of the variant of any of Embodiments 1 to 142 for producing a baked product.

- 35 Embodiment 148. Use of the variant of any of Embodiments 1 to 142 for liquefying a starch-containing material.

Embodiment 149. A method of producing liquefied starch, comprising liquefying a starch-containing material with the variant of any of Embodiments 1 to 142.

- 5 Embodiment 150. A method of producing a fermentation product, comprising
- (a) liquefying a starch-containing material with the variant of any of Embodiments 1 to 142 to produce a liquefied mash;
 - (b) saccharifying the liquefied mash to produce fermentable sugars; and
 - (c) fermenting the fermentable sugars in the presence of a fermenting organism.

- 10 Embodiment 151. The method of Embodiment 150, wherein step (a) is performed at pH 4-5.

- Embodiment 152. A method of producing a fermentation product, comprising contacting a starch substrate with the variant of any of Embodiments 1-142, a glucoamylase, and a fermenting
15 organism.

Embodiment 153. An isolated polynucleotide encoding the variant of any of Embodiments 1-142.

- 20 Embodiment 154. A nucleic acid construct comprising the polynucleotide of Embodiment 153.

Embodiment 155. An expression vector comprising the polynucleotide of Embodiment 153.

- Embodiment 156. A host cell comprising the polynucleotide of Embodiment 153.
25

Embodiment 157. A method of producing an alpha-amylase variant, comprising:

- a. cultivating the host cell of Embodiment 156 under conditions suitable for expression of the variant; and
- b. recovering the variant.

- 30 Embodiment 158. A method for obtaining an alpha-amylase variant, comprising (a) introducing into a parent alpha-amylase a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and a substitution at one or more positions corresponding to positions 15, 48, 49, 50, 107, 116, 133, 138, 156, 176, 181, 187, 188, 190, 197, 201, 205, 209, 213, 239, 241, 255, 264,
35 299, 360, 375, 416, 437, 474 and 475 of SEQ ID NO: 1; and (b) recovering the variant.

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

5

Examples

Assays for measurement of amylolytic activity (alpha-amylase activity)

PNP-G7 assay:

10 The alpha-amylase activity is determined by a method employing the PNP-G7 substrate. PNP-G7 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 hydrolysed substrate further to liberate a free PNP molecule which has a yellow color and thus can be measured by visible
15 spectrophotometry at $\lambda=405\text{nm}$ (400-420 nm.). Kits containing PNP-G7 substrate and alpha-glucosidase is manufactured by Roche/Hitachi (cat. No.11876473).

REAGENTS:

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.

20 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 reagent with 0.2 ml of the G7-PNP substrate. This substrate working solution is made immediately before use.

Dilution buffer: 50 mM EPPS, 0.01% (w/v) Triton X100™ (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (C₁₄H₂₂O(C₂H₄O)_{*n*} (n = 9-10))), 1mM CaCl₂, pH 7.0.
25

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 20 μl diluted enzyme samples to 96 well microtiter plate and adding 80 μl substrate working solution. The solution is mixed and pre-incubated 1 minute at room temperature and absorption is measured every 20 sec. over 5 minutes
30 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:

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. 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 amylase sample to be analysed is diluted in activity buffer with the desired pH. One substrate tablet is suspended in 5mL activity buffer and mixed on magnetic stirrer. 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 amylase sample should be diluted so that the absorbance at 620nm is between 0 and 2.2, and is within the linear range of the activity assay.

20

Reducing sugar activity assay:

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 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 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.

The 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.

5

EnzChek® assay:

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

25

Reference alpha-amylase and variants thereof

The reference alpha-amylase is LE399 (previously disclosed in, e.g., WO 2002/010355). LE399 comprises amino acids 1-37 of the alpha-amylase from *Bacillus amyloliquefaciens* (SEQ ID NO: 6) and amino acids 40-483 of the alpha-amylase from *Bacillus licheniformis* (SEQ ID NO: 1) with the following substitutions G48A T49I G107A H156Y A181T N190F I201F A209V Q264S.

The variants tested are variants of LE399, so the substitutions in each variant as listed in the tables below are substitutions as compared to LE399. The position numbering is according to SEQ ID NO: 1.

Example 1**Thermostability of Alpha-Amylase Variants at pH 4.5**

The thermostability of a reference alpha-amylase and alpha-amylase variants thereof was determined by incubating the reference alpha-amylase and variants at pH 4.5 and temperatures of 90°C and 95°C with 0.125 mM CaCl₂ followed by residual activity determination using the EnzChek® substrate (EnzChek® Ultra Amylase assay kit, E33651, Molecular Probes).

Purified enzyme samples were diluted to working concentrations of 5 and 10 ppm (micrograms/ml) in enzyme dilution buffer (10 mM acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 5.0). Twenty microliters enzyme sample was transferred to 96-well PCR MTP and 180 microliters stability buffer (150 mM acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 4.5 or 4.8) was added to each well and mixed. The assay was performed using two concentrations of enzyme in duplicates. Before incubation at 90°C or 95°C, 20 microliters was withdrawn and stored on ice as unstressed control samples. Incubation was performed in a PCR machine for 2 or 8 minutes (pH 4.5 and 90°C) and 30 minutes (pH 4.5 and 95°C). Incubation time (minutes) is selected so that residual activity is between 10-90%.

