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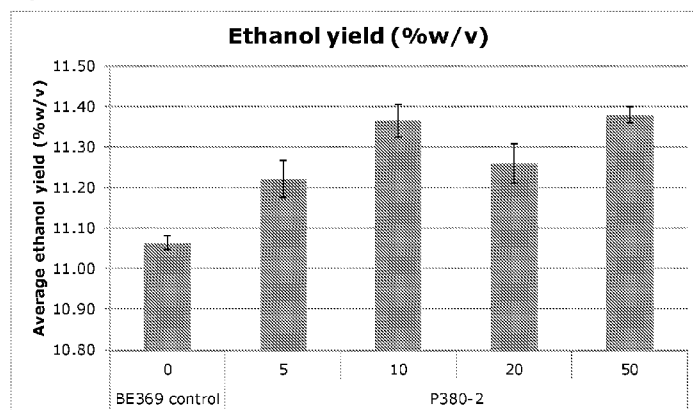
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(54) Title: POLYPEPTIDES HAVING PULLULANASE ACTIVITY SUITABLE FOR USE IN LIQUEFACTION

Figure 1



(57) Abstract: The present invention relates to a variant pullulanase, wherein the pullulanase comprises at least the following combination of substitutions: N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C, and optionally further comprises N222P+Q252A+Q256R; wherein the variant has pullulanase activity, and wherein the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3. Further aspect the present invention relates to a process for liquefying starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase and a thermo-stable pullulanase of the invention.

**POLYPEPTIDES HAVING PULLULANASE ACTIVITY SUITABLE FOR USE IN
LIQUEFACTION**

Reference to a Sequence Listing

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

Field of the Invention

10 The present invention relates to use of thermo-stable pullulanase variants in a process for producing fermentation products from starch-containing material and to polypeptides having pullulanase activity.

Background of the Invention

15 Starch usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages. Amylopectin is partially degraded by alpha-amylase, which hydrolyzes the 1,4-alpha-glucosidic linkages to produce branched and linear oligosaccharides. Prolonged degradation of amylopectin by alpha-amylase results in the formation of so-called alpha-limit dextrins that are not susceptible to further hydrolysis by the alpha-amylase. Branched oligosaccharides can be hydrolyzed into linear oligosaccharides by a debranching enzyme. The remaining branched oligosaccharides can be depolymerized to D-glucose by glucoamylase, which hydrolyzes linear oligosaccharides into D-glucose.

20 Debranching enzymes which can attack amylopectin are divided into two classes: isoamylases (E.C. 3.2.1.68) and pullulanases (E.C. 3.2.1.41), respectively. Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by
25 their limited action on alpha-limit dextrins.

30 It is well-known in the art to add isoamylases or pullulanases in starch conversion processes. Pullulanase is a starch debranching enzyme having pullulan 6-glucano-hydrolase activity (EC3.2.1.41) that catalyzes the hydrolyses the α -1,6-glycosidic bonds in pullulan, releasing maltotriose with reducing carbohydrate ends. Usually pullulanase is used in combination with an alpha amylase and/or a glucoamylase.

 Pullulanases are known in the art. US 6,074,854 and US 5,817,498 disclose a pullulanase from *Bacillus deramificans*. WO2009/075682 discloses a pullulanase derived from *Bacillus acidopullulyticus*.

35 WO 2015/007639 discloses a hybrid pullulanase obtained by combining an N-terminal fragment of a pullulanase from *Bacillus acidopullulyticus* fused to a C-terminal fragment of a pullulanase from *Bacillus deramificans*. Prior art pullulanases derived from *Bacillus sp.* have so

far not been sufficiently thermos-stable in order to be added during liquefaction in conventional starch conversion processes.

It is an object of the present invention to provide pullulanase variants having increased thermo-activity suitable for use in liquefaction of starch containing material.

5

Summary of the Invention

The present invention relates to a variant pullulanase, wherein the pullulanase comprises at least the following combination of substitutions:

N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C, and optionally further comprises N222P+Q252A+Q256R; wherein the variant has pullulanase activity, and
10 wherein the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3.

Further aspect the present invention relates to a process for liquefying starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase
15 and a thermo-stable pullulanase of the invention.

Thus in second aspect the 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 using an alpha-amylase and a variant pullulanase of the invention;
- 20 b) saccharifying using a glucoamylase.

In a third aspect the present invention relates to a process for producing fermentation products from starch-containing material comprising the steps of:

- a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase and a variant pullulanase of the invention;
- 25 b) saccharifying using a glucoamylase;
- c) fermenting using a fermenting organism.

In a fourth aspect the present invention relates to compositions comprising the variant pullulanase of the invention and a stabilizer.

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

Brief Description of the Figures

Figure 1 shows average ethanol yields (in %w/v) for the BE369 amylase only control and four doses of the thermostable pullulanase, P380-2, in an 80°C liquefaction and standard lab
35 scale fermentation assays. All of the P380-2 doses produced statistically more ethanol than the

BE369 control as determined by JMP software. The percent ethanol increase with the control set to 100% was 1.4 – 2.9% for the P380-2 treatments.

Figure 2 shows ethanol yields from experiment testing addition of P380-2 to an 80°C slurry after 30 minutes of liquefaction with amylase alone. With removal of several outliers, JMP statistical analysis shows the two doses of P380-2 are higher in ethanol yield than amylase control.

Figure 3 shows average ethanol yields (in %w/v) for the two amylase controls (Alpha-amylase BE369 (AA369) and Alpha-amylase blend AA) and the five doses of either P598 or P604 after an 80°C liquefaction and standard lab scale fermentation assays. The 50 microgram enzyme protein per gram dry solids dose of P604 produced statistically more ethanol than the Alpha-amylase blend AA control as determined by the ANOVA and Tukey-Kramer tests in SAS JMP software. All of the P604 doses produced statistically more ethanol than the AA369 control. The 5 and 50 micrograms enzyme protein per gram dry solids of P598 produced statistically more ethanol than the AA369 control.

Figure 4 shows average residual DP4+ concentrations for the two controls (AA369 and Alpha-amylase blend AA) and the five doses of P598 and P604 after the 80°C liquefaction and 54 hour fermentation assays. The DP4+ concentrations for the 10, 20 and 50 microgram enzyme protein per gram dry solids doses of P604 were statistically lower than the Alpha-amylase blend AA control after 54 hours of fermentation.

Figure 5 shows average residual DP3 concentrations for the two controls (AA369 and Alpha-amylase blend AA) and the five doses of P598 and P604 after the 80°C liquefaction and 54 hour fermentation assays. The 50 microgram enzyme protein per gram dry solids dose of P598 and the 5, 10, 20 and 50 micrograms enzyme protein per gram dry solids doses of P604 had statistically lower residual DP3 concentrations than the Alpha-amylase blend AA control.

Figure 6 shows average residual DP2 concentrations for the two controls (AA369 and Alpha-amylase blend AA) and the five doses of P598 and P604 after the 80°C liquefaction and 54 hour fermentation assays. All doses of P604 had statistically significantly less DP2 remaining at the end of fermentation than the Alpha-amylase blend AA control. The 50 micrograms enzyme protein per gram dry solids dose of P598 had statistically lower DP2 than the Alpha-amylase blend AA control.

Definitions

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.

Catalytic domain: The term “catalytic domain” means the region of an enzyme containing the catalytic machinery of the enzyme.

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 polypeptide. 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 mature polypeptide 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 polypeptide 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 polypeptide.

Expression: The term “expression” includes any step involved in the production of a polypeptide 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 polypeptide 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 or domain; wherein the fragment has pullulanase activity.

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

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

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

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

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

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

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

Pullulanase: The term "pullulanase" means a starch debranching enzyme having pullulan 6-glucano-hydrolase activity (EC 3.2.1.41) that catalyzes the hydrolysis the α -1,6-glycosidic bonds in pullulan, releasing maltotriose with reducing carbohydrate ends. For purposes of the present invention, pullulanase activity can be determined according to the procedure described in the Examples. In the context of the present invention the variant pullulanases have increased thermo-activity. Increased thermo-activity was determined as relative activity when measured at 76-81.5°C relative to activity at 65°C or 75°C using the PHADEBAS assay as described in the examples.

In particular the pullulanase variants suitable for the process of the invention have at least 30% relative activity when measured at 76°C relative to activity at 65°C, more particularly

at least 40%, more particularly at least 50%, more particularly at least 60%, more particularly at least 70%, more particularly at least 80%, more particularly at least 90%, more particularly at least 95%. More particularly pullulanase variants suitable for the process of the invention have at least 50% relative activity when measured at 79°C relative to activity at 75°C, more particularly at least 60%, more particularly at least 70%, more particularly at least 80%, more particularly at least 90%, more particularly at least 95%.

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

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

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

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

The term “low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern

blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 50°C.

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

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

The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

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

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

S8A Protease: The term "S8A protease" means an S8 protease belonging to subfamily A. Subtilisins, EC 3.4.21.62, are a subgroup in subfamily S8A, however, the present S8A protease from *Thermococcus* sp PK is a subtilisin-like protease, which has not yet been included in the IUBMB classification system. The S8A protease according to the invention hydrolyses the substrate Suc-Ala-Ala-Pro-Phe-pNA. The release of p-nitroaniline (pNA) results in an increase of absorbance at 405 nm and is proportional to the enzyme activity. pH optimum = pH 8, and Temperature optimum = 60°C.

Variant: The term "variant" means a polypeptide having pullulanase activity comprising an alteration, i.e., a substitution, insertion, and/or deletion, at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position. In describing variants, the nomenclature described below is adapted for

ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviations are employed.

In the context of the present invention the variant pullulanases has increased thermo-activity. Increased thermo-activity was determined as relative activity when measured at 76-79°C relative to activity at 65°C using the PHADEBAS assay as described in the examples, or measured at 78-81.5°C relative to activity at 75°C using the PHADEBAS assay as described in the examples.

In particular the pullulanase variants suitable for the process of the invention have at least 30% relative activity when measured at 76°C relative to activity at 65°C, more particularly at least 40%, more particularly at least 50%, more particularly at least 60%, more particularly at least 70%, more particularly at least 80%, more particularly at least 90%, more particularly at least 95%. More particularly pullulanase variants suitable for the process of the invention have at least 50% relative activity when measured at 79°C relative to activity at 75°C, more particularly at least 60%, more particularly at least 70%, more particularly at least 80%, more particularly at least 90%, more particularly at least 95%.

Conventions for Designation of Variants

For purposes of the present invention, the mature hybrid pullulanase polypeptide disclosed as SEQ ID NO: 3 is used to determine the corresponding amino acid residue in another pullulanase. The amino acid sequence of another pullulanase is aligned with the mature polypeptide disclosed as SEQ ID NO: 3, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed as SEQ ID NO: 3 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

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

When the other enzyme has diverged from the polypeptide of SEQ ID NO: 3 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* 295: 613-615), other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that
5 utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Atschul *et al.*, 1997, *Nucleic Acids Res.* 25: 3389-3402). Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such
10 as GenTHREADER (Jones, 1999, *J. Mol. Biol.* 287: 797-815; McGuffin and Jones, 2003, *Bioinformatics* 19: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough *et al.*, 2000, *J. Mol. Biol.* 313: 903-919, can be used to align a sequence of unknown structure with
15 the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

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

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

30 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
35 (S) with phenylalanine (F), respectively.

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

Ser411*" or "G195* + S411*".

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

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

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

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

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

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

Throughout the present description in some embodiments the variants of the invention have been described by giving the amino acid present at the specified position in SEQ ID NO: 3 as well as the amino acid present after substitution. This does not mean that the starting amino acid in the specified position cannot be a different one. The starting amino acid in a specific position of course depends on the choice of the parent pullulanase. The essential feature of the present invention is the resulting amino acid present after the substitution. In case the parent pullulanase already has the desired amino acid in a specific position this means that it should be maintained. E.g., the parent pullulanase disclosed as SEQ ID NO: 3 has alanine in position 492. Therefore according to the invention position 492 should also have 492A in the variants according to the invention.

Detailed Description of the Invention

The present invention relates to variant pullulanases derived from a hybrid parent pullulanase. The hybrid parent pullulanase was constructed by fusing the N-terminal amino acids 1-451 from a wild type pullulanase (SEQ ID NO: 1) isolated from *Bacillus acidopullulyticus* with the C-terminal amino acids 452-828 from another wild type pullulanase (SEQ ID NO: 2) isolated from *Bacillus deramificans*. The resulting hybrid pullulanase, disclosed as SEQ ID NO: 3 herein, was used as the parent pullulanase. The polynucleotide sequence encoding the parent pullulanase is included herein as SEQ ID NO: 4, wherein nucleotides 1-99 encode a signal peptide, and nucleotides 100-2583 encode the mature polypeptide disclosed in SEQ ID NO: 3.

The variants according to the present invention have improved properties compared to the parent. The improved properties are in one embodiment increased thermo-activity. The positions to be substituted in order to obtain increased thermo-activity will be described in detail below. The increase in thermo-activity may be determined as relative activity measured in the range of 65-81.5°C, pH 5.0 by the PHADEBAS assay described herein in the pullulanase assay section. In a particular embodiment the variants according to the invention have at least 30% relative activity when measured at 76°C relative to activity at 65°C. In another embodiment the variants have at least 50% relative activity when measured at 78°C relative to activity at 65°C. In another embodiment the variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C. In another embodiment the variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In one aspect the present invention therefore relates to a pullulanase variant, wherein the variant comprises at least one of the following combinations of substitutions:

368G+ 393A+ 431E+ 432F+ 492A,S+ 610R+ 624S+ 631S+ 632C;

368G+393A+431E+432F+492A,S+610R+624S+631S+632C+20G+28K

+80Y+187R+310A+311K+387L+459G+586S+699R+798R;

222P+252A+256R+368G+393A+431E+432F+492A,S+610R+624S+ 631S+632C;

222P+252A+256R+368G+393A+431E+432F+492A,S+610R+624S+631S+632C+20G

+28K+80Y+187R+310A+311K+ 387L+ 459G+ 586S+699R+ 798R;

wherein the variant has pullulanase activity, and wherein the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3.

