



(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2021/02/09
(87) Date publication PCT/PCT Publication Date: 2021/08/19
(85) Entrée phase nationale/National Entry: 2022/07/27
(86) N° demande PCT/PCT Application No.: US 2021/017173
(87) N° publication PCT/PCT Publication No.: 2021/163011
(30) Priorités/Priorities: 2020/02/10 (US62/972,132);
2020/06/01 (US63/032,779); 2020/09/03 (US63/073,968)

(51) Cl.Int./Int.Cl. *C12N 9/18* (2006.01)
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(54) Titre : VARIANTS D'ALPHA-AMYLASE ET POLYNUCLEOTIDES CODANT POUR CEUX-CI
(54) Title: ALPHA-AMYLASE VARIANTS AND POLYNUCLEOTIDES ENCODING SAME

(57) Abrégé/Abstract:

The present invention relates to isolated alpha-amylase variants of a parent alpha-amylase, comprising an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. The alpha-amylase variants of the invention have increased stability at pH 4.0 and 32°C compared to the parent alpha-amylase.



Date Submitted: 2022/07/27

CA App. No.: 3166233

Abstract:

The present invention relates to isolated alpha-amylase variants of a parent alpha-amylase, comprising an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4. The alpha-amylase variants of the invention have increased stability at pH 4.0 and 32°C compared to the parent alpha-amylase.

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

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

Background of the Invention**Field of the Invention**

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

Description of the Related Art

Alpha-amylases (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1) constitute a group of
enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic
15 oligo- and polysaccharides.

Alpha-amylases are well known in industrial applications, e.g., in producing syrups or
ethanol. One known alpha-amylase derived from *Bacillus* sp. belonging to the GH13_28 family is
known to have some disadvantages for industrial applications because of poor stability at low pH,
e.g., at pH below 5. There is therefore a need for improving pH stability of amylases belonging to
20 the GH13_28 family, in order to improve the industrial applicability, e.g., in an ethanol production
process from starch-containing material; either in a conventional starch to ethanol process or in
a raw starch hydrolysis process.

Ethanol production from raw starch is normally performed as a one step process in which
starch hydrolysis and fermentation is performed simultaneously, typically using an alpha-amylase
25 and a glucoamylase to hydrolyze the raw starch and a yeast to ferment the glucose to produce
ethanol. Process conditions are typically around 32°C and at a pH from 4-5.

WO17/037614 (US2018265853 AA) discloses an alpha-amylase (SEQ ID NO: 6) having
about 99% sequence identity to SEQ ID NO: 1 of the present disclosure.

US2019010473 discloses an alpha-amylase (SEQ ID NO: 34) having 87% sequence identity
30 to SEQ ID NO: 1 of the present disclosure.

US9090887 BB discloses variants of an alpha-amylase (AmyE/SEQ ID NO: 2), wherein AmyE
shares about 92% sequence identity with SEQ ID NO: 1 of the present disclosure.

WO17/133974 discloses an alpha-amylase (SEQ ID NO: 1) having about 97% sequence
identity to SEQ ID NO: 1 of the present disclosure.

35 WO18/002360 discloses an alpha-amylase (SEQ ID NO: 2) having about 98% sequence
identity to SEQ ID NO: 1 of the present disclosure.

The present invention provides alpha-amylase variants having improved properties compared to its parent.

Summary of the Invention

5 The present invention relates to isolated alpha-amylase variants of a parent alpha-amylase, comprising an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 10 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and wherein the variant has at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 15 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

The present invention also relates to isolated polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of 20 producing the variants.

The present invention also relates to a process of producing a fermentation product from starch-containing material comprising the steps of: (a) liquefying starch-containing material above the initial gelatinization temperature of said starch-containing material in the presence of an alpha amylase; (b) saccharifying the liquefied material; and (c) fermenting with a fermenting organism; 25 wherein step (b) is carried out in the presence of at least a variant alpha-amylase of the invention, and optionally a glucoamylase.

The present invention also relates to a process of producing a syrup product from starch-containing material, comprising the step of: (a) liquefying starch-containing material at a temperature above the initial gelatinization temperature of said starch-containing material in the 30 presence of an alpha-amylase; (b) saccharifying the liquefied material in the presence of at least a variant alpha-amylase of the invention, and optionally a glucoamylase.

In further aspects the present invention relates to a composition comprising the variant alpha-amylase, and to use of the variant alpha-amylase for production of syrup and/or a fermentation product. 35

Definitions

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 measuring residual activity after stressing the sample at pH 4.0 using a commercial alpha-amylase activity assay kit, such as kits containing G7-pNP substrate and alpha-Glucosidase, e.g., manufactured by Roche/Hitachi (cat. No.11876473) or Sigma-Aldrich (Catalog number MAK009).

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.

Carbohydrate Binding Module: The term "carbohydrate binding module" means a polypeptide amino acid sequence which binds preferentially to a poly- or oligosaccharide (carbohydrate), frequently - but not necessarily exclusively - to a water-insoluble (including crystalline) form thereof. A carbohydrate-binding module (CBM), is often referred to, a carbohydrate-binding domain (CBD).

CBMs derived from starch degrading enzymes are often referred to as starch-binding modules or SBMs (which may occur in certain amylolytic enzymes, such as certain glucoamylases (GA), or in enzymes such as cyclodextrin glucanotransferases, or in alpha-amylases). SBMs are often referred to as SBDs (Starch Binding Domains).

The parent alpha-amylase and the variant amylases of the invention preferably comprises a CBM, and in one embodiment the CBM comprises or consists of amino acids 527-626 of SEQ ID NO: 1.

Amino acids 439-526 of SEQ ID NO: 1 comprises or consists of a linker region.

In one embodiment the variant according to the invention comprises a heterologous CBM, i.e., a CBM which is foreign (not naturally occurring in the parent wt amylase enzyme) to the parent alpha-amylase used as the starting point for the variants of the invention. Such a heterologous CBM is preferably a CBM of Family 20 or a CBM-20 module. Alternatively, the heterologous CBM can be selected from Carbohydrate-Binding Module Family 21, 25, 26, 34, 41, or 48.

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this disclosure defined as a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al. (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises about 100

amino acids of the polypeptide, *i.e.*, the subsequence from amino acid 582 to amino acid 683. The numbering of Glycoside Hydrolase Families applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* at URL: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *"Genetics, Biochemistry and Ecology of Cellulose Degradation"*, K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23 and Bourne, Y. & Henrissat, B. 2001; Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600.

Examples of enzymes which comprise a CBM suitable for use in the context of the invention are alpha-amylases, maltogenic alpha-amylases, glucoamylases, beta-amylases, pullulanases, cellulases, xylanases, mannanases, arabinofuranosidases, acetylerases and chitinases.

In one embodiment the CBM comprises or consists of amino acids 527-626 of SEQ ID NO: 1. This CBM belongs to Family 26 or CBM-26.

Catalytic domain: The term "catalytic domain" means the region of an enzyme containing the catalytic machinery of the enzyme. In one embodiment the catalytic domain comprises or consists of amino acids 12-438 of SEQ ID NO: 1.

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 embodiment a fragment comprises or consists of amino acids 12-438 of SEQ ID NO: 1. In another embodiment a fragment comprises or consists of amino acids 1-438 of SEQ ID NO: 1.

Fusion polypeptide: The term “fusion polypeptide” is a polypeptide in which one polypeptide is fused at the N-terminus or the C-terminus of a variant 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 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 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.

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.

Hybrid polypeptide: The term “hybrid polypeptide” means a polypeptide comprising domains from two or more polypeptides, e.g., a binding module from one polypeptide and a

catalytic domain from another polypeptide. The domains may be fused at the N-terminus or the C-terminus.

Hybridization: The term "hybridization" means the pairing of substantially complementary strands of nucleic acids, using standard Southern blotting procedures.

Hybridization may be performed under medium, medium-high, high or very high stringency conditions. Medium stringency conditions means prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide for 12 to 24 hours, followed by washing three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 55°C. Medium-high stringency conditions means prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide for 12 to 24 hours, followed by washing three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 60°C. High stringency conditions means prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide for 12 to 24 hours, followed by washing three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 65°C. Very high stringency conditions means prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide for 12 to 24 hours, followed by washing three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 70°C.

Half-life: For a given variant of the invention, the enzyme activity (measured as residual activity of a sample after incubation at pH 4.0 at 37°C for 18-24 hours) of the stressed sample was divided by the enzyme activity of the unstressed sample, to compute residual activity (see examples for exact procedure). From this, the half-life in hours of the enzyme candidate is computed as the negative of the incubation-time in hours divided by log2 of the residual activity.

Improvement Factor (IF): Improvement factor (IF) was calculated from the estimated half-life ($T_{1/2}$), by dividing the estimated $T_{1/2}$ for variants with the $T_{1/2}$ of the wild type enzyme (SEQ ID NO:1).

Improved property: The term "improved property" means a characteristic associated with a variant that is improved, such as increased stability, compared to the parent. Such improved properties include, but are not limited to, pH stability. Increased pH stability may be determined as % residual activity of the variants according to the invention after stressing the enzyme by incubation at low pH, e.g., pH 4.0 at 37°C for 18-24 hours. Increased pH stability may also be determined as % residual activity of the variants according to the invention after stressing the enzyme by incubation at low pH, e.g., pH 4.0 at 32°C for 24 hours or 96 hours. Residual activity determined for the variants of the invention may be determined as an improvement factor compared to the parent alpha-amylase of SEQ ID NO: 1.

Isolated: The term "isolated" means a polypeptide, nucleic acid, cell, or other specified material or component that is separated from at least one other material or component with which

it is naturally associated as found in nature, including but not limited to, for example, other proteins, nucleic acids, cells, etc. An isolated polypeptide includes, but is not limited to, a culture broth containing the secreted polypeptide.

5 **Mature polypeptide:** The term "mature polypeptide" means a polypeptide in its mature form following N-terminal processing (*e.g.*, removal of signal peptide). In one embodiment the mature polypeptide is amino acids 1-626 of the polypeptide disclosed as SEQ ID NO: 1.

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

Mutant: The term "mutant" means a polynucleotide encoding a variant.

10 **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. The one or more control sequences may be heterologous or foreign to the polynucleotide encoding the variant polypeptide of the invention.

15 **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.

20 **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.

25 **Purified:** The term "purified" means a nucleic acid or polypeptide that is substantially free from other components as determined by analytical techniques well known in the art (*e.g.*, a purified polypeptide or nucleic acid may form a discrete band in an electrophoretic gel, chromatographic eluate, and/or a media subjected to density gradient centrifugation). A purified nucleic acid or polypeptide is at least about 50% pure, usually at least about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 99.5%, about 99.6%, about 99.7%, about 99.8% or more pure (*e.g.*, percent by weight on a molar basis). In a related sense, a composition is enriched for a molecule when there is a substantial increase in the concentration of the molecule after application of a purification or enrichment technique. The term "enriched" refers to a compound, polypeptide, cell, nucleic acid, amino acid, or other specified material or component that is present in a composition at a relative or absolute concentration that is higher than a starting composition.

35 **Recombinant:** The term "recombinant," when used in reference to a cell, nucleic acid, protein or vector, means that it has been modified from its native state. Thus, for example,

recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, or express native genes at different levels or under different conditions than found in nature. Recombinant nucleic acids differ from a native sequence by one or more nucleotides and/or are operably linked to heterologous sequences, e.g., a heterologous promoter in an expression vector. Recombinant proteins may differ from a native sequence by one or more amino acids and/or are fused with heterologous sequences. A vector comprising a nucleic acid encoding a polypeptide is a recombinant vector. The term "recombinant" is synonymous with "genetically modified" and "transgenic".

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 as the output of "longest identity" 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 6.6.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. In order for the Needle program to report the longest identity, the -nobrief option must be specified in the command line. The output of Needle labeled "longest identity" is calculated as follows:

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

For purposes of the present invention, the sequence identity between two polynucleotide sequences is determined as the output of "longest identity" using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 6.6.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. In order for the Needle program to report the longest identity, the nobrief option must be specified in the command line. The output of Needle labeled "longest identity" is calculated as follows:

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

The sequence identity between two polynucleotide sequences can be determined using the same Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 6.6.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EDNAFULL (EMBOSS

version of NCBI NUC4.4) substitution matrix. The percent sequence identity is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of the Alignment})$$

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

Variant: The term “variant” means a polypeptide having alpha-amylase activity comprising an alteration, e.g., a substitution, an insertion, and/or a 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 an amino acid adjacent to and immediately following the amino acid occupying a position. The variants of the present invention have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the alpha-amylase activity of the parent alpha-amylase. The variant alpha-amylases according to the invention has increased stability at pH 4.0 compared to a parent alpha-amylase, and wherein increased pH stability at pH 4.0 can be determined as % residual alpha-amylase activity (%RA) after incubation of the variant amylase at pH 4.0, 32 °C, for 18-24 hours. The Residual activity (RA%) after stress was calculated by dividing activity of stressed samples with unstressed samples and multiplying with 100. Alpha-amylase activity may e.g., be determined using the pNP-G7 alpha-amylase activity assay as described in the examples and in the material and methods section.

In one embodiment the parent alpha-amylase is preferably selected from polypeptide of SEQ ID NO: 1.

Raw Starch Material: The term “raw starch material” means primary starch-based grains, which has not been subjected to temperatures above 57°C for more than 10 minutes.

Raw Starch Hydrolysis (RSH): The term “raw starch hydrolysis” means the degradation of starch to polysaccharides from primary starch-based grains which has not been subjected to temperatures above 57°C for more than 10 minutes.

Raw Starch Hydrolysis and fermentation process: The term “raw starch hydrolysis and fermentation process” means the fermentation of starch to ethanol from primary starch-based grains which has not been subjected to temperatures above 57°C for more than 10 minutes.

Wild-type: The term “wild-type” in reference to an amino acid sequence or nucleic acid sequence means that the amino acid sequence or nucleic acid sequence is a native or naturally occurring sequence. As used herein, the term “naturally occurring” refers to anything (e.g., proteins, amino acids, or nucleic acid sequences) that is found in nature. Conversely, the term “non-naturally occurring” refers to anything that is not found in nature (e.g., recombinant nucleic

acids and protein sequences produced in the laboratory or modification of the wild- type sequence).

Conventions for Designation of Variants

For purposes of the present invention, the polypeptide disclosed in SEQ ID NO: 1 is used to determine the corresponding amino acid position in another alpha-amylase. The amino acid sequence of another alpha-amylase is aligned with the 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 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.

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.

Alterations as used herein includes substitutions, deletions and insertions as described below.

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

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

Insertions. For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly, the insertion of lysine after glycine at position 195 is designated "Gly195GlyLys" or "G195GK". An insertion of multiple amino acids is described as: 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

Variants

The present invention relates to variant alpha-amylases having increased pH stability at acidic pH, such as at pH 4.0 – 5.5 compared to a parent alpha-amylase. The parent alpha-amylase is in one embodiment the mature polypeptide disclosed herein as SEQ ID NO: 1. In other embodiments of the invention the parent polypeptide is selected from alpha-amylases disclosed herein as SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

The present invention relates to isolated alpha-amylase variants of a parent alpha-amylase, comprising an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

In a further particular embodiment the present invention relates to isolated alpha-amylase variants, comprising a substitution at one or more positions corresponding to positions 64, 96, 150, 179, 196, 199, 207, 222, 284 and 603 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

The variant alpha-amylases according to the invention have increased pH stability at pH 4.0 compared to a parent alpha-amylase, and wherein increased pH stability at pH 4.0 can be determined as % residual alpha-amylase activity (%RA) after incubation of the variant amylase at pH 4.0, 32 °C, for 18-24 hours. The Residual activity (RA%) after stress was calculated by dividing activity of stressed samples with unstressed samples and multiplying with 100. Alpha-amylase activity may e.g., be determined using the pNP-G7 alpha-amylase activity assay as described in the examples and in the material and methods section.

In particular the alterations are selected from alterations selected from the group consisting of: E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, V9*, V9D, V9L, T10*, T10I, A11*, S12*, S12P, S13*, V14*, V14I, K15*, N16*, N16S, N28R, N28W, R38H, R38Y, D39R, A43D, A43T, A43V, K54I, G56P, G56W, N57P, R64S, Y67T, Y67W, W68S, W68Y, Y70F, Q71E, Q71N, Q86R, K89R, D90E, A94D, E96H, E96K, G99N, K101R, I103Y, V107T, I108L, I108P, H110D, S113D, S113F, S113G, S113H, S113Q, S113W, S113Y, D114Q, A117T, N127D, Q134E, Q134L, Q134M, Q134N, Q134T, Q134W, W138Y, W142E, L150F, L150H, L150M, L150S, L150V, L150W, L150Y, G151F, G151S, G151W, G151Y, L152M, N156K, N156R, F169H, E171Q, L174I, D179G, D179S, Y183F, Y183I, D193S, D193DQ, D193DY, D193DQY, D193SQY, N196W, S199G, Q200W, N204D, I205Y, N207W, T208N, T208S, S209L, F212W, L218F, L218W, S221N, A222E, A222I, A222V, R224K, N233S, H241N, S245N, H259Y, S275L, S275N, T278N, T278W, T278Y, N281Q, N281S, D282P, D283*, D283A, D283P, E284Q, E285V, T308M, T308Y, R323K, S335K, S335Q, S335R, T348K, E359Y, A382T, S386D, S388W, N392R, N392W, S394K, K396S, Q412W, A414K, K417W, K417Y, A424P, A428S, Q457L, Q457R, T459M, A466V, Q479QP, L489Q, E511D, G533H, Y534H, Q542K, V543P, A545P, I547Y, K549*, K549Y, H550*, H550Y, D551*, G560P, A566P, N570H, M574MW, M574W, Y575W, T576Y, L577Y, T578Y, P580*, E581*, N582*, K589F, F592FK, V599W, N603W, P605S, D608Y, L614W, G619W, and H626* using SEQ ID NO: 1 for numbering or corresponding substitutions in another parent alpha-amylase.

In one embodiment alterations such as substitutions are selected from R64S, E96K, L150Y, L150W, L150H, L150M, L150F, D179S, N196W, S199G, N207W, A222E, A222I, A222V, E284Q and N603W using SEQ ID NO: 1 for numbering or corresponding substitutions in another parent alpha-amylase.

5 In case the parent alpha-amylase is different from SEQ ID NO: 1, the amino acid in a given position may be different from the amino acid present in the corresponding position in SEQ ID NO: 1. This, however, is understood to be within the scope of the present invention since the only essential feature is the amino acid in a given position after substitution.

10 In an embodiment, the variant has a sequence identity of 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%, to the amino acid sequence of the parent alpha-amylase.

15 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%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the polypeptide of SEQ ID NO: 1.

In one aspect, the number of alterations in the variants of the present invention is 1-20, *e.g.*, 1-10 and 1-5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 alterations.

20 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%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the polypeptide of SEQ ID NO: 2

25 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%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the polypeptide of SEQ ID NO: 3

30 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%, such as at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity to the polypeptide of SEQ ID NO: 4

In another aspect, a variant comprises a substitution, at one or more positions corresponding to positions 64, 96, 150, 179, 196, 199, 207, 222, 284 and 603 of the polypeptide of SEQ ID NO: 1.

35 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 196. In another aspect, the amino acid at a position corresponding to position 196 is substituted with Trp. In another aspect, the variant comprises or consists of the substitution N196W of the polypeptide of SEQ ID NO: 1.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 199. In another aspect, the amino acid at a position corresponding to position 199 is substituted with Gly. In another aspect, the variant comprises or consists of the substitution S199G of the polypeptide of SEQ ID NO: 1.

5 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 222. In another aspect, the amino acid at a position corresponding to position 222 is substituted with Val. In another aspect, the variant comprises or consists of the substitution A222V of the polypeptide of SEQ ID NO: 1.

10 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 222. In another aspect, the amino acid at a position corresponding to position 222 is substituted with Ile. In another aspect, the variant comprises or consists of the substitution A222I of the polypeptide of SEQ ID NO: 1.

15 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 222. In another aspect, the amino acid at a position corresponding to position 222 is substituted with Glu. In another aspect, the variant comprises or consists of the substitution A222E of the polypeptide of SEQ ID NO: 1.

20 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 207. In another aspect, the amino acid at a position corresponding to position 207 is substituted with Trp. In another aspect, the variant comprises or consists of the substitution N207W of the polypeptide of SEQ ID NO: 1.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 603. In another aspect, the amino acid at a position corresponding to position 603 is substituted with Trp. In another aspect, the variant comprises or consists of the substitution N603W of the polypeptide of SEQ ID NO: 1.

25 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 150. In another aspect, the amino acid at a position corresponding to position 150 is substituted with Tyr. In another aspect, the variant comprises or consists of the substitution L150Y of the polypeptide of SEQ ID NO: 1.

30 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 150. In another aspect, the amino acid at a position corresponding to position 150 is substituted with Trp. In another aspect, the variant comprises or consists of the substitution L150W of the polypeptide of SEQ ID NO: 1.

35 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 150. In another aspect, the amino acid at a position corresponding to position 150 is substituted with His. In another aspect, the variant comprises or consists of the substitution L150H of the polypeptide of SEQ ID NO: 1.

In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 150. In another aspect, the amino acid at a position corresponding to position 150 is substituted with Met. In another aspect, the variant comprises or consists of the substitution L150M of the polypeptide of SEQ ID NO: 1.

5 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 150. In another aspect, the amino acid at a position corresponding to position 150 is substituted with Phe. In another aspect, the variant comprises or consists of the substitution L150F of the polypeptide of SEQ ID NO: 1.

10 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 64. In another aspect, the amino acid at a position corresponding to position 64 is substituted with Ser. In another aspect, the variant comprises or consists of the substitution R64S of the polypeptide of SEQ ID NO: 1.

15 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 96. In another aspect, the amino acid at a position corresponding to position 96 is substituted with Lys. In another aspect, the variant comprises or consists of the substitution E96K of the polypeptide of SEQ ID NO: 1.

20 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 179. In another aspect, the amino acid at a position corresponding to position 179 is substituted with Ser. In another aspect, the variant comprises or consists of the substitution D179S of the polypeptide of SEQ ID NO: 1.

25 In another aspect, the variant comprises or consists of a substitution at a position corresponding to position 284. In another aspect, the amino acid at a position corresponding to position 284 is substituted with Gln. In another aspect, the variant comprises or consists of the substitution E284Q of the polypeptide of SEQ ID NO: 1.

30 In another aspect, the variant comprises or consists of one or more substitutions selected from the group consisting of R64S, E96K, L150Y, L150W, L150H, L150M, L150F, D179S, N196W, S199G, N207W, A222E, A222I, A222V, E284Q and N603W using SEQ ID NO: 1 for numbering.

In one specific embodiment the present invention relates to alpha-amylase variants, comprising a substitution at one or more positions corresponding to positions 150, 196, 199, 207, 222, and 603 of the polypeptide of SEQ ID NO: 1, comprising a substitution or a combination of substitutions selected from:

35 A222I;
A222V;
A222E;

S199G;
 N196W;
 N207W;
 N603W;
 5 L150Y;
 L150W;
 L150H;
 L150M;
 L150F;
 10 R64S;
 E96K;
 D179S;
 E284Q;
 N207W + N603W;
 15 N196W+N207W;
 N196W+N603W;
 N196W+N207W + N603W;
 A222I + S199G + N196W;
 A222V + S199G + N196W;
 20 A222E + S199G + N196W;
 A222V + S199G + N196W + L150Y;
 L150H + S199G + A222I;
 L150M + S199G + A222V;
 N196W + S199G + A222V + N603W;
 25 L150F + N196W + S199G + A222I;
 L150M + N196W + S199G + A222V;
 L150W + S199G + A222V;
 L150H + N196W + S199G + A222V;
 L150W + N196W + S199G + A222I;
 30 L150Y + S199G + A222V;
 E96K + D179S + N196W + S199G + A222V + E284Q;
 R64S + E96K + N196W + S199G + A222V;

wherein the variant has alpha-amylase activity and 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 95%, at
 35 least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100%
 sequence identity, to the polypeptide of SEQ ID NO: 1, and wherein the variant alpha-amylase

has increased pH stability at pH 4.0, 32 °C or 37°C compared to a the alpha-amylase disclosed as SEQ ID NO: 1.

In one specific embodiment the present invention relates to alpha-amylase variants, comprising a substitution at one or more positions corresponding to positions 150, 196, 199, 207, 222, and 603 of the polypeptide of SEQ ID NO: 1, comprising a substitution or a combination of substitutions selected from:

A222I;

A222V;

A222E;

S199G;

N196W;

N207W;

N603W;

L150Y;

L150W;

L150H;

L150M;

L150F;

R64S;

E96K;

D179S;

E284Q;N207W + N603W;

N196W+N207W;

N196W+N603W;

N196W+N207W + N603W;

A222I + S199G + N196W;

A222V + S199G + N196W;

A222E + S199G + N196W;

A222V + S199G + N196W + L150Y;

L150H + S199G + A222I;

L150M + S199G + A222V;

N196W + S199G + A222V + N603W;

L150F + N196W + S199G + A222I;

L150M + N196W + S199G + A222V;

L150W + S199G + A222V;

L150H + N196W + S199G + A222V;

L150W + N196W + S199G + A222I;

L150Y + S199G + A222V;

E96K + D179S + N196W + S199G + A222V + E284Q;

R64S + E96K + N196W + S199G + A222V;

wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 2, and wherein the variant alpha-amylase has increased pH stability at pH 4.0, 37°C compared to a the alpha-amylase disclosed as SEQ ID NO: 2.