After incubation samples were diluted to 15 ng/ml in residual activity buffer (100 mM Acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 5.5) and 25 microliters diluted enzyme was transferred to black 384-well MTP. Residual activity was determined using the EnzChek® substrate by adding 25 microliters substrate solution (100 micrograms/ml) to each well. Fluorescence was determined at 25°C every minute for 15 minutes using excitation filter at 485-P nm and emission filter at 555 nm (fluorescence reader is Polarstar, BMG). The residual activity was normalized to unstressed control samples for each setup.

Assuming exponential decay half life time ($T_{1/2}$ (min)) was calculated using the equation: $T_{1/2}$ (min) = $T(\text{min}) \cdot \text{LN}(0.5) / \text{LN}(\% \text{RA} / 100)$, where T is assay incubation time in minutes, and %RA is % residual activity determined in assay.

Using this assay setup the half life time was determined as a measure of thermostability for the reference alpha-amylase and variants thereof as shown in Tables 1 and 2.

The reference amylase was not tested at pH 4.5, 0.125 mM CaCl₂ at 95°C for 30 minutes since there is no residual activity (half life time is 1.2 minutes at 90°C).

30

Table 1

Conditions: pH 4.5, 0.125 mM CaCl₂, 90°C for 2 or 8 minutes

Mutations	T½(min) (pH 4.5, 0.125 mM CaCl₂, 90°C)
Reference amylase	1.2
K176L F201Y H205Y K213T Q360S D416V R437W	8
K176L E185P F201Y H205Y K213T Q360S D416V R437W	54
K176L	6
E185P	26
K176L E185P	100% residual activity

Table 2

Conditions: pH 4.5, 0.125 mM CaCl₂, 95°C for 30minutes

Mutations	T½(min) (pH 4.5, 0.125 mM CaCl₂, 95°C)
K176L E185P	20
T116G K176L E185P	23

Example 2**Thermostability of Alpha-Amylase Variants at pH 4.8**

10 The thermostability of a reference alpha-amylase and alpha-amylase variants thereof was determined by incubating the reference alpha-amylase and variants at pH 4.8 and temperatures of 90°C and 95°C with 0.125 mM CaCl₂ followed by residual activity determination using the EnzChek® substrate (EnzChek® Ultra Amylase assay kit, E33651, Molecular Probes).

15 Purified enzyme samples were diluted to working concentrations of 5 and 10 ppm (micrograms/ml) in enzyme dilution buffer (10 mM acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 5.0). Twenty microliters enzyme sample was transferred to 96-well PCR MTP and 180 microliters stability buffer (150 mM acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 4.5 or 4.8) was added to each well and mixed. The assay was performed using two concentrations of enzyme in duplicates. Before incubation at 90°C or 95°C, 20 microliters was withdrawn and stored on ice as
 20 unstressed control samples. Incubation was performed in a PCR machine for 12, 20, or 25 minutes (pH 4.8 and 90°C) or 50 minutes (pH 4.8 and 95°C). Incubation time (minutes) is selected so that residual activity is between 10-90%.

After incubation samples were diluted to 10-20 ng/ml in residual activity buffer (100 mM Acetate, 0.01% Triton X100, 0.125 mM CaCl₂, pH 5.5) and 25 microliters diluted enzyme was
 25 transferred to black 384-well MTP. Residual activity was determined using the EnzChek® substrate by adding 25 microliters substrate solution (100 micrograms/ml) to each well. Fluorescence was determined at 25°C every minute for 15 minutes using excitation filter at 485-P nm and emission

filter at 555 nm (fluorescence reader is Polarstar, BMG). The residual activity was normalized to control samples for each setup.

Assuming exponential decay half life time ($T_{1/2}$ (min)) was calculated using the equation: $T_{1/2}$ (min) = $T(\text{min}) * \text{LN}(0.5) / \text{LN}(\% \text{RA} / 100)$, where T is assay incubation time in minutes, and %RA is % residual activity determined in assay.

Using this assay setup the half life time was determined as a measure of thermostability for the reference alpha-amylase and variants thereof as shown in Tables 3 and 4.

The reference amylase was not tested at pH 4.8, 0.125 mM CaCl_2 at 95°C for 50 minutes since there is no residual activity (half life time is 4.6 minutes at 90°C).

Table 3

Conditions: pH 4.8, 0.125 mM CaCl_2 , 90°C for 12, 20 or 25 minutes

Mutations	$T_{1/2}(\text{min})$ (pH 4.8, 0.125 mM CaCl_2, 90°C)
Reference amylase	4.6
K176L F201Y H205Y K213T Q360S D416V R437W	25
K176L E185P F201Y H205Y K213T Q360S D416V R437W	53
K176L	21
E185P	75
K176L E185P	127

Table 4

Conditions: pH 4.8, 0.125 mM CaCl_2 , 95°C for 50minutes

Mutations	$T_{1/2}(\text{min})$ (pH 4.8, 0.125 mM CaCl_2, 95°C)
K176L E185P	42
T116G K176L E185P	55
K176L E185P L241D	49
K176L E185P R375V	52
K176L E185P R375G	51
I49T K176L E185P	47
I49H K176L E185P	48
I49G K176L E185P	43
I49L S50T K176L E185P	48

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.