In another aspect the present invention relates to a pullulanase variant, wherein the variant comprises at least one of the following combinations of substitutions:

N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C;

N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+T631S+S632C+N20G+Y28K

+H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+S798R;

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+

T631S+S632C;

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+
G624S+T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+ Q387L+ Q459G+
D586S+E699R+ S798R;

wherein the variant has pullulanase activity, and wherein the variants have at least 85%, at least
5 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
sequence identity to the polypeptide of SEQ ID NO: 3.

The variants according to the invention have at least 30% relative pullulanase activity
when measured at 76°C relative to activity at 65°C.

In one embodiment the invention therefore relates to a pullulanase variant, wherein the
10 variant comprises the following combination of substitutions:

N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C; and
wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
sequence identity to the polypeptide of SEQ ID NO: 3, and wherein the variant has at least 30%
15 relative pullulanase activity when measured at 76°C relative to activity at 65°C.

In another embodiment the invention therefore relates to a pullulanase variant, wherein
the variant comprises the following combination of substitutions:

N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+T631S+S632C+N20G+Y28K
+H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+S798R; and
20 wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
sequence identity to the polypeptide of SEQ ID NO: 3 and wherein the variant has at least 50%
relative activity when measured at 78°C relative to activity at 65°C.

In another embodiment the invention therefore relates to a pullulanase variant, wherein
25 the variant comprises the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
30 sequence identity to the polypeptide of SEQ ID NO: 3, and wherein the variant has at least 30%
relative pullulanase activity when measured at 76°C relative to activity at 65°C.

In another embodiment the invention therefore relates to a pullulanase variant, wherein
the variant comprises the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
35 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%

sequence identity to the polypeptide of SEQ ID NO: 3, and wherein the variant has at least 50% relative activity when measured at 78°C relative to activity at 65°C.

Starting from one of the above variants, thermo-activity has been further increased. In a still further embodiment the present invention therefore relates to a pullulanase variant, wherein

5 the variant comprises the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

10 and further the variant comprises at least one of the following combinations of deletions and substitutions:

P30* +V31* +N32*;
P30*+ V31* +N32*+D57N+D58P;
Q29G+P30* +V31*+N32*+D57N+D58P;
P30* +V31* +N32* +D57N+D58P+A195G;
P30* +V31* +N32* +D57N+D58P+N197T ;
P30* +V31* +N32* +D57N+D58P+N202K;
P30* +V31* +N32* +D57N+D58P+A345P;
P30* +V31* +N32* +D57N+D58P+M402S;
P30* +V31* +N32* +D57N+D58P+F456W;
P30* +V31* +N32* +D57N+D58P+I460V;
P30* +V31* +N32* +D57N+D58P+N479H;
P30* +V31* +N32* +D57N+D58P+I514V;
P30* +V31* +N32* +D57N+D58P+E560R;
P30* +V31* +N32* +D57N+D58P+D615E;
P30* +V31* +N32* +D57N+D58P+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+I514V;
P30* +V31* +N32* +D57N+D58P+A345P+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+F456W;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R+D615E;

P30* +V31*+N32* +D57N+D58P+A195G+A345P+M402S+I460V+I514V+E560R;
 P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I514V;
 P30* +V31* +N32* +D57N+D58P+A195G+A345P;
 P30* +V31* +N32* +D57N+D58P+A195G+A345P+F456W;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+ F456W+
 I460V+ I514V ;
 P30* +V31* +N32* +D57N+D58P+N479H ;
 P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
 I460V +I514V+E560R;
 P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+N479H+I514V
 +E560R;
 P30* +V31*+N32* +D57N+D58P+N197T+A345P+M402S+I460V+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A252I +N202K+A345P
 +M402S+I460V+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+N202K+A345P+
 M402S+I460V+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+
 F456W+ I460V+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+A345P+M402S
 +F456W+I460V+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
 I460V+N479H+I514V+E560R;
 Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P
 +M402S +F456W+ I460V +I514V+E560R
 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+
 I460V+N479H+I514V+E560R
 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T +N202K+ A345P +M402S
 +F456W+I460V+N479H+I514V+E560R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
 least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
 sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least
 50%, particularly at least 60%, more particularly at least 70%, even more particularly at least
 5 80% relative activity when measured at 78°C relative to activity at 65°C.

In more particular embodiments the present invention relates to pullulanase variants,
 wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 10 D586S+E699R+ S798R;

and further the variant comprises at least one of the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R;

5 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R;

Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+
I460V +I514V+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+

10 I460V+N479H+I514V+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T +N202K+ A345P +M402S
+F456W+I460V+N479H+I514V+E560R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
15 sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least
70% relative activity when measured at 78°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants,
wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+

20 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;

and wherein the variant has pullulanase activity, and the variants have at least 85%, at least
25 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least
70% relative activity when measured at 78°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants,
wherein the variants comprise the following combination of substitutions:

30 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+

T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+

D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V

35 +E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 78°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
5 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
10 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
15 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
20 least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
25 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+
30 I460V +I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at
least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said
variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
35 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V

+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In one particular embodiment the present invention relates to pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+N202K+A345P+M402S+F456W+I460V

+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

Polynucleotides

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

either homologous or heterologous to the host cell.

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

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

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

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

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

The control sequence may also be a 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.

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.

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

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

It may also be desirable to add regulatory sequences that regulate expression of the variant relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used.

Expression Vectors

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

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

closed circular plasmid.

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

5 The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

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

Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* 15 *dal* genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin or tetracycline resistance.

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.

20 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 25 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 30 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 35 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*.

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.

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 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
5 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
10 *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),
15 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.

Methods of Production

20 The present invention also relates to methods of producing a variant, comprising: (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant; and (b) recovering the variant.

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

The variant may be detected using methods known in the art. For example, an enzyme
35 assay may be used to determine the activity of the variant. See the Assay section for suitable pullulanase activity assays.

The variant may be recovered using methods known in the art. For example, the variant may be recovered from the nutrient medium by conventional procedures including, but not

limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

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

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

Fermentation Broth Formulations or Cell Compositions

The present invention also relates to a fermentation broth formulation or a cell composition comprising a polypeptide 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 polypeptide of the present invention which are used to produce the polypeptide of interest), cell debris, biomass, fermentation media and/or fermentation products. In some embodiments, the composition is a cell-killed whole broth containing organic acid(s), killed cells and/or cell debris, and culture medium.

The term "fermentation broth" as used herein refers to a preparation produced by cellular fermentation that undergoes no or minimal recovery and/or purification. For example, fermentation broths are produced when microbial cultures are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (e.g., expression of enzymes by host 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.

5 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.

10 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
15 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.

20 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

25 The present invention also relates to compositions comprising a pullulanase variant of the invention and a suitable stabilizer.

The compositions may comprise the pullulanase variant as the major enzymatic component, e.g., a mono-component composition. Alternatively, the compositions may comprise multiple enzymatic activities, such as one or more (e.g., several) enzymes selected from the group consisting of alpha-amylase, glucoamylase, beta-amylase, protease.

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. The compositions may be stabilized in accordance with methods known in the art.

Examples are given below of preferred uses of the compositions of the present invention. In a particular embodiment the composition further comprises an alpha-amylase.

35 The alpha-amylase is preferably a bacterial acid stable alpha-amylase. Particularly the alpha-amylase is from an *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: 5 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
5 positions I181 + G182, R179 + G180, G180 + I181, R179 + I181, or G180 + G182, preferably I181 + G182, and optionally a N193F substitution, (using SEQ ID NO: 5 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
10 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 in addition to a double deletion in the region from position 179 to 182, particularly I181*+G182* and optionally N193F:

-V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- 59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;

- K220P+N224L+S242Q+Q254S;
- M284V;
- V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V.

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; and
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 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: 5.

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: 5 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: 5, 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.

Protease Present and/or Added During Liquefaction

In a preferred embodiment the enzyme composition of the invention, further comprises a protease.

According to the invention a thermostable protease may optionally be present and/or added during liquefaction together with a variant pullulanase of the invention and an alpha-amylase, such as a thermostable alpha-amylase.

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.

In an embodiment the thermostable protease has at least 20%, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 100% of the protease activity of the Protease 196 variant or Protease Pfu determined by the AZCL-casein assay described in the “Materials & Methods” section.

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 a preferred 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 an embodiment the thermostable protease is a variant of the mature part of the metallo protease shown in SEQ ID NO: 2 disclosed in WO 2003/048353 or the mature part of SEQ ID NO: 1 in WO 2010/008841 and shown as SEQ ID NO: 6 herein further with mutations selected from below list:

- S5*+D79L+S87P+A112P+D142L;
- D79L+S87P+A112P+T124V+D142L;
- S5*+N26R+D79L+S87P+A112P+D142L;
- N26R+T46R+D79L+S87P+A112P+D142L;
- T46R+D79L+S87P+T116V+D142L;

- D79L+P81R+S87P+A112P+D142L;
- A27K+D79L+S87P+A112P+T124V+D142L;
- D79L+Y82F+S87P+A112P+T124V+D142L;
- D79L+Y82F+S87P+A112P+T124V+D142L;
- 5 - D79L+S87P+A112P+T124V+A126V+D142L;
- D79L+S87P+A112P+D142L;
- D79L+Y82F+S87P+A112P+D142L;
- S38T+D79L+S87P+A112P+A126V+D142L;
- D79L+Y82F+S87P+A112P+A126V+D142L;
- 10 - A27K+D79L+S87P+A112P+A126V+D142L;
- D79L+S87P+N98C+A112P+G135C+D142L;
- D79L+S87P+A112P+D142L+T141C+M161C;
- S36P+D79L+S87P+A112P+D142L;
- A37P+D79L+S87P+A112P+D142L;
- 15 - S49P+D79L+S87P+A112P+D142L;
- S50P+D79L+S87P+A112P+D142L;
- D79L+S87P+D104P+A112P+D142L;
- D79L+Y82F+S87G+A112P+D142L;
- S70V+D79L+Y82F+S87G+Y97W+A112P+D142L;
- 20 - D79L+Y82F+S87G+Y97W+D104P+A112P+D142L;
- S70V+D79L+Y82F+S87G+A112P+D142L;
- D79L+Y82F+S87G+D104P+A112P+D142L;
- D79L+Y82F+S87G+A112P+A126V+D142L;
- Y82F+S87G+S70V+D79L+D104P+A112P+D142L;
- 25 - Y82F+S87G+D79L+D104P+A112P+A126V+D142L;
- A27K+D79L+Y82F+S87G+D104P+A112P+A126V+D142L;
- A27K+Y82F+S87G+D104P+A112P+A126V+D142L;
- A27K+D79L+Y82F+ D104P+A112P+A126V+D142L;
- A27K+Y82F+D104P+A112P+A126V+D142L;
- 30 - A27K+D79L+S87P+A112P+D142L;
- D79L+S87P+D142L.

In an 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 or SEQ ID NO: 6 herein 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 or SEQ ID NO: 6 herein.

The thermostable protease may also be derived from any bacterium as long as the protease has the thermo-stability properties defined according to the description.

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 the one shown as SEQ ID NO: 1 in US patent No. 6,358,726-B1 (Takara Shuzo Company) or SEQ ID NO: 7 herein.

In another embodiment the thermostable protease is one disclosed in SEQ ID NO: 7 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: 7 herein. The *Pyrococcus furiosus* protease can be purchased from Takara Bio, Japan.

Thus in a particular embodiment of the invention the enzyme composition further comprises a protease selected from a *Pyrococcus* sp protease, e.g. a *Pyrococcus furiosus* protease (SEQ ID NO: 7), a *Thermococcus* sp. S8A protease (SEQ ID NO: 8), e.g. a *Thermococcus litoralis* S8A protease, or a *Thermoascus* sp protease, e.g., a *Thermoascus aurantiacus* protease, particularly a variant of a *Thermoascus aurantiacus* protease, shown as SEQ ID NO: 6, comprising one of the specific combinations of substitutions in the D79L+S87P+A112P+D142L; D79L+S87P+D142L; or A27K+ D79L+ Y82F+S87G+D104P+A112P+A126V+D142L.

In an embodiment the composition of the invention comprises:

- i) a *Bacillus stearothermophilus* alpha-amylase, or a variant thereof;
- ii) a variant pullulanase of the invention;
- iii) optionally a protease; and

wherein the ratio between alpha-amylase and protease is in the range from 1:1 and 1:50 (micro gram alpha-amylase : micro gram protease).

In an embodiment the ratio between alpha-amylase and protease is in the range between 1:3 and 1:40, such as around 1:4 (micro gram alpha-amylase: micro gram protease).

In an embodiment the ratio between alpha-amylase and pullulanase is between 1:1 and 1:10, such as around 1:2.5 or 1:5 (micro gram alpha-amylase: micro gram pullulanase).