In one specific embodiment the present invention relates to alpha-amylase variants, comprising a substitution at one or more positions corresponding to positions 150, 196, 199, 207, 222, and 603 of the polypeptide of SEQ ID NO: 1, comprising a substitution or a combination of substitutions selected from:

A222I;

A222V;

A222E;

S199G;

N196W;

N207W;

N603W;

L150Y;

L150W;

L150H;

L150M;

L150F;

R64S;

E96K;

D179S;

E284Q;

N207W + N603W;

N196W+N207W;

N196W+N603W;

N196W+N207W + N603W;

A222I + S199G + N196W;

A222V + S199G + N196W;

A222E + S199G + N196W;

A222V + S199G + N196W + L150Y;

L150H + S199G + A222I;
 L150M + S199G + A222V;
 N196W + S199G + A222V + N603W;
 L150F + N196W + S199G + A222I;
 5 L150M + N196W + S199G + A222V;
 L150W + S199G + A222V;
 L150H + N196W + S199G + A222V;
 L150W + N196W + S199G + A222I;
 L150Y + S199G + A222V;

10 E96K + D179S + N196W + S199G + A222V + E284Q;
 R64S + E96K + N196W + S199G + A222V;

wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100%
 15 sequence identity, to the polypeptide of SEQ ID NO: 3, and wherein the variant alpha-amylase has increased pH stability at pH 4.0, 37°C compared to a the alpha-amylase disclosed as SEQ ID NO: 3.

In one specific embodiment the present invention relates to alpha-amylase variants,
 20 comprising a substitution at one or more positions corresponding to positions 150, 196, 199, 207, 222, and 603 of the polypeptide of SEQ ID NO: 1, comprising a substitution or a combination of substitutions selected from:

A222I;
 A222V;
 25 A222E;
 S199G;
 N196W;
 N207W;
 N603W;
 30 L150Y;
 L150W;
 L150H;
 L150M;
 L150F;
 35 R64S;
 E96K;
 D179S;

E284Q;
 N207W + N603W;
 N196W+N207W;
 N196W+N603W;
 5 N196W+N207W + N603W;
 A222I + S199G + N196W;
 A222V + S199G + N196W;
 A222E + S199G + N196W;
 A222V + S199G + N196W + L150Y;
 10 L150H + S199G + A222I;
 L150M + S199G + A222V;
 N196W + S199G + A222V + N603W;
 L150F + N196W + S199G + A222I;
 L150M + N196W + S199G + A222V;
 15 L150W + S199G + A222V;
 L150H + N196W + S199G + A222V;
 L150W + N196W + S199G + A222I;
 L150Y + S199G + A222V;
 E96K + D179S + N196W + S199G + A222V + E284Q;
 20 R64S + E96K + N196W + S199G + A222V;

wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 4, and wherein the variant alpha-amylase
 25 has increased pH stability at pH 4.0, 37°C compared to a the alpha-amylase disclosed as SEQ ID NO: 4.

The variants of the invention have been selected based on improved pH 4 stability. More particularly the variants have increased pH stability at pH 4.0, 32 °C or 37°C, compared to a parent
 30 alpha-amylase particularly the alpha-amylase disclosed as SEQ ID NO: 1.

The increased pH stability at pH 4.0 can be determined as residual alpha-amylase activity after incubation of the variant amylase at pH 4.0, 32 °C or 37°C, for 18-24 hours and calculation of enzyme half-life in hours or % residual alpha-amylase activity. The increase in pH stability may
 35 in another embodiment be determined at pH 4.0, 32 °C, for 24 hours or 96 hours. Thus, in this embodiment the variant alpha-amylase of the invention has an improved property relative to the parent, wherein the improved property is increased pH stability at pH 4.0, 32°C compared to a

parent alpha-amylase particularly the alpha-amylase disclosed as SEQ ID NO: 1. Also in this case the improved stability may be calculated as enzyme half-life in hours.

In a particular aspect the half-life is increased compared to the amylase of SEQ ID NO: 1 of at least a factor 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, such as at least 8.0.

It is well known in the art that possessing of the signal peptide may result in a distribution of different forms of mature polypeptides, thus in one embodiment the mature polypeptide will start from other positions than position 1 of SEQ ID NO: 1. E.g., the alpha-amylase variants of the present invention may comprise an N-terminal deletion, more particularly comprising at least amino acids 11 to 626 of SEQ ID NO: 1, at least amino acids 12 to 626 of SEQ ID NO: 1, such as at least amino acids 13 to 626 of SEQ ID NO: 1.

The variants of the invention may also comprise C-terminal deletions. E.g., in one embodiment the alpha-amylase variants of the invention comprise a C-terminal deletion, particularly H626*.

More particularly, the variant alpha-amylases, may comprise combinations of substitutions selected from:

S199G + H626*;

N196W + S199G + A222V + H626*;

N196W + H626*

N196W + N207W + H626*;

L150Y + N196W + S199G + A222V

E96K + D179S + N196W + S199G + A222V + E284Q;

R64S + E96K + N196W + S199G + A222V;

and wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

In another particular embodiment the variant alpha-amylase of the invention, comprising an alteration or a combination of alterations selected from:

N28W;

N196W;

S199G;

N196W+ V599W;

N196W+ H550Y+ P605S;

- N196W+ A545P+ T576Y;
N196W+ K549Y+ G560P;
I108P+ Y183I+ N196W+ I205Y;
N196W+ R323K;
5 N196W+ D283P;
W138Y+ N196W;
L150W;
N196W+ N392W+ K417W;
N196W+ N392R+ K417W;
10 N196W+ K549*+ H550*+ D551*;
N196W+ P580*+ E581*+ N582*;
N196W+ F592FK;
N28W+ N196W+ N207W+ S386D+ N603W;
R38Y+ N196W;
15 N196W+ H259Y;
N196W+ Q412W;
N196W+ F212W;
N196W+ V599W;
N196W+ H550Y+ P605S;
20 N196W+ H550Y+ K589F;
N196W+ H550Y+ D608Y;
N196W+ M574W+ L614W;
N196W+ G533H+ M574W+ L614W;
N196W+ V543P+ N570H;
25 N196W+ G533H+ Y575W+ L614W;
N196W+ A545P+ T576Y;
N196W+ A566P+ T578Y;
N196W+ K549Y+ G560P;
N196W+ A566P+ L577Y;
30 N196W+ I547Y+ G560P;
N196W+ M574MW;
N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK;
N196W+ L614W+ G619W;
N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W;
35 N28W+ I108P+ N196W+ N207W+ S386D+ N603W;
N196W+ D282P+ D283*;
W138Y+ N196W;

- N28W+ N196W+ N207W+ S386D+ N603W;
N196W+ S388W+ A424P;
N196W+ S388W+ A424P+ L489Q;
A117T+ N196W+ H550Y+ D608Y;
5 N196W+ Q457R+ Y575W+ L614W;
N196W+ S199G;
N196W+ A222V;
N196W+ A222E;
N196W+ A222I;
10 S199G+ A222V;
S199G+ A222E;
S199G+ A222I;
N196W+ S199G+ A222I;
Q134L;
15 L150W+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156K+ N196W+ S199G+ A222I;
L150W+ N196W+ S199G+ A222I+ A428S;
L150F+ N156K+ N196W+ S199G+ A222I;
L150Y+ N156R+ N196W+ A222V;
20 L150M+ N156R+ N196W+ S199G+ A222I;
L150M+ N156R+ N196W+ A222V;
L150Y+ N156R+ N196W+ S199G+ A222V;
L150M+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W+ A222I;
25 L150H+ N156R+ N196W+ S199G+ A222I;
L150H+ N156K+ N196W+ A222V;
L150W+ N156R+ N196W+ A222I;
L150F+ N156R+ N196W+ A222I;
L150F+ N156K+ N196W+ S199G+ A222V;
30 L150H+ N156K+ N196W+ S199G+ A222V;
L150F+ N156R+ S199G+ A222I;
L150M+ N156K+ N196W+ A222V;
L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
35 L150Y+ N156R+ S199G+ A222I;
L150M+ N156R+ S199G+ A222I;
L150W+ N156K+ S199G+ A222V;

- L150W+ N156R+ N196W+ S199G+ A222V;
 L150Y+ N156R+ N196W;
 N156K+ N196W+ A222V;
 N156K+ N196W+ S199G;
 5 N156R+ S199G+ A222V;
 S113H+ N196W+ S199G+ A222V;
 Q71E+ S113H+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ D283A;
 N196W+ S199G+ A222V+ D283P;
 10 W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 S113Q+ Q134E+ N196W+ S199G+ A222V;
 15 S113D+ Q134N+ N196W+ S199G+ A222V;
 S113F+ N196W+ S199G+ A222V;
 E171Q+ N196W+ S199G+ N204D+ A222V;
 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
 N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
 20 N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 V107T+ H110D+ N196W+ S199G+ A222V;
 Q134T+ L150Y+ N196W+ S199G+ A222V;
 25 S113F+ L150W+ N196W+ S199G+ A222V;
 S113F+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 30 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 N28R+ Q86R+ N196W+ S199G+ A222V;
 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
 35 E96K+ N196W+ S199G+ A222V;
 T10I+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V;

- R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ N196W+ S199G+ A222V;
 D39R+ E96K+ N196W+ S199G+ A222V;
 5 R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
 R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 10 T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 E96K+ N196W+ S199G+ A222V+ E284Q;
 T10I+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q;
 15 R64S+ N196W+ S199G+ A222V+ E284Q;
 T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
 D193SQY+ N196W+ S199G+ A222V;
 Q134T+ N196W+ S199G+ A222V;
 L174I+ N196W+ S199G+ T208N+ A222V;
 20 Y183F+ N196W+ S199G+ T208S+ A222V;
 N127D+ N156R+ N196W+ S199G+ A222V;
 Q134T+ L150W+ N196W+ S199G+ A222V;
 N57P+ N196W+ S199G+ A222V;
 N196W+ S199G+ Q200W+ A222V;
 25 T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
 T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
 D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
 T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
 30 T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;
 D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
 D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
 R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
 D39R+ R64S+ N196W+ S199G+ A222V;
 35 S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;
 S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
 V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;

- S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
 N28W+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N392W+ K417W;
 T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
- 5 V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
 S113F+ L150Y+ N156K+ N196W+ S199G+ A222V;
 S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V;
 S113W+ L150Y+ N156K+ N196W+ S199G+ A222V;
 S113F+ N156K+ N196W+ S199G+ A222V;
- 10 S113Y+ N156K+ N196W+ S199G+ A222V;
 S113W+ N156K+ N196W+ S199G+ A222V;
 W138Y+ L150V+ N196W+ S199G+ A222V;
 W138Y+ L150V+ D179G+ N196W+ S199G+ A222V;
 W138Y+ L150V+ N196W+ S199G+ L218W+ A222V;
- 15 E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q;
 E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V;
- 20 R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
 R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
 L150F+ N196W+ S199G+ A222I;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222I;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;
- 25 Q134L+ L150M+ N156K+ N196W+ S199G+ A222I;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L;
 Q134M+ L150W+ N156K+ N196W+ S199G+ A222I;
 Q134W+ L150W+ N156K+ N196W+ S199G+ A222I;
 L150W+ L152M+ N156K+ N196W+ S199G+ A222I;
- 30 S113F+ L150W+ N156K+ N196W+ S199G+ A222I;
 S113Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151W+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
 S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
- 35 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;

- L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;
 S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222I;
 5 L150W+ G151F+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151Y+ N156K+ N196W+ S199G+ A222I;
 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T;
 10 K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V;
 K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V;
 Q134L+ N196W+ S199G+ A222V;
 V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V;
 15 N196W+ S199G+ A222V+ N392W+ K417W;
 V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
 E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q;
 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150Y+ N196W+ S199G+ A222V;
 20 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150F+ N156R+ N196W+ S199G+ A222V;
 L150H+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222V;
 25 Q134L+ L150F+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
 L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
 30 Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V;
 L150Y+ L152M+ N156K+ N196W+ S199G+ A222V;
 L150S+ N196W+ S199G+ A222V;
 35 N196W+ S199G+ A222V+ N281S;
 Y67T+ N196W+ S199G+ A222V;
 Q71N+ N196W+ S199G+ A222V;

- Q71N+ A94D+ N196W+ S199G+ A222V;
 N196W+ S199G+ L218F+ A222V;
 N196W+ S199G+ L218W+ A222V;
 N196W+ S199G+ A222V+ T278W;
 5 N196W+ S199G+ A222V+ T278W+ T459M;
 N196W+ S199G+ A222V+ T278Y;
 N196W+ S199G+ A222V+ S275N;
 N196W+ S199G+ A222V+ S275L;
 N196W+ S199G+ A222V+ S335Q;
 10 N196W+ S199G+ A222V+ S335K;
 N196W+ S199G+ A222V+ S335R;
 N196W+ S199G+ L218W+ A222V+ S335K;
 N196W+ S199G+ L218W+ A222V+ S335Q;
 Y67W+ N196W+ S199G+ A222V;
 15 N196W+ S199G+ A222V+ N281Q;
 L150M+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150M+ N156K+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
 20 D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
 D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 25 N196W+ N207W;
 N196W+ N207W+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
 L150Y+ N196W+ S199G+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
 30 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
 S199G+ T208N+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;
 35 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+
 N207W+ A222I+ E284Q;
 E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
 R64S+ E96K+ N196W+ S199G+ N207W+ A222V;
 N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q;

5 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

L150Y+ N196W+ S199G+ N207W+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;

10 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
 S199G+ N207W+ T208N+ A222V+ E284Q;

Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

15 R64S+ E96K+ N196W+ S199G+ A222V;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

and wherein the variant has alpha-amylase activity and 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

20 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than
 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or
 SEQ ID NO: 4, and wherein the variant has increased pH stability at pH 4.0, 32°C compared to
 a parent alpha-amylase particularly the alpha-amylase disclosed as SEQ ID NO: 1, and optionally
 the variant further comprises the C-terminal deletion H626*.

25

In another particular embodiment the alpha-amylase variants of the invention comprise a
 combination of alterations selected from:

N28W+ H626*;

S199G+ H626*;

30 N196W+ V599W+ H626*;

N196W+ H550Y+ P605S+ H626*;

N196W+ A545P+ T576Y+ H626*;

N196W+ K549Y+ G560P+ H626*;

I108P+ Y183I+ N196W+ I205Y+ H626*;

35 N196W+ R323K+ H626*;

N196W+ D283P+ H626*;

W138Y+ N196W+ H626*;

- L150W+ H626*;
 N196W+ N392W+ K417W+ H626*;
 N196W+ N392R+ K417W+ H626*;
 N196W+ K549*+ H550*+ D551*+ H626*;
 5 N196W+ P580*+ E581*+ N582*+ H626*;
 N196W+ F592FK+ H626*;
 N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
 R38Y+ N196W+ H626*;
 N196W+ H259Y+ H626*;
 10 N196W+ Q412W+ H626*;
 N196W+ F212W+ H626*;
 N196W+ V599W+ H626*;
 N196W+ H550Y+ P605S+ H626*;
 N196W+ H550Y+ K589F+ H626*;
 15 N196W+ H550Y+ D608Y+ H626*;
 N196W+ M574W+ L614W+ H626*;
 N196W+ G533H+ M574W+ L614W+ H626*;
 N196W+ V543P+ N570H+ H626*;
 N196W+ G533H+ Y575W+ L614W+ H626*;
 20 N196W+ A545P+ T576Y+ H626*;
 N196W+ A566P+ T578Y+ H626*;
 N196W+ K549Y+ G560P+ H626*;
 N196W+ A566P+ L577Y+ H626*;
 N196W+ I547Y+ G560P+ H626*;
 25 N196W+ M574MW+ H626*;
 N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK + H626*;
 N196W+ L614W+ G619W+ H626*;
 N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W+ H626*;
 N28W+ I108P+ N196W+ N207W+ S386D+ N603W+ H626*;
 30 N196W+ D282P+ D283*+ H626*;
 W138Y+ N196W+ H626*;
 N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
 N196W+ S388W+ A424P+ H626*;
 N196W+ S388W+ A424P+ L489Q+ H626*;
 35 A117T+ N196W+ H550Y+ D608Y+ H626*;
 N196W+ Q457R+ Y575W+ L614W+ H626*;
 N196W+ S199G;

- N196W+ A222V;
N196W+ A222E;
N196W+ A222I;
S199G+ A222V;
5 S199G+ A222E;
S199G+ A222I;
N196W+ S199G+ A222I;
Q134L+ H626*;
L150W+ N156K+ N196W+ S199G+ A222V;
10 L150Y+ N156K+ N196W+ S199G+ A222I;
L150W+ N196W+ S199G+ A222I+ A428S;
L150F+ N156K+ N196W+ S199G+ A222I;
L150Y+ N156R+ N196W+ A222V;
L150M+ N156R+ N196W+ S199G+ A222I;
15 L150M+ N156R+ N196W+ A222V;
L150Y+ N156R+ N196W+ S199G+ A222V;
L150M+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W+ A222I;
L150H+ N156R+ N196W+ S199G+ A222I;
20 L150H+ N156K+ N196W+ A222V;
L150W+ N156R+ N196W+ A222I;
L150F+ N156R+ N196W+ A222I;
L150F+ N156K+ N196W+ S199G+ A222V;
L150H+ N156K+ N196W+ S199G+ A222V;
25 L150F+ N156R+ S199G+ A222I;
L150M+ N156K+ N196W+ A222V;
L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ S199G+ A222I;
30 L150M+ N156R+ S199G+ A222I;
L150W+ N156K+ S199G+ A222V;
L150W+ N156R+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W;
N156K+ N196W+ A222V;
35 N156K+ N196W+ S199G;
N156R+ S199G+ A222V;
S113H+ N196W+ S199G+ A222V;

- Q71E+ S113H+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ D283A;
 N196W+ S199G+ A222V+ D283P;
 W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
- 5 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 S113Q+ Q134E+ N196W+ S199G+ A222V;
 S113D+ Q134N+ N196W+ S199G+ A222V;
- 10 S113F+ N196W+ S199G+ A222V;
 E171Q+ N196W+ S199G+ N204D+ A222V;
 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
 N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
 N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
- 15 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 V107T+ H110D+ N196W+ S199G+ A222V;
 Q134T+ L150Y+ N196W+ S199G+ A222V;
 S113F+ L150W+ N196W+ S199G+ A222V;
- 20 S113F+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
- 25 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 N28R+ Q86R+ N196W+ S199G+ A222V;
 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
 E96K+ N196W+ S199G+ A222V;
- 30 T10I+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V;
 R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ N196W+ S199G+ A222V;
- 35 D39R+ E96K+ N196W+ S199G+ A222V;
 R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
 R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;

- R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
5 R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ N196W+ S199G+ A222V+ E284Q;
D39R+ N196W+ S199G+ A222V+ E284Q;
R64S+ N196W+ S199G+ A222V+ E284Q;
10 T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
D193SQY+ N196W+ S199G+ A222V;
Q134T+ N196W+ S199G+ A222V;
L174I+ N196W+ S199G+ T208N+ A222V;
Y183F+ N196W+ S199G+ T208S+ A222V;
15 N127D+ N156R+ N196W+ S199G+ A222V;
Q134T+ L150W+ N196W+ S199G+ A222V;
N57P+ N196W+ S199G+ A222V;
N196W+ S199G+ Q200W+ A222V;
T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
20 T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;
25 D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;
30 S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
N28W+ N196W+ S199G+ A222V;
N196W+ S199G+ A222V+ N392W+ K417W;
35 T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
S113F+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;

- S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113F+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 5 S113W+ N156K+ N196W+ S199G+ A222V+ H626*;
 W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
 W138Y+ L150V+ D179G+ N196W+ S199G+ A222V+ H626*;
 W138Y+ L150V+ N196W+ S199G+ L218W+ A222V+ H626*;
 E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 10 E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+
 15 H626*;
 R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ H626*;
 L150F+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ H626*;
 20 Q134L+ L150M+ N156K+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L+ H626*;
 Q134M+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 Q134W+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ L152M+ N156K+ N196W+ S199G+ A222I+ H626*;
 25 S113F+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 S113Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;
 S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;
 30 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;
 35 S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151F+ N156K+ N196W+ S199G+ A222I+ H626*;

- L150W+ G151Y+ N156K+ N196W+ S199G+ A222I+ H626*;
Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T+ H626*;
5 K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V+ H626*;
K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V+ H626*;
K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
Q134L+ N196W+ S199G+ A222V+ H626*;
V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V+ H626*;
10 N196W+ S199G+ A222V+ N392W+ K417W+ H626*;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K+ H626*;
E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q+ H626*;
Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
L150Y+ N196W+ S199G+ A222V+ H626*;
15 Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
20 Q134L+ L150F+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150H+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
25 Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V+ H626*;
L150Y+ L152M+ N156K+ N196W+ S199G+ A222V+ H626*;
L150S+ N196W+ S199G+ A222V+ H626*;
30 N196W+ S199G+ A222V+ N281S+ H626*;
Y67T+ N196W+ S199G+ A222V+ H626*;
Q71N+ N196W+ S199G+ A222V+ H626*;
Q71N+ A94D+ N196W+ S199G+ A222V+ H626*;
N196W+ S199G+ L218F+ A222V+ H626*;
35 N196W+ S199G+ L218W+ A222V+ H626*;
N196W+ S199G+ A222V+ T278W+ H626*;
N196W+ S199G+ A222V+ T278W+ T459M+ H626*;

- N196W+ S199G+ A222V+ T278Y+ H626*;
 N196W+ S199G+ A222V+ S275N+ H626*;
 N196W+ S199G+ A222V+ S275L+ H626*;
 N196W+ S199G+ A222V+ S335Q+ H626*;
 5 N196W+ S199G+ A222V+ S335K+ H626*;
 N196W+ S199G+ A222V+ S335R+ H626*;
 N196W+ S199G+ L218W+ A222V+ S335K+ H626*;
 N196W+ S199G+ L218W+ A222V+ S335Q+ H626*;
 Y67W+ N196W+ S199G+ A222V+ H626*;
 10 N196W+ S199G+ A222V+ N281Q+ H626*;
 L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150M+ N156K+ N196W+ S199G+ A222V+ H626*;
 D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
 15 D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
 D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q+
 20 H626*;
 N196W+ N207W;
 N196W+ N207W+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
 L150Y+ N196W+ S199G+ A222V+ H626*;
 25 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
 S199G+ T208N+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q+ H626*;
 30 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+
 H626*;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+
 N207W+ A222I+ E284Q;
 E96K+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 35 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
 R64S+ E96K+ N196W+ S199G+ N207W+ A222V;
 N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

D39R+ N196W+ S199G+ A222V+ E284Q+ H626*;

D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

5 L150Y+ N196W+ S199G+ N207W+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ H626*;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V+ E284Q+ H626*;

10 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

R64S+ E96K+ N196W+ S199G+ A222V+ H626*;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

15 and wherein the variant has alpha-amylase activity and 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
95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than
100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or
SEQ ID NO: 4, and wherein the variant has increased pH stability at pH 4.0, 32°C compared to
20 a parent alpha-amylase particularly the alpha-amylase disclosed as SEQ ID NO: 1.

The variants of the invention may in a particular embodiment, further comprise a substitution corresponding to K8N. More particularly when expressing the variants in a yeast host cell.

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

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 variant polypeptide of the invention 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.

E.g., in one embodiment the catalytic domain of the variant alpha-amylases according to the invention may be fused to a Carbohydrate Binding Module (CBM) from another enzyme thus forming a hybrid alpha-amylase, wherein the catalytic core and the CBM are heterologous meaning that they do not occur in nature and that the CBM is foreign or heterologous to the catalytic domain.

Therefore, a further embodiment of the invention relates to a variant catalytic domain fragment, comprising a catalytic domain corresponding to at least amino acids 12-438 of SEQ ID NO: 1, preferably amino acids 1-438 of SEQ ID NO: 1, wherein optionally the linker and/or a carbohydrate binding module, CBM, has been replaced with a heterologous CBM.

Heterologous meaning that the catalytic core and the CBM is not naturally occurring in the same polypeptide or enzyme.

In one embodiment, the CBM comprises amino acids 527-626 of SEQ ID NO: 1, and amino acids 439-526 comprises a linker region.

In a further embodiment, the heterologous CBM is selected from a CBM belonging to Family 20, 21, 25, 26, 34, 41 or 48. Preferably, the CBM is a Family 20 CBM.