Claims

1. An alpha-amylase variant comprising a substitution with proline at a position corresponding to position 185 of SEQ ID NO: 1 and further comprising a substitution with leucine at a position corresponding to position 176 of SEQ ID NO: 1, wherein the variant has at least 90% and less than 100% sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14, and wherein the variant has alpha-amylase activity, and wherein the variant has a residual activity half life, when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.8, which is at least 10 minutes.
2. The variant of claim 1, wherein the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 90°C and pH 4.5, which is at least 2 minutes.
3. The variant of claim 1 or 2, wherein the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.5, which is at least 5 minutes.
4. The variant of any one of claims 1 to 3, wherein the variant has a residual activity half-life, when incubated with 0.125 mM CaCl₂ at 95°C and pH 4.8, which is at least 10 minutes.
5. The variant of any one of claims 1 to 4, which further comprises one or more of the following substitutions:
 - a substitution at a position corresponding to position 15 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;
 - a substitution at a position corresponding to position 48 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;
 - a substitution at a position corresponding to position 49 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val;
 - a substitution at a position corresponding to position 50 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 107 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 116 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr or Val;

a substitution at a position corresponding to position 133 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 138 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr or Val;

a substitution at a position corresponding to position 156 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 181 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 187 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 188 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 190 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 197 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 201 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 205 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 209 of SEQ ID NO: 1 with Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 213 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 239 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 241 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 255 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 264 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 299 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 360 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 375 of SEQ ID NO: 1 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 416 of SEQ ID NO: 1 with Ala, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 437 of SEQ ID NO: 1 with Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val;

a substitution at a position corresponding to position 474 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val; and

a substitution at a position corresponding to position 475 of SEQ ID NO: 1 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val.

6. The variant of claim 5, wherein

the substitution at a position corresponding to position 15 of SEQ ID NO: 1 is with Leu, Ser or Thr;

the substitution at a position corresponding to position 48 of SEQ ID NO: 1 is with Ala;

the substitution at a position corresponding to position 49 of SEQ ID NO: 1 is Gly, His, Ile or Leu;

the substitution at a position corresponding to position 50 of SEQ ID NO: 1 is with Thr;

the substitution at a position corresponding to position 107 of SEQ ID NO: 1 is with Ala;

the substitution at a position corresponding to position 116 of SEQ ID NO: 1 is with Gly;

the substitution at a position corresponding to position 133 of SEQ ID NO: 1 is with Tyr;

the substitution at a position corresponding to position 138 of SEQ ID NO: 1 is with Phe or Tyr;

the substitution at a position corresponding to position 156 of SEQ ID NO: 1 is with Tyr;

the substitution at a position corresponding to position 181 is with Asp, Glu or Thr;

the substitution at a position corresponding to position 187 is with Asp;

the substitution at a position corresponding to position 188 is with Ser or Thr;

the substitution at a position corresponding to position 190 is with Phe;

the substitution at a position corresponding to position 197 of SEQ ID NO: 1 is with Ile, Leu, Ser, Thr or Val;

the substitution at a position corresponding to position 201 of SEQ ID NO: 1 is with Phe or Tyr;

the substitution at a position corresponding to position 205 of SEQ ID NO: 1 is with Tyr;

the substitution at a position corresponding to position 209 of SEQ ID NO: 1 is with Val;

the substitution at a position corresponding to position 213 of SEQ ID NO: 1 is with Thr;

the substitution at a position corresponding to position 239 of SEQ ID NO: 1 is with Ala, Asn, Asp, Cys, Gln, Glu or Met;

the substitution at a position corresponding to position 241 of SEQ ID NO: 1 is with Asp;

the substitution at a position corresponding to position 255 of SEQ ID NO: 1 is with Gly or Pro;

the substitution at a position corresponding to position 264 of SEQ ID NO: 1 is with Ser;

the substitution at a position corresponding to position 299 of SEQ ID NO: 1 is with Arg;
the substitution at a position corresponding to position 360 of SEQ ID NO: 1 is with Ser;
the substitution at a position corresponding to position 375 of SEQ ID NO: 1 is with Gly
or Val;

the substitution at a position corresponding to position 416 of SEQ ID NO: 1 is with Val;
the substitution at a position corresponding to position 437 of SEQ ID NO: 1 is with Trp;
the substitution at a position corresponding to position 474 of SEQ ID NO: 1 is with Arg,
Gln, Glu or Lys; and

the substitution at a position corresponding to position 475 of SEQ ID NO: 1 is with Arg,
Gln, Glu or Lys.

7. The variant of any one of claims 1 to 4, which further comprises one or more substitutions selected from the group consisting of M15T, M15S, M15L, G48A, T49H, T49G, T49L, T49I, S50T, G107A, T116G, H133Y, W138Y, W138F, H156Y, A181T, A181E, A181D, S187D, N188S, N188T, N190F, M197S, M197T, M197V, M197L, M197I, I201Y, I201F, H205Y, A209V, K213T, S239Q, S239E, S239N, S239D, S239A, S239M, S239C, L241D, E255P, E255G, Q264S, G299R, Q360S, R375V, R375G, D416V, R437W, G474K, G474R, G474E, G474Q, G475K, G475R, G475E and G475Q.

