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(54) **Title**: ALPHA-AMYLASES, COMPOSITIONS & METHODS

(57) **Abstract**:

ALPHA-AMYLASES, COMPOSITIONS & METHODS

CROSS-REFERENCE TO RELATED APPLICATION

[001] This application claims the benefit of International Application No. PCT/CN2016/078291, filed April 1, 2016, which is incorporated herein by reference.

FIELD OF THE INVENTION

[002] The present disclosure relates to isolated, derived or derivable (synthetic or engineered) polypeptides having alpha-amylase activity and compositions comprising such polypeptides. The present disclosure further relates to polynucleotides encoding such polypeptides, engineered nucleic acid constructs, vectors and host cells comprising genes encoding such polypeptides, which may also enable the production of such polypeptides. Moreover, the disclosure relates to methods of recombinantly producing such polypeptides or such compositions, as well as methods of using or applying the polypeptides or compositions thus produced in industrial settings, for example, for starch liquefaction and saccharification, removing starchy stains, textile desizing, and food or beverage preparation.

BACKGROUND

[003] Starch consists of a mixture of amylose (15-30% w/w) and amylopectin (70-85% w/w). Amylose consists of linear chains of alpha-1,4-linked glucose units having a molecular weight (MW) from about 60,000 to about 800,000. Amylopectin is a branched polymer containing alpha-1,6 branch points every 24-30 glucose units; its MW may be as high as 100 million.

[004] Alpha-amylases hydrolyze starch, glycogen, and related polysaccharides by cleaving internal alpha-1,4-glucosidic bonds at random. Alpha-amylases, particularly from *Bacilli*, have been used for a variety of different purposes, including starch liquefaction and saccharification, textile desizing, starch modification in the paper and pulp industry, brewing, baking, production of syrups for the food industry, production of feedstocks for fermentation processes, and in animal feed to increase digestability. Alpha-amylases have also been used to remove starchy soils and stains during dishwashing and laundry washing.

[005] Depending on the industrial applications, alpha-amylases suitable for these industrial processes can be diverse. There is as such always a need in the art for alternative alpha-amylases with improved or different properties such as pH optimum, temperature optimum, substrate specificities, and/or thermostability.

[006] It is an object of the present disclosure to provide certain polypeptides having alpha-amylase activity, polynucleotides encoding the polypeptides, nucleic acid constructs that can be used to produce such polypeptides, compositions comprising thereof, as well as methods of making and using such polypeptides.

SUMMARY

[007] The present compositions and methods relate to alpha-amylase polypeptides, and methods of use, thereof. Aspects and embodiments of the present compositions and methods are summarized in the following separately-numbered paragraphs:

1. In a first aspect, the present disclosure provides an isolated, synthetic, or recombinant polypeptide having alpha-amylase activity, selected from the group consisting of:

   a) a polypeptide comprising an amino acid sequence that is at least about 85% identical to that of SEQ ID NO: 10, at least about 92% identical to that of SEQ ID NO: 12, at least about 83% identical to that of SEQ ID NO: 14, or at least about 94% identical to that of SEQ ID NO: 16; and

   b) a polypeptide comprising an amino acid sequence that is at least about 87% identical to that of amino acids 1-440 of SEQ ID NO: 10; at least 94% identical to that of amino acids 1-440 of SEQ ID NO: 12; at least 87% identical to that of amino acids 1-441 of SEQ ID NO: 14; or at least 94% identical to that of amino acids 1-433 of SEQ ID NO: 16; and

   c) (i) a polypeptide encoded by a polynucleotide that hybridizes under at least medium-high stringency conditions with the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (ii) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (iii) a full-length complementary strand of (i) or (ii); or under at least high stringency conditions with (iv) the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (v) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide

coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (vi) a full-length complementary strand of (iv) or (v); and

d) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least about 85%, at least about 90%, at least about 95% identical to the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; and

e) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the polypeptide of SEQ ID NO: 10, 12, 14, or 16; and

f) the polypeptide of (a), (b), (c), (d), or (e) having alpha-amylase activity but lacking a signal sequence or a carbohydrate binding module; and

g) an active fragment of the polypeptide of (a), (b), (c), (d), (e), or (f) having alpha-amylase activity.