The pullulanase may according to the invention be added in an effective amount which include the preferred amount of about 2-100 micro gram enzyme protein per gram DS, preferably 5-50 micro gram enzyme protein per gram DS. Pullulanase activity may be determined as NPUN. An Assay for determination of NPUN is described in the assay section.

5 In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

10 and further the variant comprises at least one of the following combinations of substitutions:
Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;
Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R;

15 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R; and

20 wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

25 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;

30 and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

35 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
5 least 70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants,
wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
10 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
15 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants,
wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
20 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
25 least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants,
wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
30 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+
35 I460V +I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at
least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said
variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
5 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V

+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least
10 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

15 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+N202K+A345P+M402S+F456W+I460V

20 +N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

25 **Processes of the invention**

The present invention relates to processes for producing fermentation products from starch-containing material. In particular the product is an alcohol, more particularly ethanol.

The inventors have found that an increased ethanol yield can be obtained when a pullulanase variant according to the invention, having increased thermo-activity, is present or
30 added during liquefaction together with at least an alpha-amylase.

Process Of Producing A Fermentation Product Of The Invention

In a particular aspect the invention relates to a process for producing a syrup from starch-containing material comprising the steps of:

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

In another particular aspect the invention relates to processes for producing fermentation products from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

- 5 an alpha-amylase and a variant pullulanase of the invention.;
- b) saccharifying using a glucoamylase;
- c) fermenting using a fermenting organism.

In a preferred embodiment the fermentation product is recovered after fermentation, such as by distillation. In an embodiment the fermentation product is an alcohol, preferably
10 ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

Alpha-Amylases Present And/Or Added In Liquefaction

The alpha-amylase added during liquefaction step a) in a process of the invention may be any alpha-amylase. Preferred are bacterial alpha-amylases, which typically are stable at a temperature used in liquefaction.

15 In an embodiment the alpha-amylase is from a strain of the genus *Exiguobacterium* or *Bacillus*.

In a preferred embodiment the alpha-amylase is from a strain of *Bacillus stearothermophilus*, such as the sequence shown in SEQ ID NO: 3 in WO99/019467 or in SEQ ID NO: 5 herein. In an embodiment the alpha-amylase is the *Bacillus stearothermophilus* alpha-
20 amylase shown in SEQ ID NO: 5 herein, such as one having at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99% identity to SEQ ID NO: 5 herein.

In an embodiment the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably at the C-terminal, preferably truncated to have around 491 amino acids,
25 such as from 480-495 amino acids.

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: 5 for numbering).

30 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 in addition to a double
35 deletion in the region from position 179 to 182, particularly I181*+G182*, and optionally N193F:

- V59A+Q89R+G112D+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+D269E+D281N;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+I270L;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+H274K;
- V59A+Q89R+E129V+K177L+R179E+K220P+N224L+Q254S+Y276F;
- V59A+E129V+R157Y+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+H208Y+K220P+N224L+S242Q+Q254S;
- 59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+H274K;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+D281N;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- V59A+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+G416V;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S;
- V59A+E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- A91L+M96I+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+Y276F+L427M;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+M284T;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S+N376*+I377*;
- E129V+K177L+R179E+K220P+N224L+Q254S;
- E129V+K177L+R179E+K220P+N224L+Q254S+M284T;
- E129V+K177L+R179E+S242Q;
- E129V+K177L+R179V+K220P+N224L+S242Q+Q254S;
- K220P+N224L+S242Q+Q254S;
- M284V;
- V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V.

In a preferred embodiment the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- 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; and
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using

SEQ ID NO: 5 for numbering).

According to the invention the alpha-amylase variant 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%, but less than 100% identity to the polypeptide of SEQ ID NO: 5 herein.

The alpha-amylase may according to the invention be present and/or added in a concentration of 0.1-100 micro gram per gram DS, such as 0.5-50 micro gram per gram DS, such as 1-25 micro gram per gram DS, such as 1-10 micro gram per gram DS, such as 2-5 micro gram per gram DS.

5 In an embodiment the ratio between alpha-amylase and pullulanase is between 1:1 and 1:10, such as around 1:2.5 or 1:5 (micro gram alpha-amylase: micro gram pullulanase).

The pullulanase may according to the invention be added in an effective amount which include the preferred amount of about 2-100 micro gram enzyme protein per gram DS, preferably 5-50 micro gram enzyme protein per gram DS. Pullulanase activity may be
10 determined as NPUN. An Assay for determination of NPUN is described in the assay section.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+

15 D586S+E699R+ S798R;

and further the variant comprises at least one of the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;
Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R;

20 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at
25 least 95%, 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, and wherein said variants have at least
70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

30 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;

35 and wherein the variant has pullulanase activity, and the variants have at least 85%, at least
90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%
sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least
70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
5 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
10 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 78°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
15 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
20 least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
25 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
30 +E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
35 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+I460V +I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
 5 but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

10 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

15 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V
 +N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

20 In a particular embodiment the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

25 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+N202K+A345P+M402S+F456W+I460V
 +N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at
 30 75°C.

Protease Present and/or Added During Liquefaction

In a preferred embodiment the processes of the invention, further comprises adding a protease in liquefaction.

35 According to the invention a thermostable protease may optionally be present and/or added during liquefaction together with a variant pullulanase of the invention and an alpha-amylase, such as a thermostable alpha-amylase.

For more details on suitable proteases see the composition section above.

In an 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 or SEQ ID NO: 6 herein 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 or SEQ ID NO: 6 herein.

The thermostable protease may also be derived from any bacterium as long as the protease has the thermo-stability properties defined according to the description.

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 the one shown as SEQ ID NO: 1 in US patent No. 6,358,726-B1 (Takara Shuzo Company) or SEQ ID NO: 7 herein.

In another embodiment the thermostable protease is one disclosed in SEQ ID NO: 7 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: 7 herein. The *Pyrococcus furiosus* protease can be purchased from Takara Bio, Japan.

Thus in a particular embodiment of the invention the enzyme composition further comprises a protease selected from a *Pyrococcus* sp protease, e.g. a *Pyrococcus furiosus* protease (SEQ ID NO: 7), a *Thermococcus* sp. S8A protease (SEQ ID NO: 8), e.g. a *Thermococcus litoralis* S8A protease, or a *Thermoascus* sp protease, e.g., a *Thermoascus aurantiacus* protease, particularly a variant of a *Thermoascus aurantiacus* protease, shown as SEQ ID NO: 6, comprising one of the specific combinations of substitutions in the D79L+S87P+A112P+D142L;

D79L+S87P+D142L; or

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

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. sepiarium* or *G. trabeum*, or a strain of the *Nigrofores*.

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: 9 herein,

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

- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 9 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: 9 herein.

In an embodiment the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus sanguineus* described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6), such as the one shown as SEQ ID NO: 4 in WO 2011/066576 or SEQ ID NO: 10 herein.

In an embodiment the glucoamylase is derived from a strain of the genus *Gloeophyllum*, such as a strain of *Gloeophyllum sepiarium* or *Gloeophyllum trabeum*, in particular a strain of *Gloeophyllum* as described in WO 2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16). In a preferred embodiment the glucoamylase is the *Gloeophyllum sepiarium* shown in SEQ ID NO: 2 in WO 2011/068803 or SEQ ID NO: 11 herein.

In a preferred embodiment the glucoamylase is derived from *Gloeophyllum sepiarium*, such as the one shown in SEQ ID NO: 11 herein. In an embodiment the glucoamylase is selected from the group consisting of:

- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 11 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: 11 herein.

In another embodiment the glucoamylase is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 12 herein. In an embodiment the glucoamylase is selected from the group consisting of:

- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 12 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: 12 herein.

In an embodiment the glucoamylase is derived from a strain of the genus *Trametes*, in particular a strain of *Trametes cingulata* disclosed in WO 2006/069289, and herein as SEQ ID NO: 13.

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.

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).

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

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

In an embodiment an alpha-amylase 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. 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 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-bonding domain, such as the one shown in SEQ ID NO: 14 herein, or a variant thereof.

In an embodiment the alpha-amylase present and/or added in saccharification and/or fermentation is selected from the group consisting of:

- (i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 14 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: 14 herein.

In a preferred embodiment the alpha-amylase is a variant of the alpha-amylase shown in

SEQ ID NO: 14 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; 5 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: 11 for numbering).

10 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: 14 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 14 for numbering).

15 In an embodiment the 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: 14 herein.

20 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.

Further Aspects Of Processes Of The Invention

25 Prior to liquefaction step a), processes of the invention, including processes of extracting/recovering oil and processes for producing fermentation products, may comprise the steps of:

- i) reducing the particle size of the starch-containing material, preferably by dry milling;
- ii) forming a slurry comprising the starch-containing material and water.

30 In an embodiment at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

In an embodiment the pH during liquefaction is between above 4.5-6.5, such as 4.5-5.0, 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.

35 In an embodiment the temperature during liquefaction is above the initial gelatinization temperature, preferably in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, especially around 85°C.

In an embodiment a jet-cooking step is carried out before liquefaction in step a). In an

embodiment the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

5 In a preferred embodiment saccharification and fermentation is carried out sequentially or simultaneously.

In an embodiment saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

10 In an embodiment fermentation or simultaneous saccharification and fermentation (SSF) is carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment fermentation is ongoing for 6 to 120 hours, in particular 24 to 96 hours.

In a preferred embodiment the fermentation product is recovered after fermentation, such as by distillation.

15 In an embodiment the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

In an embodiment the starch-containing starting material is whole grains. In an embodiment the starch-containing material is selected from the group of corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca, sorghum, rice, and potatoes.

20 In an embodiment the fermenting organism is yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisiae*.

In an embodiment the temperature in step (a) is above the initial gelatinization temperature, such as at a temperature between 80-90°C, such as around 85°C.

25 In an embodiment a process of the invention further comprises a pre-saccharification step, before saccharification step b), carried out for 40-90 minutes at a temperature between 30-65°C. In an embodiment saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5. In an embodiment fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C. In an embodiment the 30 fermentation step c) or simultaneous saccharification and fermentation (SSF) (i.e., steps b) and c)) are ongoing for 6 to 120 hours, in particular 24 to 96 hours.

In an embodiment separation in step e) is carried out by centrifugation, preferably a decanter centrifuge, filtration, preferably using a filter press, a screw press, a plate-and-frame press, a gravity thickener or decker.

35 In an embodiment the fermentation product is recovered by distillation.

Examples of Specific Process Embodiments Of The Invention

Producing Fermentation Products:

In one embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

- 5 - an alpha-amylase derived from *Bacillus stearothermophilus* having 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 optional substitution N193F; further one of the following set of substitutions:
 - E129V+K177L+R179E;
 - 10 - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
 - V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;
 - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%,
 - 15 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: 5;
 - a variant pullulanase, wherein the variants comprise the following combination of substitutions:
 - N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
 - 20 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 - D586S+E699R+ S798R;
- and further the variant comprises the following combinations of substitutions:
 - Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;
 - and wherein the variant has pullulanase activity, and the variants have at least 85%, at least
 - 25 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using *Saccharomyces cerevisiae*.

30 In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

- 35 - an alpha-amylase derived from *Bacillus stearothermophilus* having 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 optional substitution N193F; further one of the following set of substitutions:
 - E129V+K177L+R179E;

- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
 - V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;
 - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has at least 85%, more preferably at least 90%,
 5 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: 5;

- a variant pullulanase, wherein the variants comprise the following combination of substitutions:

10 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
 15 +E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C;

b) saccharifying using a glucoamylase enzyme;

20 c) fermenting using *Saccharomyces cerevisiae*.

In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

25 - an alpha-amylase derived from *Bacillus stearothermophilus* having 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 optional substitution N193F; further one of the following set of substitutions:

- E129V+K177L+R179E;

30 - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

- V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, even more preferably at least 93%,
 35 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: 5;

- a variant pullulanase, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

- 5 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C;
- 10 b) saccharifying using a glucoamylase enzyme;
c) fermenting using *Saccharomyces cerevisiae*.

In another embodiment the invention relates to processes for producing ethanol from
starch-containing material comprising the steps of:

- a) liquefying the starch-containing material at a temperature above the initial gelatinization
15 temperature using:
- an alpha-amylase derived from *Bacillus stearothermophilus* having 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 optional substitution N193F; further one of the following set of substitutions:
- 20 - E129V+K177L+R179E;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;
- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for
numbering), and wherein the alpha-amylase has at least 85%, more preferably at least 90%,
25 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: 5;
- a variant pullulanase, wherein the variants comprise the following combination of substitutions:
- 30 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

- Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
35 +E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C;

- b) saccharifying using a glucoamylase enzyme;
- c) fermenting using *Saccharomyces cerevisiae*.

In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

- 5 a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:
 - an alpha-amylase derived from *Bacillus stearothermophilus* having 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
 - 10 I181 + G182,, and optional substitution N193F; further one of the following set of substitutions:
 - E129V+K177L+R179E;
 - V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
 - V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;
 - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for
 - 15 numbering), and wherein the alpha-amylase has 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: 5;
 - 20 - a variant pullulanase, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

 - 25 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V+
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C;
- 30 b) saccharifying using a glucoamylase enzyme;
- c) fermenting using *Saccharomyces cerevisiae*.

In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

- 35 a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:
 - an alpha-amylase derived from *Bacillus stearothermophilus* having 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 optional substitution N193F; further one of the following set of substitutions:

- E129V+K177L+R179E;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

5 - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has 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
10 SEQ ID NO: 5;

- a variant pullulanase, wherein the variants comprise the following combination of substitutions:
N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

15 and further the variant comprises the following combinations of substitutions:
Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+
I460V +I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at
least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said
20 variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C;
b) saccharifying using a glucoamylase enzyme;
c) fermenting using *Saccharomyces cerevisiae*.