In one particular embodiment the CBM is selected from the group consisting of:

i) a polypeptide of SEQ ID NO: 14, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 14;

ii) a polypeptide of SEQ ID NO: 15, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 15;

iii) a polypeptide of SEQ ID NO: 16, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 16;

iv) a polypeptide of SEQ ID NO: 17, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 17;

v) a polypeptide of SEQ ID NO: 18, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 18; and

vi) a polypeptide of SEQ ID NO: 19, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 19.

It is well known in the art that linker regions may differ in length among different alpha-amylases and glucoamylases, and it is common that the length may vary in the range from about 1 amino acid to about 100 amino acids. Thus, in one embodiment the linker is selected to be in the range from 1-100 amino acids.

In an embodiment, the variant has increased pH stability at pH 4, 37°C, compared to the parent enzyme. In another embodiment the variant has increased pH stability at pH 4.0, 32°C, compared to the parent enzyme.

Parent alpha-amylases

The parent alpha-amylase may in a preferred embodiment be a polypeptide having at least 60% sequence identity to the polypeptide of SEQ ID NO: 1.

In an aspect, the parent has a sequence identity to the polypeptide of SEQ ID NO: 1 of 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%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by up to 10 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, from the polypeptide of SEQ ID NO: 1. In another aspect, the parent comprises or consists

of the amino acid sequence of SEQ ID NO: 1. In another aspect, the parent is a fragment of the polypeptide of SEQ ID NO: 1 containing at least the catalytic domain.

The parent alpha-amylase may be a polypeptide having at least 60% sequence identity to the polypeptide of SEQ ID NO: 2.

5 In an aspect, the parent has a sequence identity to the polypeptide of SEQ ID NO: 1 of 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%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by up to 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9,
10 or 10, from the polypeptide of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent is a fragment of the polypeptide of SEQ ID NO: 2 containing at least the catalytic domain.

The parent alpha-amylase may be a polypeptide having at least 60% sequence identity to the polypeptide of SEQ ID NO: 3.

15 In an aspect, the parent has a sequence identity to the polypeptide of SEQ ID NO: 1 of 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%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by up to 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9,
20 or 10, from the polypeptide of SEQ ID NO: 3. In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 3. In another aspect, the parent is a fragment of the polypeptide of SEQ ID NO: 3 containing at least the catalytic domain.

The parent alpha-amylase may be a polypeptide having at least 60% sequence identity to the polypeptide of SEQ ID NO: 4.

25 In an aspect, the parent has a sequence identity to the polypeptide of SEQ ID NO: 1 of 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%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by up to 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9,
30 or 10, from the polypeptide of SEQ ID NO: 4. In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 4. In another aspect, the parent is a fragment of the polypeptide of SEQ ID NO: 4 containing at least the catalytic domain.

35 The parent polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide. *E.g.*, in one embodiment the catalytic domain of the variant alpha-amylases according to the invention may be fused to a Carbohydrate Binding Module (CBM) from another enzyme thus forming a hybrid

alpha-amylase, wherein the catalytic core and the CBM are heterologous meaning that they do not occur in nature and that the CBM is foreign or heterologous to the catalytic domain.

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 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 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.

The parent may be obtained from bacteria of the genus *Bacillus*. 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 extracellularly.

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

In one particular aspect, the parent is a *Bacillus licheniformis*.

In another aspect, the parent is a *Bacillus amyloliquefaciens* alpha-amylase, e.g., the alpha-amylase of SEQ ID NO: 1.

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

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 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*).

Preparation of Variants

The present invention also relates to methods for obtaining a variant having an increased stability at pH 4, 32 °C or 37°C, and having alpha-amylase activity, comprising: (a) introducing into a parent alpha-amylase an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1 (or in another related parent alpha-amylase), wherein the variant has alpha-amylase activity; and (b) recovering the variant.

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

Site-directed mutagenesis is a technique in which one or more 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.

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

10 Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, 15 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 20 (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

Semi-synthetic gene construction is accomplished by combining aspects of synthetic 25 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, while yet other regions may be subjected to error-prone PCR or non-error prone PCR 30 amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

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

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

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

The control sequence may be a promoter, a polynucleotide recognized by a host cell for expression of a polynucleotide encoding a variant of the present invention. 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 V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor, 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. Other promoters are described in U.S. Patent No. 6,011,147.

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 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 in the present invention.

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

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, *Fusarium oxysporum* trypsin-like protease, *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 V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor.

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.

5 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).

10 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 is operably linked to the 5'-terminus of the polynucleotide encoding the variant. Any leader that is functional in the host cell may be used.

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

15 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).

20 The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide 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.

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

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

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Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

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

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

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

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

It may also be desirable to add regulatory sequences that regulate expression of the variant relative to the growth of the host cell. Examples of regulatory sequences 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 sequences in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter, *Trichoderma reesei* cellobiohydrolase I promoter, and *Trichoderma reesei* cellobiohydrolase II 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 to the regulatory sequence.

Expression Vectors

5 The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant of the present invention, a promoter, and transcriptional and 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.

10 Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

15 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.

20 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
25 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.

30 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.

35 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, *adeA* (phosphoribosylaminoimidazole-succinocarboxamide synthase), *adeB* (phosphoribosylaminoimidazole synthase), *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. Preferred for use in a *Trichoderma* cell are *adeA*, *adeB*, *amdS*, *hph*, and *pyrG* genes.

The selectable marker may be a dual selectable marker system as described in WO 2010/039889. In one aspect, the dual selectable marker is a *hph-tk* dual selectable marker system.

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

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

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

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

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

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175;

WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

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

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

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.

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*. 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 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.

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

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

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

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

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

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*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *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 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) cultivating a recombinant host cell of the present invention under conditions conducive for production of the variant; and optionally (b) recovering the variant.

The recombinant host cells are cultivated in a nutrient medium suitable for production of the variant using methods known in the art. For example, the cells 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 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 variants may be detected using methods known in the art that are specific for the variants. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the variant.

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

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.

Fermentation Broth Formulations or Cell Compositions

The present invention also relates to a fermentation broth formulation or a cell composition comprising a variant of the present invention. The fermentation broth product further comprises additional ingredients used in the fermentation process, such as, for example, cells (including, the host cells containing the gene encoding the variant of the present invention which are used to produce the variant of interest), cell debris, biomass, fermentation media and/or fermentation products. In some embodiments, the composition is a cell-killed whole broth containing organic acid(s), killed cells and/or cell debris, and culture medium.

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

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

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

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

The cell-killed whole broth or composition may contain the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the cell-killed whole broth or composition contains the spent culture medium and cell debris present after the microbial cells (e.g., filamentous fungal cells) are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis. In some embodiments, the cell-killed whole broth or composition

contains the spent cell culture medium, extracellular enzymes, and killed filamentous fungal cells. In some embodiments, the microbial cells present in the cell-killed whole broth or composition can be permeabilized and/or lysed using methods known in the art.

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

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

Enzyme Compositions

The present invention also relates to compositions comprising a variant of the present invention. Preferably, the compositions are enriched in such a variant. The term "enriched" indicates that the alpha-amylase activity of the composition has been increased, *e.g.*, with an enrichment factor of at least 1.1.

The compositions may comprise a variant of the present invention as the major enzymatic component, *e.g.*, a mono-component composition. Alternatively, the compositions may comprise multiple enzymatic activities, such as one or more enzymes selected from the group consisting of hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an alpha-galactosidase, alpha-glucosidase, aminopeptidase, amylase, beta-galactosidase, beta-glucosidase, beta-xylosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, glucoamylase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase.

In a particular embodiment, the composition comprises the variant alpha-amylase of the invention and a glucoamylase.

In another embodiment, the composition comprises the variant alpha-amylase of the invention and another alpha-amylase.

In another embodiment, the composition comprises the variant alpha-amylase of the invention, a glucoamylase, and another alpha-amylase.

In an embodiment the other alpha-amylase is of fungal or bacterial origin. In a preferred embodiment the other alpha-amylase is a fungal acid stable alpha-amylase. A fungal acid stable alpha-amylase is an alpha-amylase that has activity in the pH range of 3.0 to 7.0 and preferably in the pH range from 3.5 to 6.5, including activity at a pH of about 4.0, 4.5, 5.0, 5.5, and 6.0.

In a preferred embodiment the other alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Rhizomucor*, preferably

a strain the *Rhizomucor pusillus*, such as one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-binding domain, such as the one shown in SEQ ID NO: 12 herein, or a variant thereof.

In an embodiment the other alpha-amylase is selected from the group consisting of:

- (i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 12 herein;
- (ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 12 herein.

In a preferred embodiment the other alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 12 having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H + Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C (using SEQ ID NO: 12 for numbering).

In an embodiment the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 12 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 12 for numbering).

In an embodiment the other alpha-amylase variant present and/or added in saccharification and/or fermentation has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the polypeptide of SEQ ID NO: 12 herein.

In an embodiment, the glucoamylase comprised in the composition is of fungal origin, preferably from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii* or a strain of *Trametes*, preferably *T. cingulata*, or a strain of *Pycnoporus*, preferable *P. sanguineus*, or a strain of *Gloeophyllum*, such as *G. serpiarium* or *G. trabeum*, or a strain of the *Nigrofores*.

In an embodiment the glucoamylase is derived from *Trametes*, such as a strain of *Trametes cingulata*, such as the one shown in SEQ ID NO: 7 herein.

In an embodiment the glucoamylase is selected from the group consisting of:

- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 7 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 7 herein.

5 In an embodiment the glucoamylase is derived from *Talaromyces*, such as a strain of *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 8 herein.

In an embodiment the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 8 herein;

10 (ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 8 herein.

15 Glucoamylases may in an embodiment be added to the saccharification and/or fermentation in an amount of 0.0001-20 AGU/g DS, preferably 0.001-10 AGU/g DS, especially between 0.01-5 AGU/g DS, such as 0.1-2 AGU/g DS.

20 Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U, SPIRIZYME™ ULTRA, SPIRIZYME™ EXCEL and AMG™ E (from Novozymes A/S); OPTIDEX™ 300, GC480, GC417 (from DuPont.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from DuPont).

In addition to a glucoamylase the composition may further comprise a protease. In particular an endoprotease of family S53, more particular an S53 protease derived from *Meripilus giganteus*.

25 In a preferred embodiment, the ratio between glucoamylase and alpha-amylase present and/or added during saccharification and/or fermentation may preferably be in the range from 500:1 to 1:1, such as from 250:1 to 1:1, such as from 100:1 to 1:1, such as from 100:2 to 100:50, such as from 100:3 to 100:70.

30 The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the composition may be in the form of granulate or microgranulate. The variant may be stabilized in accordance with methods known in the art.

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

35 The enzyme composition of the present invention may be in any form suitable for use, such as, for example, a crude fermentation broth with or without cells removed, a cell lysate with

or without cellular debris, a semi-purified or purified enzyme composition, or a host cell, as a source of the enzymes.

The enzyme composition may be a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a stabilized protected enzyme. Liquid enzyme compositions may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, and/or lactic acid or another organic acid according to established processes.

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

Methods of using the alpha-amylase of the invention - Industrial Applications

The variant alpha-amylases of the present invention possess valuable properties allowing for a variety of industrial applications. In particular, the alpha-amylases may be used in ethanol production, and starch conversion processes.

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

In one embodiment, the present invention relates to a use of the alpha-amylase according to the invention in a saccharification process, particularly a simultaneous saccharification and fermentation process.

Starch Processing

Native starch consists of microscopic granules, which are insoluble in water at room temperature. When aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. At temperatures up to about 50°C to 75°C the swelling may be reversible. However, with higher temperatures an irreversible swelling called "gelatinization" begins. During this "gelatinization" process there is a dramatic increase in viscosity. Granular starch to be processed may be a highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5% pure or it may be a more crude starch-containing materials comprising (e.g., milled) whole grains including non-starch fractions such as germ residues and fibers. The raw material, such as whole grains, may be reduced in particle size, e.g., by milling, in order to open up the structure and allowing for further processing. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolyzate is used in the production of, e.g., syrups. Both dry and wet milling is well known in the art of starch

processing and may be used in a process of the invention. Methods for reducing the particle size of the starch containing material are well known to those skilled in the art.

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.

Liquefaction is carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase and/or acid fungal alpha-amylase. In an embodiment, a phytase is also present during liquefaction. In an embodiment, viscosity reducing enzymes such as a xylanase and/or beta-glucanase is also present during liquefaction.

During liquefaction, the long-chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. Liquefaction may be carried out as a three-step hot slurry process. The slurry is heated to between 60-95°C (e.g., 70-90°C, such as 77-86°C, 80-85°C, 83-85°C) and an alpha-amylase is added to initiate liquefaction (thinning).

The slurry may in an embodiment be jet-cooked at between 95-140°C, e.g., 105-125°C, for about 1-15 minutes, e.g., about 3-10 minutes, especially around 5 minutes. The slurry is then cooled to 60-95°C and more alpha-amylase is added to obtain final hydrolysis (secondary liquefaction). The jet-cooking process is carried out at pH 4.5-6.5, typically at a pH between 5 and 6. The alpha-amylase may be added as a single dose, e.g., before jet cooking, or be partly dosed before jet cooking and partly dosed after.

The liquefaction process is carried out at between 70-95°C, such as 80-90°C, such as around 85°C, for about 10 minutes to 5 hours, typically for 1-2 hours. The pH is between 4 and 7, such as between 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, calcium may optionally be added (to provide 1-60 ppm free calcium ions, such as about 40 ppm free calcium ions). After such treatment, the liquefied starch will typically have a "dextrose equivalent" (DE) of 10-16.

Generally, liquefaction and liquefaction conditions are well known in the art.

Alpha-amylases for use in liquefaction are preferably bacterial acid stable alpha-amylases. Particularly the alpha-amylase is from a *Cytophaga* sp., *Exiguobacterium* sp. or a *Bacillus* sp. such as e.g., *Bacillus stearothermophilus* or *Bacillus licheniformis*.

In an embodiment the alpha-amylase is from the genus *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in SEQ ID NO: 3 in WO 99/019467 or SEQ ID NO: 9 herein.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a double deletion of two amino acids in the region from position 179 to 182, more particularly a double deletion at positions I181 + G182, R179 + G180, G180 + I181, R179 + I181, or G180 + G182, preferably I181 + G182, and optionally a N193F substitution, (using SEQ ID NO: 9 for numbering).

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

In an embodiment the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations:

- I181*+G182*+N193F+E129V+K177L+R179E;
- I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V;
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S; and
- V59A +E129V +K177L +R179S +I181* +G182* +A184Q +E188P +T191N +N193F +V212T +S242Y +Q254S +Y268G +K279I +M284V +N293Y +T297N; (using SEQ ID NO: 9 for numbering).

In an embodiment the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the polypeptide of SEQ ID NO: 9.

In another embodiment the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants with the following mutations:

- N126Y F153W R178* G179* T180H E187P I203Y S362A R377Y;
- N126Y F153W R178* G179* T180H E187P I203Y Y303A S362A R377Y; and
- N126Y F153W R178* G179* T180H I203Y S241Q; (using SEQ ID NO: 13 for numbering), and wherein the alpha-amylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the polypeptide of SEQ ID NO: 9 or the polypeptide of SEQ ID NO: 13.

It should be understood that when referring to *Bacillus stearothermophilus* alpha-amylase and variants thereof they are normally produced in truncated form. In particular, the truncation may be so that the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 99/19467 or SEQ ID NO: 9 herein, or variants thereof, are truncated in the C-terminal preferably to have around 490 amino acids, such as from 482-493 amino acids. Preferably the *Bacillus stearothermophilus* variant alpha-amylase is truncated, preferably after position 484 of SEQ ID NO: 9, particularly after position 485, particularly after position 486, particularly after

position 487, particularly after position 488, particularly after position 489, particularly after position 490, particularly after position 491, particularly after position 492, more particularly after position 493.

5 Saccharification may be carried out using conditions well-known in the art with a carbohydrate-source generating enzyme, in particular an alpha-amylase according to the present invention and a glucoamylase. For instance, a full saccharification step may last from about 24 to about 72 hours. However, it is common 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
10 during fermentation in a simultaneous saccharification and fermentation (SSF) process. Saccharification is typically carried out at a temperature in the range of 20-75°C, e.g., 25-65°C and 40-70°C, typically around 60°C, and at a pH between about 4 and 5, normally at about pH 4.5.

15 The saccharification and fermentation steps may be carried out either sequentially or simultaneously. In an embodiment, saccharification and fermentation are performed simultaneously (referred to as "SSF"). 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
20 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 optionally a debranching enzyme, such as an isoamylase (U.S. Patent No. 4,335,208) or a pullulanase. The temperature is lowered to
25 60°C, prior to the addition of the glucoamylase and debranching enzyme. The saccharification process 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.
30 Normally, about 0.2-0.5% of the saccharification product is the branched trisaccharide panose (Glc pα1-6Glc pα1-4Glc), 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.

35 Other fermentation products may be fermented at conditions and temperatures well known to persons skilled in the art, suitable for the fermenting organism in question.

The fermentation product may be recovered by methods well known in the art, *e.g.*, by distillation.

In a particular embodiment, the process of the invention further comprises, prior to the conversion of a starch-containing material to sugars/dextrins the steps of:

- (x) reducing the particle size of the starch-containing material; and
- (y) forming a slurry comprising the starch-containing material and water.

In an embodiment, the starch-containing material is milled to reduce the particle size. In an embodiment the particle size is reduced to between 0.05-3.0 mm, preferably 0.1-0.5 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the starch-containing material fits through a sieve with a 0.05-3.0 mm screen, preferably 0.1-0.5 mm screen.

The aqueous slurry may contain from 10-55 wt. % dry solids (DS), preferably 25-45 wt. % dry solids (DS), more preferably 30-40 wt. % dry solids (DS) of starch-containing material.

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.

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.

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

Fermentable sugars (*e.g.*, dextrins, monosaccharides, particularly glucose) are produced 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, 3-HP and lactic acid), sugar alcohols (*e.g.*, glycerol), ascorbic acid intermediates (*e.g.*, gluconate, 2-keto-D-gluconate, 2,5-diketo-D-gluconate, 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 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. 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 are 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 un-gelatinized 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 (often referred to as a "raw starch hydrolysis" process). 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 an alpha-amylase of the invention and 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 un-gelatinized (i.e., uncooked), preferably milled, cereal grains, such as corn.

Accordingly, in one aspect the invention relates to processes for producing a fermentation product from starch-containing material comprising simultaneously saccharifying and fermenting starch-containing material using a carbohydrate-source generating enzymes and a fermenting organism at a temperature below the initial gelatinization temperature of said starch-containing material in the presence of an alpha-amylase of the invention.

Saccharification and fermentation may also be separate. Thus, in another aspect the invention relates to processes of producing fermentation products, comprising the following steps:

- (i) saccharifying a raw starch-containing material at a temperature below the initial gelatinization temperature; and
 - (ii) fermenting using a fermentation organism;
- wherein step (i) is carried out using at least an alpha-amylase of the invention, and optionally a glucoamylase.

In one embodiment, the fermenting organism, preferably a *Saccharomyces cerevisiae*, expresses the alpha-amylase of the invention and/or a glucoamylase.

In one embodiment, the fermenting organism, preferably a *Saccharomyces cerevisiae*, expresses the alpha-amylase of the invention and another alpha-amylase.

In an embodiment the other alpha-amylase is of fungal or bacterial origin. In a preferred embodiment the other alpha-amylase is a fungal acid stable alpha-amylase. A fungal acid stable alpha-amylase is an alpha-amylase that has activity in the pH range of 3.0 to 7.0 and preferably in the pH range from 3.5 to 6.5, including activity at a pH of about 4.0, 4.5, 5.0, 5.5, and 6.0.

In a preferred embodiment the other alpha-amylase expressed by the fermenting organism in fermentation is derived from a strain of the genus *Rhizomucor*, preferably a strain the *Rhizomucor pusillus*, such as one shown in SEQ ID NO: 3 in WO 2013/006756, such as a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-binding domain, such as the one shown in SEQ ID NO: 12 herein, or a variant thereof.

In an embodiment the other alpha-amylase is selected from the group consisting of:

- (i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 12 herein;
- (ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 12 herein.

In a preferred embodiment the other alpha-amylase is a variant of the alpha-amylase shown in SEQ ID NO: 12 having at least one of the following substitutions or combinations of substitutions: D165M; Y141W; Y141R; K136F; K192R; P224A; P224R; S123H + Y141W; G20S + Y141W; A76G + Y141W; G128D + Y141W; G128D + D143N; P219C + Y141W; N142D + D143N; Y141W + K192R; Y141W + D143N; Y141W + N383R; Y141W + P219C + A265C; Y141W + N142D + D143N; Y141W + K192R V410A; G128D + Y141W + D143N; Y141W + D143N + P219C; Y141W + D143N + K192R; G128D + D143N + K192R; Y141W + D143N + K192R + P219C; G128D + Y141W + D143N + K192R; or G128D + Y141W + D143N + K192R + P219C (using SEQ ID NO: 12 for numbering).

In an embodiment the alpha-amylase is derived from a *Rhizomucor pusillus* with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 12 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 12 for numbering).

In an embodiment the other alpha-amylase variant present and/or added in saccharification and/or fermentation has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the polypeptide of SEQ ID NO: 12 herein.

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, particularly the alpha-amylase of the invention. 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 at a temperature above the initial gelatinization temperature in the presence of an alpha-amylase;

(b) saccharifying the liquefied material obtained in step (a) using a variant alpha-amylase of the invention, and optionally a glucoamylase; and

(c) fermenting using a fermenting organism.

In an embodiment, a protease, such as a thermo-stable serine protease, an acid fungal protease or a metallo protease 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 another embodiment the protease is a bacterial protease, particularly a serine protease, e.g., an S8 protease, more particularly a protease derived from a strain of *Pyrococcus* or *Thermococcus*, more particularly from *Pyrococcus furiosus* disclosed in US 6,358,726, or SEQ ID NO: 10 herein.

In an embodiment the glucoamylase is 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*; or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *G. serpiarium* or *G. trabeum*, or a strain of the *Nigrofores*; or a mixture thereof. Saccharification step (b) and fermentation step (c) may be carried out either sequentially or simultaneously. A pullulanase and/or protease 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 protease 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 preferred embodiment, the yeast is expressing the variant glucoamylase of the invention. 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 5 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 10 5 minutes. The slurry is cooled to 60-95°C and more alpha-amylase and optionally pullulanase and/or protease, preferably metalloprotease, is(are) added to finalize hydrolysis (secondary liquefaction). The liquefaction process is usually carried out at pH 4.5-6.5, such as around 4.8, or a pH between 5.0-6.2, such as 5.0-6.0, such as between 5.0-5.5, such as around 5.2, such as around 5.4, such as around 5.6, such as around 5.8. Saccharification step (b) may be carried out 15 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 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 25 to 40°C, such as from 28°C to 36°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.

Processes for producing syrup from gelatinized starch-containing material

In this aspect the fermentation step is left out, however, conditions are generally as 30 described above for "Processes for producing fermentation products from gelatinized starch-containing material". Thus, in this aspect the present invention relates to a process for producing a syrup from starch-containing material comprising the steps of:

- a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature in the presence of an alpha-amylase; and
- 35 b) saccharifying the product of step a) in the presence of a glucoamylase and a variant alpha-amylase of the invention.

Protease Present and/or Added During Liquefaction

According to the invention a thermostable protease may in one embodiment be present and/or added during liquefaction together with an alpha-amylase, such as a thermostable alpha-amylase, and optionally a carbohydrate-source generating enzyme, in particular a thermostable glucoamylase or thermostable pullulanase.

Proteases are classified on the basis of their catalytic mechanism into the following groups: Serine proteases (S), Cysteine proteases (C), Aspartic proteases (A), Metallo proteases (M), and Unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998), in particular the general introduction part.

In a preferred embodiment the thermostable protease used according to the invention is a "metallo protease" defined as a protease belonging to EC 3.4.24 (metalloendopeptidases); preferably EC 3.4.24.39 (acid metallo proteinases).

To determine whether a given protease is a metallo protease or not, reference is made to the above "Handbook of Proteolytic Enzymes" and the principles indicated therein. Such determination can be carried out for all types of proteases, be it naturally occurring or wild-type proteases; or genetically engineered or synthetic proteases.

Protease activity can be measured using any suitable assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Assay-pH and assay-temperature are likewise to be adapted to the protease in question. Examples of assay-pH-values are pH 6, 7, 8, 9, 10, or 11. Examples of assay-temperatures are 30, 35, 37, 40, 45, 50, 55, 60, 65, 70 or 80°C.

Examples of protease substrates are casein, such as Azurine-Crosslinked Casein (AZCL-casein). Two protease assays are described below in the "Materials & Methods"-section, of which the so-called "AZCL-Casein Assay" is the preferred assay.

There are no limitations on the origin of the protease used in a process of the invention as long as it fulfills the thermostability properties defined below.

The protease may be a variant of, e.g., a wild-type protease as long as the protease has the thermostability properties defined herein.