2. In certain embodiments of the polypeptide of paragraph 1, further comprising a heterologous amino acid sequence.

3. In some embodiments of the polypeptide of paragraph 2, wherein the heterologous amino acid sequence comprises:

a) a heterologous signal sequence, a heterologous carbohydrate binding module, a heterologous catalytic domain (CD), or a combination thereof;

b) the sequence of a), wherein the heterologous signal sequence, carbohydrate binding module or heterologous catalytic domain (CD) is derived from a heterologous enzyme, a tag, an epitope, a targeting peptide, a cleavable sequence, a detectable moiety or an enzyme; or

c) the sequence of a), wherein the heterologous carbohydrate binding module (CBM) comprises is a xylan binding module, a cellulose binding module, a lignin binding module, a xylose binding module, a mannan binding module, a xyloglucan-specific module arabinofuranosidase binding module, or another carbohydrate binding module.

4. In a second aspect, the present disclosure provides a polynucleotide encoding the polypeptide of any one of paragraphs 1-3.

5. In certain embodiments of the polynucleotide of paragraph 4, wherein the polynucleotide hybridizes under stringent conditions to a nucleic acid that is complementary to a polynucleotide sequence of SEQ ID NO: 1, 3, 5 or 7.

6. In some embodiments of the polynucleotide of paragraph 4 or 5, comprising a polynucleotide sequence that is at least about 85%, at least about 90%, at least about 95% identical to that of SEQ ID NO:1, 3, 5 and 7, together with at least one transcriptional or translational regulatory sequence that allows the polynucleotide sequence to be expressed by a host cell.

7. In some embodiments of the polynucleotide of paragraph 6, wherein the transcriptional or translational regulatory sequence is one that is heterologous to the microorganism from which the polynucleotide sequence is derived.

8. In a third aspect, the present disclosure provides a vector comprising the polynucleotide of any one of paragraphs 4-7.

9. In a fourth aspect, the present disclosure provides a host cell comprising the vector of paragraph 8.

10. In certain embodiments of the host cell comprising the polynucleotide of any one of paragraphs 4-7.

11. In some embodiments of the host cell of paragraph 9 or 10, which is a *Trichoderma, Aspergillus* or *Myceliopthora* cell.

12. In some embodiments of the host cell of paragraph 9 or 10, which is an *E.coli, Bacillus, Streptomyces,* or *Pseudomonas* cell.

13. In some embodiments of the host cell of paragraph 9 or 10, which is an ethanologenic microorganisms.

14. In some embodiments of the host cell of any one of paragraphs 9-13, which further expresses and secretes one or more starch degrading enzymes, and one or more additional enzymes selected from the group comprising protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, phytase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase.

15. In some embodiments of the host cell of any one of paragraphs 9-13, which can be fermented to produce ethanol, other biochemicals, or biomaterials.

16. In a fifth aspect, the present disclosure provides composition comprising the polypeptide having alpha-amylase activity of any one of paragraphs 1-3.

17. In certain embodiments of the composition of paragraph 16, wherein the composition is for liquifying a composition comprising starch.

18. In some embodiments of the composition of paragraph 17, wherein the liquefaction comprises a primary and/or secondary liquefaction step, further comprising adding additional substrate to the slurry in the primary and/or secondary liquefaction step.

19. In some embodiments of the composition of paragraph 18, wherein the composition is for liquifying pullulan, or a composition comprising starch and pullulan.

20. In some embodiments of the composition of any one of paragraphs 17-19, wherein the composition is for producing a fermented beverage.

21. In some embodiments of the composition of any one of paragraphs 17-19, wherein the composition is for producing a baked food product.

22. In certain embodiments of the composition of paragraph 16, wherein the composition is for saccharifying a composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

23. In certain embodiments of the composition of paragraph 16, wherein the composition is for raw starch hydrolysis process.

24. In certain embodiments of the composition of paragraph 16, wherein the composition is for treating the fermented mash.