In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

25 a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

- an alpha-amylase derived from *Bacillus stearothermophilus* having 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

30 I181 + G182,, and optional substitution N193F; further one of the following set of substitutions:

- E129V+K177L+R179E;
- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

35 - E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has 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: 5;

- a variant pullulanase, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
5 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+

I460V+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at
10 least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C;

b) saccharifying using a glucoamylase enzyme;

c) fermenting using *Saccharomyces cerevisiae*.

15 In another embodiment the invention relates to processes for producing ethanol from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using:

- an alpha-amylase derived from *Bacillus stearothermophilus* having a double deletion of two
20 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 optional substitution N193F; further one of the following set of substitutions:

- E129V+K177L+R179E;

- V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;

25 - V59A+Q89R+E129V+K177L+R179E+Q254S+M284V;

- E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 herein for numbering), and wherein the alpha-amylase has 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
30 96%, at least 97%, at least 98%, at least 99%, but less than 100% identity to the polypeptide of SEQ ID NO: 5;

- a variant pullulanase, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
35 D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+N202K+A345P+M402S+F456W

+I460V+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the

variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C;

- 5 b) saccharifying using a glucoamylase enzyme;
- c) fermenting using *Saccharomyces cerevisiae*.

In a preferred embodiment saccharification in step b) is performed using a glucoamylase and an alpha-amylase selected as a *Rhizomucor pusillus* alpha-amylase with an *Aspergillus niger* glucoamylase linker and starch-binding domain (SBD), preferably disclosed as SEQ ID NO: 14 herein, preferably having one or more of the following substitutions: G128D, D143N, preferably G128D+D143N (using SEQ ID NO: 14 for numbering);

c) fermenting using a fermenting organism.

Fermentation Medium

15 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
20 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
25 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*.
30

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
35 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).

5

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.

15 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.

The invention is further defined in the following numbered embodiments:

Embodiment 1. A variant pullulanase, wherein the pullulanase comprises at least the following combination of substitutions:

N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C, and optionally further comprises N222P+Q252A+Q256R; wherein the variant has pullulanase activity, and
 5 wherein the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3.

Embodiment 2. The variant according to embodiment 1, wherein said variants have at least 30%
 10 relative activity when measured at 76°C relative to activity at 65°C.

Embodiment 3. The variant according to embodiment 1, wherein the variant pullulanase further comprises N20G+Y28K +H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+
 15 S798R;

Embodiment 4. The variant according to embodiments 1 and 3, wherein said variants have at least 50% relative activity when measured at 78°C relative to activity at 65°C.

Embodiment 5. The variant according to embodiment 3, wherein the variant comprises the following combination of substitutions:

20 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A+N610R+G624S+
 T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
 D586S+E699R+ S798R;

and further the variant comprises one of the following combinations of deletions and substitutions:

P30* +V31* +N32*;

P30*+ V31* +N32*+D57N+D58P;

Q29G+P30* +V31*+N32*+D57N+D58P;

P30* +V31* +N32* +D57N+D58P+A195G;

P30* +V31* +N32* +D57N+D58P+N197T ;

P30* +V31* +N32* +D57N+D58P+N202K;

P30* +V31* +N32* +D57N+D58P+A345P;

P30* +V31* +N32* +D57N+D58P+M402S;

P30* +V31* +N32* +D57N+D58P+F456W;

P30* +V31* +N32* +D57N+D58P+I460V;

P30* +V31* +N32* +D57N+D58P+N479H;

P30* +V31* +N32* +D57N+D58P+I514V;

P30* +V31* +N32* +D57N+D58P+E560R;

P30* +V31* +N32* +D57N+D58P+D615E;

P30* +V31* +N32* +D57N+D58P+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+I514V;
P30* +V31* +N32* +D57N+D58P+A345P+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+F456W;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R+D615E;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+I514V+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I514V;
P30* +V31* +N32* +D57N+D58P+A195G+A345P;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+F456W;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+ F456W+
I460V+ I514V ;
P30* +V31* +N32* +D57N+D58P+N479H ;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
I460V +I514V+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+N479H+I514V
+E560R;
P30* +V31* +N32* +D57N+D58P+N197T+A345P+M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A252I +N202K+A345P
+M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+N202K+A345P+
M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+
F456W+ I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+A345P+M402S
+F456W+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
I460V+N479H+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P
+M402S +F456W+ I460V +I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+F456W+

I460V+N479H+I514V+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T +N202K+ A345P +M402S

+F456W+I460V+N479H+I514V+E560R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 50%, particularly at least 60%, more particularly at least 70%, even more particularly at least 80% relative activity when measured at 78°C relative to activity at 65°C.

Embodiment 6. The variants according to embodiment 5, wherein the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V+E560R;

and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C.

Embodiment 7. The variants according to embodiment 5, wherein the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 70% relative activity when measured at 78°C relative to activity at 75°C.

Embodiment 8. The variants according to embodiment 5, wherein the variant pullulanase is selected from pullulanase variants, wherein the variants comprise the following combination of substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

- 5 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+A345P+M402S+F456W+I460V+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

10

Embodiment 9. The variants according to embodiment 5, wherein the variant pullulanase is
selected from pullulanase variants, wherein the variants comprise the following combination of
substitutions:

- 15 N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

- Q29G+P30*+V31*+N32*+D57N+D58P+A195G+A345P+M402S+F456W+I460V+N479H+I514V
+E560R; and wherein the variant has pullulanase activity, and the variants have at least 85%, at
20 least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than
100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at
least 70% relative activity when measured at 79°C relative to activity at 75°C.

- Embodiment 10. The variants according to embodiment 5, wherein the variant pullulanase is
25 selected from pullulanase variants, wherein the variants comprise the following combination of
substitutions:

- N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

- 30 and further the variant comprises the following combinations of substitutions:

- Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+
I460V +I514V+E560R; and wherein the variant has pullulanase activity, and the variants have at
least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said
35 variants have at least 70% relative activity when measured at 79°C relative to activity at 75°C.

Embodiment 11. The variants according to embodiment 5, wherein the variant pullulanase is
selected from pullulanase variants, wherein the variants comprise the following combination of

substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

5 and further the variant comprises the following combinations of substitutions:

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+I460V
+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have
at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least
99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein
10 said variants have at least 70% relative activity when measured at 79°C relative to activity at
75°C.

Embodiment 12. The variants according to embodiment 5, wherein the variant pullulanase is
selected from pullulanase variants, wherein the variants comprise the following combination of
15 substitutions:

N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A,S+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;

and further the variant comprises the following combinations of substitutions:

20 Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T+N202K+A345P+M402S+F456W+I460V
+N479H+I514V+E560R; and wherein the variant has pullulanase activity, and the variants have
at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least
99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein
said variants have at least 70% relative activity when measured at 79°C relative to activity at
25 75°C.

Embodiment 13. A polynucleotide encoding the pullulanase of any of the embodiments 1-12.

Embodiment 14. A nucleic acid construct or expression vector comprising the polynucleotide of
30 embodiment 13 operably linked to one or more control sequences that direct the production of
the polypeptide in an expression host.

Embodiment 15. A recombinant host cell comprising the polynucleotide of embodiment 13
operably linked to one or more control sequences that direct the production of the polypeptide.

35 Embodiment 16. A whole broth formulation or cell culture composition comprising the
polypeptide of any of embodiments 1-12.

Embodiment 17. A composition comprising the variant pullulanase of any of embodiments 1-12 and a stabilizer.

5 Embodiment 18. The composition according to embodiment 17, further comprising an alpha-amylase.

Embodiment 19. The composition according to embodiment 18, wherein the alpha-amylase is from the genus *Exiguobacterium* or *Bacillus*, such as a strain of *Bacillus stearothermophilus*, in particular a variant of a *Bacillus stearothermophilus* alpha-amylase, such as the one shown in
10 SEQ ID NO: 5.

Embodiment 20. The composition of embodiment 16, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably after position 484 of SEQ ID NO: 5, particularly after position 485, particularly after position 486, particularly after position 487,
15 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..

Embodiment 21. The composition of any of embodiments 19 or 20, wherein the *Bacillus stearothermophilus* alpha-amylase has 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: 5 for numbering).
20

Embodiment 22. The composition of any of embodiments 19-21, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.
25

Embodiment 23. The composition of any of embodiments 19-22, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.
30

Embodiment 24. The composition of any of embodiments 19-23, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- I181*+G182*+N193F+E129V+K177L+R179E;
- 35 - I181*+G182*+N193F+V59A+Q89R+E129V+K177L+R179E+H208Y+K220P+N224L+Q254S;
- I181*+G182*+N193F +V59A Q89R+ E129V+ K177L+ R179E+ Q254S+ M284V; and
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID

NO: 5 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: 5.

Embodiment 25. The composition according to any of embodiments 17-24, further comprising a protease, preferably a protease selected from a *Pyrococcus* sp protease, e.g. a *Pyrococcus furiosus* protease shown as SEQ ID NO: 7, a *Thermococcus* sp. S8A protease shown as SEQ ID NO: 8, e.g. a *Thermococcus litoralis* S8A protease, or a *Thermoascus* sp protease, e.g., a *Thermoascus aurantiacus* protease, particularly a variant of a *Thermoascus aurantiacus* protease, SEQ ID NO: 6, comprising one of the specific combinations of substitutions in the D79L+S87P+A112P+D142L;

D79L+S87P+D142L; or

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

Embodiment 26. A method of producing a polypeptide according to any of the embodiments 1-12, comprising cultivating the host cell of embodiment 15 under conditions conducive for production of the polypeptide.

Embodiment 27. 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 using an alpha-amylase and a variant pullulanase of any of the embodiments 1-12;
- b) saccharifying using a glucoamylase.

Embodiment 28. A process for producing fermentation products from starch-containing material comprising the steps of:

- a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase and a variant pullulanase of any of the embodiments 1-12;
- b) saccharifying using a glucoamylase;
- c) fermenting using a fermenting organism.

Embodiment 29. The process according to any of embodiments 27-28, wherein the alpha-amylase is from the genus *Exiguobacterium* or *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: 5.

Embodiment 30. The process of embodiment 29, wherein the *Bacillus stearothermophilus* alpha-amylase or variant thereof is truncated, preferably after position 484 of SEQ ID NO: 5, 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.

Embodiment 31. The process of any of embodiments 29-30, wherein the *Bacillus stearothermophilus* alpha-amylase has 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: 5 for numbering).

Embodiment 32. The process of any of embodiments 29-31, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position S242, preferably S242Q substitution.

Embodiment 33. The process of any of embodiments 29-32, wherein the *Bacillus stearothermophilus* alpha-amylase has a substitution at position E188, preferably E188P substitution.

Embodiment 34. The process of any of embodiments 29-33, wherein the alpha-amylase is selected from the group of *Bacillus stearothermophilus* alpha-amylase variants:

- 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; and
- I181*+G182*+N193F+E129V+K177L+R179E+K220P+N224L+S242Q+Q254S (using SEQ ID NO: 5 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: 5.

Embodiment 35. The process of any of embodiments 27-34, further comprising that a protease is present in step a), preferably a protease selected from a *Pyrococcus* sp protease, e.g. a *Pyrococcus furiosus* protease shown as SEQ ID NO: 7, a *Thermococcus* sp. S8A protease, shown as SEQ ID NO: 8, e.g. a *Thermococcus litoralis* S8A protease, or a *Thermoascus* sp protease, e.g., a *Thermoascus aurantiacus* protease, particularly a variant of a *Thermoascus*

aurantiacus protease, SEQ ID NO: 6, comprising one of the specific combinations of substitutions in the D79L+S87P+A112P+D142L;

D79L+S87P+D142L; or

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

5

Embodiment 36. The process of any of embodiments 27-35, wherein the glucoamylase present and/or added in saccharification step b) and/or fermentation step c) 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. sepiarium* or *G. trabeum*, or a strain of *Nigrofores*.

10

Embodiment 37. The process of embodiment 36, wherein the glucoamylase is derived from *Talaromyces emersonii*, such as the one shown in SEQ ID NO: 9 herein.

15

Embodiment 38. The process of embodiment 37, wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 9 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: 9.

20

Embodiment 39. The process of embodiments 36, wherein the glucoamylase is derived from *Gloeophyllum sepiarium*, such as the one shown in SEQ ID NO: 11.

25

Embodiment 40. The process of embodiments 39, wherein the glucoamylase is selected from the group consisting of:

(i) a glucoamylase comprising the polypeptide of SEQ ID NO: 11;

(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: 11.

30

Embodiment 41. The process of embodiments 36, wherein the glucoamylase is derived from *Gloeophyllum trabeum* such as the one shown in SEQ ID NO: 12.

35

Embodiment 42. The process of embodiment 41, wherein the glucoamylase is selected from the

group consisting of:

- (i) a glucoamylase comprising the polypeptide of SEQ ID NO: 12;
- (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%,
5 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 43. The process of any of embodiments 27-42, wherein a glucoamylase in combination with an alpha-amylase is present in saccharification and/or fermentation.

10

Embodiment 44. The process of embodiment 43, wherein the alpha-amylase present in saccharification and/or fermentation is of fungal or bacterial origin.

Embodiment 45. The process of embodiment 43 or 44, wherein the alpha-amylase present
15 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 a *Rhizomucor pusillus* alpha-amylase hybrid having an *Aspergillus niger* linker and starch-bonding domain, such as the one shown in SEQ ID NO: 14.