In an embodiment the protease has a thermostability above 60%, such as above 90%, such as above 100%, such as above 110% at 85°C as determined using the Zein-BCA assay.

In an embodiment protease has a thermostability between 60-120, such as between 70-120%, such as between 80-120%, such as between 90-120%, such as between 100-120%, such as 110-120% at 85°C as determined using the Zein-BCA assay.

In one embodiment the thermostable protease is a variant of a metallo protease as defined above. In an embodiment the thermostable protease used in a process of the invention is of fungal origin, such as a fungal metallo protease, such as a fungal metallo protease derived from a strain of the genus *Thermoascus*, preferably a strain of *Thermoascus aurantiacus*, especially *Thermoascus aurantiacus* CGMCC No. 0670 (classified as EC 3.4.24.39).

In a preferred embodiment the thermostable protease is a variant of the metallo protease disclosed as the mature part of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 with the following mutations:

D79L+S87P+A112P+D142L;

D79L+S87P+D142L; or

A27K+ D79L+ Y82F+S87G+D104P+A112P+A126V+D142L.

In an embodiment the protease variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841.

The thermostable protease may also be derived from a bacterium, particularly a serine protease, more particularly an S8 protease, more particularly an S8 protease from *Pyrococcus* sp or *Thermococcus* sp.

In an embodiment the thermostable protease is derived from a strain of the bacterium *Pyrococcus*, such as a strain of *Pyrococcus furiosus* (pfu protease).

In an embodiment the protease is one shown as SEQ ID NO: 1 in US patent No. 6,358,726-B1 (Takara Shuzo Company) and SEQ ID NO: 10 herein.

In another embodiment the thermostable protease is one disclosed in SEQ ID NO: 10 herein or a protease having at least 80% identity, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 1 in US patent no. 6,358,726-B1 or SEQ ID NO: 10 herein.

Glucoamylase Present And/Or Added In Liquefaction

In an embodiment a glucoamylase is present and/or added in liquefaction step a) in a process of the invention (i.e., oil recovery process and fermentation product production process).

In a preferred embodiment the glucoamylase present and/or added in liquefaction step a) is derived from a strain of the genus *Penicillium*, especially a strain of *Penicillium oxalicum* disclosed as SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 11 herein.

In an embodiment the glucoamylase has at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99% or 100% identity to the mature polypeptide shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 11 herein.

In a preferred embodiment the glucoamylase is a variant of the *Penicillium oxalicum* glucoamylase shown in SEQ ID NO: 2 in WO 2011/127802 or SEQ ID NO: 11 herein having a K79V substitution (using the mature sequence shown in SEQ ID NO: 11 for numbering), such as a variant disclosed in WO 2013/053801.

In a preferred embodiment the glucoamylase present and/or added in liquefaction is the *Penicillium oxalicum* glucoamylase having a K79V substitution and preferably further one of the following substitutions:

- P11F + T65A + Q327F;
- P2N + P4S + P11F + T65A + Q327F (using SEQ ID NO: 11 for numbering).

In an embodiment the glucoamylase variant has at least 75% identity preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%, most preferably at least 94%, and even most preferably at least 95%, such as even at least 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the mature part of the polypeptide of SEQ ID NO: 11 herein.

The glucoamylase may be added in amounts from 0.1- 100 micro grams EP/g, such as 0.5-50 micro grams EP/g, such as 1-25 micrograms EP/g, such as 2-12 micrograms EP/g DS.

Glucoamylase Present And/Or Added In Saccharification And/Or Fermentation

A glucoamylase is present and/or added in saccharification and/or fermentation, preferably simultaneous saccharification and fermentation (SSF), in a process of the invention (i.e., oil recovery process and fermentation product production process).

In an embodiment the glucoamylase present and/or added in saccharification and/or fermentation is of fungal origin, preferably from a strain of *Aspergillus*, preferably *A. niger*, *A. awamori*, or *A. oryzae*; or a strain of *Trichoderma*, preferably *T. reesei*; or a strain of *Talaromyces*, preferably *T. emersonii* or a strain of *Trametes*, preferably *T. cingulata*, or a strain of *Pycnoporus*, or a strain of *Gloeophyllum*, such as *G. serpiarium* or *G. trabeum*, or a strain of the *Nigrofores*.

In an embodiment the glucoamylase is derived from *Trametes*, such as a strain of *Trametes cingulata*, such as the one shown in SEQ ID NO: 7 herein.

- In an embodiment the glucoamylase is selected from the group consisting of:
- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 7 herein;

(ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 7 herein.

5 In an embodiment the glucoamylase is derived from *Talaromyces*, such as a strain of *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 8 herein.

In an embodiment the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 8 herein;

10 (ii) a glucoamylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 8 herein.

15 Glucoamylases may in an embodiment be added to the saccharification and/or fermentation in an amount of 0.0001-20 AGU/g DS, preferably 0.001-10 AGU/g DS, especially between 0.01-5 AGU/g DS, such as 0.1-2 AGU/g DS.

20 Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U, SPIRIZYME™ ULTRA, SPIRIZYME™ EXCEL and AMG™ E (from Novozymes A/S); OPTIDEX™ 300, GC480, GC417 (from DuPont.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from DuPont).

25 According to a preferred embodiment of the invention the glucoamylase is present and/or added in saccharification and/or fermentation in combination with a variant alpha-amylase of the invention. Examples of suitable alpha-amylase are described below.

Alpha-Amylase Present and/or Added In Saccharification And/Or Fermentation

30 In an embodiment a variant alpha-amylase of the invention is present and/or added in saccharification and/or fermentation in a process of the invention. In a preferred embodiment the alpha-amylase is of fungal or bacterial origin. In a preferred embodiment the alpha-amylase is a fungal acid stable alpha-amylase of the invention. A fungal acid stable alpha-amylase is an alpha-amylase that has activity in the pH range of 3.0 to 7.0 and preferably in the pH range from 3.5 to 6.5, including activity at a pH of about 4.0, 4.5, 5.0, 5.5, and 6.0.

35 In a preferred embodiment the alpha-amylase present and/or added in saccharification and/or fermentation is derived from a strain of the genus *Bacillus*, preferably a strain the *Bacillus licheniformis*, such as one shown in SEQ ID NO: 1 herein.

In an embodiment the alpha-amylase present and/or added in saccharification and/or fermentation is selected from a variant alpha-amylase of the invention or a variant alpha-amylase of the invention having 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% identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 4.

In a preferred embodiment, the ratio between glucoamylase and variant alpha-amylase present and/or added during saccharification and/or fermentation may preferably be in the range from 500:1 to 1:1, such as from 250:1 to 1:1, such as from 100:1 to 1:1, such as from 100:2 to 100:50, such as from 100:3 to 100:70.

Fermentation Medium

The environment in which fermentation is carried out is often referred to as the "fermentation media" or "fermentation medium". The fermentation medium includes the fermentation substrate, that is, the carbohydrate source that is metabolized by the fermenting organism. According to the invention the fermentation medium may comprise nutrients and growth stimulator(s) for the fermenting organism(s). Nutrient and growth stimulators are widely used in the art of fermentation and include nitrogen sources, such as ammonia; urea, vitamins and minerals, or combinations thereof.

Fermenting Organisms

The term "fermenting organism" refers to any organism, including bacterial and fungal organisms, especially yeast, suitable for use in a fermentation process and capable of producing the desired fermentation product. Especially suitable fermenting organisms are able to ferment, i.e., convert, sugars, such as glucose or maltose, directly or indirectly into the desired fermentation product, such as ethanol. Examples of fermenting organisms include fungal organisms, such as yeast. Preferred yeast includes strains of *Saccharomyces* spp., in particular, *Saccharomyces cerevisiae*.

Suitable concentrations of the viable fermenting organism during fermentation, such as SSF, are well known in the art or can easily be determined by the skilled person in the art. In one embodiment the fermenting organism, such as ethanol fermenting yeast, (e.g., *Saccharomyces cerevisiae*) is added to the fermentation medium so that the viable fermenting organism, such as yeast, count per mL of fermentation medium is in the range from 10^5 to 10^{12} , preferably from 10^7 to 10^{10} , especially about 5×10^7 .

Examples of commercially available yeast includes, e.g., RED STAR™ and ETHANOL RED™ yeast (available from Fermentis/Lesaffre, USA), FALI (available from Fleischmann's Yeast, USA), SUPERSTART and THERMOSACC™ fresh yeast (available from Ethanol Technology, WI, USA), BIOFERM AFT and XR (available from NABC - North American

Bioproducts Corporation, GA, USA), GERT STRAND (available from Gert Strand AB, Sweden), and FERMIOL (available from DSM Specialties).

Starch-Containing Materials

Any suitable starch-containing material may be used according to the present invention. The starting material is generally selected based on the desired fermentation product. Examples of starch-containing materials, suitable for use in a process of the invention, include whole grains, corn, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, beans, or sweet potatoes, or mixtures thereof or starches derived therefrom, or cereals. Contemplated are also waxy and non-waxy types of corn and barley. In a preferred embodiment the starch-containing material, used for ethanol production according to the invention, is corn or wheat.

Fermentation Products

The term "fermentation product" means a product produced by a process including a fermentation step using a fermenting organism. Fermentation products contemplated according to the invention include alcohols (e.g., ethanol, methanol, butanol; polyols such as glycerol, sorbitol and inositol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, succinic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H₂ and CO₂); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B₁₂, beta-carotene); and hormones. In a preferred embodiment the fermentation product is ethanol, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol or products used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Preferably processes of the invention are used for producing an alcohol, such as ethanol. The fermentation product, such as ethanol, obtained according to the invention, may be used as fuel, which is typically blended with gasoline. However, in the case of ethanol it may also be used as potable ethanol.

Recovery of Fermentation Products

Subsequent to fermentation, or SSF, the fermentation product may be separated from the fermentation medium. The slurry may be distilled to extract the desired fermentation product (e.g., ethanol). Alternatively, the desired fermentation product may be extracted from the fermentation medium by micro or membrane filtration techniques. The fermentation product may also be recovered by stripping or other method well known in the art.

Plants

The present invention also relates to isolated plants, e.g., a transgenic plant, plant part, or plant cell, comprising a polynucleotide of the present invention so as to express and produce the variant in recoverable quantities. The variant may be recovered from the plant or plant part.

Alternatively, the plant or plant part containing the variant may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor, or for reducing the amount of alpha-amylase needed in saccharification of starch, and optionally fermenting the glucose to a fermentation product, such as an alcohol.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family Brassicaceae), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seed coats.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

The transgenic plant or plant cell expressing a variant may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by incorporating one or more expression constructs encoding a variant into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

The expression construct is conveniently a nucleic acid construct that comprises a polynucleotide encoding a variant operably linked with appropriate regulatory sequences required for expression of the polynucleotide in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying plant cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences, is determined, for example, on the basis of when, where, and how the variant is desired to be expressed. For instance, the expression of the gene encoding a variant may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are, for example, described by Tague *et al.*, 1988, *Plant Physiology* 86: 506.

For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, or the rice actin 1 promoter may be used (Franck *et al.*, 1980, *Cell* 21: 285-294; Christensen *et al.*, 1992, *Plant Mol. Biol.* 18: 675-689; Zhang *et al.*, 1991, *Plant Cell* 3: 1155-1165). Organ-specific promoters may be, for example, a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards and Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito *et al.*, 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu *et al.*, 1998, *Plant Cell Physiol.* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad *et al.*, 1998, *J. Plant Physiol.* 152: 708-711), a promoter from a seed oil body protein (Chen *et al.*, 1998, *Plant Cell Physiol.* 39: 935-941), the storage protein *napA* promoter from *Brassica napus*, or any other seed specific promoter known in the art, *e.g.*, as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcs* promoter from rice or tomato (Kozuka *et al.*, 1993, *Plant Physiol.* 102: 991-1000), the chloroella virus adenine methyltransferase gene promoter (Mitra and Higgins, 1994, *Plant Mol. Biol.* 26: 85-93), the *aldP* gene promoter from rice (Kagaya *et al.*, 1995, *Mol. Gen. Genet.* 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu *et al.*, 1993, *Plant Mol. Biol.* 22: 573-588). Likewise, the promoter may be induced by abiotic treatments such as temperature, drought, or alterations in salinity or induced by exogenously applied substances that activate the promoter, *e.g.*, ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

A promoter enhancer element may also be used to achieve higher expression of a variant in the plant. For instance, the promoter enhancer element may be an intron that is placed between the promoter and the polynucleotide encoding a variant. For instance, Xu *et al.*, 1993, *supra*, disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation,

and electroporation (Gasser *et al.*, 1990, *Science* 244: 1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto *et al.*, 1989, *Nature* 338: 274).

Agrobacterium tumefaciens-mediated gene transfer is a method for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Mol. Biol.* 19: 15-38) and for transforming monocots, although other transformation methods may be used for these plants. A method for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant J.* 2: 275-281; Shimamoto, 1994, *Curr. Opin. Biotechnol.* 5: 158-162; Vasil *et al.*, 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh *et al.*, 1993, *Plant Mol. Biol.* 21: 415-428. Additional transformation methods include those described in U.S. Patent Nos. 6,395,966 and 7,151,204 (both of which are herein incorporated by reference in their entirety).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using, for example, co-transformation with two separate T-DNA constructs or site-specific excision of the selection gene by a specific recombinase.

In addition to direct transformation of a particular plant genotype with a construct of the present invention, transgenic plants may be made by crossing a plant having the construct to a second plant lacking the construct. For example, a construct encoding a variant can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the present invention encompasses not only a plant directly regenerated from cells which have been transformed in accordance with the present invention, but also the progeny of such plants. As used herein, progeny may refer to the offspring of any generation of a parent plant prepared in accordance with the present invention. Such progeny may include a DNA construct prepared in accordance with the present invention. Crossing results in the introduction of a transgene into a plant line by cross pollinating a starting line with a donor plant line. Non-limiting examples of such steps are described in U.S. Patent No. 7,151,204.

Plants may be generated through a process of backcross conversion. For example, plants include plants referred to as a backcross converted genotype, line, inbred, or hybrid.

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed

to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

5 The present invention also relates to methods of producing a variant of the present invention comprising: (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the variant under conditions conducive for production of the variant; and (b) recovering the variant.

10 The present invention is further illustrated by the following numbered embodiments.

Embodiment 1. An alpha-amylase variant of a parent alpha-amylase, comprising an alteration at one or more positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113, 114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204, 205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284, 285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459, 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576, 577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

Embodiment 2. An alpha-amylase variant according to embodiment 1, comprising a substitution at one or more positions corresponding to positions 196, 199, 64, 96, 150, 179, 207, 222, 284 and 603 of the polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

Embodiment 3. The variant of embodiment 1, which comprises an alteration selected from the group consisting of: E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, V9*, V9D, V9L, T10*, T10I, A11*, S12*, S12P, S13*, V14*, V14I, K15*, N16*, N16S, N28R, N28W, R38H, R38Y, D39R, A43D, A43T, A43V, K54I, G56P, G56W, N57P, R64S, Y67T, Y67W, W68S, W68Y, Y70F, Q71E, Q71N,

Q86R, K89R, D90E, A94D, E96H, E96K, G99N, K101R, I103Y, V107T, I108L, I108P, H110D, S113D, S113F, S113G, S113H, S113Q, S113W, S113Y, D114Q, A117T, N127D, Q134E, Q134L, Q134M, Q134N, Q134T, Q134W, W138Y, W142E, L150F, L150H, L150M, L150S, L150V, L150W, L150Y, G151F, G151S, G151W, G151Y, L152M, N156K, N156R, F169H, E171Q, L174I, D179G, D179S, Y183F, Y183I, D193S, D193DQ, D193DY, D193DQY, D193SQY, N196W, S199G, Q200W, N204D, I205Y, N207W, T208N, T208S, S209L, F212W, L218F, L218W, S221N, A222E, A222I, A222V, R224K, N233S, H241N, S245N, H259Y, S275L, S275N, T278N, T278W, T278Y, N281Q, N281S, D282P, D283*, D283A, D283P, E284Q, E285V, T308M, T308Y, R323K, S335K, S335Q, S335R, T348K, E359Y, A382T, S386D, S388W, N392R, N392W, S394K, K396S, Q412W, A414K, K417W, K417Y, A424P, A428S, Q457L, Q457R, T459M, A466V, Q479QP, L489Q, E511D, G533H, Y534H, Q542K, V543P, A545P, I547Y, K549*, K549Y, H550*, H550Y, D551*, G560P, A566P, N570H, M574MW, M574W, Y575W, T576Y, L577Y, T578Y, P580*, E581*, N582*, K589F, F592FK, V599W, N603W, P605S, D608Y, L614W, G619W, and H626*.

Embodiment 4. The variant of embodiment 2, which comprises an alteration selected from the group consisting of: R64S, E96K, L150Y, L150W, L150H, L150M, L150F, D179S, N196W, S199G, N207W, A222E, A222I, A222V, E284Q, N603W, and H626*.

Embodiment 5. The variant of any one of embodiments 1-4, which has an improved property relative to the parent, wherein the improved property is increased pH stability at pH 4.0, 32 °C to 37°C, preferably 32 °C, compared to a parent alpha-amylase, particularly the alpha-amylase disclosed as SEQ ID NO: 1.

Embodiment 6. The variant of any of embodiments 1-5, comprising a substitution or a combination of substitutions selected from:

A222I;

A222V;

A222E;

S199G;

N196W;

N207W;

N603W;

L150Y;

L150W;

L150H;

L150M;

L150F;

R64S;

E96K;

D179S;

5 E284Q;

N207W + N603W;

N196W+N207W;

N196W+N603W;

N196W+N207W + N603W;

10 A222I + S199G + N196W;

A222V + S199G + N196W;

A222E + S199G + N196W;

A222V + S199G + N196W + L150Y;

L150H + S199G + A222I;

15 L150M + S199G + A222V;

N196W + S199G + A222V + N603W;

L150F + N196W + S199G + A222I;

L150M + N196W + S199G + A222V;

L150W + S199G + A222V;

20 L150H + N196W + S199G + A222V;

L150W + N196W + S199G + A222I;

L150Y + S199G + A222V;

E96K + D179S + N196W + S199G + A222V + E284Q;

R64S + E96K + N196W + S199G + A222V;

25 wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

30

Embodiment 7. The variant of any of embodiments 1-6, comprising a N-terminal deletion, more particularly comprising at least amino acids 11 to 626 of SEQ ID NO: 1, at least amino acids 12 to 626 of SEQ ID NO: 1, such as at least amino acids 13 to 626 of SEQ ID NO: 1.

35 Embodiment 8. The variant of any of embodiments 1-7, wherein the alpha-amylase further comprises a C-terminal deletion, particularly H626*.

Embodiment 9. The variant alpha-amylase of any of embodiments 1-5, comprising an alteration or a combination of alterations selected from:

N28W;

N196W;

5 S199G;

N196W+ V599W;

N196W+ H550Y+ P605S;

N196W+ A545P+ T576Y;

N196W+ K549Y+ G560P;

10 I108P+ Y183I+ N196W+ I205Y;

N196W+ R323K;

N196W+ D283P;

W138Y+ N196W;

L150W;

15 N196W+ N392W+ K417W;

N196W+ N392R+ K417W;

N196W+ K549*+ H550*+ D551*;

N196W+ P580*+ E581*+ N582*;

N196W+ F592FK;

20 N28W+ N196W+ N207W+ S386D+ N603W;

R38Y+ N196W;

N196W+ H259Y;

N196W+ Q412W;

N196W+ F212W;

25 N196W+ V599W;

N196W+ H550Y+ P605S;

N196W+ H550Y+ K589F;

N196W+ H550Y+ D608Y;

N196W+ M574W+ L614W;

30 N196W+ G533H+ M574W+ L614W;

N196W+ V543P+ N570H;

N196W+ G533H+ Y575W+ L614W;

N196W+ A545P+ T576Y;

N196W+ A566P+ T578Y;

35 N196W+ K549Y+ G560P;

N196W+ A566P+ L577Y;

N196W+ I547Y+ G560P;

- N196W+ M574MW;
 N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK;
 N196W+ L614W+ G619W;
 N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W;
 5 N28W+ I108P+ N196W+ N207W+ S386D+ N603W;
 N196W+ D282P+ D283*;
 W138Y+ N196W;
 N28W+ N196W+ N207W+ S386D+ N603W;
 N196W+ S388W+ A424P;
 10 N196W+ S388W+ A424P+ L489Q;
 A117T+ N196W+ H550Y+ D608Y;
 N196W+ Q457R+ Y575W+ L614W;
 N196W+ S199G;
 N196W+ A222V;
 15 N196W+ A222E;
 N196W+ A222I;
 S199G+ A222V;
 S199G+ A222E;
 S199G+ A222I;
 20 N196W+ S199G+ A222I;
 Q134L;
 L150W+ N156K+ N196W+ S199G+ A222V;
 L150Y+ N156K+ N196W+ S199G+ A222I;
 L150W+ N196W+ S199G+ A222I+ A428S;
 25 L150F+ N156K+ N196W+ S199G+ A222I;
 L150Y+ N156R+ N196W+ A222V;
 L150M+ N156R+ N196W+ S199G+ A222I;
 L150M+ N156R+ N196W+ A222V;
 L150Y+ N156R+ N196W+ S199G+ A222V;
 30 L150M+ N156K+ N196W+ S199G+ A222V;
 L150Y+ N156R+ N196W+ A222I;
 L150H+ N156R+ N196W+ S199G+ A222I;
 L150H+ N156K+ N196W+ A222V;
 L150W+ N156R+ N196W+ A222I;
 35 L150F+ N156R+ N196W+ A222I;
 L150F+ N156K+ N196W+ S199G+ A222V;
 L150H+ N156K+ N196W+ S199G+ A222V;

- L150F+ N156R+ S199G+ A222I;
L150M+ N156K+ N196W+ A222V;
L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
- 5 L150Y+ N156R+ S199G+ A222I;
L150M+ N156R+ S199G+ A222I;
L150W+ N156K+ S199G+ A222V;
L150W+ N156R+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W;
- 10 N156K+ N196W+ A222V;
N156K+ N196W+ S199G;
N156R+ S199G+ A222V;
S113H+ N196W+ S199G+ A222V;
Q71E+ S113H+ N196W+ S199G+ A222V;
- 15 N196W+ S199G+ A222V+ D283A;
N196W+ S199G+ A222V+ D283P;
W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
- 20 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
S113Q+ Q134E+ N196W+ S199G+ A222V;
S113D+ Q134N+ N196W+ S199G+ A222V;
S113F+ N196W+ S199G+ A222V;
E171Q+ N196W+ S199G+ N204D+ A222V;
- 25 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
- 30 V107T+ H110D+ N196W+ S199G+ A222V;
Q134T+ L150Y+ N196W+ S199G+ A222V;
S113F+ L150W+ N196W+ S199G+ A222V;
S113F+ L150Y+ N196W+ S199G+ A222V;
E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
- 35 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

- E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 N28R+ Q86R+ N196W+ S199G+ A222V;
 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
- 5 E96K+ N196W+ S199G+ A222V;
 T10I+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V;
 R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V;
- 10 T10I+ D39R+ N196W+ S199G+ A222V;
 D39R+ E96K+ N196W+ S199G+ A222V;
 R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
 R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
- 15 T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 E96K+ N196W+ S199G+ A222V+ E284Q;
- 20 T10I+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q;
 R64S+ N196W+ S199G+ A222V+ E284Q;
 T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
 D193SQY+ N196W+ S199G+ A222V;
- 25 Q134T+ N196W+ S199G+ A222V;
 L174I+ N196W+ S199G+ T208N+ A222V;
 Y183F+ N196W+ S199G+ T208S+ A222V;
 N127D+ N156R+ N196W+ S199G+ A222V;
 Q134T+ L150W+ N196W+ S199G+ A222V;
- 30 N57P+ N196W+ S199G+ A222V;
 N196W+ S199G+ Q200W+ A222V;
 T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
 T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
- 35 D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
 T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
 T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;

- D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V;
- 5 S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;
S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
N28W+ N196W+ S199G+ A222V;
- 10 N196W+ S199G+ A222V+ N392W+ K417W;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
S113F+ L150Y+ N156K+ N196W+ S199G+ A222V;
S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V;
- 15 S113W+ L150Y+ N156K+ N196W+ S199G+ A222V;
S113F+ N156K+ N196W+ S199G+ A222V;
S113Y+ N156K+ N196W+ S199G+ A222V;
S113W+ N156K+ N196W+ S199G+ A222V;
W138Y+ L150V+ N196W+ S199G+ A222V;
- 20 W138Y+ L150V+ D179G+ N196W+ S199G+ A222V;
W138Y+ L150V+ N196W+ S199G+ L218W+ A222V;
E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q;
E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
- 25 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V;
R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V;
R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
L150F+ N196W+ S199G+ A222I;
- 30 Q134L+ L150F+ N156R+ N196W+ S199G+ A222I;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;
Q134L+ L150M+ N156K+ N196W+ S199G+ A222I;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L;
Q134M+ L150W+ N156K+ N196W+ S199G+ A222I;
- 35 Q134W+ L150W+ N156K+ N196W+ S199G+ A222I;
L150W+ L152M+ N156K+ N196W+ S199G+ A222I;
S113F+ L150W+ N156K+ N196W+ S199G+ A222I;

- S113Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151W+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
 S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
- 5 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;
- 10 S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151F+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151Y+ N156K+ N196W+ S199G+ A222I;
 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
- 15 G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V;
 K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V;
- 20 Q134L+ N196W+ S199G+ A222V;
 V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N392W+ K417W;
 V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
 E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q;
- 25 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150Y+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150F+ N156R+ N196W+ S199G+ A222V;
 L150H+ N156R+ N196W+ S199G+ A222V;
- 30 Q134L+ L150F+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
- 35 L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V;

- Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V;
 L150Y+ L152M+ N156K+ N196W+ S199G+ A222V;
 L150S+ N196W+ S199G+ A222V;
 5 N196W+ S199G+ A222V+ N281S;
 Y67T+ N196W+ S199G+ A222V;
 Q71N+ N196W+ S199G+ A222V;
 Q71N+ A94D+ N196W+ S199G+ A222V;
 N196W+ S199G+ L218F+ A222V;
 10 N196W+ S199G+ L218W+ A222V;
 N196W+ S199G+ A222V+ T278W;
 N196W+ S199G+ A222V+ T278W+ T459M;
 N196W+ S199G+ A222V+ T278Y;
 N196W+ S199G+ A222V+ S275N;
 15 N196W+ S199G+ A222V+ S275L;
 N196W+ S199G+ A222V+ S335Q;
 N196W+ S199G+ A222V+ S335K;
 N196W+ S199G+ A222V+ S335R;
 N196W+ S199G+ L218W+ A222V+ S335K;
 20 N196W+ S199G+ L218W+ A222V+ S335Q;
 Y67W+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N281Q;
 L150M+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V;
 25 Q134L+ L150M+ N156K+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
 D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
 30 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 N196W+ N207W;
 N196W+ N207W+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
 35 L150Y+ N196W+ S199G+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;

Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

5 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ N207W+ A222I+ E284Q;

E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

R64S+ E96K+ N196W+ S199G+ N207W+ A222V;

10 N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

D39R+ N196W+ S199G+ A222V+ E284Q;

D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

15 L150Y+ N196W+ S199G+ N207W+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V+ E284Q;

20 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

R64S+ E96K+ N196W+ S199G+ A222V;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

25 and wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

30

Embodiment 10. The variant of embodiment 9, wherein the alpha-amylase further comprises a C-terminal deletion, particularly H626*.