25. In some embodiments of the composition of paragraph 24, wherein the fermented mash is derived from a process of producing a fermentation product utilizing starch-containing material as feedstock.

26. In some embodiments of the composition of paragraph 24 or 25, wherein the fermented mash comprises beer well, whole stillage, thin stillage, wet cake, DDG or DDGS.

27. In certain embodiments of the composition of paragraph 16, wherein the composition can be used in dry milling or wet milling.

28. In certain embodiments of the composition of paragraph 16, wherein the composition is effective for removing starchy stains from laundry, dishes, textiles, or hard surfaces.

29. In some embodiments of the composition of paragraph 28, further comprising a surfactant.

30. In some embodiments of the composition of paragraph 28 or 29, wherein the composition is a detergent composition.

31. In some embodiments of the composition of any one of paragraphs 28-30, wherein the composition is selected from the group comprising a laundry detergent, a laundry detergent additive, a manual or automatic dishwashing detergent, or a dishwashing machine cleaning composition.

32. In some embodiments of the composition of any one of paragraphs 28-31, further comprising one or more additional enzymes selected from the group comprising protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, phytase, pullulanase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, and an alpha-amylase other than the alpha-amylase of any one of paragraphs 1-3.

33. In certain embodiments of the composition of paragraph 16, wherein the composition is for textile desizing.

34. In a sixth aspect, the present disclosure provides a method for liquefying a composition comprising starch comprising:

    a) adding a polypeptide having alpha-amylase activity of any one of paragraphs 1-3; and

    b) liquefying the composition comprising starch to form a solubilized liquid or a slurry.

35. In certain embodiments of the method of paragraph 34, wherein the starch is obtained from plant material, corn, milo, rye, barley, wheat, sorghum, oats, rice, brans, cassava, millet, potato, sweet potato, or tapioca.

36. In some embodiments of the method of paragraph 35, wherein the starch is granular starch from either a dry or wet milling process.

37. In some embodiments of the method of any one of paragraphs 34-36, further comprising a primary and/or secondary liquefaction step, further comprising adding additional substrate to the slurry in the primary and/or secondary liquefaction step.

38. In some embodiments of the method of any one of paragraphs 34-37, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of paragraphs 1-3, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch comprising solution.

39. In a seventh aspect, the present disclosure provides a liquefied starch produced by the method of any one of paragraphs 34-38.

40. In an eighth aspect, the present disclosure provides a method of saccharifying a composition comprising starch to produce a composition comprising glucose, wherein the method comprises:

    a) contacting the composition comprising starch with effective amount of the polypeptide having alpha-amylase activity of any one of paragraphs 1-3; and

    b) saccharifying the composition comprising starch to produce the composition comprising glucose; wherein the alpha-amylase catalyzes the saccharification of the starch to glucose.

41. In certain embodiments of the method of paragraph 40, wherein the composition comprising starch comprises liquefied starch, gelatinized starch, or granular starch.

42. In some embodiments of the method of paragraph 40 or 41, further comprising fermenting the glucose composition to produce an end of fermentation (EOF) product.

43. In some embodiments of the method of paragraph 42, wherein the fermentation is a simultaneous saccharification and fermentation (SSF) reaction.

44. In some embodiments of the method of paragraph 42 or 43, wherein the EOF product comprises ethanol.

45. In some embodiments of the method of paragraph 42 or 43, wherein the EOF product comprises a metabolite.

46. In some embodiments of the method of paragraph 45, wherein the metabolite is citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, an organic acid, glucono delta-lactone, sodium erythorbate, omega 3 fatty acid, butanol, iso-butanol, an amino acid, lysine, tyrosine, threonine, glycine, itaconic acid, 1,3-propanediol, vitamins, or isoprene or other biochemicals or biomaterials.

47. In some embodiments of the method of any one of paragraphs 40-46, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of paragraphs 1-3, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch comprising composition.

48. In some embodiments of the method of any one of paragraphs 40-47, wherein the composition comprising starch further comprises pullulan, and wherein the alpha-amylase catalyzes the saccharification of the pullulan.

49. In some embodiments of the method of any one of paragraphs 34-48, wherein the alpha-amylase is expressed and secreted by a host cell.