8. The variant of any one of claims 1 to 4, which comprises a set of substitutions selected from the group consisting of:

T49H+K176L+E185P,

T49G+K176L+E185P,

T49L+S50T+K176L+E185P,

T116G+K176L+E185P,

K176L+E185P,

K176L+E185P+I201Y+H205Y+K213T+Q360S+D416V+R437W,

K176L+E185P+L241D,

K176L+E185P+R375V, and

K176L+E185P+R375G.

9. The variant of any one of claims 1 to 8, wherein the variant has at least 91%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ

ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

10. The variant of claim 9, wherein the variant has at least 92%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

11. The variant of claim 9, wherein the variant has at least 93%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

12. The variant of claim 8, wherein the variant has at least 94%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

13. The variant of claim 9, wherein the variant has at least 95%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

14. The variant of claim 9, wherein the variant has at least 96%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

15. The variant of claim 9, wherein the variant has at least 97%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

16. The variant of claim 9, wherein the variant has at least 98%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to

485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

17. The variant of claim 9, wherein the variant has at least 99%, but less than 100%, sequence identity to (i) the mature polypeptide of any of SEQ ID NOs: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14, or (ii) amino acids 1 to 483 of SEQ ID NO: 1, amino acids 1 to 483 of SEQ ID NO: 2, amino acids 1 to 482 of SEQ ID NO: 4, amino acids 1 to 484 of SEQ ID NO: 5, amino acids 1 to 483 of SEQ ID NO: 6, amino acids 1 to 485 of SEQ ID NO: 7, amino acids 1 to 485 of SEQ ID NO: 8, amino acids 1 to 485 of SEQ ID NO: 9, amino acids 1 to 485 of SEQ ID NO: 10, amino acids 1 to 485 of SEQ ID NO: 11, amino acids 1 to 480 of SEQ ID NO: 12, amino acids 1 to 483 of SEQ ID NO: 13 or amino acids 1 to 481 of SEQ ID NO: 14.

18. The variant of any one of claims 1 to 17, which is an isolated alpha-amylase variant.

19. Use of the variant of any one of claims 1 to 18 for liquefying a starch-containing material.

20. An isolated polynucleotide encoding the variant of any one of claims 1 to 18.

21. A nucleic acid construct comprising the polynucleotide of claim 20.

22. An expression vector comprising the polynucleotide of claim 20.

23. A host cell comprising the polynucleotide of claim 20.

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

- a. cultivating the host cell of claim 23 under conditions suitable for expression of the variant; and
- b. recovering the variant.