20 Embodiment 46. The process of any of embodiments 43-45, wherein the alpha-amylase present in saccharification and/or fermentation is selected from the group consisting of:

- (i) an alpha-amylase comprising the polypeptide of SEQ ID NO: 14;
- (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
25 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: 14.

Embodiment 47. The process of embodiment 46, wherein the alpha-amylase comprises one or more of the following substitutions: G128D, D143N, preferably G128D+D143N.

30

Embodiment 48. The process of any of embodiments 27-47, further comprising, prior to the liquefaction step a), the steps of:

- i) reducing the particle size of the starch-containing material, preferably by dry milling;
- ii) forming a slurry comprising the starch-containing material and water.

35

Embodiment 49. The process of any of embodiments 27-48, wherein at least 50%, preferably at least 70%, more preferably at least 80%, especially at least 90% of the starch-containing material fit through a sieve with # 6 screen.

Embodiment 50. The process of any of embodiments 27-49, wherein the pH in liquefaction is between above 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.

5

Embodiment 51. The process of any of embodiments 27-50, wherein the temperature in liquefaction is above the initial gelatinization temperature, such as in the range from 70-100°C, such as between 75-95°C, such as between 75-90°C, preferably between 80-90°C, especially around 85°C.

10

Embodiment 52. The process of any of embodiments 27-51, wherein a jet-cooking step is carried out before liquefaction in step a).

Embodiment 53. The process of embodiment 52, wherein the jet-cooking is carried out at a temperature between 110-145°C, preferably 120-140°C, such as 125-135°C, preferably around 130°C for about 1-15 minutes, preferably for about 3-10 minutes, especially around about 5 minutes.

Embodiment 54. The process of any of embodiments 27-53, wherein saccharification is carried out at a temperature from 20-75°C, preferably from 40-70°C, such as around 60°C, and at a pH between 4 and 5.

Embodiment 55. The process of any of embodiments 28-54, wherein fermentation or simultaneous saccharification and fermentation (SSF) is carried out at a temperature from 25°C to 40°C, such as from 28°C to 35°C, such as from 30°C to 34°C, preferably around about 32°C.

Embodiment 56. The process of any of embodiments 28-55, wherein the fermentation product is recovered after fermentation, such as by distillation.

30

Embodiment 57. The process of any of embodiments 28-56, wherein the fermentation product is an alcohol, preferably ethanol, especially fuel ethanol, potable ethanol and/or industrial ethanol.

Embodiment 58. The process of any of embodiments 27-57, wherein the starch-containing starting material is whole grains.

35

Embodiment 59. The process of any of embodiments 27-58, wherein the starch-containing material is derived from corn, wheat, barley, rye, milo, sago, cassava, manioc, tapioca,

sorghum, rice or potatoes.

Embodiment 60. The process of any of embodiments 28-59, wherein the fermenting organism is yeast, preferably a strain of *Saccharomyces*, especially a strain of *Saccharomyces cerevisiae*.

5

Embodiment 61. The recombinant host cell according to embodiment 15, wherein the host cell is a yeast host cell, particularly a strain of *Saccharomyces*, more particularly *Saccharomyces cerevisiae*.

10 Embodiment 62. A use of the host cell according to embodiment 61, in saccharification of starch.

Embodiment 63. A use of the variant pullulanase of any of the embodiments 1-12 in a brewing process.

15

Embodiment 64. A method of producing a brewer's wort comprising adding to a mash, a pullulanase of any of embodiments 1-12.

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

Examples

Enzymes

Protease PfuS: Protease derived from *Pyrococcus furiosus* shown in SEQ ID NO: 7.

25 Alpha-Amylase BE369 (AA369): *Bacillus stearothermophilus* alpha-amylase disclosed herein as SEQ ID NO: 5, and further having the mutations: I181* +G182* +N193F+ V59A+ Q89R+E129V+K177L+R179E+Q254S+M284V truncated to 491 amino acids (using SEQ ID NO: 5 for numbering).

30 Alpha-amylase blend AA: Blend comprising Alpha-amylase AA369, and protease PfuS (dosing: 2.1 µg EP /g DS AA369, 3.0 µg EP/g DS PfuS, where EP is enzyme protein and DS is total dry solids).

Glucoamylase A: Blend comprising *Talaromyces emersonii* glucoamylase (Te AMG) disclosed as SEQ ID NO: 34 in WO99/28448, *Trametes cingulata* glucoamylase (Tc AMG) disclosed as SEQ ID NO: 2 in WO 06/69289, and *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and starch binding domain (SBD) (Rp AA) disclosed in 35 SEQ ID NO: 14 herein having the following substitutions G128D+D143N using SEQ ID NO: 14 for numbering (activity ratio in AGU:AGU:FAU-F is about 29:8:1).

Glucoamylase B: Same as glucoamylase blend A further having a cellulase composition containing a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* cellobiohydrolase I (WO 2011/057140), *Aspergillus fumigatus* cellobiohydrolase II (WO 2011/057140), *Aspergillus fumigatus* beta-glucosidase variant (WO 2012/044915), and
 5 *Penicillium* sp. (*emersonii*) GH61 polypeptide (WO 2011/041397) (dosing: Te AMG 60 µg EP/gDS; Tc AMG 20 µg EP/gDS; Rp AA 11 µg EP/gDS; Cellulase composition 30 µg EP/gDS).

Yeast: ETHANOL RED™ from Fermentis, USA

Assays

10 Protease assays

1) Kinetic Suc-AAPF-pNA assay:

pNA substrate : Suc-AAPF-pNA (Bachem L-1400).

Temperature : Room temperature (25°C)

Assay buffers : 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS,

15 1mM CaCl₂, 150mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 with HCl or NaOH.

20µl protease (diluted in 0.01% Triton X-100) was mixed with 100µl assay buffer. The assay was started by adding 100µl pNA substrate (50mg dissolved in 1.0ml DMSO and further
 20 diluted 45x with 0.01% Triton X-100). The increase in OD₄₀₅ was monitored as a measure of the protease activity.

2) Endpoint Suc-AAPF-pNA AK assay:

pNA substrate : Suc-AAPF-pNA (Bachem L-1400).

25 Temperature : controlled (assay temperature).

Assay buffer : 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS,
 1mM CaCl₂, 150mM KCl, 0.01% Triton X-100, pH 7.0.

200µl pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with the
 30 Assay buffer) were pipetted in an Eppendorf tube and placed on ice. 20µl protease sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm.). The incubation was stopped by transferring the tube back to the ice bath and
 35 adding 600µl 500mM Succinic acid/NaOH, pH 3.5. After mixing the Eppendorf tube by vortexing 200µl mixture was transferred to a microtiter plate. OD₄₀₅ was read as a measure of protease activity. A buffer blind was included in the assay (instead of enzyme).

Glucoamylase activity (AGU)

Glucoamylase activity may be measured in Glucoamylase Units (AGU).

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

<u>AMG incubation:</u>	
Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

<u>Color reaction:</u>	
GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD:	0.21 mM
Buffer:	phosphate 0.12 M; 0.15 M NaCl
pH:	7.60 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Wavelength:	340 nm

A folder ([EB-SM-0131.02/01](#)) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

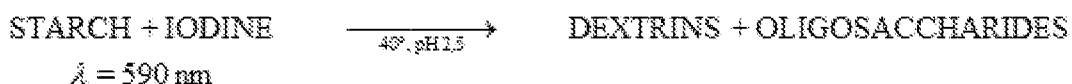
Alpha-amylase activity

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 AFAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrans and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch.

Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

ALPHA - AMYLASE

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I ₂):	0.03 g/L
CaCl ₂ :	1.85 mM
pH:	2.50 ± 0.05
Incubation	40°C

temperature:

Reaction time:	23 seconds
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Wavelength:	590 nm
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Enzyme	0.025 AFAU/mL
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concentration:

Enzyme working	0.01-0.04 AFAU/mL
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range:

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Determination of FAU-F

FAU-F Fungal Alpha-Amylase Units (Fungamyl) is measured relative to an enzyme standard of a declared strength.

Reaction conditions	
Temperature	37°C
pH	7.15

Wavelength	405 nm
Reaction time	5 min
Measuring time	2 min

A folder (EB-SM-0216.02) describing this standard method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

5 Alpha-amylase activity (KNU)

The alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e., at 37°C +/- 0.05; 0.0003 M Ca²⁺; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum soluble.

15 A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

pNP-G7 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)-□,D-maltoheptaoside, a blocked oligosaccharide which can be cleaved by an endo-amylase, such as an alpha-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the hydrolysed substrate further to liberate a free PNP molecule which has a yellow color and thus can be measured by visible spectrophotometry at lambda=405nm (400-420 nm.). Kits containing G7-pNP substrate and alpha-Glucosidase is manufactured by Roche/Hitachi (cat. No.11876473).

REAGENTS:

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

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).

35 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.

5 Dilution buffer: 50 mM MOPS, 0.05% (w/v) Triton X100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether ($C_{14}H_{22}O(C_2H_4O)_n$ ($n = 9-10$))), 1mM $CaCl_2$, pH8.0.

PROCEDURE:

10 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.

15 The slope (absorbance per minute) of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question under the given set of conditions. The amylase sample should be diluted to a level where the slope is below 0.4 absorbance units per minute.

Phadebas activity assay:

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

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

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

Reducing sugar activity assay:

The alpha-amylase activity may also be determined by reducing sugar assay with for example corn starch substrate. The number of reducing ends formed by the alpha-

amylase hydrolysing the alpha-1,4-glycosidic linkages in starch is determined by reaction with p-Hydroxybenzoic acid hydrazide (PHBAH). After reaction with PHBAH the number of reducing ends can be measured by absorbance at 405nm and the concentration of reducing ends is proportional to the alpha-amylase activity in the sample.

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

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

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

EnzChek® assay:

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

20 The substrate is a corn starch derivative, DQ™ starch, which is corn starch labeled with BODIPY® FL dye to such a degree that fluorescence is quenched. One vial containing approx. 1 mg lyophilized substrate is dissolved in 100 microliters of 50 mM sodium acetate (pH 4.0). The vial is vortexed for 20 seconds and left at room temperature, in the dark, with occasional mixing until dissolved. Then 900 microliters of 100 mM acetate, 0.01% (w/v) TRITON® X100, 0.125

25 mM CaCl₂, pH 5.5 is added, vortexed thoroughly and stored at room temperature, in the dark until ready to use. The stock substrate working solution is prepared by diluting 10-fold in residual activity buffer (100 mM acetate, 0.01% (w/v) TRITON® X100, 0.125 mM CaCl₂, pH 5.5). Immediately after incubation the enzyme is diluted to a concentration of 10-20 ng enzyme protein/ml in 100 mM acetate, 0.01% (W/v) TRITON® X100, 0.125 mM CaCl₂, pH 5.5.

30 For the assay, 25 microliters of the substrate working solution is mixed for 10 second with 25 microliters of the diluted enzyme in a black 384 well microtiter plate. The fluorescence intensity is measured (excitation: 485 nm, emission: 555 nm) once every minute for 15 minutes in each well at 25°C and the V_{max} is calculated as the slope of the plot of fluorescence intensity against time. The plot should be linear and the residual activity assay has been adjusted so that

35 the diluted reference enzyme solution is within the linear range of the activity assay.

Pullulanase Assays

Pullulanase Activity (NPUN) assay

Endo-pullulanase activity in NPUN is measured relative to a Novozymes pullulanase standard. One pullulanase unit (NPUN) is defined as the amount of enzyme that releases 1 micro mol glucose per minute under the standard conditions (0.7% red pullulan (Megazyme), pH 5, 40°C, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

5

1 mL diluted sample or standard is incubated at 40°C for 2 minutes. 0.5 mL 2% red pullulan, 0.5 M KCl, 50 mM citric acid, pH 5 are added and mixed. The tubes are incubated at 40°C for 20 minutes and stopped by adding 2.5 ml 80% ethanol. The tubes are left standing at room temperature for 10-60 minutes followed by centrifugation 10 minutes at 4000 rpm. OD of the supernatants is then measured at 510 nm and the activity calculated using a standard curve.

10

Red-pullulan assay (Megazyme)

Substrate solution

0.1g red-pullulan (megazyme S-RPUL)

15 0.75ml 2M sodium acetate, pH5.5

14.25ml H₂O

10 µl of enzyme samples were mixed with 80 µl of substrate solution and incubated at set temperatures (ex. 55, 60, 65°C) for 20min. 50 µl of ethanol was added to the reaction mixtures and centrifuge for 10 min. at 3500 rpm.

20 The supernatants were carefully taken out and the absorbance, A₅₁₀ was determined.

PAHBAH-pullulan assay

Substrate solution

0.15g BH4-pullulan

25 25ml 50mM Na acetate buffer, pH5.5

PAHBAH solution

0.0552g Bismuth (III)-acetate

0.2g PAHBAH

0.5g Potassium sodium tartrate, tetrahydrate

30 10 ml 500 mM NaOH

10 µl of enzyme samples were mixed with 110 µl of substrate solution and incubated at set temperatures (e.g., 55, 60, 65°C) for 20min. 40 µl of PAHBAH solution was added to the reaction mixtures, incubated for another 20 min at 50°C and the absorbance, A₄₀₅ was determined.

35

Lintner soluble waxy starch assay

Substrate solution

0.2 g Lintner's waxy corn starch

2.5 ml 2M sodium acetate

97.5 ml H₂O

5 μ l of enzyme samples were mixed with 100 μ l of substrate solution and incubated at set temperatures (e.g., 55, 60, 65, 70, 75°C) for 20 min. 100 μ l of 0.15% I₂/1.5% KI solution was added to the reaction mixtures and the absorbance, A₆₁₀ was determined.