50. In some embodiments of the method of paragraph 49, wherein the composition comprising starch is contacted with the host cell.

51. In some embodiments of the method of paragraph 49 or 50, wherein the host cell further expresses and secretes a glucoamylase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of paragraphs 1-3 or other enzymes.

52. In some embodiments of the method of any one of paragraphs 49-51, wherein the host cell is capable of fermenting the composition.

53. In a ninth aspect, the present disclosure provides a method for removing a starchy stain or soil from a surface, comprising:

    a) contacting the surface with a composition comprising an effective amount of the polypeptide having alpha-amylase activity of any one of paragraphs 1-3; and

b) allowing the alpha-amylase to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in aqueous solution; thereby removing the starchy stain from the surface.

54. In certain embodiments of the method of paragraph 53, wherein the aqueous composition further comprises a surfactant.

55. In some embodiments of the method of paragraph 53 or 54, wherein the surface is a textile surface or a surface on dishware.

56. In some embodiments of the method for any of paragraphs 53-55, wherein the composition further comprises at least one additional enzymes selected from protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phytase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, metalloproteinase, amadoriase, and an alpha-amylase other than the alpha-amylase of any one of paragraphs 1-3.

57. In a tenth aspect, the present disclosure provides a method for desizing a textile comprising:

a) contacting a sized textile with an effective amount of the polypeptide having alpha-amylase activity of any one of paragraphs 1-3; and

b) allowing the alpha-amylase to hydrolyze starch components in the size to produce smaller starch-derived molecules that dissolve in aqueous solution; thereby removing the size from the textile.

58. In a eleventh aspect, the present disclosure provides a method for preparing a foodstuff or beverage comprising:

a) contacting a foodstuff or beverage comprising starch with an effective amount of the polypeptide having alpha-amylase activity of any one of paragraphs 1-3; and

b) allowing the alpha-amylase to hydrolyze the starch to produce smaller starch-derived molecules.

59. In certain embodiments of the method of paragraph 58, further comprising contacting the foodstuff or beverage with glucoamylase, hexokinase, xylanase, glucose isomerase,

xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of paragraphs 1-3, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, α-glucosidase, beta-glucosidase, or a combination thereof.

60. In a twelveth aspect, the present disclosure provides a foodstuff or beverage produced by the method of paragraph 58 or 59.

61. In a thirteenth aspect, the present disclosure provides a method of making the polypeptide of any one of paragraphs 1-3 or the composition of any one of paragraphs 16-33 comprising cultivating the host cell of any one of paragraphs 9-15.

62. In certain embodiments of the method of paragraph 61, further comprising a step during which the polypeptide is recovered, enriched and/or purified.

63. In a fourteenth aspect, the present disclosure provides a whole broth formulation or cell culture composition comprising the polypeptide of any one of paragraphs 1-3.

BRIEF DESCRIPTION OF THE DRAWINGS

[008] Figure 1 is a map of the expression plasmid made to express BpuAmy1.

[009] Figure 2 is a map of the expression plasmid made to express BsuAmy3.

[0010] Figure 3 is a map of the expression plasmid made to express BspAmy33.

[0011] Figure 4 is a map of the expression plasmid made to express BsuAmy4.

DETAILED DESCRIPTION

[0012] Described are isolated, derived or derivable (synthetic or engineered) polypeptides from *Bacillus* having alpha-amylase activity and compositions comprising such polypeptides. The present disclosure further relates to polynucleotides encoding such polypeptides, engineered nucleic acid constructs, vectors and host cells comprising genes encoding such polypeptides, which may also enable the production of such polypeptides. Moreover, the disclosure relates to methods of recombinantly producing such polypeptides or such compositions, as well as methods of using or applying the polypeptides or compositions thus produced in industrial settings, for example, for starch liquefaction and saccharification, removing starchy stains, textile desizing, and food or beverage preparation. These and other aspects of the compositions and methods are described in detail, below.

[0013] Prior to describing the various aspects and embodiments of the present compositions and methods, the following definitions and abbreviations are described.