		1	50
SEQ ID NO: 1	(1)	---ANLNGTLMQYFEWYMPNDGQHWRLQND SAYLAEHGITAVWIPPAYK	
SEQ ID NO: 2	(1)	--AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYK	
SEQ ID NO: 3	(1)	ANTAPINETMMQYFEWDLPNDDGTLWTKVKNEAANNLSSLGITALWLPPAYK	
SEQ ID NO: 4	(1)	---VPVNGTMMQYFEWYLPDDGTLWTKVANNAQSLANLGITALWLPPAYK	
SEQ ID NO: 5	(1)	-GSVPVNGTMMQYFEWYLPDDGTLWTKVANNAQSLANLGITALWLPPAYK	
SEQ ID NO: 6	(1)	-----VNGTLMQYFEWYTPNDGQHWKRLQND AEHLSDIGITAVWIPPAYK	
SEQ ID NO: 7	(1)	-HHNGTNGTMMQYFEWYLPNDGNHWNRLRDDAANLKS KGITAVWIPPAYK	
SEQ ID NO: 8	(1)	-HHNGTNGTMMQYFEWYLPNDGNHWNRLRDDASNLRNRGITAIWIPPAYK	
SEQ ID NO: 9	(1)	-HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASN LKDKGISAVWIPPAYK	
SEQ ID NO: 10	(1)	-HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASN LKDKGITAVWIPPAYK	
SEQ ID NO: 11	(1)	-HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASN LKSKGITAVWIPPAYK	
SEQ ID NO: 12	(1)	---DGLNGTMMQYFEWYLPNDGNHWNRLHDDAAALSDAGITAIWIPPAYK	
SEQ ID NO: 13	(1)	---ANLNGTLMQYFEWYMPNDGQHWKRLQND SAYLAEHGITAVWIPPAYK	
SEQ ID NO: 14	(1)	-----VNGTLMQYFEWYTPNDGQHWKRLQND AEHLSDIGITAVWIPPAYK	
		51	100
SEQ ID NO: 1	(48)	GTSQADVGYGAYDLYDLGEFHQKGTVRTKYGT KGELQSAIKSLHSRDINV	
SEQ ID NO: 2	(49)	GTSRSDVGYGVYDLYDLGEFNQKGTVRTKYGT KAQYLQAIQAAHAAGMQV	
SEQ ID NO: 3	(51)	GTSQSDVGYGVYDLYDLGEFNQKGTIRTKYGT KTQYIQAIQAAKAAGMQV	
SEQ ID NO: 4	(48)	GTSSSDVGYGVYDLYDLGEFNQKGTVRTKYGT KTQYIQAIQAAHTAGMQV	
SEQ ID NO: 5	(50)	GTSSSDVGYGVYDLYDLGEFNQKGTVRTKYGT KTQYIQAIQAAHTAGMQV	
SEQ ID NO: 6	(46)	GLSQSDNGYGPYDLYDLGEFQKGTVRTKYGT KSELQDAIGSLHSRNVQV	
SEQ ID NO: 7	(50)	GTSQNDVGYGAYDLYDLGEFNQKGTVRTKYGT RNLQAAVTSLKNNGIQV	
SEQ ID NO: 8	(50)	GTSQNDVGYGAYDLYDLGEFNQKGTVRTKYGT RSQLESAIAHALKNNGVQV	
SEQ ID NO: 9	(50)	GASQNDVGYGAYDLYDLGEFNQKGTIRTKYGT RNLQAAVNALKSNGIQV	
SEQ ID NO: 10	(50)	GASQNDVGYGAYDLYDLGEFNQKGTVRTKYGT RNLQAAVTALKSNGIQV	
SEQ ID NO: 11	(50)	GASQNDVGYGAYDLYDLGEFNQKGTVRTKYGT RSQLQAAVTSLKNNGIQV	
SEQ ID NO: 12	(48)	GNSQADVGYGAYDLYDLGEFNQKGTVRTKYGT KAQLERAIGSLKSNDINV	
SEQ ID NO: 13	(48)	GTSQADVGYGAYDLYDLGEFHQKGTVRTKYGT KGELQSAIKSLHSRDINV	
SEQ ID NO: 14	(46)	AISQADVGYGAYDLYDLGEFHQKGTVRTKYGT KGELQSAIKSLHSRDINV	
		101	150
SEQ ID NO: 1	(98)	YGDVVINHKGADATEDVTAVEVDPADRNRVIS GEHLIKAWTHFHFPGRG	
SEQ ID NO: 2	(99)	YADVVDHKGADGTEWVD AVEVNP SDRNQEISGTYQIQAWTKFDFPGRG	
SEQ ID NO: 3	(101)	YADVFNHKGADGTEFVDAVEVDP SDRNQETSGTYQIQAWTKFDFPGRG	
SEQ ID NO: 4	(98)	YADVFNHKGADGTELVDAVEVNP SDRNQEISGTYQIQAWTKFDFPGRG	
SEQ ID NO: 5	(100)	YADVFNHKGADGTELVDAVEVNP SDRNQEISGTYQIQAWTKFDFPGRG	
SEQ ID NO: 6	(96)	YGDVVLNHKGADATEDVTAVEVNPANRNQETSEEYQIKAWTDFRFPGRG	
SEQ ID NO: 7	(100)	YGDVVMNHKGADGTEIVNAVEVNRSNRNQETSGEYIAEAWTKFDFPGRG	
SEQ ID NO: 8	(100)	YGDVVMNHKGADATENVLAVEVNPNNRNQETSGDYTIEAWTKFDFPGRG	
SEQ ID NO: 9	(100)	YGDVVMNHKGADATEMVR AVEVNPNNRNQEVSGEYTIEAWTKFDFPGRG	
SEQ ID NO: 10	(100)	YGDVVMNHKGADATEWVR AVEVNPNNRNQEVSGDYTIEAWTKFDFPGRG	
SEQ ID NO: 11	(100)	YGDVVMNHKGADATEMVR AVEVNPNNRNQEVSGEYTIEAWTRFDFPGRG	
SEQ ID NO: 12	(98)	YGDVVMNHKMGADFT EAVQAVQVNPTNRWQDISGAYTIDAWTGDFDFSGRN	
SEQ ID NO: 13	(98)	YGDVVINHKGADATEDVTAVEVDPADRNRVIS GEYLIKAWTHFHFPGRG	
SEQ ID NO: 14	(96)	YGDVVINHKGADATEDVTAVEVDPADRNRVIS GEHLIKAWTHFHFPGRG	