35

Embodiment 11. The variant alpha-amylase of any of embodiments 1-5, comprising a combination of alterations selected from:

N28W+ H626*;

S199G+ H626*;

- N196W+ V599W+ H626*;
N196W+ H550Y+ P605S+ H626*;
N196W+ A545P+ T576Y+ H626*;
N196W+ K549Y+ G560P+ H626*;
5 I108P+ Y183I+ N196W+ I205Y+ H626*;
N196W+ R323K+ H626*;
N196W+ D283P+ H626*;
W138Y+ N196W+ H626*;
L150W+ H626*;
10 N196W+ N392W+ K417W+ H626*;
N196W+ N392R+ K417W+ H626*;
N196W+ K549*+ H550*+ D551*+ H626*;
N196W+ P580*+ E581*+ N582*+ H626*;
N196W+ F592FK+ H626*;
15 N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
R38Y+ N196W+ H626*;
N196W+ H259Y+ H626*;
N196W+ Q412W+ H626*;
N196W+ F212W+ H626*;
20 N196W+ V599W+ H626*;
N196W+ H550Y+ P605S+ H626*;
N196W+ H550Y+ K589F+ H626*;
N196W+ H550Y+ D608Y+ H626*;
N196W+ M574W+ L614W+ H626*;
25 N196W+ G533H+ M574W+ L614W+ H626*;
N196W+ V543P+ N570H+ H626*;
N196W+ G533H+ Y575W+ L614W+ H626*;
N196W+ A545P+ T576Y+ H626*;
N196W+ A566P+ T578Y+ H626*;
30 N196W+ K549Y+ G560P+ H626*;
N196W+ A566P+ L577Y+ H626*;
N196W+ I547Y+ G560P+ H626*;
N196W+ M574MW+ H626*;
N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK + H626*;
35 N196W+ L614W+ G619W+ H626*;
N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W+ H626*;
N28W+ I108P+ N196W+ N207W+ S386D+ N603W+ H626*;

- N196W+ D282P+ D283*+ H626*;
W138Y+ N196W+ H626*;
N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
N196W+ S388W+ A424P+ H626*;
5 N196W+ S388W+ A424P+ L489Q+ H626*;
A117T+ N196W+ H550Y+ D608Y+ H626*;
N196W+ Q457R+ Y575W+ L614W+ H626*;
N196W+ S199G;
N196W+ A222V;
10 N196W+ A222E;
N196W+ A222I;
S199G+ A222V;
S199G+ A222E;
S199G+ A222I;
15 N196W+ S199G+ A222I;
Q134L+ H626*;
L150W+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156K+ N196W+ S199G+ A222I;
L150W+ N196W+ S199G+ A222I+ A428S;
20 L150F+ N156K+ N196W+ S199G+ A222I;
L150Y+ N156R+ N196W+ A222V;
L150M+ N156R+ N196W+ S199G+ A222I;
L150M+ N156R+ N196W+ A222V;
L150Y+ N156R+ N196W+ S199G+ A222V;
25 L150M+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W+ A222I;
L150H+ N156R+ N196W+ S199G+ A222I;
L150H+ N156K+ N196W+ A222V;
L150W+ N156R+ N196W+ A222I;
30 L150F+ N156R+ N196W+ A222I;
L150F+ N156K+ N196W+ S199G+ A222V;
L150H+ N156K+ N196W+ S199G+ A222V;
L150F+ N156R+ S199G+ A222I;
L150M+ N156K+ N196W+ A222V;
35 L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ S199G+ A222I;

- L150M+ N156R+ S199G+ A222I;
 L150W+ N156K+ S199G+ A222V;
 L150W+ N156R+ N196W+ S199G+ A222V;
 L150Y+ N156R+ N196W;
 5 N156K+ N196W+ A222V;
 N156K+ N196W+ S199G;
 N156R+ S199G+ A222V;
 S113H+ N196W+ S199G+ A222V;
 Q71E+ S113H+ N196W+ S199G+ A222V;
 10 N196W+ S199G+ A222V+ D283A;
 N196W+ S199G+ A222V+ D283P;
 W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 15 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 S113Q+ Q134E+ N196W+ S199G+ A222V;
 S113D+ Q134N+ N196W+ S199G+ A222V;
 S113F+ N196W+ S199G+ A222V;
 E171Q+ N196W+ S199G+ N204D+ A222V;
 20 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
 N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
 N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 25 V107T+ H110D+ N196W+ S199G+ A222V;
 Q134T+ L150Y+ N196W+ S199G+ A222V;
 S113F+ L150W+ N196W+ S199G+ A222V;
 S113F+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 30 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 N28R+ Q86R+ N196W+ S199G+ A222V;
 35 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
 E96K+ N196W+ S199G+ A222V;

- T10I+ N196W+ S199G+ A222V;
D39R+ N196W+ S199G+ A222V;
R64S+ N196W+ S199G+ A222V;
T10I+ D39R+ R64S+ N196W+ S199G+ A222V;
- 5 T10I+ D39R+ N196W+ S199G+ A222V;
D39R+ E96K+ N196W+ S199G+ A222V;
R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
- 10 T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
E96K+ N196W+ S199G+ A222V+ E284Q;
- 15 T10I+ N196W+ S199G+ A222V+ E284Q;
D39R+ N196W+ S199G+ A222V+ E284Q;
R64S+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
D193SQY+ N196W+ S199G+ A222V;
- 20 Q134T+ N196W+ S199G+ A222V;
L174I+ N196W+ S199G+ T208N+ A222V;
Y183F+ N196W+ S199G+ T208S+ A222V;
N127D+ N156R+ N196W+ S199G+ A222V;
Q134T+ L150W+ N196W+ S199G+ A222V;
- 25 N57P+ N196W+ S199G+ A222V;
N196W+ S199G+ Q200W+ A222V;
T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
- 30 D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;
D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
- 35 R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;

- S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
N28W+ N196W+ S199G+ A222V;
- 5 N196W+ S199G+ A222V+ N392W+ K417W;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
S113F+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
- 10 S113W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
S113F+ N156K+ N196W+ S199G+ A222V+ H626*;
S113Y+ N156K+ N196W+ S199G+ A222V+ H626*;
S113W+ N156K+ N196W+ S199G+ A222V+ H626*;
W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
- 15 W138Y+ L150V+ D179G+ N196W+ S199G+ A222V+ H626*;
W138Y+ L150V+ N196W+ S199G+ L218W+ A222V+ H626*;
E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
- 20 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V+ H626*;
R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V+ H626*;
R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ H626*;
R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ H626*;
- 25 L150F+ N196W+ S199G+ A222I+ H626*;
Q134L+ L150F+ N156R+ N196W+ S199G+ A222I+ H626*;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ H626*;
Q134L+ L150M+ N156K+ N196W+ S199G+ A222I+ H626*;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L+ H626*;
- 30 Q134M+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
Q134W+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
L150W+ L152M+ N156K+ N196W+ S199G+ A222I+ H626*;
S113F+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
S113Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
- 35 L150W+ G151W+ N156K+ N196W+ S199G+ A222I+ H626*;
L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;
S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;

- Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;
L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
5 L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;
S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
Q134L+ L150F+ N156K+ N196W+ S199G+ A222I+ H626*;
L150W+ G151F+ N156K+ N196W+ S199G+ A222I+ H626*;
L150W+ G151Y+ N156K+ N196W+ S199G+ A222I+ H626*;
10 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T+ H626*;
K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V+ H626*;
K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V+ H626*;
15 K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
Q134L+ N196W+ S199G+ A222V+ H626*;
V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V+ H626*;
N196W+ S199G+ A222V+ N392W+ K417W+ H626*;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K+ H626*;
20 E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q+ H626*;
Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
L150Y+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
25 L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150F+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150H+ N156K+ N196W+ S199G+ A222V+ H626*;
30 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
35 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V+ H626*;
L150Y+ L152M+ N156K+ N196W+ S199G+ A222V+ H626*;
L150S+ N196W+ S199G+ A222V+ H626*;

- N196W+ S199G+ A222V+ N281S+ H626*;
Y67T+ N196W+ S199G+ A222V+ H626*;
Q71N+ N196W+ S199G+ A222V+ H626*;
Q71N+ A94D+ N196W+ S199G+ A222V+ H626*;
5 N196W+ S199G+ L218F+ A222V+ H626*;
N196W+ S199G+ L218W+ A222V+ H626*;
N196W+ S199G+ A222V+ T278W+ H626*;
N196W+ S199G+ A222V+ T278W+ T459M+ H626*;
N196W+ S199G+ A222V+ T278Y+ H626*;
10 N196W+ S199G+ A222V+ S275N+ H626*;
N196W+ S199G+ A222V+ S275L+ H626*;
N196W+ S199G+ A222V+ S335Q+ H626*;
N196W+ S199G+ A222V+ S335K+ H626*;
N196W+ S199G+ A222V+ S335R+ H626*;
15 N196W+ S199G+ L218W+ A222V+ S335K+ H626*;
N196W+ S199G+ L218W+ A222V+ S335Q+ H626*;
Y67W+ N196W+ S199G+ A222V+ H626*;
N196W+ S199G+ A222V+ N281Q+ H626*;
L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
20 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
Q134L+ L150M+ N156K+ N196W+ S199G+ A222V+ H626*;
D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
25 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q+
H626*;
N196W+ N207W;
30 N196W+ N207W+ E284Q;
E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
L150Y+ N196W+ S199G+ A222V+ H626*;
E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;
35 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
S199G+ T208N+ A222V;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q+ H626*;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ H626*;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ N207W+ A222I+ E284Q;

5 E96K+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
R64S+ E96K+ N196W+ S199G+ N207W+ A222V;

N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

D39R+ N196W+ S199G+ A222V+ E284Q+ H626*;

10 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

L150Y+ N196W+ S199G+ N207W+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ H626*;

15 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V+ E284Q+ H626*;

Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

20 R64S+ E96K+ N196W+ S199G+ A222V+ H626*;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

and wherein the variant has alpha-amylase activity and 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

25 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than
100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or
SEQ ID NO: 4.

Embodiment 12. The variant of any of embodiments 1-8, wherein the alpha-amylase comprises
30 combinations of alterations selected from:

S199G + H626*;

N196W + H626*

N196W + S199G + A222V + H 626*;

N196W + N207W + H626*;

35 L150Y + N196W + S199G + A222V

E96K + D179S + N196W + S199G + A222V + E284Q;

R64S + E96K + N196W + S199G + A222V;

and wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

Embodiment 13. The variant of any of embodiments 1-12, wherein increased pH stability at pH 4.0 can be determined as % residual alpha-amylase activity (%RA) after incubation of the variant amylase at pH 4.0, 32 °C, for 18-24 hours.

Embodiment 14. The variant of any of embodiments 1-12, wherein increased pH stability at pH 4.0 can be determined as residual alpha-amylase activity after incubation of the variant amylase at pH 4.0, 32°C, for 96 hours, and calculation of enzyme half-life in hours.

Embodiment 15. The variant of any of embodiments 1- 14, wherein half-life is increased compared to the parent amylase of SEQ ID NO: 1, 2, 3, or 4 of at least a factor 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, such as at least 8.0.

Embodiment 16. The variant of any of embodiments 1-10, further comprising a substitution corresponding to K8N.

Embodiment 17. A variant alpha-amylase of any of the preceding embodiments, comprising at least the catalytic domain, comprised in amino acids 12-438 of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and wherein the catalytic domain has 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%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, and wherein the variant optionally has a CBM.

Embodiment 18. The variant of any of embodiments 1-17, wherein the linker and/or a carbohydrate binding module, CBM, has been replaced with a heterologous CBM.

Embodiment 19. The variant of embodiment 18, wherein the CBM comprises amino acids 527-626 of SEQ ID NO: 1, and amino acids 439-526 comprises a linker region.

Embodiment 20. The variant of any of embodiments 17-19, wherein the heterologous CBM is selected from a heterologous CBM belonging to Family 20, 21, 25, 26, 34, 41 or 48.

Embodiment 21. The variant of embodiment 20, wherein the CBM is a Family 20 CBM.

Embodiment 22. The variant of any of the embodiments 18-21, wherein the CBM is selected from the group consisting of:

- 5 i) a polypeptide of SEQ ID NO: 14, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 14;
- ii) a polypeptide of SEQ ID NO: 15, or a polypeptide having 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 10 98%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 15;
- iii) a polypeptide of SEQ ID NO: 16, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 16;
- iv) a polypeptide of SEQ ID NO: 17, or a polypeptide having at least 65%, at least 70%, at least 15 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 17;
- v) a polypeptide of SEQ ID NO: 18, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 18; and
- 20 vi) a polypeptide of SEQ ID NO: 19, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 19.

25 Embodiment 23. The variant of any of embodiments 18-22, wherein the linker is between 1 to 100 amino acids.

Embodiment 24. An isolated polynucleotide encoding the variant of any one of embodiments 1-23.

30 Embodiment 25. A nucleic acid construct or expression vector comprising the polynucleotide of embodiment 24 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

35 Embodiment 26. A recombinant host cell comprising the polynucleotide of embodiment 24 operably linked to one or more heterologous control sequences that direct the production of the polypeptide.

Embodiment 27. The host cell according to embodiment 26, wherein the host cell is a yeast cell, particularly a *Saccharomyces*, such as *Saccharomyces cerevisiae*.

Embodiment 28. A method of producing a variant alpha-amylase of any of embodiments 1-23, comprising: cultivating the recombinant host cell of embodiment 26 under conditions suitable for expression of the variant; and optionally recovering the variant.

Embodiment 29. A composition comprising the variant alpha-amylase of any of embodiments 1-23.

Embodiment 30. The composition of embodiment 29 further comprising at least one glucoamylase.

Embodiment 31. A whole broth formulation or cell culture composition comprising the variant alpha-amylase of any of embodiments 1-23.

Embodiment 32. The composition of embodiment 29, further comprising a surfactant.

Embodiment 33. The composition of embodiment 32, wherein the composition comprises a surfactant or surfactant system wherein the surfactant can be selected from nonionic surfactants, anionic surfactants, cationic surfactants, ampholytic surfactants, zwitterionic surfactants, semi-polar nonionic surfactants and mixtures thereof.

Embodiment 34. The composition of embodiment 33, wherein the composition comprises an anionic surfactant, in particular linear alkylbenzene sulfonate (LAS) and/or alcohol ethoxysulfate (AEOS).

Embodiment 35. The composition of embodiment 33, wherein the composition comprises a nonionic surfactant, such as alcohol ethoxylate (AEO).

Embodiment 36. The composition of any of embodiments 32-35, wherein the composition comprises one or more anionic and/or one or more nonionic surfactants.

Embodiment 37. The composition of any of embodiments 32-36, wherein the composition comprises one or more of surfactants, in particular linear alkylbenzenesulfonic acid (LAS), sodium laureth sulfate (SLES) and/or alcohol ethoxylate (AEO).

Embodiment 38. A use of a variant alpha-amylase of any of embodiments 1-23 for production of syrup and/or a fermentation product.

Embodiment 39. A process of producing a fermentation product from starch-containing material comprising the steps of: (a) liquefying starch-containing material above the initial gelatinization temperature of said starch-containing material in the presence of an alpha amylase; (b) saccharifying the liquefied material; and (c) fermenting with a fermenting organism; wherein step (b) is carried out in the presence of at least a variant alpha-amylase of any of embodiments 1-23, and optionally a glucoamylase.

Embodiment 40. The process of embodiment 39, wherein step (b) and step (c) are carried out simultaneously.

Embodiment 41. The process of any of the embodiments 39-40, wherein the host cell of any of embodiments 26-27 is applied as the fermenting organism.

Embodiment 42. The process of embodiment 39, wherein the host cell further is expressing a glucoamylase.

Embodiment 43. The process according to any of embodiments 41-42, wherein the host cell further is expressing an alpha-amylase derived from a strain of the genus *Rhizomucor*, preferably a strain of *Rhizomucor pusillus*, such as a *Rhizomucor pusillus* alpha-amylase hybrid having a linker and starch-binding domain from an *Aspergillus niger* glucoamylase.

Embodiment 44. The process of embodiment 43, wherein the further alpha-amylase expressed by the host cell in fermentation is selected from the group consisting of:

- (i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 12;
- (ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 12.

Embodiment 45. The process of embodiment 44, wherein the further alpha-amylase comprises one or more of the following substitutions: G128D, D143N, preferably G128D+D143N, using SEQ ID NO: 12 for numbering.

Embodiment 46. The process of any of embodiments 39-45, wherein the fermenting organism or host cell is a yeast cell, particularly a *Saccharomyces* cell, such as *Saccharomyces cerevisiae*.

Embodiment 47. The process of any of embodiments 39-46, wherein the fermentation product is an alcohol, such as ethanol.

Embodiment 48. The process of any of embodiments 39-47, wherein the starch-containing material is corn.

Embodiment 49. A process of producing a syrup product from starch-containing material, comprising the step of: (a) liquefying starch-containing material at a temperature above the initial gelatination temperature of said starch-containing material in the presence of an alpha-amylase; (b) saccharifying the liquefied material in the presence of at least a variant alpha-amylase of any of the embodiments 1-23, and optionally a glucoamylase.

Embodiment 50. The process of embodiment 49, wherein step a) is carried out using at least a variant alpha-amylase of any of embodiments 1-22, and a second alpha-amylase.

Embodiment 51. The variant alpha-amylase according to any of embodiments 1-23, wherein the variant is isolated.

Embodiment 52. A transgenic plant, plant part or plant cell comprising the variant alpha-amylase of any of embodiments 1- 23.

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

Examples**Strains**

Bacillus amyloliquefaciens strain used in the examples was isolated from soil in Virginia in the USA in 2011.

- 5 *Saccharomyces cerevisiae* strain MBG5012 (deposited under Accession No. NRRL Y67700 at the Agricultural Research Service Patent Culture Collection 5 (NRRL), USA, and described in WO19/161227.

Materials and Methods

- 10 Material:

TB-GLY media

MSA-SUB-FS-057

Raw material	Amount	
Tryptone	13,3	g
Yeast extract	26,6	g
Glycerol anhydrous pure	5,5	g
Ion exchanged water up to	1000	ml

- 15 The 100 mM BR buffer is prepared from a 1M BR stock by adding 50 μ L of a 2M CaCl_2 solution, and 1000 μ L of a 10% Brij35 stock solution to 100 mL 1M stock, and 900 mL milliQ water to a concentration of 0.1mM CaCl_2 and 0.01% Brij35.

The 1M BR stock is prepared by dissolving 136 g Sodium acetate dihydrate (CAS 6131-90-4), 142 g Disodium hydrogen phosphate (CAS 7558-79-4), and 61.8g Boric acid (CAS 10043-35-3)

- 20 in milliQ water to a total volume of 1L.

After dilution, the pH of the 100 mM BR buffer is adjusted to pH 4 or pH 7 by adding HCL or NaOH as needed.

Assays:

- 25 **pNP-G7 alpha-amylase activity assay**

The alpha-amylase activity may be determined by a method employing the G7-pNP substrate. G7-pNP which is an abbreviation for 4,6-ethylidene(G₇)-p-nitrophenyl(G₁)- α ,D-maltoheptaoside, a blocked oligosaccharide which can be cleaved by an endo-amylase, such as an alpha-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the hydrolyzed substrate

30 further to liberate a free PNP molecule which has a yellow color and thus can be measured by visible spectrophotometry at $\lambda=405\text{nm}$ (400-420 nm.). Kits containing G7-pNP substrate and alpha-Glucosidase is manufactured by Roche/Hitachi (cat. No.11876473) or Sigma-Aldrich (Catalog

number MAK009).

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) .

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

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 μ l diluted enzyme samples to 96 well microtiter plate and adding 80 μ l 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 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.

Amylase pH 4 stability screening assay

Sample preparation

The expression clones are grown between 18 hours and 24 hours under good expression conditions in TB-GLY medium in 2.2 mL deep-well plates at 37°C at 700 RPM in a TH 15 TiMix microplate shaker from Edmund Bühler GmbH.

After fermentation, the samples can be spun down to reduce the number of cells in the supernatant.

Sample incubation

From each variant supernatant sample, a subsample was incubated at pH 4 and 37°C for 18 to 24 hours in a 100 mM BR-buffer adjusted to pH 4 (hereafter called the stressed sample), while an equivalent subsample was stored at pH 7 at 4°C or below in a 100 mM BR buffer adjusted to

pH 7 (hereafter called the unstressed sample). Incubation was performed in a 96-well Nunc MicroWell polypropylene plate (Sigma Aldrich P6866) sealed with an Agilent PlateLoc plate-sealer.

10 μ L of variant supernatant sample was diluted 10 times by mixing it into 90 μ L buffer for incubation.

Sample analysis

After incubation, the samples were prediluted 20 before activity was measured. Amylase activity was determined using a G7-pNP kit (Sigma-Aldrich Catalog number MAK009). Sample dilution was performed in the pH 7 BR buffer. A further dilution was done to a total in-assay dilution of 1000x, by mixing 10 μ L of diluted sample with 40 μ L G7 substrate in a 380-well PerkinElmer SpectraPlate reader-plate.

The stressed and unstressed sample of a given variant were read on the same reader-plate. The enzyme activity was evaluated according to the G7 assay protocol. The solution was mixed and pre-incubated 1 minute at room temperature and absorption was measured every 20 sec. over 30 minutes at OD 405 nm, and the highest measured slope was used as a measure of enzyme activity. A well with no enzyme sample added was used as control, and any slope in that well was subtracted from all the measured activities on the same plate.

Data evaluation – determination of half-life

For a given variant, the enzyme activity of the stressed sample was divided by the enzyme activity of the unstressed sample, to compute residual activity. From this, the half-life in hours of the enzyme candidate is computed as the negative of the incubation-time in hours divided by log2 of the residual activity.

Example 1: Cloning and expression of the parent wild type *Bacillus amyloliquefaciens* alpha-amylase and variants thereof

The strain *Bacillus amyloliquefaciens* was isolated from soil in Virginia in the USA in 2011. Chromosomal DNA from the strain was subjected to full genome sequencing using Illumina technology. The GH13 subfamily 28 amylase was identified in the genome by analysing for glycosyl hydrolase domains (according to the CAZY definition).