PHADEBAS assay (used for determining relative activity)

Substrate solution:

1 tablet of PHADEBAS alpha-amylase tablet

10 5ml 50mM Na acetate buffer, pH5

40sec. microwave oven up to boiling

Stop solution:

18% acetic acid

Assay method:

15 Enzyme reaction in 96 well PCR tube

10ul of enzyme samples were mixed with 100ul of substrate solution and incubated at set temperatures (eg., 55, 60, 65°C) for 20min. 50ul of stop solution was added to the reaction mixtures and centrifuge for 10minutes at 3500rpm. The supernatants were carefully taken out and the absorbance at A₆₀₀ was read.

20

EXAMPLE 1: Construction of pullulanase variants P380 and P507 from a hybrid parent pullulanase

A hybrid pullulanase was constructed by fusing the N-terminal amino acids 1-451 from a natural pullulanase (SEQ ID NO: 1) isolated from *Bacillus acidopullulyticus* with the C-terminal amino acids 452-828 from a natural pullulanase (SEQ ID NO: 2) isolated from *Bacillus deramificans*. This hybrid pullulanase, disclosed as SEQ ID NO: 3 herein, was used as the parent pullulanase for constructing variant pullulanases P380 (and P380-2) and P507 (and P507-2). The substitutions introduced into the hybrid parent enzyme are disclosed in table 1 below.

25

30 Table 1. Variant pullulanases P380 and P507

Variant	Substitution using SEQ ID NO: 3 for numbering
P380	N368G+ N393A+ Q431E+ L432F+ A492A+ N610R+ G624S+ T631S+ S632C
P507	N368G+N393A+Q431E+L432F+A492A+N610R+G624S+T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+S798R
P380-2	N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A+N610R+G624S+T631S+S632C
P507-2	N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A+N610R+G624S+

	T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+ S798R
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EXAMPLE 2: Relative activity measurements of pullulanase variants, P380-2 and P507-2

Relative activity measurements of selected pullulanase variants was carried out in the range of 65-79°C, pH 5.0 by the PHADEBAS assay described in the pullulanase assay section.

5 The results are shown in the table 2 below.

Table 2.

Variant	Relative activity		
	76°C/65°C	78°C/65°C	79°C/65°C
P380-2	32%	7%	
P507-2	88%	74%	38%

EXAMPLE 3: Construction of pullulanase libraries

10 Genomic DNAs from *Bacillus subtilis* strains harboring pullulanase genes of pullulanase variants P380-2 (SEQ ID NO: 15/16) or P507-2 (SEQ ID NO: 17/18) described in Example 1 above were isolated using NucleoSpin® Tissue kit [MACHEREY-NAGEL] according to its procedure.

Pullulanase libraries were constructed as follows.

A reverse or forward primer having NNK or desired mutation(s) at target site(s) with 15bp overlaps each other were designed and two PCRs were carried out using Primer1F or 2F and the reverse primer and the forward primer and Primer 1R or 2R using appropriate template genomic DNAs under the following conditions. The resultant PCR fragments were purified by NucleoSpin® Gel and PCR Clean-up kit [MACHEREY-NAGEL] and ligated to vectors by In fusion cloning (Clontech). The *in fusion* mixture was then introduced into *E.coli* DH5α, Jet
 15 Competent *E. coli* Cell, BDL.
 20

Primer F1 atgtattatggagctctataaaaaatgaggaggggaaccgaatgtccctaatacgttctag (SEQ ID NO: 19)

Primer R1 TTATTGATTAACGCGTTTAATTTTGATCAATGACATC (SEQ ID NO: 20)

Primer F2 atgtattatggagctctataaaaaatgaggaggggaaccgaatggctaaaaaactaatttatg (SEQ ID NO: 21)

25 Primer R2 TTATTGATTAACGCGTTTACTTTTTACCGTGGTCTG (SEQ ID NO: 22)

Phusion polymerase (thermo scientific)

Total 20μl

1,0μl Template (100ng/μl)

30 4,8μl H₂O

4μl Phusion HF Buffer

1,6μl dNTP (2,5mM)

0,2µl Reverse primer (20µM)
 0,4µl Phusion (2U/µl)
 8,0µl Forward mutation primer(1µM)

5 PCR-program:

98°C/30sec

30x(98°C/10sec, 60°C/20sec, 72°C/3min)

72°C/5min

4°C/∞

10

EXAMPLE 4: Screening for thermoactivity

Bacillus libraries constructed as in Example 3 were fermented in 96 well MTPs containing TB-gly medium (13.3 g/L Bacto™ Tryptone, 26.6 g/L Bacto™ Yeast extract D, 4.4 g/L Glycerol) with 6mg/L chloramphenicol at 220rpm, 37°C and pullulanase activities were measured at
 15 several temperatures by Lintner soluble starch assay and/or Phadebas assay described in the pullulanase assay section.

Table 3a Lists the relative activity of pullulanase variants, showing higher thermoactivity than their parental pullulanases.

	Relative activity of 78°C/65°C (%)	Relative activity of 79°C/65°C (%)
P380-2	8%	2%
P507-2	68%	30%
P523	64%	34%
P530	60%	45%
P564	78%	52%

20

Table 3b Lists the relative activity of pullulanase variants, showing higher thermoactivity than their parental pullulanases.

	Relative activity of 78°C/75°C (%)	Relative activity of 79.5°C/75°C (%)
P530	71%	43%
P576	88%	55%
P578	80%	65%
P558	80%	62%

P591	99%	67%
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Table 3c Lists the relative activity of pullulanase variants, showing higher thermoactivity than their parental pullulanases.

	Relative activity of 78°C/75°C (%)	Relative activity of 79.5°C/75°C (%)
P530	71%	43%
P544	92%	
P546	86%	
P547	76%	
P548	96%	
P549	87%	
P579	106%	82%
P592	92%	63%

	Relative activity of 78°C/75°C (%)	Relative activity of 79.5°C/75°C (%)
P530	72%	47%
P576	81%	56%
P588	79%	56%
P590	84%	68%
P585	83%	69%
P585	82%	75%
P594	88%	70%
P595	83%	62%
P583	80%	77%

5

	Relative activity of 78.5°C/75°C (%)	Relative activity of 80°C/75°C (%)
P530	88%	53%
P554	84%	65%
P555	95%	59%
P556	86%	70%
P567	89%	64%

P571	85%	59%
P572	86%	62%
P573	82%	68%
P574	91%	64%
P581	88%	72%
P582	86%	78%
P584	86%	76%
P586	90%	73%
P587	86%	60%
P593	79%	71%
P596	87%	68%
P597	88%	77%
P598	91%	68%

	Relative activity of 79°C/75°C (%)	Relative activity of 81.5°C/75°C (%)
P380	2%	2%
P507	38%	6%
P530	65%	12%
P599	88%	49%
P600	84%	44%

Table 4. Substitutions of thermo-stabilized variants of P507-2

5

Variant	Substitutions starting from variant P507-2 (position numbering is according to SEQ ID NO: 3)
P523	P30* +V31* +N32*
P530	P30*+ V31* +N32*+D57N+D58P
P578	Q29G+P30* +V31*+N32*+D57N+D58P
P558	P30* +V31* +N32* +D57N+D58P+A195G
P591	P30* +V31* +N32* +D57N+D58P+N197T
P564	P30* +V31* +N32* +D57N+D58P+N202K
P544	P30* +V31* +N32* +D57N+D58P+A345P
P546	P30* +V31* +N32* +D57N+D58P+M402S
P576	P30* +V31* +N32* +D57N+D58P+F456W
P548	P30* +V31* +N32* +D57N+D58P+I460V
P592	P30* +V31* +N32* +D57N+D58P+N479H

P547	P30* +V31* +N32* +D57N+D58P+I514V
P549	P30* +V31* +N32* +D57N+D58P+E560R
P579	P30* +V31* +N32* +D57N+D58P+D615E
P556	P30* +V31* +N32* +D57N+D58P+A345P+E560R
P554	P30* +V31* +N32* +D57N+D58P+A345P+I514V
P555	P30* +V31* +N32* +D57N+D58P+A345P+I460V+I514V
P567	P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V
P571	P30* +V31* +N32* +D57N+D58P+N202K+A345P+E560R
P572	P30* +V31* +N32* +D57N+D58P+A345P+M402S+E560R
P573	P30* +V31* +N32* +D57N+D58P+N202K+A345P+M402S+E560R
P574	P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V
P576	P30* +V31* +N32* +D57N+D58P+F456W
P581	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V
P582	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V +I514V
P583	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V +I514V+E560R
P584	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V +I514V+E560R+D615E
P585	P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+I514V+E560R
P586	P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I514V
P587	P30* +V31* +N32* +D57N+D58P+A195G+A345P
P588	P30* +V31* +N32* +D57N+D58P+A195G+A345P+F456W
P590	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+ F456W+ I460V+ I514V
P592	P30* +V31* +N32* +D57N+D58P+N479H
P593	P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+ I460V +I514V+E560R
P594	P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+N479H+I514V +E560R
P595	P30* +V31* +N32* +D57N+D58P+N197T+A345P+M402S+I460V+I514V+E560R
P596	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A252I +N202K+A345P +M402S+I460V+I514V+E560R
P597	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+N202K+A345P +M402S+I460V+I514V+E560R
P598	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+ F456W+ I460V+I514V+E560R
P599	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+A345P+M402S +F456W+I460V+I514V+E560R
P600	Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+ I460V+N479H+I514V+E560R

P602	Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P +M402S +F456W+ I460V +I514V+E560R
P603	Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+ I460V+N479H+I514V+E560R
P604	Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T +N202K+ A345P +M402S +F456W+I460V+N479H+I514V+E560R

EXAMPLE 5: Fermentation of the Bacillus strains

B. subtilis strains expressing the variants were fermented on a rotary shaking table in 500 ml baffled flasks containing 100ml TB-gly with 6mg/L chloramphenicol at 220rpm, 37°C. The cultures was centrifuged (20000 x g, 20min) and the supernatants were carefully decanted from the precipitates. The supernatants were filtered through a 0.45um filter unit to remove the rest of the *Bacillus* host cells.

EXAMPLE 6: Purification of pullulanases

Purification of pullulanases was carried out by β -cyclodextrin affinity column and followed by anion exchange column chromatography. After purification, pullulanases were dialyzed against 20 mM sodium acetate buffer (pH 5.5) and concentrated.

EXAMPLE 7: Enzyme thermostability measurement

Purified enzyme was diluted with 50 mM sodium acetate pH 5.0 or 4.3 to 0.5 mg/ml and mixed with the equal volume of SYPRO Orange (Invitrogen) diluted with Milli-Q water. Thirty microliters of mixture solution was transfer to LightCycler 480 Multiwell Plate 96 (Roche Diagnostics) and the plate was sealed.

Equipment parameters of TSA:

Apparatus: LightCycler 480 Real-Time PCR System (Roche Applied Science)

Scan rate: 0.02°C/sec

Scan range: 37 - 96°C

Scan rate: 1.26°C/min

Integration time: 0.5 sec

Excitation wave length 465 nm

Emission wave length 580 nm

The obtained fluorescence signal was normalized into a range of 0 and 1. The Melting temperature (T_m) was defined as the temperature where the normalized value is closest to 0.5.

	TSA	
	T _m (°C)	
	pH4.3	pH5.0
P380-2	75.5	76.7

P507-2	80.4	80.7
P530	80	80.4
P558	79.9	80.5
P567	79.6	80.4
P574	80.6	80.8
P576	78	78.6
P578	78.1	78.2
P583	80	80.5
P598	80.6	81.3
P599	-	-

EXAMPLE 8: liquefaction and fermentation testing of pullulanase variant P380-2

The thermostabilized pullulanase variant, P380-2, was application tested in lab scale liquefaction and fermentation assays. Ground corn and backset were obtained from industrial ethanol plants. A corn slurry was made to 30.5% dry solids (% DS) with a backset inclusion rate of 30%. The slurries were made in the stainless steel Labomat canisters. The Labomat is the machine used to make lab scale liquefacts as it uses sealed canisters thus eliminating water evaporation, provides constant mixing and can operate at the elevated temperatures needed for corn liquefaction. The slurries were equilibrated for 15-30 minutes before pH measurement and adjustment. The pH was adjusted to between 4.95 and 5.05 for all slurries. The amylase used in this experiment was BE369 dosed at 2.1 µg/g DS. The P380-2 pullulanase was tested at 4 different doses (5, 10, 20 and 50 micrograms enzyme protein per gram dry solids). The control was BE369 amylase alone. After the slurry pH was adjusted, the enzymes were added at the appropriate doses. The canisters were sealed and mixed prior to insertion in the Labomat. The Labomat settings were: ramp to 80°C, hold at 80°C for 120 minutes total and mixing for 30 seconds clockwise then 30 seconds counterclockwise. After the liquefaction, the canisters were removed and incubated in an ice and water bath for 10-20 minutes to aid in rapid cooling to room temperature. The liquefacts had urea added to a final concentration of 1000 ppm and penicillin added to a final concentration of 3 ppm. The pH was checked and re-adjusted if needed to be between 4.95 and 5.05. Approximately 5 grams of each liquefact was aliquoted into a pre-weighed, drilled 15 mL flip top centrifuge tube. For each liquefact, there were 5 replicate tubes created. After the liquefacts were aliquoted, the filled tubes were weighed again. The glucoamylase used in this experiment was a Glucoamylase A blend dosed at 0.5 AGU per gram dry solids. The yeast used was Ethanol Red. It was rehydrated by adding 5.5 grams of active dry yeast to 100 milliliters of warm tap water and incubating at 32°C for 30 minutes with occasional mixing by hand. To start the fermentations, each tube was dosed with Glucoamylase A, water and rehydrated yeast. The fermentations were incubated in a 32°C water bath for 54 hours with mixing twice a day by hand. The fermentations were stopped with the addition of 10 microliters per gram of mash of 40% sulfuric acid. The tubes were then

vortexed to mix and centrifuged at 3000 RPM for 10 minutes to remove the solids. The liquid samples were filtered through a 0.45 micron syringe filter into HPLC vials. The HPLC (using the standard fuel program) was used to quantify ethanol, residual dextrans (DP1 – 4+, fructose), organic acids (acetate and lactate) and glycerol. Statistical analysis was done using SAS JMP software (version 11).