Fig. 1

		151	200
SEQ ID NO: 1	(148)	STYSDFKWHWYHFDGTDWDESR-KLNRIYKFQG--KAWDWEVSNENGNYD	
SEQ ID NO: 2	(149)	NTYSSFKWRWYHFDGVDWDESR-KLSRIYKFRGIGKAWDWEVDTENGNYD	
SEQ ID NO: 3	(151)	NTYSSFKWRWYHFDGTDWDESR-KLNRIYKFRSTGKAWDWEVDTENGNYD	
SEQ ID NO: 4	(148)	NTYSSFKWRWYHFDGTDWDESR-KLNRIYKFRGTGKAWDWEVDTENGNYD	
SEQ ID NO: 5	(150)	NTYSSFKWRWYHFDGTDWDESR-KLNRIYKFRGTGKAWDWEVDTENGNYD	
SEQ ID NO: 6	(146)	NTYSDFKWHWYHFDGADWDESR-KISRIKFGRGEGKAWDWEVSSENGNYD	
SEQ ID NO: 7	(150)	NNHSSFKWRWYHFDGTDWDQSRQLQNKIYKFRGTGKAWDWEVDTENGNYD	
SEQ ID NO: 8	(150)	NTYSDFKWRWYHFDGVDWDQSRQFQNRIYKFRGDGKAWDWEVDSENGNYD	
SEQ ID NO: 9	(150)	NTHSNFKWRWYHFDGVDWDQSRKLNNRIYKFRGDGKGWDWEVDTENGNYD	
SEQ ID NO: 10	(150)	NTHSNFKWRWYHFDGVDWDQSRQLQNRIYKFRGDGKGWDWEVDTENGNYD	
SEQ ID NO: 11	(150)	NTHSSFKWRWYHFDGVDWDQSRRLNNRIYKFRGHGKAWDWEVDTENGNYD	
SEQ ID NO: 12	(148)	NAYSDFKWRWFHFNQVDWDQRY-QENHIFRFAN--TNWNWRVDEENGNYD	
SEQ ID NO: 13	(148)	STYSDFKWWYHFDGTDWDESR-KLNRIYKFQG--KYWDWEVSNENGNYD	
SEQ ID NO: 14	(146)	STYSDFKWWYHFDGTDWDESR-KLNRIYKFQG--KTWDWEVSNENFGNYD	
		201	250
SEQ ID NO: 1	(195)	YLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDW	
SEQ ID NO: 2	(198)	YLMYADLDMDHPEVVTELKNWGKQWYVNTTNIDGFRLDAVKHIKFSFFPDW	
SEQ ID NO: 3	(200)	YLMFADLDMDHPEVVTELKNWGWTWYVNTTNIDGFRLDAVKHIKYSFFPDW	
SEQ ID NO: 4	(197)	YLMYADLDMDHPEVVSELKNWGKQWYVTTTNIDGFRLDAVKHIKYSFFPDW	
SEQ ID NO: 5	(199)	YLMYADLDMDHPEVVSELKNWGKQWYVITTNIDGFRLDAVKHIKYSFFPDW	
SEQ ID NO: 6	(195)	YLMYADVDDYDHPDVVAETKKWGIWYANELSLDGFRLDAVKHIKFSFLRDW	
SEQ ID NO: 7	(200)	YLMYADVDMMDHPEVIHELNRNWGVWYTNLTNLNLDGFRLDAVKHIKYSFTRDW	
SEQ ID NO: 8	(200)	YLMYADVDMMDHPEVVNELRRWGEWYTNLTNLNLDGFRLDAVKHIKYSFTRDW	
SEQ ID NO: 9	(200)	YLMYADIDMDHPEVVNELRNWGVWYTNLTGLDGFRLDAVKHIKYSFTRDW	
SEQ ID NO: 10	(200)	YLMYADIDMDHPEVVNELRNWGVWYTNLTGLDGFRLDAVKHIKYSFTRDW	
SEQ ID NO: 11	(200)	YLMYADIDMDHPEVVNELRNWGVWYTNLTGLDGFRLDAVKHIKYSFTRDW	
SEQ ID NO: 12	(195)	YLLGSNIDFSHPEVQDELKDWGSWFTDELDDLGYRLDAIKHIPFWYTSDW	
SEQ ID NO: 13	(195)	YLMYADIDYDHPDVAAEIKRWGTWYANELQLDGNRLDAVKHIKFSFLRDW	
SEQ ID NO: 14	(193)	YLMYADFDYDHPDVVAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDW	
		251	300
SEQ ID NO: 1	(245)	VNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHVSFVDVPLHYQFHA	
SEQ ID NO: 2	(248)	LSYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYT	
SEQ ID NO: 3	(250)	LTYVRNQTGKNLFAVGFEWSYDVNKLHNYITKTNGSMSLFDAPLHNNFYT	
SEQ ID NO: 4	(247)	LSYVRTQTQKPLFAVGFEWSYDISKLHNYITKTNGSMSLFDAPLHNNFYI	
SEQ ID NO: 5	(249)	LSYLRTQTQKPLFAVGFEWSYDINKLHNYITKTNGSMSLFDAPLHNNFYI	
SEQ ID NO: 6	(245)	VQAVRQATGKEMFTVAEYWQNNAGKLENYLNKTSFNQSVFVDVPLHFNLQA	
SEQ ID NO: 7	(250)	LTHVRNTTGKPMFAVAEFWKNDLGALENYLNKTSWNHVSFVDVPLHYNLYN	
SEQ ID NO: 8	(250)	LTHVRNATGKEMFAVAEFWKNDLGALENYLNKTNWNHVSFVDVPLHYNLYN	
SEQ ID NO: 9	(250)	INHVRSATGKNMFVAVAEFWKNDLGALENYLNKTNWNHVSFVDVPLHYNLYN	
SEQ ID NO: 10	(250)	LTHVRNTTGKNMFVAVAEFWKNDLGALENYLSKTNWNHVSFVDVPLHYNLYN	
SEQ ID NO: 11	(250)	INHVRSATGKNMFVAVAEFWKNDLGALENYLQKTNWNHVSFVDVPLHYNLYN	
SEQ ID NO: 12	(245)	VRHQREADQDLFVVGEYWKDDVGALEFYLDENMWEMSFLFDVPLNYNFYR	
SEQ ID NO: 13	(245)	VNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHVSFVDVPLHYQFYA	
SEQ ID NO: 14	(243)	VNHVREKTGKEMFTVAEYWSNDLGALENYLNKTNFNHVSFVDVPLHYQFHA	

Fig. 1 cont.