A linear integration vector-system was used for the expression cloning of the amylase from *Bacillus amyloliquefaciens*. The linear integration construct was a PCR fusion product made by fusion of the gene between two *Bacillus subtilis* homologous chromosomal regions along with a

strong promoter and a chloramphenicol resistance marker. The fusion was made by SOE PCR (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Engineering hybrid genes without the use of restriction enzymes, gene splicing by overlap extension Gene 77: 61-68). The SOE PCR method is also described in patent application WO 2003/095658. The gene was expressed under the control of a triple promoter system (as described in WO 1999/43835), consisting of the promoters from *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), and the *Bacillus thuringiensis* cryIIIA promoter including stabilizing sequence. The gene coding for chloramphenicol acetyl-transferase was used as marker (described in e.g. Diderichsen, B.; Poulsen, G.B.; Joergensen, S.T. 1993, *Plasmid*, "A useful cloning vector for *Bacillus subtilis*" 30:312. The final gene construct was integrated in the *Bacillus* chromosome by homologous recombination into the pectate lyase locus.

The gene encoding the amylase was amplified from chromosomal DNA of the strains with gene specific primers containing overhang to the two flanking vector fragments. The amylase was expressed with a *Bacillus clausii* secretion signal (amino acids 1-27 of SEQ ID NO: 6) replacing the genes native secretion signal and with a histidine tag of 6 histidines fused directly to the C-terminal of the protein. The polynucleotide sequence used for expression was included herein as SEQ ID NO: 5. The upstream and downstream vector fragments were amplified from genomic DNA of the strain MB1361 (based on strain PL3598 described in patent application WO 2003095658). The 2 linear vector fragments and the gene fragment were assembled into one linear vector construct by SOE PCR. An aliquot of the PCR product was transformed into *Bacillus subtilis*. Transformants were selected on LB plates supplemented with 6 µg of chloramphenicol per ml. A recombinant *Bacillus subtilis* clone containing the sequence confirmed integrated expression construct was cultivated in liquid culture on a rotary shaking table in 500 mL baffled Erlenmeyer flasks each containing 100 ml yeast extract-based media. The clone was cultivated for 4 days at 30°C. The enzyme containing supernatant was harvested and the enzyme purified as described in Example 2.

Example 2: Purification of the His-tagged amylase from *Bacillus amyloliquefaciens*

The pH of the supernatant was adjusted to pH 8 with 3 M Tris, left for 1 hour, and then filtered using a filtration unit equipped with a 0.2 µm filter (Nalgene). The filtered supernatant was applied to a 5 ml HisTrap™ Excel column (GE Healthcare Life Sciences) pre-equilibrated with 5 column volumes (CV) of 50 mM Tris/HCl pH 8. Unbound protein was eluted by washing the column with 8 CV of 50 mM Tris/HCl pH 8. The amylase was eluted with 50 mM HEPES pH 7-10 mM imidazole and elution was monitored by absorbance at 280 nm. The eluted amylase was desalted on a HiPrep™ 26/10 desalting column (GE Healthcare Life Sciences) pre-equilibrated with 3 CV of 50 mM HEPES pH 7-100 mM NaCl. The amylase was eluted from the column using the same buffer at a flow rate of 10 ml/minute. Relevant fractions were selected and pooled based on the

chromatogram and SDS-PAGE analysis using 4-12% Bis-Tris gels (Invitrogen) and 2-(N-morpholino)ethanesulfonic acid (MES) SDS-PAGE running buffer (Invitrogen). The gel was stained with InstantBlue (Novexin) and destained using milliQ water. The concentration of the purified enzyme was determined by absorbance at 280 nm.

Example 3: Determination of pH stability for variant alpha-amylases

The pH stability of the amylase variants according to the invention were tested as described above using the G7-pNP kit (Sigma-Aldrich Catalog number MAK009). After growing the variants as described, supernatant samples of each variant were tested for increased stability at pH 4 over the parent amylase. A subsample was incubated at pH 4 and 37°C for 19.5 or 21 hours in a 100 mM BR-buffer adjusted to pH 4 (hereafter called the stressed sample), while an equivalent subsample was stored at pH 7 at 4°C or below in a 100 mM BR buffer adjusted to pH 7 (hereafter called the unstressed sample). Incubation was performed in a 96-well Nunc MicroWell polypropylene plate (Sigma Aldrich P6866) sealed with an Agilent PlateLoc plate-sealer.

After incubation, the samples were prediluted 20 to 200 times before activity was measured. Amylase activity was determined using a G7-pNP kit (Sigma-Aldrich Catalog number MAK009). Sample dilution was performed in the pH 7 BR buffer. A further dilution was done to a total in-assay dilution of 100x to 1000x. 10 µL of diluted sample is mixed with 40 µL G7 substrate in a 380-well PerkinElmer SpectraPlate reader-plate.

The stressed and unstressed sample of each variant were read on the same reader-plate. The enzyme activity was evaluated according to the G7 assay protocol. The solution was mixed and pre-incubated 1 minute at room temperature and absorption was measured every 20 sec. over 30 minutes at OD 405 nm, and the highest measured slope was used as a measure of enzyme activity. A well with no enzyme sample added was used as control, and any slope in that well was subtracted from all the measured activities on the same plate.

The results of the stability test are shown in Table 1 and 2 below.

Table 1. Half-life for variants stressed at pH 4.0, 37°C for 19.5 hours

Variant substitutions	IF	HIF	Half Life (hours)
A222I	8.1	4.0	19.4
A222V	7.9	3.9	18.7
S199G	7.8	3.8	18.6
N196W, N207W, N603W	7.2	3.4	16.7
N196W, N603W	7.1	3.4	16.5

N196W, N207W	6.8	3.2	15.6
A222E	4.8	2.3	11.1
N207W, N603W	1.4	1.1	5.5
N603W	1.3	1.1	5.4
Wt parent amylase SEQ ID NO: 1	1.0	1.0	4.8

Table 2. Half-life for variants stressed at pH 4.0, 37°C for 21 hours

Variant substitution	HIF	Half Life (hours)
S199G	3.94	16.82
A222V	3.86	16.46
A222I	3.85	16.42
N196W	3.48	14.84
A222E	2.90	12.38
Wt parent amylase SEQ ID NO: 1	1.08	4.61

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The results show that all tested variants had increased stability at pH 4.0 compared to the wild type parent alpha-amylase of SEQ ID NO: 1.

Example 4: Determination of pH 4 stability for variant alpha-amylases in presence of raw starch

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Amylase variants were tested as supernatants from fermentation and purified samples. Purified enzyme samples were diluted in water with 0.01 % Brij to a concentration of 5 μ M. For the assay, 10 μ l of supernatant or diluted purified sample was mixed with 90 μ l 10% raw corn starch in 100 mM BR buffer, pH 4. Each sample was prepared on two separate plates, where one was stressed by incubation for 18 hours at 37°C, 850 rpm (hereafter called stressed samples) and the second was incubated for 2 minutes at room temperature, 850 rpm (hereafter called unstressed samples). After respective incubation, both plates were spun down for 2 min at 2000 rpm and supernatants were stored at -20°C until analysis. Defrosted supernatants were diluted 10 x in 100 mM BR buffer, pH 7 and enzyme activity was evaluated using G7-pNP assay protocol (Roche/Hitachi, cat. no.11876473). Briefly, 20 μ l diluted enzyme sample was mixed with 100 μ l G7-pNP solution and absorbance was followed at 405 nm for 20 min at room temperature. Initial slopes (0-2min), after blank subtraction, were used as activity measure. Residual activity after stress was calculated by dividing activity of stressed samples with unstressed samples. From

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this, the half-life ($T_{1/2}$) in hours of the enzyme candidate was computed as the incubation-time in hours divided by $\log_2$ of the residual activity multiplied by -1.

Improvement factor (IF) was calculated from the estimated half-life ($T_{1/2}$), by dividing the estimated $T_{1/2}$ for variants with the $T_{1/2}$ of the wild type enzyme (SEQ ID NO:1).

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Table 3. Half-life for variants stressed at pH 4.0, 37°C for 18 hours in presence of raw starch

	Supernatant samples			Purified samples		
	RA @ 18h	$T_{1/2}$ (hour)	IF ($T_{1/2}$)		RA @ 18h	$T_{1/2}$ (hour)
Wt parent amylase SEQ ID NO: 1	7%	4,9	1,0	Ref.	0%	
N196W	28%	9,8	2,0	N196W	23%	8,7

The results show that listed variants had increased stability at pH 4.0 compared to the wild type parent alpha-amylase of SEQ ID NO: 1.

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Example 5: Impact of protein engineering on *B. amyloliquefaciens* alpha-amylase (SEQ ID NO: 1) to improve ethanol yield and increase kinetics during raw-starch corn mash fermentation

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This example describes the evaluation of the alpha-amylase of SEQ ID NO: 1 containing a mutation for improved ethanol and kinetics during raw-starch corn mash simultaneous saccharification and fermentation (SSF). Particularly, the ethanol and kinetics during fermentation are compared among the enzymes listed in Table 4.

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Table 4. List of enzymes used

Enzymes
Wt AA <i>Bacillus amyloliquefaciens</i> (SEQ ID NO: 1)
N196W variant AA

Seed culture:

Cryo-preserved culture of *Saccharomyces cerevisiae* strain MBG5012 (deposited under Accession No. NRRL Y67700 at the Agricultural Research Service Patent Culture Collection 5 (NRRL), USA, and described in WO19/161227 (incorporated herein by reference in its entirety) was first grown in liquid YPD media (Yeast extract, 10 g. Peptone, 20 g. Dextrose, 60 g. dissolve

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in 1 L of distilled water). Cultivation was done aseptically in a sterile 125-ml Erlenmeyer flask filled with 50 ml YPD media and inoculated with 100 µl of cryo-preserved culture. Flask was incubated in a shaking incubator at 32°C for 16 h with shaking at 150 rpm. The YPD grown seed cultures (40 ml) were centrifuged at 3,500 rpm for 10 min at 22°C, and the resulting cell pellet was washed and resuspended in tap water and glycerol. The resuspended cells were used to inoculate the corn mash at the beginning of simultaneous saccharification and fermentation (SSF).

Corn mash:

Corn kernels were ground using the Turkish grind setting on a Bunn Coffee Grinder. The %dried solids (DS) of the corn flour was 84.50%. Using the Turkish ground corn and tap water, a slurry targeting 37.50%DS was prepared. The corn slurry was supplemented with 1000 ppm urea and 3 ppm of antibiotic LACTROL™ and its pH was adjusted to 4.5 prior to use in SSF. Final dried solids level was determined to be 37.50%DS.

Simultaneous Saccharification and Fermentation (SSF)

All fermentations were carried out in 12mL round-bottom tubes with caps having a drilled hole. Tubes were filled with 3.8-4.5 g of corn slurry and inoculated with seed culture at 10 million cells per gram mash. A glucoamylase from *Trametes cingulate* (disclosed herein as SEQ ID NO: 7) was added to the tubes at 88 µg enzyme protein per g of dry corn solids. Amylases in Table 4 were added to the tubes at 32 µg enzyme protein per g of dry corn solids. All tubes contained the same glucoamylase and one amylase from Table 4 was added per tube. Tubes were incubated in an incubator at 32°C. Tubes were vortexed two times per day. Fermentation was run for 72 hours.

Weight loss analysis

Initial weight of tubes prior to fermentation was recorded. Weight of tubes was recorded after 18, 24, 48 and 72 h of fermentation. The difference in weight between 0 h and 18, 24, 48 and 72 h of fermentation was calculated. Weight difference was divided by g DS per tube resulting in g weight loss/g DS.

Ethanol analysis

After 72 hours of fermentation, 100 µl of 40% v/v H₂SO₄ was added to each sample tube, samples were vortexed, and centrifuged at 3,500 rpm for 10 min at 22°C. The resulting supernatant was filtered through a 0.2 µm syringe filter. Filtered samples were stored at 4°C prior to and during HPLC analysis. Analysis of ethanol was conducted using an HPLC (Agilent 1100/1200 series) machine equipped with a guard column (Bio-Rad, Micro-Guard Cation H+

Cartridge, 30 x 4.6mm) and an analytical column (Bio-Rad, Aminex HPX-87H, 300 x 7.8mm) using 5mM Sulfuric Acid as a mobile phase with a flow rate of 0.8mL/min. Column temperature was maintained at 65°C, and ethanol was detected using a Refractive Index detector at 55°C.

Results

Table 5 and Table 6 show the CO₂ g weight loss kinetics/gDS and ethanol titers, respectively, for the amylases during corn mash fermentation. Results indicate that the fermentation kinetics and ethanol titer increase when the fermentation is run with a variant alpha-amylase comprising a N196W substitution versus the parent wt amylase. The difference in kinetics and ethanol titer can be attributed to the mutation present in the variant amylase. It can be concluded that the N196W substitution present in the variant provides improvement in fermentation performance by enabling increased activity on the starch substrate present in the raw-starch corn slurry.

Table 5. Gram weight loss/g DS kinetics during raw-starch fermentation run with

Amylase	18h g Weight Loss/g DS Mean	24h g Weight Loss/g DS Mean	48h g Weight Loss/g DS Mean	72h g Weight Loss/g DS Mean
SEQ ID NO: 1	0.159	0.215	0.353	0.460
N196W	0.173	0.231	0.379	0.468

Table 6. Ethanol titer after 72 h of fermentation in raw-starch slurry with amylases from Table 4.

Amylase	Mean Ethanol % (v/v)	Std Dev
SEQ ID NO: 1	18.32	0.13
N196W	19.01	0.18

Example 6. Determination of pH 4 stability for variant alpha-amylases in presence and absence of raw starch

Purified amylase variants were diluted in water with 0.01 % Brij to a concentration of 5 µM. For the assay, 10 µl diluted purified sample was mixed with 90 µl 100 mM BR buffer, pH or with 10% raw corn starch in 100 mM BR buffer, pH 4. Each sample was prepared on two separate plates, where one was stressed by incubation for 96 hours at 32°C, 850 rpm (hereafter called stressed

samples) and the second was incubated for 2 minutes at room temperature, 850 rpm (hereafter called unstressed samples). After respective incubation, both plates were spun down for 2 min at 2000 rpm and supernatants were stored at -20°C until analysis. Defrosted supernatants were diluted 10 x in 100 mM BR buffer, pH 7 and enzyme activity was evaluated using G7-pNP assay protocol (Roche/Hitachi, cat. no.11876473). Briefly, 20 µl diluted enzyme sample was mixed with 100 µl G7-pNP solution and absorbance was followed at 405 nm for 20 min at room temperature. Initial slopes (0-2min), after blank subtraction, were used as activity measure. Residual activity after stress was calculated by dividing activity of stressed samples with unstressed samples.

Table 7. Residual activities for variants stressed at pH 4.0, 32°C for 96 hours in absence or presence of raw starch.

Mutations relative to WT parent amylase SEQ ID NO: 1	Residual Activity after incubation in buffer pH 4, with 10% starch	Residual Activity after incubation in buffer pH 4
S199G H626*	9	0
L150H S199G A222I	>95	83
L150M S199G A222V	86	88
N196W S199G A222V N603W	82	91
L150Y N196W S199G A222V	>95	93
L150F N196W S199G A222I	>95	>95
L150M N196W S199G A222V	>95	>95
L150W S199G A222V	>95	>95
L150H N196W S199G A222V	>95	>95
N196W S199G A222V H626*	>95	>95
L150W N196W S199G A222I	>95	>95
N196W S199G A222V	>95	>95
L150Y N196W S199G A222V	>95	>95
L150Y S199G A222V	>95	>95

The results show that listed variants had increased stability at pH 4.0 compared to the wild type parent alpha-amylase of SEQ ID NO: 1.

Example 7. Determination of pH 4 stability for variant alpha-amylases

Purified amylase variants were diluted in water with 0.01% Triton-X-100 to a concentration of 5 μ M. For the assay, the 5 μ M samples were diluted 10X into either stress buffer (222 mM sodium acetate buffer pH 4, 0.56mM CaCl₂ and 0.01% Brij-35) or dilution buffer (0.01% Triton-X-100).

5 Samples in the stress buffer were incubated at 32°C (850 rpm) for 24H (hereafter called stressed samples). The other samples mixed with dilution buffer were stored at 5°C until further analysis, max. 2 days (hereafter called unstressed samples).

After the respective incubations, samples were diluted 10-50X with assay buffer (500 mM Hepes pH 7, 0.5 mM CaCl₂, 0.01% Brij-35) and enzyme activity was evaluated using G7-pNP assay
10 protocol (Roche/Hitachi, cat. no.11876473). Briefly, 20 μ l diluted enzyme sample was mixed with 100 μ l G7-pNP solution and absorbance was measured at 405 nm for 20 min at room temperature. Initial slopes (lag time: 2 min, max absorbance = 2) were calculated and blank subtracted. These slopes – blank were used as activity measure. Residual activity (RA%) after
15 stress was calculated by dividing activity of stressed samples with unstressed samples and multiplying with 100.

Table 8. Residual activities (RA%) for variants stressed at pH 4.0, 32°C for 24 hours.

Variant substitutions	%RA after 24hrs at pH 4
<i>Wt parent amylase SEQ ID # 1</i>	0
A222V, H626*	13.8
A222I, H626*	14.4
A222E, H626*	4.2
N196W, H626*	10.2
L150H, H626*	1.6
L150M, H626*	0.70
L150Y, H626*	6.3
L150W	7.9
S199G	18.1
N196W, N603W, H626*	8.3
N196W, N207W, H626*	11.3
N196W, N207W, N603W, H626*	9.4
N196W, S199G, A222V	65.1
N196W, S199G, A222V, H626*	64.0
N196W, S199G, A222V, N603W	66.6
L150F, N196W, S199G, A222I	94.7
L150H, S199G, A222I	55.6
L150M, S199G, A222V	65.5
L150M, N196W, S199G, A222V	64.2
L150W, S199G, A222V	61.7
L150W, N196W, S199G, A222I	90.3
L150Y, S199G, A222V	58.2
L150Y, N196W, S199G, A222V	80.9
L150H, N196W, S199G, A222V	71.0
E96K, D179S, N196W, S199G, A222V, E284Q	77.9
R64S, E96K, N196W, S199G, A222V	86.6

The results show that the listed variants had increased stability at pH 4.0 compared to the wild type parent alpha-amylase of SEQ ID NO: 1.

Example 8. Determination of pH 4 stability for variant alpha-amylases

Purified amylase variants were diluted in water with 0.01% Triton-X-100 to a concentration of 5 μ M. For the assay, the 5 μ M samples were diluted 10X into either stress buffer (222 mM sodium acetate buffer pH 4, 0.56mM CaCl₂ and 0.01% Brij-35) or dilution buffer (0.01% Triton-X-100). Samples in the stress buffer were incubated at 32°C (850 rpm) for 24Hours (hereafter called stressed samples). The other samples mixed with dilution buffer were stored at 5°C until further analysis (hereafter called unstressed samples).

After incubation, the stressed samples were diluted 10-50X with assay buffer (500 mM Hepes pH 7, 0.5 mM CaCl₂, 0.01% Brij-35). The unstressed samples were diluted between 10-100X to get minimum four different dilutions and thus minimum 4 different concentrations. Enzyme activity was evaluated for all diluted samples (stressed and unstressed) using G7-pNP assay protocol (Roche/Hitachi, cat. no.11876473). Briefly, 20 μ l diluted enzyme sample was mixed with 100 μ l G7-pNP solution and absorbance was measured at 405 nm for 20 min at room temperature. Initial slopes (lag time: 2 min, max absorbance = 1.5) were calculated. For the unstressed samples, a nonlinear fit (e.g. Michaelis Menten) was made using initial slopes as Y and enzyme concentration as X. Based on this fit, the concentration of residual active enzyme was estimated in the stressed samples. The level of residual activity (RA%) was calculated by dividing the estimated concentration of residual active enzyme with the initial concentration of enzyme (at start) and multiplying with 100.

The results are given in Table 9 below. The listed alterations were introduced into the parent alpha-amylase of SEQ ID NO: 1, and the results show that the listed variants had increased stability at pH 4.0 compared to the wild type parent alpha-amylase of SEQ ID NO: 1.

Table 9. Residual activities (RA%) for variants stressed at pH 4.0, 32°C for 24 hours.

Variant substitutions	%RA after 24hrs at pH 4
Wt parent amylase of SEQ ID NO: 1	0
N28W, H626*	12.8
S199G, H626*	21.6
N196W, V599W, H626*	11
N196W, H550Y, P605S, H626*	12.5
N196W, A545P, T576Y, H626*	8

N196W, K549Y, G560P, H626*	8.7
I108P, Y183I, N196W, I205Y, H626*	16.9
N196W, R323K, H626*	9.6
N196W, D283P, H626*	11.4
W138Y, N196W, H626*	9.6
L150W, H626*	7.2
N196W, N392W, K417W, H626*	8.3
N196W, N392R, K417W, H626*	15.6
N196W, K549*, H550*, D551*, H626*	14.6
N196W, P580*, E581*, N582*, H626*	14
N196W, F592FK, H626*	10.9
N28W, N196W, N207W, S386D, N603W, H626*	9.3
R38Y, N196W, H626*	16.4
N196W, H259Y, H626*	11.4
N196W, Q412W, H626*	10
N196W, F212W, H626*	11.7
N196W, V599W, H626*	10
N196W, H550Y, P605S, H626*	13.4
N196W, H550Y, K589F, H626*	11.7
N196W, H550Y, D608Y, H626*	11.8
N196W, M574W, L614W, H626*	14.4
N196W, G533H, M574W, L614W, H626*	9.5
N196W, V543P, N570H, H626*	16.8
N196W, G533H, Y575W, L614W, H626*	11.1
N196W, A545P, T576Y, H626*	14.4
N196W, A566P, T578Y, H626*	11.9
N196W, K549Y, G560P, H626*	15.2
N196W, A566P, L577Y, H626*	14.4
N196W, I547Y, G560P, H626*	13.2
N196W, M574MW, H626*	16.5
N196W, K549*, H550*, D551*, M574MW, P580*, E581*, N582*, F592FK, H626*	13.1
N196W, L614W, G619W, H626*	14.4
N28W, I108P, N196W, N207W, S386D, A466V, Q542K, N603W, H626*	19.5
N28W, I108P, N196W, N207W, S386D, N603W, H626*	16.2
N196W, D282P, D283*, H626*	7.7
W138Y, N196W, H626*	6.6
N28W, N196W, N207W, S386D, N603W, H626*	19.5
N196W, S388W, A424P, H626*	15.9
N196W, S388W, A424P, L489Q, H626*	16
A117T, N196W, H550Y, D608Y, H626*	7.8
N196W, Q457R, Y575W, L614W, H626*	13.4
N196W, S199G	41.7
N196W, A222V	40.4
N196W, A222E	44.4
N196W, A222I	32.4
S199G, A222V	55.6
S199G, A222E	40.7
S199G, A222I	48.5
N196W, S199G, A222I	72.5
Q134L, H626*	Below 5
L150W, N156K, N196W, S199G, A222V	71.7

L150Y, N156K, N196W, S199G, A222I	63.2
L150W, N196W, S199G, A222I, A428S	73.1
L150F, N156K, N196W, S199G, A222I	67.7
L150Y, N156R, N196W, A222V	70.6
L150M, N156R, N196W, S199G, A222I	64.9
L150M, N156R, N196W, A222V	61.2
L150Y, N156R, N196W, S199G, A222V	69.8
L150M, N156K, N196W, S199G, A222V	51.1
L150Y, N156R, N196W, A222I	94.2
L150H, N156R, N196W, S199G, A222I	63.1
L150H, N156K, N196W, A222V	75
L150W, N156R, N196W, A222I	87.6
L150F, N156R, N196W, A222I	Above 95
L150F, N156K, N196W, S199G, A222V	81.6
L150H, N156K, N196W, S199G, A222V	60.8
L150F, N156R, S199G, A222I	65.9
L150M, N156K, N196W, A222V	57.5
L150W, N156K, N196W, S199G, A222I	72.2
N156K, N196W, S199G, A222V	65.3
L150Y, N156R, S199G, A222I	88.7
L150M, N156R, S199G, A222I	64.4
L150W, N156K, S199G, A222V	74.6
L150W, N156R, N196W, S199G, A222V	58.4
L150Y, N156R, N196W	52.1
N156K, N196W, A222V	59
N156K, N196W, S199G	46.4
N156R, S199G, A222V	82.2
S113H, N196W, S199G, A222V	55.5
Q71E, S113H, N196W, S199G, A222V	52.9
N196W, S199G, A222V, D283A	78
N196W, S199G, A222V, D283P	88.5
W142E, D193SQY, N196W, S199G, A222V, R224K	13.5
E96K, K101R, L150W, N156R, D179S, N196W, S199G, T208N, A222V	68.3
E96K, K101R, L150Y, N156R, D179S, N196W, S199G, T208N, A222V, E284Q	72.8
E96K, K101R, L150M, N156R, D179S, N196W, S199G, T208N, A222V, E284Q	58.2
S113Q, Q134E, N196W, S199G, A222V	50.5
S113D, Q134N, N196W, S199G, A222V	67
S113F, N196W, S199G, A222V	70.5
E171Q, N196W, S199G, N204D, A222V	81.9
N196W, S199G, A222V, H241N, S245N, T278N, E284Q, E285V	80
N196W, S199G, A222V, S394K, A414K, K417Y	56.9
N196W, S199G, A222V, E359Y, S394K, K396S, A414K, K417Y	Above 95
R38H, E96K, G99N, K101R, D179S, N196W, S199G, A222V	83.1
R38H, E96K, G99N, K101R, D179S, N196W, S199G, A222V, E284Q	80.3
V107T, H110D, N196W, S199G, A222V	66.9
Q134T, L150Y, N196W, S199G, A222V	86.2
S113F, L150W, N196W, S199G, A222V	68.6
S113F, L150Y, N196W, S199G, A222V	Above 95
E96K, K101R, N156R, D179S, N196W, S199G, T208N, A222V	69.4
E96K, K101R, L150Y, N156R, D179S, N196W, S199G, T208N, A222V	61.3