The result, as seen in figure 1, shows a clear increase in ethanol yield when the thermostable pullulanase variant, P380-2, was added to the liquefaction mix.

EXAMPLE 9: Liquefaction and fermentation testing of pullulanase variant P380-2

A second experiment was done where corn slurries with an amylase added were heated in an 80°C water bath for 30 minutes prior to the addition of the P380-2 pullulanase. The corn slurries were made in the same way as described above (in a capped Nalgene bottle rather than the stainless steel Labomat canister). The BE369 amylase was dosed at 2.1 µg/g DS, the slurries capped and incubated in the 80°C water bath with manual shaking every 2-3 minutes for the first 30 minutes. The P380-2 pullulanase was dosed at 0, 5 or 10 micrograms per gram dry solids. The liquefactions were continued for another 90 minutes with occasional mixing by hand. After liquefaction, the mashers were cooled to room temperature and urea added to 1000 ppm and penicillin added to 3 ppm as before. Small scale fermentations were done as described above with Glucoamylase A dosed at 0.5 AGU per gram dry solids.

The result, shown in figure 2, clearly indicates an increase in ethanol yield also when the thermostable pullulanase variant, P380-2, is added after the mash has reached 80°C.

EXAMPLE 10: Liquefaction and fermentation test of pullulanase variants P598 and P604

The thermo-stabilized pullulanase variants, P598 and P604, were application tested in lab scale liquefaction and fermentation assays. Ground corn was obtained from an industrial ethanol plant. A corn slurry was made to 32 % dry solids (% DS). The slurries were made in the stainless steel Labomat canisters. The Labomat is the machine used to make lab scale liquefacts as it uses sealed canisters thus eliminating water evaporation, provides constant mixing and can operate at the elevated temperatures needed for corn liquefaction. The slurries were equilibrated for 15-30 minutes before pH measurement and adjustment. The pH was adjusted to between 5.0 and 5.2 for all slurries. The amylase product used in this experiment (for all pullulanase treatments) was Alpha-amylase blend AA dosed at 0.021 % weight enzyme product per weight of ground corn. The P598 and P604 pullulanases were tested at 5 different doses (1, 5, 10, 20 and 50 micrograms enzyme protein per gram dry solids). The controls were AA369 product alone dosed at 0.0857 KNU(AH) per gram dry solids or Alpha-amylase blend AA alone dosed at 0.021 % weight of product per weight of corn. After the slurry pH was adjusted, the enzymes were added at the appropriate doses. The canisters were sealed and mixed prior to insertion in the Labomat. The Labomat settings were: ramp to 80°C, hold at 80°C for 120

minutes total and mixing for 30 seconds clockwise then 30 seconds counterclockwise. After the liquefaction, the canisters were removed and incubated in an ice and water bath for 10-20 minutes to aid in rapid cooling to room temperature. The liquefacts had urea added to a final concentration of 250 ppm for all Alpha-amylase blend AA containing mashes including the pullulanase treatments or 1000 ppm for the AA369 control. Penicillin was added to a final concentration of 3 ppm. The pH was checked but not re-adjusted. Approximately 5 grams of each liquefact was aliquoted into a pre-weighed, drilled 15 mL flip top centrifuge tube. For each liquefact, there were 4 replicate tubes created. After the liquefacts were aliquoted, the filled tubes were weighed again. The glucoamylase used in this experiment was Glucoamylase blend B dosed at 0.6 AGU per gram dry solids. The yeast used was Fermentis Ethanol Red. It was rehydrated by adding 2.75 grams of active dry yeast to 50 milliliters of warm tap water and incubating at 32°C for 30 minutes with occasional mixing by hand. To start the fermentations, each tube was dosed with Glucoamylase blend B, water and rehydrated yeast (added 100 microliters of rehydrated yeast to each tube). The fermentations were incubated in a 32°C water bath for 54 hours with mixing twice a day by hand. The fermentations were stopped with the addition of 10 microliters per gram of mash of 40% sulfuric acid. The tubes were vortexed to mix and centrifuged at 3000 RPM for 10 minutes to remove the solids. The liquid samples were filtered through a 0.45 micron syringe filter into HPLC vials. The HPLC (using the standard fuel program) was used to quantify ethanol, residual dextrans (DP1 – 4+, fructose), organic acids (acetate and lactate) and glycerol. Statistical analysis was done using SAS JMP software (version 11)

Claims

1. A variant pullulanase, wherein the pullulanase comprises at least the following combination of substitutions:
N368G+ N393A+ Q431E+ L432F+ A492A,S+ N610R+ G624S+ T631S+ S632C, and optionally
5 further comprises N222P+Q252A+Q256R; wherein the variant has pullulanase activity, and wherein the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3.
- 10 2. The variant according to claim 1, wherein said variants have at least 30% relative activity when measured at 76°C relative to activity at 65°C.
3. The variant according to claim 1, wherein the variant pullulanase further comprises N20G+Y28K +H80Y+Q187R+E310A+D311K+Q387L+Q459G+D586S+E699R+S798R;
15
4. The variant according to claims 1 and 3, wherein said variants have at least 50% relative activity when measured at 78°C relative to activity at 65°C.
5. The variant according to claim 3, wherein the variant comprises the following combination of
20 substitutions:
N222P+Q252A+Q256R+N368G+N393A+Q431E+L432F+A492A+N610R+G624S+
T631S+S632C+N20G+Y28K+H80Y+Q187R+E310A+D311K+Q387L+Q459G+
D586S+E699R+ S798R;
and further the variant comprises one of the following combinations of deletions and
25 substitutions:
P30* +V31* +N32*;
P30*+ V31* +N32*+D57N+D58P;
Q29G+P30* +V31*+N32*+D57N+D58P;
P30* +V31* +N32* +D57N+D58P+A195G;
P30* +V31* +N32* +D57N+D58P+N197T ;
P30* +V31* +N32* +D57N+D58P+N202K;
P30* +V31* +N32* +D57N+D58P+A345P;
P30* +V31* +N32* +D57N+D58P+M402S;
P30* +V31* +N32* +D57N+D58P+F456W;
P30* +V31* +N32* +D57N+D58P+I460V;
P30* +V31* +N32* +D57N+D58P+N479H;
P30* +V31* +N32* +D57N+D58P+I514V;
P30* +V31* +N32* +D57N+D58P+E560R;
P30* +V31* +N32* +D57N+D58P+D615E;

P30* +V31* +N32* +D57N+D58P+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+I514V;
P30* +V31* +N32* +D57N+D58P+A345P+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+E560R;
P30* +V31* +N32* +D57N+D58P+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+N202K+A345P+M402S+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V+I514V;
P30* +V31* +N32* +D57N+D58P+F456W;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+I460V+I514V ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R ;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+I460V
+I514V+E560R+D615E;
P30* +V31*+N32* +D57N+D58P+A195G+A345P+M402S+I460V+I514V+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I514V;
P30* +V31* +N32* +D57N+D58P+A195G+A345P;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+F456W;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+ F456W+
I460V+ I514V ;
P30* +V31* +N32* +D57N+D58P+N479H ;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
I460V +I514V+E560R;
P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+I460V+N479H+I514V
+E560R;
P30* +V31*+N32* +D57N+D58P+N197T+A345P+M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A252I +N202K+A345P
+M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+N202K+A345P+
M402S+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N202K+A345P+M402S+
F456W+ I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+N197T+A345P+M402S
+F456W+I460V+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G+A345P+M402S+F456W+
I460V+N479H+I514V+E560R;
Q29G+P30* +V31* +N32* +D57N+D58P+A195G +N197T +N202K + A345P
+M402S +F456W+ I460V +I514V+E560R;
Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N202K+A345P+M402S+F456W+

I460V+N479H+I514V+E560R;

Q29G+P30*+V31*+N32*+D57N+D58P+A195G+N197T +N202K+ A345P +M402S

+F456W+I460V+N479H+I514V+E560R; and

wherein the variant has pullulanase activity, and the variants have at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100% sequence identity to the polypeptide of SEQ ID NO: 3, and wherein said variants have at least 50%, particularly at least 60%, more particularly at least 70%, even more particularly at least 80% relative activity when measured at 78°C relative to activity at 65°C.

6. A polynucleotide encoding the pullulanase of any of the claims 1-5.

7. A nucleic acid construct or expression vector comprising the polynucleotide of claim 6 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.

8. A recombinant host cell comprising the polynucleotide of claim 6 operably linked to one or more control sequences that direct the production of the polypeptide.

9. A composition comprising the variant pullulanase of any of claims 1-5 and a stabilizer.

10. A method of producing a polypeptide according to any of the claims 1-5, comprising cultivating the host cell of claim 15 under conditions conducive for production of the polypeptide.

11. 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 using an alpha-amylase and a variant pullulanase of any of the claims 1-5;
b) saccharifying using a glucoamylase.

12. A process for producing fermentation products from starch-containing material comprising the steps of:

a) liquefying the starch-containing material at a temperature above the initial gelatinization temperature using an alpha-amylase and a variant pullulanase of any of the claims 1-5;
b) saccharifying using a glucoamylase;
c) fermenting using a fermenting organism.