3/4

		301	350
SEQ ID NO: 1	(295)	ASTQGGGYDMRKLLNGTVVSKHPLKSVTFVDNHDTPGQSLESTVQTWFK	
SEQ ID NO: 2	(298)	ASKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFK	
SEQ ID NO: 3	(300)	ASKSSGYFDMRYLLNNTLMKDDPSLAVTLVDNHDTPGQSLQSWVEPWFK	
SEQ ID NO: 4	(297)	ASKSGGYFDMRTLNNNTLMKDDQPTLAVTLVDNHDTEPGQSLQSWVEPWFK	
SEQ ID NO: 5	(299)	ASKSGGYFDMRTLNNNTLMKEQPTLSVTLVDNHDTEPGQSLQSWVEPWFK	
SEQ ID NO: 6	(295)	ASSQGGGYDMRRLLDGTVVSRHPEKAVTFVENHDTPGQSLESTVQTWFK	
SEQ ID NO: 7	(300)	ASNSGGYDMRNILNGSVVQKHPHAVTFVDNHDSPGEALESFVQQWFK	
SEQ ID NO: 8	(300)	ASNSGGNYDMAKLLNGTVVQKHPHMAVTFVDNHDSPGESLESFVQEWFK	
SEQ ID NO: 9	(300)	ASKSGGNYDMRQIFNGTVVQRHPHMAVTFVDNHDSPEEALESFVEEWFK	
SEQ ID NO: 10	(300)	ASRSGGNYDMRQIFNGTVVQRHPHMAVTFVDNHDSPEEALESFVEEWFK	
SEQ ID NO: 11	(300)	ASKSGGNYDMRNIFNGTVVQRHPHMAVTFVDNHDSPEEALESFVEEWFK	
SEQ ID NO: 12	(295)	ASQQGGSYDMRNILRGLSVEAHMPHMAVTFVDNHDTPGESLESWVADWFK	
SEQ ID NO: 13	(295)	ASTQGGGYDMRKLLNDTVVSKHPLKSVTFVDNHDTPGQSLESTVQTWFK	
SEQ ID NO: 14	(293)	ASTQGGGYDMRKLLNGTVVSKHPLKSVTFVDNHDTPGQSLESTVQTWFK	
		351	400
SEQ ID NO: 1	(345)	PLAYAFILTRESGYPQVFYGDYMGTKGDSQREIPALKHKIEPILKARKQY	
SEQ ID NO: 2	(348)	PLAYAFILTRQEGYPCVFYGDYYPQYN---IPSLKSKIDPLLIARRDY	
SEQ ID NO: 3	(350)	PLAYAFILTRQEGYPCVFYGDYYPKYN---IPGLKSKIDPLLIARRDY	
SEQ ID NO: 4	(347)	PLAYAFILTRQEGYPCVFYGDYYPKYN---IPALKSKLDPLLIARRDY	
SEQ ID NO: 5	(349)	PLAYAFILTRQEGYPCVFYGDYYPKYN---IPALKSKLDPLLIARRDY	
SEQ ID NO: 6	(345)	PLAYAFILTRESGYPQVFYGDYMGTKGTSPKEIPSLKDNIPIPKARKEY	
SEQ ID NO: 7	(350)	PLAYALVLTREQGYPSVFYGDYYPKYN---VPAMKSKIDPLLIARRDY	
SEQ ID NO: 8	(350)	PLAYALILTREQGYPSVFYGDYYPKYN---VPAMKAKIDPILEARQNF	
SEQ ID NO: 9	(350)	PLAYALTLTREQGYPSVFYGDYYPKYN---VPAMKSKIDPILEARQKY	
SEQ ID NO: 10	(350)	PLACALTLTREQGYPSVFYGDYYPKYN---VPAMKSKIDPILEARQKY	
SEQ ID NO: 11	(350)	PLAYALTLTREQGYPSVFYGDYYPKYN---VPAMKSKIDPILEARQKY	
SEQ ID NO: 12	(345)	PLAYATILTREGGYPNVFYGDYYPKYN---ISAKKDMIDELLDARQNY	
SEQ ID NO: 13	(345)	PLAYAFILTRESGYPQVFYGDYMGTKGDSQREIPALKHKIEPILKARKQY	
SEQ ID NO: 14	(343)	PLAYAFILTRESGYPQVFYGDYMGTKGDSQREIPALKHKIEPILKARKQY	
		401	450
SEQ ID NO: 1	(395)	AYGAQHDFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMVYVGRQN	
SEQ ID NO: 2	(395)	AYGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWYVVGKQH	
SEQ ID NO: 3	(397)	AYGTQRDYIDHQDIIGWTREGIDTKPNSGLAALITDGPGGSKWYVVGKQH	
SEQ ID NO: 4	(394)	AYGTQHDYIDSADIIGWTREGVAEKANSGLAALITDGPGGSKWYVVGKQH	
SEQ ID NO: 5	(396)	AYGTQHDYIDNADIIGWTREGVAEKANSGLAALITDGPGGSKWYVVGKQH	
SEQ ID NO: 6	(395)	AYGPQHDYIDHPDIVGWTREGDSSAAKSGLAALITDGPGGSKRMVYAGLKN	
SEQ ID NO: 7	(397)	AYGTQHDYFDHDDIIGWTREGNSSHPNSGLATIMSDGPGGNKWMYVVGKKN	
SEQ ID NO: 8	(397)	AYGTQHDYFDHDDIIGWTREGNTTHPNSGLATIMSDGPGGEKWMYVVGQNK	
SEQ ID NO: 9	(397)	AYGRQNDYLDHDDIIGWTREGNTAHPNSGLATIMSDGAGGNKWMFVGRNK	
SEQ ID NO: 10	(397)	AYGKQNDYLDHDDIIGWTREGNTAHPNSGLATIMSDGPGGNKWMYVVGKKN	
SEQ ID NO: 11	(397)	AYGKQNDYLDHDDIIGWTREGNTAHPNSGLATIMSDGAGGSKWMFVGRNK	
SEQ ID NO: 12	(392)	AYGTQHDYFDHDDVVGWTREGSSSRPNSGLATIMSDGPGGSKWYVVGKKN	
SEQ ID NO: 13	(395)	AYGAQHDFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMVYVGRQN	
SEQ ID NO: 14	(393)	AYGAQHDFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMVYVGRQN	