E96K, K101R, L150M, N156R, D179S, N196W, S199G, T208N, A222V	86.7
E96K, K101R, N156R, D179S, N196W, S199G, T208N, A222V, E284Q	66.9
E96K, K101R, L150W, N156R, D179S, N196W, S199G, T208N, A222V, E284Q	72.6
N28R, Q86R, N196W, S199G, A222V	63.1
N28R, Q86R, K89R, N196W, S199G, A222V	91.9
G56P, N196W, S199G, S209L, A222V	88.2
E96K, N196W, S199G, A222V	76.4
T10I, N196W, S199G, A222V	80.7
D39R, N196W, S199G, A222V	75.8
R64S, N196W, S199G, A222V	90.4
T10I, D39R, R64S, N196W, S199G, A222V	78.2
T10I, D39R, N196W, S199G, A222V	56.8
D39R, E96K, N196W, S199G, A222V	87
R64S, D90E, E96K, N196W, S199G, A222V	85.5
R38H, D39R, E96K, G99N, K101R, D179S, N196W, S199G, A222V	82.9
R38H, R64S, E96K, G99N, K101R, D179S, N196W, S199G, A222V	90.1
T10I, R38H, R64S, E96K, G99N, K101R, D179S, N196W, S199G, A222V	47.6
T10I, R38H, R64S, D90E, E96K, G99N, K101R, D179S, N196W, S199G, A222V	51
T10I, R38H, D39R, E96K, G99N, K101R, D179S, N196W, S199G, A222V	65.4
R38H, D39R, R64S, E96K, G99N, K101R, D179S, N196W, S199G, A222V	53.5
E96K, N196W, S199G, A222V, E284Q	58.4
T10I, N196W, S199G, A222V, E284Q	58.6
D39R, N196W, S199G, A222V, E284Q	50.4
R64S, N196W, S199G, A222V, E284Q	54.4
T10I, D39R, E96K, N196W, S199G, A222V, E284Q	62.3
D193SQY, N196W, S199G, A222V	38
Q134T, N196W, S199G, A222V	47.8
L174I, N196W, S199G, T208N, A222V	63.6
Y183F, N196W, S199G, T208S, A222V	64.8
N127D, N156R, N196W, S199G, A222V	83.1
Q134T, L150W, N196W, S199G, A222V	70.6
N57P, N196W, S199G, A222V	73.1
N196W, S199G, Q200W, A222V	58.7
T10I, D39R, R64S, E96K, N196W, S199G, A222V, E284Q	67.2
T10I, D39R, R64S, N196W, S199G, A222V, E284Q	78.1
T10I, R64S, E96K, N196W, S199G, A222V, E284Q	62.1
D39R, R64S, D90E, E96K, N196W, S199G, A222V, E284Q	64.8
T10I, R64S, E96K, N196W, S199G, A222V	54.5
T10I, D39R, N196W, S199G, A222V, E284Q	70.2
D39R, E96K, N196W, S199G, A222V, E284Q	52.3
D39R, R64S, N196W, S199G, A222V, E284Q	60.7
R64S, E96K, N196W, S199G, A222V, E284Q	67.9
D39R, R64S, N196W, S199G, A222V	63
S12*, S13*, V14*, K15*, N16*, I103Y, N196W, S199G, A222V, N233S, T308Y	48.8
S12*, S13*, V14*, K15*, N16*, A43D, I103Y, N196W, S199G, A222V, N233S, T308M	49.9
V9L, S12P, V14I, N16S, A43T, N196W, S199G, A222V	70.9
S12*, S13*, V14*, K15*, N16*, N196W, S199G, A222V	54.1
N28W, N196W, S199G, A222V	66.8

N196W, S199G, A222V, N392W, K417W	63.1
T10I, D39R, E96K, N196W, S199G, A222V	70.7
V9D, R38H, N196W, S199G, A222V, T348K	70.4
S113F, L150Y, N156K, N196W, S199G, A222V, H626*	59.2
S113Y, L150Y, N156K, N196W, S199G, A222V, H626*	73.1
S113W, L150Y, N156K, N196W, S199G, A222V, H626*	68.4
S113F, N156K, N196W, S199G, A222V, H626*	51.7
S113Y, N156K, N196W, S199G, A222V, H626*	42.1
S113W, N156K, N196W, S199G, A222V, H626*	59.2
W138Y, L150V, N196W, S199G, A222V, H626*	43.9
W138Y, L150V, D179G, N196W, S199G, A222V, H626*	39.3
W138Y, L150V, N196W, S199G, L218W, A222V, H626*	29.8
E96K, Q134L, D179S, N196W, S199G, A222V, E284Q, H626*	78.5
E96K, Q134L, L150Y, N156R, D179S, N196W, S199G, A222V, E284Q, H626*	51
E96K, L150Y, N156R, D179S, N196W, S199G, A222V, E284Q, H626*	65.2
R38H, E96K, G99N, K101R, Q134L, D179S, N196W, S199G, S221N, A222V, H626*	65.1
R38H, E96K, G99N, K101R, Q134L, D179S, N196W, S199G, A222V, H626*	69.9
R38H, E96K, G99N, K101R, Q134L, L150Y, N156R, D179S, N196W, S199G, A222V, H626*	62.1
R38H, E96K, G99N, K101R, L150Y, N156R, D179S, N196W, S199G, A222V, H626*	59.4
L150F, N196W, S199G, A222I, H626*	86.7
Q134L, L150F, N156R, N196W, S199G, A222I, H626*	78.8
Q134L, L150H, N156R, N196W, S199G, A222I, H626*	83.7
Q134L, L150M, N156K, N196W, S199G, A222I, H626*	68.1
Q134L, L150Y, N156K, N196W, S199G, A222I, Q457L, H626*	80.8
Q134M, L150W, N156K, N196W, S199G, A222I, H626*	50.9
Q134W, L150W, N156K, N196W, S199G, A222I, H626*	63.2
L150W, L152M, N156K, N196W, S199G, A222I, H626*	56.9
S113F, L150W, N156K, N196W, S199G, A222I, H626*	69.2
S113Y, L150W, N156K, N196W, S199G, A222I, H626*	55.6
L150W, G151W, N156K, N196W, S199G, A222I, H626*	23.2
L150W, G151S, N156K, N196W, S199G, A222I, H626*	22.1
S113F, L150W, G151S, N156K, N196W, S199G, A222I, H626*	38.3
Y67W, W68Y, L150W, N156K, N196W, S199G, A222I, H626*	60.6
A43V, L150M, G151F, N156R, N196W, S199G, A222I	48.2
L150M, G151Y, N156R, N196W, S199G, A222I	20.2
L150M, G151W, N156R, N196W, S199G, A222I	25.5
L150M, G151S, N156R, N196W, S199G, A222I, Y534H	52.8
S113F, L150M, G151S, N156R, N196W, S199G, A222I	17.6
Q134L, L150F, N156K, N196W, S199G, A222I, H626*	53.5
L150W, G151F, N156K, N196W, S199G, A222I, H626*	50.4
L150W, G151Y, N156K, N196W, S199G, A222I, H626*	46.4
Y67W, W68Y, L150W, N156K, N196W, S199G, A222I	58.1
G56W, N57P, Y67W, W68Y, L150W, N156K, N196W, S199G, A222I, H626*	60.5
K54I, Y67W, W68S, S113G, N196W, S199G, A222V, A382T, H626*	44.3
K54I, Y67W, W68S, S113G, D114Q, L150V, N196W, S199G, A222V, H626*	24.4
K54I, Y67W, W68S, S113G, W138Y, N196W, S199G, A222V, H626*	39.6
K54I, Y67W, W68S, S113G, D114Q, W138Y, L150V, N196W, S199G, A222V, H626*	18.8

Q134L, N196W, S199G, A222V, H626*	57.7
V107T, I108L, H110D, F169H, N196W, S199G, A222V, H626*	69.9
N196W, S199G, A222V, N392W, K417W, H626*	59.8
V9D, R38H, N196W, S199G, A222V, T348K, H626*	50.4
E96H, L150Y, N156R, N196W, S199G, A222V, E284Q, H626*	69.8
Q134L, L150Y, N196W, S199G, A222V, H626*	70.4
L150Y, N196W, S199G, A222V, H626*	Above 95
Q134L, L150Y, N196W, S199G, A222V, H626*	Above 95
L150F, N156R, N196W, S199G, A222V, H626*	52.6
L150H, N156R, N196W, S199G, A222V, H626*	70.1
Q134L, L150F, N156R, N196W, S199G, A222V, H626*	Above 95
Q134L, L150H, N156R, N196W, S199G, A222V, H626*	Above 95
Q134L, L150F, N156K, N196W, S199G, A222V, H626*	Above 95
Q134L, L150H, N156K, N196W, S199G, A222V, H626*	Above 95
Q134L, L150Y, N156K, N196W, S199G, A222V, H626*	Above 95
L150Y, N156K, N196W, S199G, A222V, H626*	Above 95
Q134L, L150Y, N156K, N196W, S199G, A222V, H626*	Above 95
Q134W, L150Y, N156K, N196W, S199G, A222V, H626*	Above 95
Q134M, L150Y, N156K, N196W, S199G, A222V, H626*	Above 95
Q134M, L150Y, N156K, N196W, S199G, A222V, A466V, H626*	Above 95
L150Y, L152M, N156K, N196W, S199G, A222V, H626*	Above 95
L150S, N196W, S199G, A222V, H626*	Above 95
N196W, S199G, A222V, N281S, H626*	Above 95
Y67T, N196W, S199G, A222V, H626*	Above 95
Q71N, N196W, S199G, A222V, H626*	Above 95
Q71N, A94D, N196W, S199G, A222V, H626*	Above 95
N196W, S199G, L218F, A222V, H626*	64.8
N196W, S199G, L218W, A222V, H626*	Above 95
N196W, S199G, A222V, T278W, H626*	66.8
N196W, S199G, A222V, T278W, T459M, H626*	Above 95
N196W, S199G, A222V, T278Y, H626*	Above 95
N196W, S199G, A222V, S275N, H626*	59.8
N196W, S199G, A222V, S275L, H626*	Above 95
N196W, S199G, A222V, S335Q, H626*	Above 95
N196W, S199G, A222V, S335K, H626*	71.3
N196W, S199G, A222V, S335R, H626*	Above 95
N196W, S199G, L218W, A222V, S335K, H626*	Above 95
N196W, S199G, L218W, A222V, S335Q, H626*	92.1
Y67W, N196W, S199G, A222V, H626*	Above 95
N196W, S199G, A222V, N281Q, H626*	Above 95
L150M, N156R, N196W, S199G, A222V, H626*	Above 95
Q134L, L150M, N156R, N196W, S199G, A222V, H626*	Above 95
Q134L, L150M, N156K, N196W, S199G, A222V, H626*	Above 95
D39R, N196W, S199G, A222V, N281Q, E284Q	Above 95
D39R, N196W, S199G, A222V, E284Q, Q479QP	Above 95
D39R, Y70F, N196W, S199G, A222V, E284Q	Above 95
N28W, D39R, N196W, S199G, A222V, E284Q	Above 95
D39R, N196W, S199G, N207W, A222V, E284Q	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, D39R, N196W, S199G, N207W, A222V, E284Q, H626*	Above 95
N196W, N207W	19.2
N196W, N207W, E284Q	19

E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, N196W, N207W, E284Q	16.8
L150Y, N196W, S199G, A222V, H626*	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, L150Y, N196W, S199G, A222V, E96K, K101R, L150M, N156R, D179S, N196W, S199G, N207W, T208N, A222V	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, E96K, K101R, L150M, N156R, D179S, N196W, S199G, T208N, A222V	Above 95
Q134L, L150H, N156R, N196W, S199G, A222I, E284Q, H626*	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, Q134L, L150H, N156R, N196W, S199G, A222I, H626*	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, Q134L, L150H, N156R, N196W, S199G, N207W, A222I, E284Q	Above 95
E96K, D179S, N196W, S199G, A222V, E284Q, H626*	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, E96K, D179S, N196W, S199G, A222V, E284Q	88.4
R64S, E96K, N196W, S199G, N207W, A222V	Above 95
N28W, D39R, N196W, S199G, N207W, A222V, E284Q	Above 95
D39R, N196W, S199G, A222V, E284Q, H626*	Above 95
D39R, N196W, S199G, N207W, A222V, E284Q	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, D39R, N196W, S199G, A222V, E284Q	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, N196W, N207W, E511D	20.9
L150Y, N196W, S199G, N207W, A222V	Above 95
E96K, K101R, L150M, N156R, D179S, N196W, S199G, T208N, A222V, H626*	Above 95
E96K, K101R, L150M, N156R, D179S, N196W, S199G, T208N, A222V, E284Q	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, E96K, K101R, L150M, N156R, D179S, N196W, S199G, N207W, T208N, A222V, E284Q, H626*	Above 95
Q134L, L150H, N156R, N196W, S199G, A222I	Above 95
N28W, E96K, D179S, N196W, S199G, A222V, E284Q	Above 95
R64S, E96K, N196W, S199G, A222V, H626*	Above 95
R64S, E96K, N196W, S199G, A222V, E284Q	Above 95
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, R64S, E96K, N196W, S199G, A222V	93.7

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 of a parent alpha-amylase, comprising an alteration at one or more
5 positions corresponding to positions 196, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28,
38, 39, 43, 54, 56, 57, 64, 67, 68, 70, 71, 86, 89, 90, 94, 96, 99, 101, 103, 107, 108, 110, 113,
114, 117, 127, 134, 138, 142, 150, 151, 152, 156, 169, 171, 174, 179, 183, 193, 199, 200, 204,
205, 207, 208, 209, 212, 218, 221, 222, 224, 233, 241, 245, 259, 275, 278, 281, 282, 283, 284,
285, 308, 323, 335, 348, 359, 382, 386, 388, 392, 394, 396, 412, 414, 417, 424, 428, 457, 459,
10 466, 479, 489, 511, 533, 534, 542, 543, 545, 547, 549, 550, 551, 560, 566, 570, 574, 575, 576,
577, 578, 580, 581, 582, 589, 592, 599, 603, 605, 608, 614, 619, or 626 of the polypeptide of
SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity,
15 but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ
ID NO: 3, or SEQ ID NO: 4.

2. An alpha-amylase variant according to claim 1, comprising a substitution at one or more
positions corresponding to positions 196, 199, 64, 96, 150, 179, 207, 222, 284 and 603 of the
20 polypeptide of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and 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 95%, at least 96%, at least 97%, at least 98%, or at least 99%
sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1,
SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

3. The variant of claim 1, which comprises an alteration selected from the group consisting of:
E1*, T2*, A3*, N4*, K5*, S6*, N7*, K8*, V9*, V9D, V9L, T10*, T10I, A11*, S12*, S12P, S13*, V14*,
V14I, K15*, N16*, N16S, N28R, N28W, R38H, R38Y, D39R, A43D, A43T, A43V, K54I, G56P,
G56W, N57P, R64S, Y67T, Y67W, W68S, W68Y, Y70F, Q71E, Q71N, Q86R, K89R, D90E,
30 A94D, E96H, E96K, G99N, K101R, I103Y, V107T, I108L, I108P, H110D, S113D, S113F, S113G,
S113H, S113Q, S113W, S113Y, D114Q, A117T, N127D, Q134E, Q134L, Q134M, Q134N,
Q134T, Q134W, W138Y, W142E, L150F, L150H, L150M, L150S, L150V, L150W, L150Y,
G151F, G151S, G151W, G151Y, L152M, N156K, N156R, F169H, E171Q, L174I, D179G,
D179S, Y183F, Y183I, D193SQY, N196W, S199G, Q200W, N204D, I205Y, N207W, T208N,
35 T208S, S209L, F212W, L218F, L218W, S221N, A222E, A222I, A222V, R224K, N233S, H241N,
S245N, H259Y, S275L, S275N, T278N, T278W, T278Y, N281Q, N281S, D282P, D283*, D283A,
D283P, E284Q, E285V, T308M, T308Y, R323K, S335K, S335Q, S335R, T348K, E359Y, A382T,

S386D, S388W, N392R, N392W, S394K, K396S, Q412W, A414K, K417W, K417Y, A424P, A428S, Q457L, Q457R, T459M, A466V, Q479QP, L489Q, E511D, G533H, Y534H, Q542K, V543P, A545P, I547Y, K549*, K549Y, H550*, H550Y, D551*, G560P, A566P, N570H, M574MW, M574W, Y575W, T576Y, L577Y, T578Y, P580*, E581*, N582*, K589F, F592FK, V599W,
 5 N603W, P605S, D608Y, L614W, G619W, and H626*.

4. The variant of claim 2, which comprises an alteration selected from the group consisting of: R64S, E96K, L150Y, L150W, L150H, L150M, L150F, D179S, N196W, S199G, N207W, A222E, A222I, A222V, E284Q, N603W, and H626*.

10 5. The variant of any one of claims 1-4, which has an improved property relative to the parent, wherein the improved property is increased pH stability at pH 4.0, 32 °C to 37°C, preferably 32 °C, compared to a parent alpha-amylase, particularly the alpha-amylase disclosed as SEQ ID NO: 1.

15 6. The variant of any of claims 1-5, comprising a substitution or a combination of substitutions selected from:

A222I;

A222V;

20 A222E;

S199G;

N196W;

N207W;

N603W;

25 L150Y;

L150W;

L150H;

L150M;

L150F;

30 R64S;

E96K;

D179S;

E284Q;

N207W + N603W;

35 N196W+N207W;

N196W+N603W;

N196W+N207W + N603W;

A222I + S199G + N196W;
 A222V + S199G + N196W;
 A222E + S199G + N196W;
 A222V + S199G + N196W + L150Y;
 5 L150H + S199G + A222I;
 L150M + S199G + A222V;
 N196W + S199G + A222V + N603W;
 L150F + N196W + S199G + A222I;
 L150M + N196W + S199G + A222V;
 10 L150W + S199G + A222V;
 L150H + N196W + S199G + A222V;
 L150W + N196W + S199G + A222I;
 L150Y + S199G + A222V;
 E96K + D179S + N196W + S199G + A222V + E284Q;
 15 R64S + E96K + N196W + S199G + A222V;

wherein the variant has alpha-amylase activity and 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 95%, at
 least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than 100%
 sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID
 20 NO: 4.

7. The variant of any of claims 1-6, comprising a N-terminal deletion, more particularly comprising
 at least amino acids 11 to 626 of SEQ ID NO: 1, at least amino acids 12 to 626 of SEQ ID NO:
 1, such as at least amino acids 13 to 626 of SEQ ID NO: 1.

8. The variant of any of claims 1-7, wherein the alpha-amylase further comprises a C-terminal
 deletion, particularly H626*.

9. The variant alpha-amylase of any of claims 1-5, comprising an alteration or a combination of
 30 alterations selected from:

N28W;
 N196W;
 S199G;
 N196W+ V599W;
 35 N196W+ H550Y+ P605S;
 N196W+ A545P+ T576Y;
 N196W+ K549Y+ G560P;

- I108P+ Y183I+ N196W+ I205Y;
N196W+ R323K;
N196W+ D283P;
W138Y+ N196W;
5 L150W;
N196W+ N392W+ K417W;
N196W+ N392R+ K417W;
N196W+ K549*+ H550*+ D551*;
N196W+ P580*+ E581*+ N582*;
10 N196W+ F592FK;
N28W+ N196W+ N207W+ S386D+ N603W;
R38Y+ N196W;
N196W+ H259Y;
N196W+ Q412W;
15 N196W+ F212W;
N196W+ V599W;
N196W+ H550Y+ P605S;
N196W+ H550Y+ K589F;
N196W+ H550Y+ D608Y;
20 N196W+ M574W+ L614W;
N196W+ G533H+ M574W+ L614W;
N196W+ V543P+ N570H;
N196W+ G533H+ Y575W+ L614W;
N196W+ A545P+ T576Y;
25 N196W+ A566P+ T578Y;
N196W+ K549Y+ G560P;
N196W+ A566P+ L577Y;
N196W+ I547Y+ G560P;
N196W+ M574MW;
30 N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK;
N196W+ L614W+ G619W;
N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W;
N28W+ I108P+ N196W+ N207W+ S386D+ N603W;
N196W+ D282P+ D283*;
35 W138Y+ N196W;
N28W+ N196W+ N207W+ S386D+ N603W;
N196W+ S388W+ A424P;

- N196W+ S388W+ A424P+ L489Q;
A117T+ N196W+ H550Y+ D608Y;
N196W+ Q457R+ Y575W+ L614W;
N196W+ S199G;
5 N196W+ A222V;
N196W+ A222E;
N196W+ A222I;
S199G+ A222V;
S199G+ A222E;
10 S199G+ A222I;
N196W+ S199G+ A222I;
Q134L;
L150W+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156K+ N196W+ S199G+ A222I;
15 L150W+ N196W+ S199G+ A222I+ A428S;
L150F+ N156K+ N196W+ S199G+ A222I;
L150Y+ N156R+ N196W+ A222V;
L150M+ N156R+ N196W+ S199G+ A222I;
L150M+ N156R+ N196W+ A222V;
20 L150Y+ N156R+ N196W+ S199G+ A222V;
L150M+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W+ A222I;
L150H+ N156R+ N196W+ S199G+ A222I;
L150H+ N156K+ N196W+ A222V;
25 L150W+ N156R+ N196W+ A222I;
L150F+ N156R+ N196W+ A222I;
L150F+ N156K+ N196W+ S199G+ A222V;
L150H+ N156K+ N196W+ S199G+ A222V;
L150F+ N156R+ S199G+ A222I;
30 L150M+ N156K+ N196W+ A222V;
L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ S199G+ A222I;
L150M+ N156R+ S199G+ A222I;
35 L150W+ N156K+ S199G+ A222V;
L150W+ N156R+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W;

- N156K+ N196W+ A222V;
 N156K+ N196W+ S199G;
 N156R+ S199G+ A222V;
 S113H+ N196W+ S199G+ A222V;
- 5 Q71E+ S113H+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ D283A;
 N196W+ S199G+ A222V+ D283P;
 W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
- 10 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 S113Q+ Q134E+ N196W+ S199G+ A222V;
 S113D+ Q134N+ N196W+ S199G+ A222V;
 S113F+ N196W+ S199G+ A222V;
- 15 E171Q+ N196W+ S199G+ N204D+ A222V;
 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
 N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
 N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
- 20 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 V107T+ H110D+ N196W+ S199G+ A222V;
 Q134T+ L150Y+ N196W+ S199G+ A222V;
 S113F+ L150W+ N196W+ S199G+ A222V;
 S113F+ L150Y+ N196W+ S199G+ A222V;
- 25 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
- 30 N28R+ Q86R+ N196W+ S199G+ A222V;
 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
 E96K+ N196W+ S199G+ A222V;
 T10I+ N196W+ S199G+ A222V;
- 35 D39R+ N196W+ S199G+ A222V;
 R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V;

- T10I+ D39R+ N196W+ S199G+ A222V;
D39R+ E96K+ N196W+ S199G+ A222V;
R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
5 R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
10 E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ N196W+ S199G+ A222V+ E284Q;
D39R+ N196W+ S199G+ A222V+ E284Q;
R64S+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
15 D193SQY+ N196W+ S199G+ A222V;
Q134T+ N196W+ S199G+ A222V;
L174I+ N196W+ S199G+ T208N+ A222V;
Y183F+ N196W+ S199G+ T208S+ A222V;
N127D+ N156R+ N196W+ S199G+ A222V;
20 Q134T+ L150W+ N196W+ S199G+ A222V;
N57P+ N196W+ S199G+ A222V;
N196W+ S199G+ Q200W+ A222V;
T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
25 T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;
D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
30 D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;
S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
35 V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
N28W+ N196W+ S199G+ A222V;

- N196W+ S199G+ A222V+ N392W+ K417W;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
S113F+ L150Y+ N156K+ N196W+ S199G+ A222V;
5 S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V;
S113W+ L150Y+ N156K+ N196W+ S199G+ A222V;
S113F+ N156K+ N196W+ S199G+ A222V;
S113Y+ N156K+ N196W+ S199G+ A222V;
S113W+ N156K+ N196W+ S199G+ A222V;
10 W138Y+ L150V+ N196W+ S199G+ A222V;
W138Y+ L150V+ D179G+ N196W+ S199G+ A222V;
W138Y+ L150V+ N196W+ S199G+ L218W+ A222V;
E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q;
E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
15 E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q;
R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V;
R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V;
R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V;
20 L150F+ N196W+ S199G+ A222I;
Q134L+ L150F+ N156R+ N196W+ S199G+ A222I;
Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;
Q134L+ L150M+ N156K+ N196W+ S199G+ A222I;
Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L;
25 Q134M+ L150W+ N156K+ N196W+ S199G+ A222I;
Q134W+ L150W+ N156K+ N196W+ S199G+ A222I;
L150W+ L152M+ N156K+ N196W+ S199G+ A222I;
S113F+ L150W+ N156K+ N196W+ S199G+ A222I;
S113Y+ L150W+ N156K+ N196W+ S199G+ A222I;
30 L150W+ G151W+ N156K+ N196W+ S199G+ A222I;
L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I;
Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
35 L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;
L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;

- S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151F+ N156K+ N196W+ S199G+ A222I;
 L150W+ G151Y+ N156K+ N196W+ S199G+ A222I;
- 5 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V;
 K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V;
- 10 K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V;
 Q134L+ N196W+ S199G+ A222V;
 V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N392W+ K417W;
 V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
- 15 E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q;
 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150Y+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N196W+ S199G+ A222V;
 L150F+ N156R+ N196W+ S199G+ A222V;
- 20 L150H+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150H+ N156K+ N196W+ S199G+ A222V;
- 25 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
 L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V;
 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V;
- 30 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V;
 L150Y+ L152M+ N156K+ N196W+ S199G+ A222V;
 L150S+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N281S;
 Y67T+ N196W+ S199G+ A222V;
- 35 Q71N+ N196W+ S199G+ A222V;
 Q71N+ A94D+ N196W+ S199G+ A222V;
 N196W+ S199G+ L218F+ A222V;

- N196W+ S199G+ L218W+ A222V;
 N196W+ S199G+ A222V+ T278W;
 N196W+ S199G+ A222V+ T278W+ T459M;
 N196W+ S199G+ A222V+ T278Y;
 5 N196W+ S199G+ A222V+ S275N;
 N196W+ S199G+ A222V+ S275L;
 N196W+ S199G+ A222V+ S335Q;
 N196W+ S199G+ A222V+ S335K;
 N196W+ S199G+ A222V+ S335R;
 10 N196W+ S199G+ L218W+ A222V+ S335K;
 N196W+ S199G+ L218W+ A222V+ S335Q;
 Y67W+ N196W+ S199G+ A222V;
 N196W+ S199G+ A222V+ N281Q;
 L150M+ N156R+ N196W+ S199G+ A222V;
 15 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V;
 Q134L+ L150M+ N156K+ N196W+ S199G+ A222V;
 D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
 D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
 20 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 N196W+ N207W;
 N196W+ N207W+ E284Q;
 25 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
 L150Y+ N196W+ S199G+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
 30 S199G+ T208N+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+
 N207W+ A222I+ E284Q;
 35 E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
 R64S+ E96K+ N196W+ S199G+ N207W+ A222V;

N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

D39R+ N196W+ S199G+ A222V+ E284Q;

D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

5 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

L150Y+ N196W+ S199G+ N207W+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+

10 S199G+ N207W+ T208N+ A222V+ E284Q;

Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

R64S+ E96K+ N196W+ S199G+ A222V;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

15 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

and wherein the variant has alpha-amylase activity and 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
95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity, but less than
100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or
20 SEQ ID NO: 4.