13. A use of the host cell according to claim 8, in saccharification of starch.

14. A use of the variant pullulanase of any of the claims 1-5 in a brewing process.

15. A method of producing a brewer's wort comprising adding to a mash, a pullulanase of any of the claims 1-5.

Figure 1

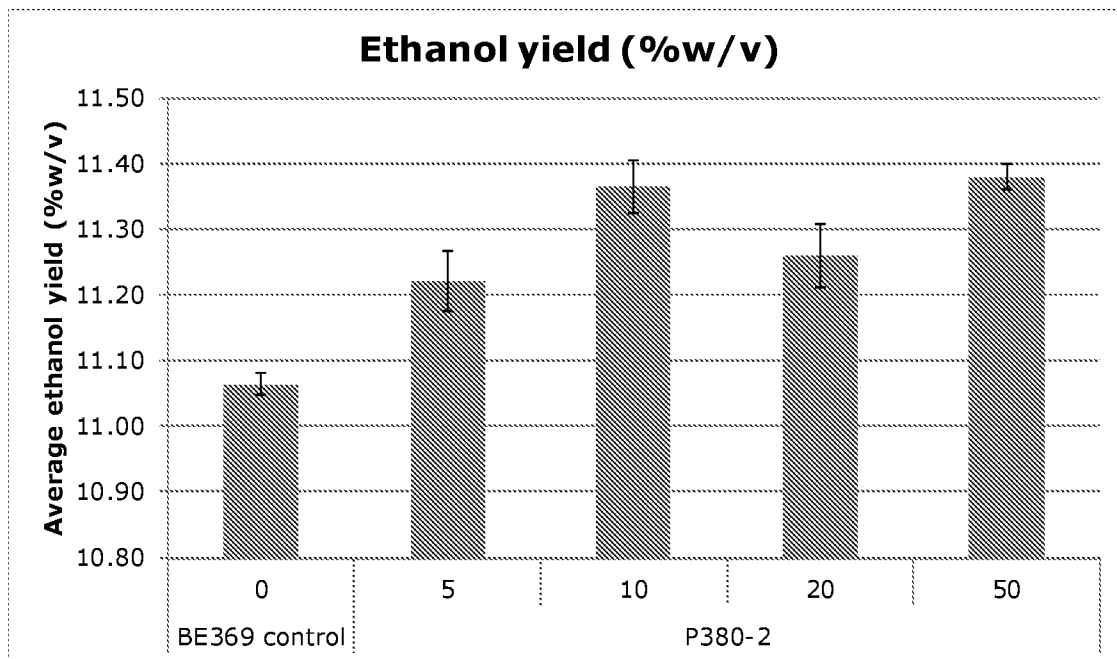


Figure 2

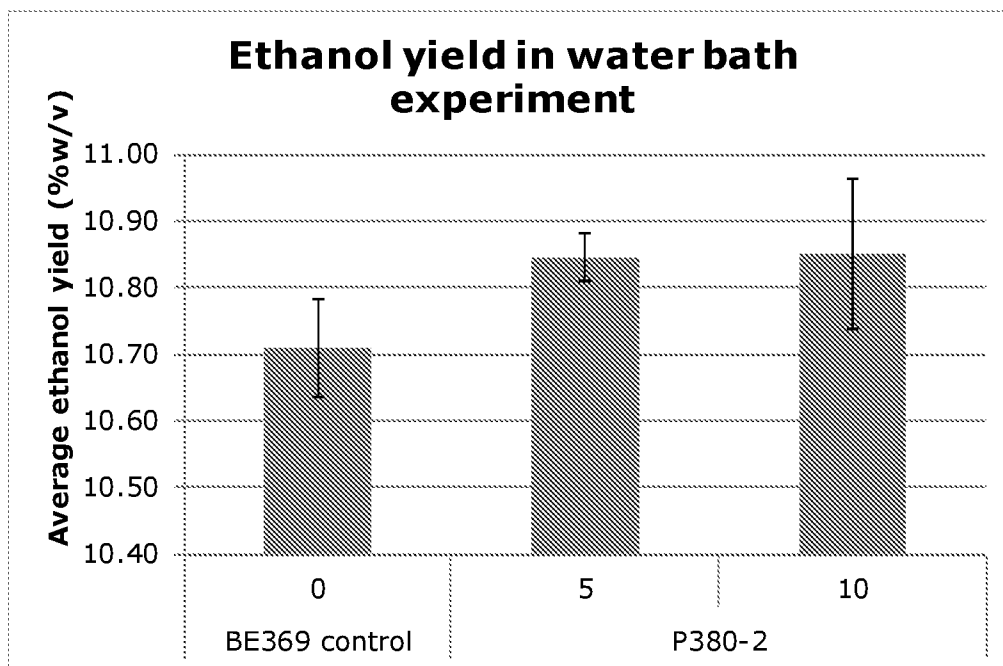


Figure 3

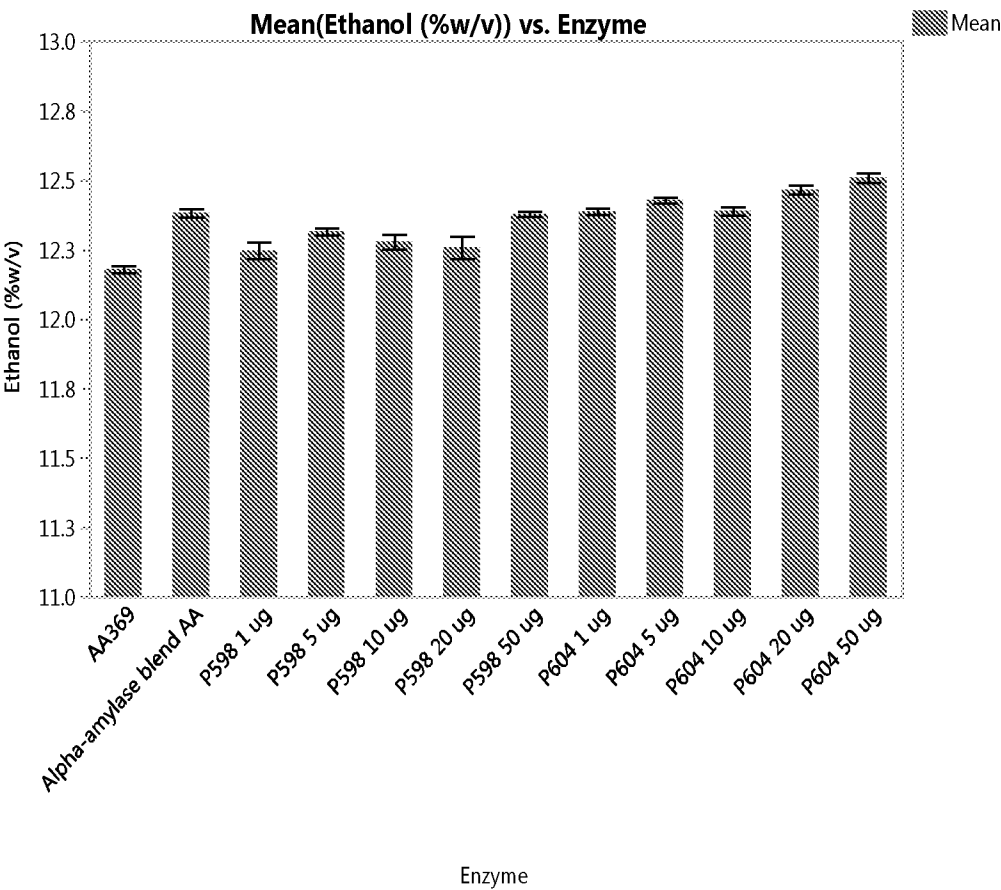


Figure 4

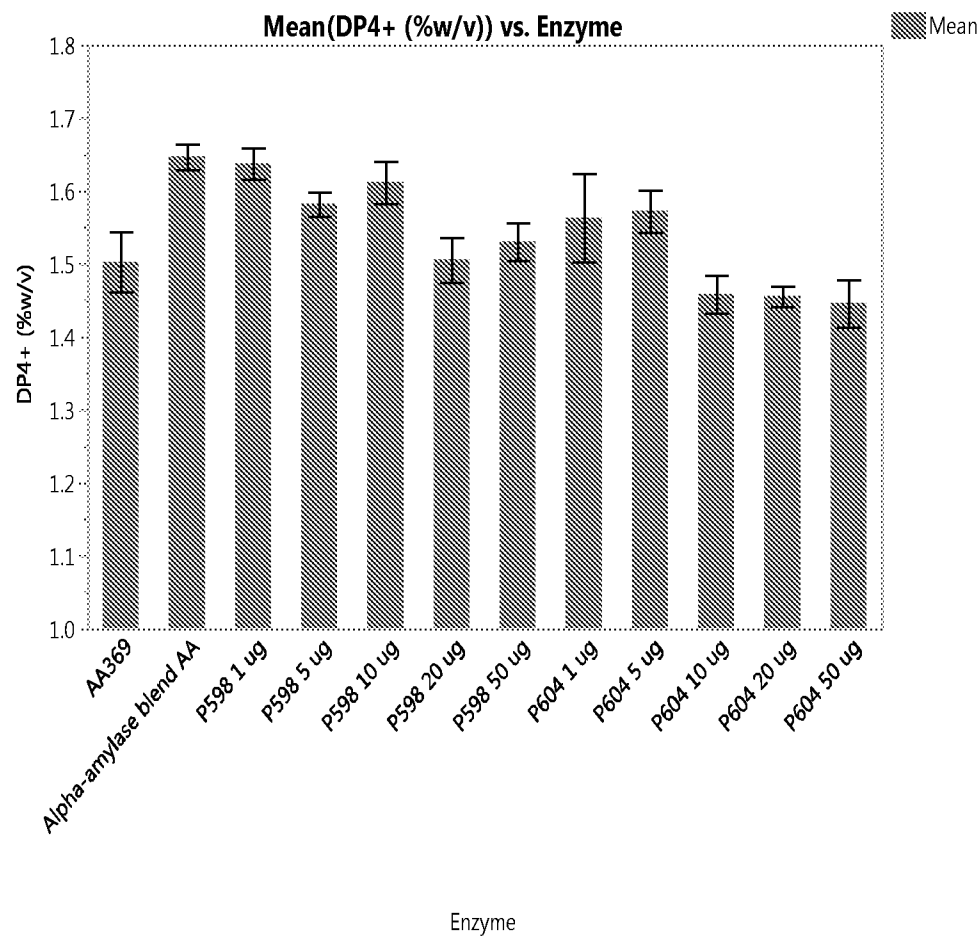


Figure 5

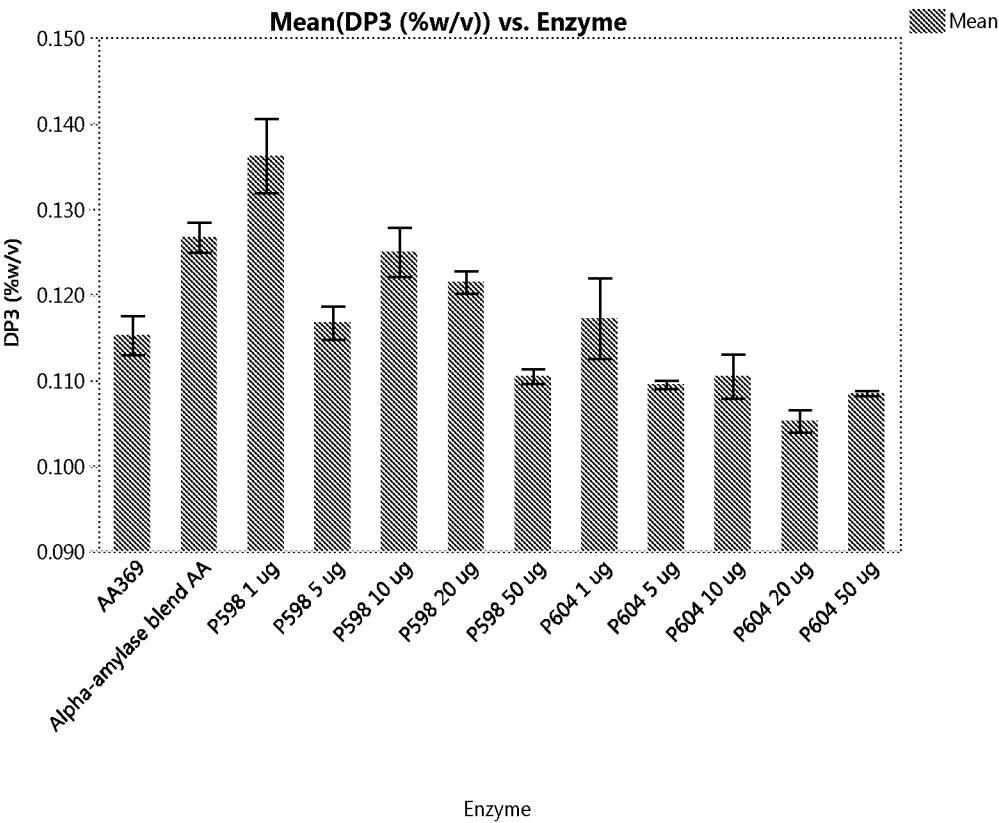
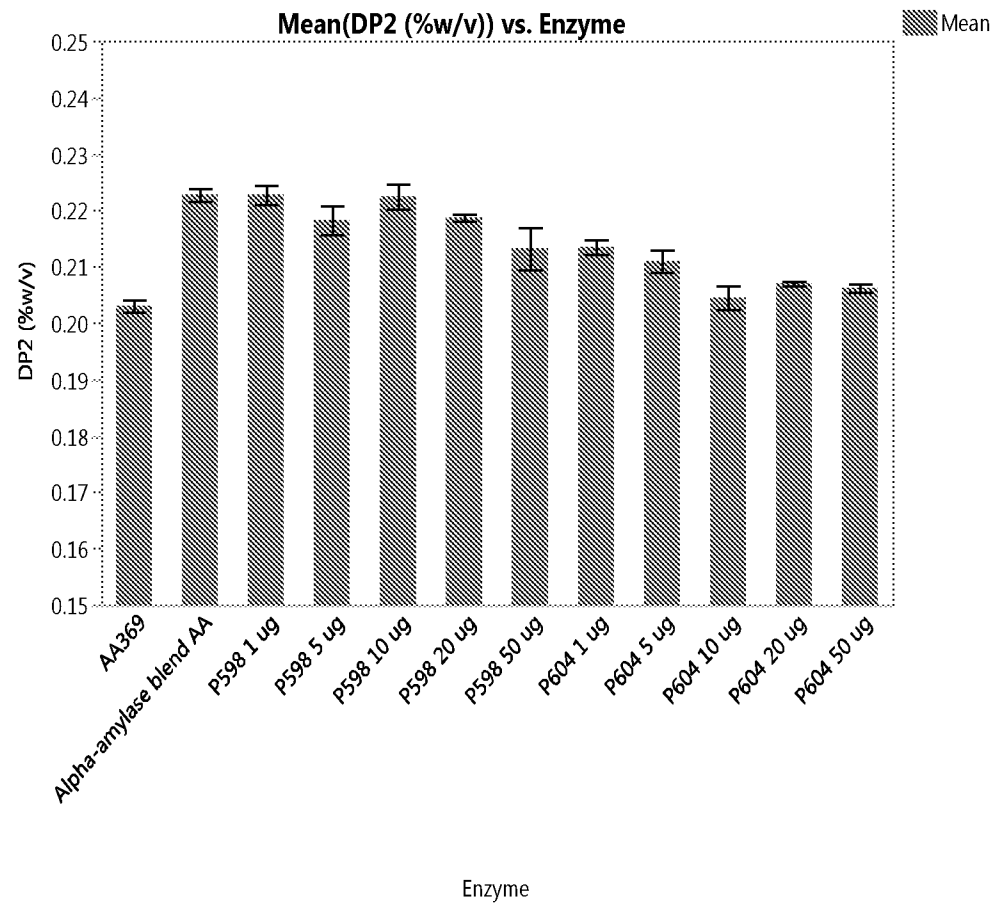


Figure 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/041737

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/44
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

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

EP0-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01/51620 A2 (NOVOZYMES AS [DK]; SVENDSEN ALLAN [DK]; ANDERSEN CARSTEN [DK]; VEDEL B) 19 July 2001 (2001-07-19) page 1, line 5 - line 12 page 6, line 6 - page 7, line 19 claims 23, 28	1-15
A	----- WO 2015/007639 A1 (NOVOZYMES AS [DK]) 22 January 2015 (2015-01-22) page 1, line 35 - page 2, line 15 page 8, line 25 - line 32 page 64, line 18 - page 65, line 4 page 66, line 24 - line 30 page 67, line 29 - page 69, line 24 examples 3, 7-8 ----- -/--	1-15



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

10 October 2016

Date of mailing of the international search report

20/10/2016

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/041737

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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			WO	2015110473 A2	30-07-2015