Fig. 1 cont.

4/4

		451	500
SEQ ID NO: 1	(445)	AGETWHDITG NRSEPVVINSEGWGEFHVNGGSVSIYVQR-----	
SEQ ID NO: 2	(445)	AGKVFDLTG NRSDTVTINSDGWGEFKVNGGSVSVVWPRKTT---VSTIA	
SEQ ID NO: 3	(447)	AGKVFDLTG NRSDTVTINADGWGEFKVNGGSVSIWVAKTSN---VTFTV	
SEQ ID NO: 4	(444)	AGKTFYDLTGNRSDTVTINADGWGEFKVNGGSVSIWVPKISTTSQITFTV	
SEQ ID NO: 5	(446)	AGKTFYDLTGNRSDTVTINADGWGEFKVNGGSVSIWVPKSTTSQITFTV	
SEQ ID NO: 6	(445)	AGETWYDITG NRSDTVKIGSDGWGEFHVNDGSVSIYVQK-----	
SEQ ID NO: 7	(447)	AGQVWRDITGNRTGTVTINADGWGNFSVNGGSVSVWVKQ-----	
SEQ ID NO: 8	(447)	AGQVWHDITGNKPGTVTINADGWANFSVNGGSVSIWVKR-----	
SEQ ID NO: 9	(447)	AGQVWTDITGNRAGTVTINADGWGNFSVNGGSVSIWVVK-----	
SEQ ID NO: 10	(447)	AGQVWRDITGNRSGTVTINADGWGNFSVNGGSVSIWVNN-----	
SEQ ID NO: 11	(447)	AGQVWSDITGNRTGTVTINADGWANFSVNGGSVSIWVVK-----	
SEQ ID NO: 12	(442)	AGQTWTDLTGNNGASVTINGDGWGEFFTNNGGSVSVYVQN-----	
SEQ ID NO: 13	(445)	AGETWYDITG NRSEPVVINSEGWGEFHVNDGSVSIYVQR-----	
SEQ ID NO: 14	(443)	AGETWHDITG NRSEPVVINSEGWGEFHVNGGSVSIYVQR-----	
		501	550
SEQ ID NO: 1	(484)	-----	
SEQ ID NO: 2	(492)	RPITTRPWTGEFVRWTEPRLVAWP-----	
SEQ ID NO: 3	(494)	NNATTTSGQNVYVVVANIPELGNWNTANA IKMNPSSYPTWKATIALPQGA	
SEQ ID NO: 4	(494)	NNATTVWGQNVYVVGNISQLGNWDPVHAVQMT PSSYPTWTVTIPLLQGQN	
SEQ ID NO: 5	(496)	NNATTVWGQNVYVVGNISQLGNWDPVNAVQMT PSSYPTWVVTVPPLPQSQN	
SEQ ID NO: 6	(484)	-----	
SEQ ID NO: 7	(486)	-----	
SEQ ID NO: 8	(486)	-----	
SEQ ID NO: 9	(486)	-----	
SEQ ID NO: 10	(486)	-----	
SEQ ID NO: 11	(486)	-----	
SEQ ID NO: 12	(481)	-----	
SEQ ID NO: 13	(484)	-----	
SEQ ID NO: 14	(482)	-----	
		551	591
SEQ ID NO: 1	(484)	-----	
SEQ ID NO: 2	(516)	-----	
SEQ ID NO: 3	(544)	IEFKFIKKDQAGNVIWESTSNRYYTVPFSSSTG SYTASWNV	
SEQ ID NO: 4	(544)	IQFKFIKKDSAGNVIWEDISNRYYTVPTAASGAYTASWNV	
SEQ ID NO: 5	(546)	IQFKFIKKDGSAGNVIWENISNRYYTVPTAASGAYTANWNV	
SEQ ID NO: 6	(484)	-----	
SEQ ID NO: 7	(486)	-----	
SEQ ID NO: 8	(486)	-----	
SEQ ID NO: 9	(486)	-----	
SEQ ID NO: 10	(486)	-----	
SEQ ID NO: 11	(486)	-----	
SEQ ID NO: 12	(481)	-----	
SEQ ID NO: 13	(484)	-----	
SEQ ID NO: 14	(482)	-----	

Fig. 1 cont.