10. The variant of claim 9, wherein the variant alpha-amylase further comprises a C-terminal
deletion, particularly H626*.

25 11. The variant alpha-amylase of any of claims 1-5, comprising a combination of alterations
selected from:

N28W+ H626*;

S199G+ H626*;

N196W+ V599W+ H626*;

30 N196W+ H550Y+ P605S+ H626*;

N196W+ A545P+ T576Y+ H626*;

N196W+ K549Y+ G560P+ H626*;

I108P+ Y183I+ N196W+ I205Y+ H626*;

N196W+ R323K+ H626*;

35 N196W+ D283P+ H626*;

W138Y+ N196W+ H626*;

L150W+ H626*;

- N196W+ N392W+ K417W+ H626*;
 N196W+ N392R+ K417W+ H626*;
 N196W+ K549*+ H550*+ D551*+ H626*;
 N196W+ P580*+ E581*+ N582*+ H626*;
 5 N196W+ F592FK+ H626*;
 N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
 R38Y+ N196W+ H626*;
 N196W+ H259Y+ H626*;
 N196W+ Q412W+ H626*;
 10 N196W+ F212W+ H626*;
 N196W+ V599W+ H626*;
 N196W+ H550Y+ P605S+ H626*;
 N196W+ H550Y+ K589F+ H626*;
 N196W+ H550Y+ D608Y+ H626*;
 15 N196W+ M574W+ L614W+ H626*;
 N196W+ G533H+ M574W+ L614W+ H626*;
 N196W+ V543P+ N570H+ H626*;
 N196W+ G533H+ Y575W+ L614W+ H626*;
 N196W+ A545P+ T576Y+ H626*;
 20 N196W+ A566P+ T578Y+ H626*;
 N196W+ K549Y+ G560P+ H626*;
 N196W+ A566P+ L577Y+ H626*;
 N196W+ I547Y+ G560P+ H626*;
 N196W+ M574MW+ H626*;
 25 N196W+ K549*+ H550*+ D551*+ M574MW + P580*+ E581*+ N582*+ F592FK + H626*;
 N196W+ L614W+ G619W+ H626*;
 N28W+ I108P+ N196W+ N207W+ S386D+ A466V+ Q542K+ N603W+ H626*;
 N28W+ I108P+ N196W+ N207W+ S386D+ N603W+ H626*;
 N196W+ D282P+ D283*+ H626*;
 30 W138Y+ N196W+ H626*;
 N28W+ N196W+ N207W+ S386D+ N603W+ H626*;
 N196W+ S388W+ A424P+ H626*;
 N196W+ S388W+ A424P+ L489Q+ H626*;
 A117T+ N196W+ H550Y+ D608Y+ H626*;
 35 N196W+ Q457R+ Y575W+ L614W+ H626*;
 N196W+ S199G;
 N196W+ A222V;

N196W+ A222E;
N196W+ A222I;
S199G+ A222V;
S199G+ A222E;
5 S199G+ A222I;
N196W+ S199G+ A222I;
Q134L+ H626*;
L150W+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156K+ N196W+ S199G+ A222I;
10 L150W+ N196W+ S199G+ A222I+ A428S;
L150F+ N156K+ N196W+ S199G+ A222I;
L150Y+ N156R+ N196W+ A222V;
L150M+ N156R+ N196W+ S199G+ A222I;
L150M+ N156R+ N196W+ A222V;
15 L150Y+ N156R+ N196W+ S199G+ A222V;
L150M+ N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W+ A222I;
L150H+ N156R+ N196W+ S199G+ A222I;
L150H+ N156K+ N196W+ A222V;
20 L150W+ N156R+ N196W+ A222I;
L150F+ N156R+ N196W+ A222I;
L150F+ N156K+ N196W+ S199G+ A222V;
L150H+ N156K+ N196W+ S199G+ A222V;
L150F+ N156R+ S199G+ A222I;
25 L150M+ N156K+ N196W+ A222V;
L150W+ N156K+ N196W+ S199G+ A222I;
N156K+ N196W+ S199G+ A222V;
L150Y+ N156R+ S199G+ A222I;
L150M+ N156R+ S199G+ A222I;
30 L150W+ N156K+ S199G+ A222V;
L150W+ N156R+ N196W+ S199G+ A222V;
L150Y+ N156R+ N196W;
N156K+ N196W+ A222V;
N156K+ N196W+ S199G;
35 N156R+ S199G+ A222V;
S113H+ N196W+ S199G+ A222V;
Q71E+ S113H+ N196W+ S199G+ A222V;

- N196W+ S199G+ A222V+ D283A;
 N196W+ S199G+ A222V+ D283P;
 W142E+ D193SQY+ N196W+ S199G+ A222V+ R224K;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 5 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 S113Q+ Q134E+ N196W+ S199G+ A222V;
 S113D+ Q134N+ N196W+ S199G+ A222V;
 S113F+ N196W+ S199G+ A222V;
 10 E171Q+ N196W+ S199G+ N204D+ A222V;
 N196W+ S199G+ A222V+ H241N+ S245N+ T278N+ E284Q+ E285V;
 N196W+ S199G+ A222V+ S394K+ A414K+ K417Y;
 N196W+ S199G+ A222V+ E359Y+ S394K+ K396S+ A414K+ K417Y;
 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 15 R38H+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V+ E284Q;
 V107T+ H110D+ N196W+ S199G+ A222V;
 Q134T+ L150Y+ N196W+ S199G+ A222V;
 S113F+ L150W+ N196W+ S199G+ A222V;
 S113F+ L150Y+ N196W+ S199G+ A222V;
 20 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V;
 E96K+ K101R+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 E96K+ K101R+ L150W+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;
 25 N28R+ Q86R+ N196W+ S199G+ A222V;
 N28R+ Q86R+ K89R+ N196W+ S199G+ A222V;
 G56P+ N196W+ S199G+ S209L+ A222V;
 E96K+ N196W+ S199G+ A222V;
 T10I+ N196W+ S199G+ A222V;
 30 D39R+ N196W+ S199G+ A222V;
 R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ R64S+ N196W+ S199G+ A222V;
 T10I+ D39R+ N196W+ S199G+ A222V;
 D39R+ E96K+ N196W+ S199G+ A222V;
 35 R64S+ D90E+ E96K+ N196W+ S199G+ A222V;
 R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
 R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;

- T10I+ R38H+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ R64S+ D90E+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
T10I+ R38H+ D39R+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
R38H+ D39R+ R64S+ E96K+ G99N+ K101R+ D179S+ N196W+ S199G+ A222V;
5 E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ N196W+ S199G+ A222V+ E284Q;
D39R+ N196W+ S199G+ A222V+ E284Q;
R64S+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
10 D193SQY+ N196W+ S199G+ A222V;
Q134T+ N196W+ S199G+ A222V;
L174I+ N196W+ S199G+ T208N+ A222V;
Y183F+ N196W+ S199G+ T208S+ A222V;
N127D+ N156R+ N196W+ S199G+ A222V;
15 Q134T+ L150W+ N196W+ S199G+ A222V;
N57P+ N196W+ S199G+ A222V;
N196W+ S199G+ Q200W+ A222V;
T10I+ D39R+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
20 T10I+ R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ D90E+ E96K+ N196W+ S199G+ A222V+ E284Q;
T10I+ R64S+ E96K+ N196W+ S199G+ A222V;
T10I+ D39R+ N196W+ S199G+ A222V+ E284Q;
D39R+ E96K+ N196W+ S199G+ A222V+ E284Q;
25 D39R+ R64S+ N196W+ S199G+ A222V+ E284Q;
R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;
D39R+ R64S+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308Y;
S12*+ S13*+ V14*+ K15*+ N16*+ A43D+ I103Y+ N196W+ S199G+ A222V+ N233S+ T308M;
30 V9L+ S12P+ V14I+ N16S+ A43T+ N196W+ S199G+ A222V;
S12*+ S13*+ V14*+ K15*+ N16*+ N196W+ S199G+ A222V;
N28W+ N196W+ S199G+ A222V;
N196W+ S199G+ A222V+ N392W+ K417W;
T10I+ D39R+ E96K+ N196W+ S199G+ A222V;
35 V9D+ R38H+ N196W+ S199G+ A222V+ T348K;
S113F+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
S113Y+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;

- S113W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113F+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 S113W+ N156K+ N196W+ S199G+ A222V+ H626*;
 5 W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
 W138Y+ L150V+ D179G+ N196W+ S199G+ A222V+ H626*;
 W138Y+ L150V+ N196W+ S199G+ L218W+ A222V+ H626*;
 E96K+ Q134L+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 E96K+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 10 E96K+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ S221N+ A222V+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ D179S+ N196W+ S199G+ A222V+ H626*;
 R38H+ E96K+ G99N+ K101R+ Q134L+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ H626*;
 15 R38H+ E96K+ G99N+ K101R+ L150Y+ N156R+ D179S+ N196W+ S199G+ A222V+ H626*;
 L150F+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ H626*;
 Q134L+ L150M+ N156K+ N196W+ S199G+ A222I+ H626*;
 20 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222I+ Q457L+ H626*;
 Q134M+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 Q134W+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ L152M+ N156K+ N196W+ S199G+ A222I+ H626*;
 S113F+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 25 S113Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151W+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;
 S113F+ L150W+ G151S+ N156K+ N196W+ S199G+ A222I+ H626*;
 Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 30 A43V+ L150M+ G151F+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151Y+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151W+ N156R+ N196W+ S199G+ A222I;
 L150M+ G151S+ N156R+ N196W+ S199G+ A222I+ Y534H;
 S113F+ L150M+ G151S+ N156R+ N196W+ S199G+ A222I;
 35 Q134L+ L150F+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151F+ N156K+ N196W+ S199G+ A222I+ H626*;
 L150W+ G151Y+ N156K+ N196W+ S199G+ A222I+ H626*;

- Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I;
 G56W+ N57P+ Y67W+ W68Y+ L150W+ N156K+ N196W+ S199G+ A222I+ H626*;
 K54I+ Y67W+ W68S+ S113G+ N196W+ S199G+ A222V+ A382T+ H626*;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ L150V+ N196W+ S199G+ A222V+ H626*;
 5 K54I+ Y67W+ W68S+ S113G+ W138Y+ N196W+ S199G+ A222V+ H626*;
 K54I+ Y67W+ W68S+ S113G+ D114Q+ W138Y+ L150V+ N196W+ S199G+ A222V+ H626*;
 Q134L+ N196W+ S199G+ A222V+ H626*;
 V107T+ I108L+ H110D+ F169H+ N196W+ S199G+ A222V+ H626*;
 N196W+ S199G+ A222V+ N392W+ K417W+ H626*;
 10 V9D+ R38H+ N196W+ S199G+ A222V+ T348K+ H626*;
 E96H+ L150Y+ N156R+ N196W+ S199G+ A222V+ E284Q+ H626*;
 Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
 L150Y+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150Y+ N196W+ S199G+ A222V+ H626*;
 15 L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
 L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150F+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150F+ N156K+ N196W+ S199G+ A222V+ H626*;
 20 Q134L+ L150H+ N156K+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 Q134W+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 25 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ H626*;
 Q134M+ L150Y+ N156K+ N196W+ S199G+ A222V+ A466V+ H626*;
 L150Y+ L152M+ N156K+ N196W+ S199G+ A222V+ H626*;
 L150S+ N196W+ S199G+ A222V+ H626*;
 N196W+ S199G+ A222V+ N281S+ H626*;
 30 Y67T+ N196W+ S199G+ A222V+ H626*;
 Q71N+ N196W+ S199G+ A222V+ H626*;
 Q71N+ A94D+ N196W+ S199G+ A222V+ H626*;
 N196W+ S199G+ L218F+ A222V+ H626*;
 N196W+ S199G+ L218W+ A222V+ H626*;
 35 N196W+ S199G+ A222V+ T278W+ H626*;
 N196W+ S199G+ A222V+ T278W+ T459M+ H626*;
 N196W+ S199G+ A222V+ T278Y+ H626*;

- N196W+ S199G+ A222V+ S275N+ H626*;
 N196W+ S199G+ A222V+ S275L+ H626*;
 N196W+ S199G+ A222V+ S335Q+ H626*;
 N196W+ S199G+ A222V+ S335K+ H626*;
 5 N196W+ S199G+ A222V+ S335R+ H626*;
 N196W+ S199G+ L218W+ A222V+ S335K+ H626*;
 N196W+ S199G+ L218W+ A222V+ S335Q+ H626*;
 Y67W+ N196W+ S199G+ A222V+ H626*;
 N196W+ S199G+ A222V+ N281Q+ H626*;
 10 L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150M+ N156R+ N196W+ S199G+ A222V+ H626*;
 Q134L+ L150M+ N156K+ N196W+ S199G+ A222V+ H626*;
 D39R+ N196W+ S199G+ A222V+ N281Q+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q+ Q479QP;
 15 D39R+ Y70F+ N196W+ S199G+ A222V+ E284Q;
 N28W+ D39R+ N196W+ S199G+ A222V+ E284Q;
 D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q+
 H626*;
 20 N196W+ N207W;
 N196W+ N207W+ E284Q;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E284Q;
 L150Y+ N196W+ S199G+ A222V+ H626*;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ L150Y+ N196W+ S199G+ A222V;
 25 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+
 S199G+ T208N+ A222V;
 Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+ E284Q+ H626*;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+ A222I+
 30 H626*;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ Q134L+ L150H+ N156R+ N196W+ S199G+
 N207W+ A222I+ E284Q;
 E96K+ D179S+ N196W+ S199G+ A222V+ E284Q+ H626*;
 E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;
 35 R64S+ E96K+ N196W+ S199G+ N207W+ A222V;
 N28W+ D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;
 D39R+ N196W+ S199G+ A222V+ E284Q+ H626*;

D39R+ N196W+ S199G+ N207W+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ D39R+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ N196W+ N207W+ E511D;

L150Y+ N196W+ S199G+ N207W+ A222V;

5 E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ H626*;

E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ T208N+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ E96K+ K101R+ L150M+ N156R+ D179S+ N196W+ S199G+ N207W+ T208N+ A222V+ E284Q+ H626*;

Q134L+ L150H+ N156R+ N196W+ S199G+ A222I;

10 N28W+ E96K+ D179S+ N196W+ S199G+ A222V+ E284Q;

R64S+ E96K+ N196W+ S199G+ A222V+ H626*;

R64S+ E96K+ N196W+ S199G+ A222V+ E284Q;

E1*+ T2*+ A3*+ N4*+ K5*+ S6*+ N7*+ K8*+ R64S+ E96K+ N196W+ S199G+ A222V;

and wherein the variant has alpha-amylase activity and wherein the variant has at least 60%,
 15 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%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

20 12. The variant of any of claims 1-8, wherein the alpha-amylase comprises combinations of alterations selected from:

S199G + H626*;

N196W + H626*

N196W + S199G + A222V + H 626*;

25 N196W + N207W + H626*:

L150Y + N196W + S199G + A222V

E96K + D179S + N196W + S199G + A222V + E284Q;

R64S + E96K + N196W + S199G + A222V;

and wherein the variant has alpha-amylase activity and wherein the variant has at least 60%,
 30 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%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

35 13. The variant of any of claims 1-12, wherein increased pH stability at pH 4.0 can be determined as % residual alpha-amylase activity (%RA) after incubation of the variant amylase at pH 4.0, 32 °C, for 18-24 hours.

14. The variant of any of claims 1-12, wherein increased pH stability at pH 4.0 can be determined as residual alpha-amylase activity after incubation of the variant amylase at pH 4.0, 32°C, for 96 hours, and calculation of enzyme half-life in hours.

15. The variant of any of claims 1-14, wherein half-life is increased compared to the parent amylase of SEQ ID NO: 1, 2, 3, or 4 of at least a factor 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, such as at least 8.0.

16. The variant of any of claims 1-15, further comprising a substitution corresponding to K8N.

17. The variant alpha-amylase of any of the preceding claims, comprising at least the catalytic domain, comprised in amino acids 12-438 of SEQ ID NO: 1, wherein the variant has alpha-amylase activity and wherein the catalytic domain has 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%, or at least 99% sequence identity, but less than 100% sequence identity, to the polypeptide of SEQ ID NO: 1, and wherein the variant optionally has a CBM.

18. The variant of any of claims 1-17, wherein a linker and/or a carbohydrate binding module, CBM, has been replaced with a heterologous CBM.

19. The variant of claim 18, wherein the CBM comprises amino acids 527-626 of SEQ ID NO: 1, and amino acids 439-526 comprises a linker region.

20. The variant of any of claims 17-19, wherein the heterologous CBM is selected from a heterologous CBM belonging to Family 20, 21, 25, 26, 34, 41 or 48.

21. The variant of claim 20, wherein the CBM is a Family 20 CBM.

22. The variant of any of the claims 18-21, wherein the CBM is selected from the group consisting of:

i) a polypeptide of SEQ ID NO: 14, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 14;

ii) a polypeptide of SEQ ID NO: 15, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 15;

iii) a polypeptide of SEQ ID NO: 16, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 16;

iv) a polypeptide of SEQ ID NO: 17, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 17;

v) a polypeptide of SEQ ID NO: 18, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 18; and

vi) a polypeptide of SEQ ID NO: 19, or a polypeptide having 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%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 19.

23. The variant of any of claims 18-22, wherein the linker is between 1 to 100 amino acids.

24. An isolated polynucleotide encoding the variant of any one of claims 1-23.

25. A nucleic acid construct or expression vector comprising the polynucleotide of claim 24 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

26. A recombinant host cell comprising the polynucleotide of claim 24 operably linked to one or more control sequences that direct the production of the polypeptide.

27. The host cell according to claim 26, wherein the host cell is a yeast cell, particularly a *Saccharomyces*, such as *Saccharomyces cerevisiae*.

28. A method of producing a variant alpha-amylase of any of claims 1-23, comprising: cultivating the recombinant host cell of claim 26 under conditions suitable for expression of the variant; and optionally recovering the variant.

29. A composition comprising the variant alpha-amylase of any of claims 1-23.

30. The composition of claim 29 further comprising at least one glucoamylase.

31. A whole broth formulation or cell culture composition comprising the variant alpha-amylase of any of claims 1-23.

32. The composition of claim 29, further comprising a surfactant.

33. The composition of claim 32, wherein the composition comprises a surfactant or surfactant system wherein the surfactant can be selected from nonionic surfactants, anionic surfactants, cationic surfactants, ampholytic surfactants, zwitterionic surfactants, semi-polar nonionic surfactants and mixtures thereof.

34. The composition of claim 33, wherein the composition comprises an anionic surfactant, in particular linear alkylbenzene sulfonate (LAS) and/or alcohol ethoxysulfate (AEOS).

35. The composition of claim 33, wherein the composition comprises a nonionic surfactant, such as alcohol ethoxylate (AEO).

36. The composition of any of claims 32-35, wherein the composition comprises one or more anionic and/or one or more nonionic surfactants.

37. The composition of any of claims 32-36, wherein the composition comprises one or more of surfactants, in particular linear alkylbenzenesulfonic acid (LAS), sodium laureth sulfate (SLES) and/or alcohol ethoxylate (AEO).

38. A use of a variant alpha-amylase of any of claims 1-23 for production of syrup and/or a fermentation product.

39. A process of producing a fermentation product from starch-containing material comprising the steps of: (a) liquefying starch-containing material above the initial gelatinization temperature of said starch-containing material in the presence of an alpha amylase; (b) saccharifying the liquefied material; and (c) fermenting with a fermenting organism; wherein step (b) is carried out in the presence of at least a variant alpha-amylase of any of claims 1-23, and optionally a glucoamylase.

40. The process of claim 39, wherein step (b) and step (c) are carried out simultaneously.

41. The process of any of the claims 39-40, wherein the host cell of any of claims 26-27 is applied as the fermenting organism.

42. The process of claim 39, wherein the host cell further is expressing a glucoamylase.

43. The process according to any of claims 41-42, wherein the host cell further is expressing an alpha-amylase derived from a strain of the genus *Rhizomucor*, preferably a strain of *Rhizomucor pusillus*, such as a *Rhizomucor pusillus* alpha-amylase hybrid having a linker and starch-binding domain from an *Aspergillus niger* glucoamylase.

44. The process of claim 43, wherein the further alpha-amylase expressed by the host cell in fermentation is selected from the group consisting of:

(i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 12;

(ii) an alpha-amylase comprising an amino acid sequence having at least 60%, at least 70%, e.g., 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% identity to the polypeptide of SEQ ID NO: 12.

45. The process of claim 44, wherein the further alpha-amylase comprises one or more of the following substitutions: G128D, D143N, preferably G128D+D143N, using SEQ ID NO: 12 for numbering.

46. The process of any of claims 39-45, wherein the fermenting organism or host cell is a yeast cell, particularly a *Saccharomyces* cell, such as *Saccharomyces cerevisiae*.

47. The process of any of claims 39-46, wherein the fermentation product is an alcohol, such as ethanol.

48. The process of any of claims 39-47, wherein the starch-containing material is corn.

49. A process of producing a syrup product from starch-containing material, comprising the step of: (a) liquefying starch-containing material at a temperature above the initial gelatination temperature of said starch-containing material in the presence of an alpha-amylase; (b) saccharifying the liquefied material in the presence of at least a variant alpha-amylase of any of the claims 1-23, and optionally a glucoamylase.

50. The process of claim 49, wherein step a) is carried out using at least a variant alpha-amylase of any of claims 1-23, and a second alpha-amylase.

51. The variant alpha-amylase according to any of the claims 1-23, wherein the variant is isolated.

52. A transgenic plant, plant part or plant cell comprising the variant alpha-amylase of any of claims 1- 23.