1. Definitions and Abbreviations

[0014] In accordance with this detailed description, the following abbreviations and definitions apply. Note that the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[0015] The present document is organized into a number of sections for ease of reading; however, the reader will appreciate that statements made in one section may apply to other sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

1.1. Abbreviations and Acronyms

[0017] The following abbreviations/acronyms have the following meanings unless otherwise specified:

ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DPn	degree of saccharide polymerization having n subunits
ds or DS	dry solids
GA	glucoamylase
GAU/g ds	glucoamylase activity unit/gram dry solids
IRS	insoluble residual starch
kDa	kiloDalton
MW	molecular weight
NCBI	National Center for Biotechnology Information
PAHBAH	p-hydroxybenzoic acid hydrazide
PEG	polyethyleneglycol
pI	isoelectric point
PI	performance index

ppm	parts per million, *e.g.*, µg protein per gram dry solid
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSF	simultaneous saccharification and fermentation
SSU/g solid	soluble starch unit/gram dry solids
sp.	species
TrGA	*Trichoderma reesei* glucoamylase
w/v	weight/volume
w/w	weight/weight
v/v	volume/volume
wt%	weight percent
°C	degrees Centigrade
H_2O	water
dH_2O or DI	deionized water
dIH_2O	deionized water, Milli-Q filtration
g or gm	grams
µg	micrograms
mg	milligrams
kg	kilograms
µL and µl	microliters
mL and ml	milliliters
M	molar
mM	millimolar
µM	micromolar
U	units
sec	seconds
min(s)	minute/minutes
hr(s)	hour/hours
DO	dissolved oxygen
ETOH	ethanol
eq.	equivalents
N	normal
PDB	Protein Database
CAZy	Carbohydrate-Active Enzymes database

Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
mS/cm	milli-Siemens/cm
CV	column volumes

1.2. Definitions

[0018] The terms "amylase" or "amylolytic enzyme" refer to an enzyme that is, among other things, capable of catalyzing the degradation of starch. Alpha-amylases are hydrolases that cleave the alpha-D-(1→4) O-glycosidic linkages in starch. Generally, alpha-amylases (EC 3.2.1.1; alpha-D-(1→4)-glucan glucanohydrolase) are defined as endo-acting enzymes cleaving alpha-D-(1→4) O-glycosidic linkages within the starch molecule in a random fashion yielding polysaccharides containing three or more (1-4)-alpha-linked D-glucose units. In contrast, the exo-acting amylolytic enzymes, such as beta-amylases (EC 3.2.1.2; alpha-D-(1→4)-glucan maltohydrolase) and some product-specific amylases like maltogenic alpha-amylase (EC 3.2.1.133) cleave the polysaccharide molecule from the non-reducing end of the substrate. Beta-amylases, alpha-glucosidases (EC 3.2.1.20; alpha-D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; alpha-D-(1→4)-glucan glucohydrolase), and product-specific amylases like the maltotetraosidases (EC 3.2.1.60) and the maltohexaosidases (EC 3.2.1.98) can produce malto-oligosaccharides of a specific length or enriched syrups of specific maltooligosaccharides.

[0019] The term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein X can be any number. The term includes plant-based materials such as grains, cereal, grasses, tubers and roots, and more specifically materials obtained from wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, milo, potato, sweet potato, and tapioca. The term "starch" includes granular starch. The term "granular starch" refers to raw, i.e., uncooked starch, e.g., starch that has not been subject to gelatinization or that has been subjected to temperatures at or below the gelatinization temperature of the starch.

[0020] The terms, "wild-type," "parental," or "reference," with respect to a polypeptide, refer to a naturally-occurring polypeptide that does not include a man-made substitution, insertion, or deletion at one or more amino acid positions. Similarly, the terms "wild-type," "parental," or "reference," with respect to a polynucleotide, refer to a naturally-occurring polynucleotide

that does not include a man-made nucleoside change. However, note that a polynucleotide encoding a wild-type, parental, or reference polypeptide is not limited to a naturally-occurring polynucleotide, and encompasses any polynucleotide encoding the wild-type, parental, or reference polypeptide.

[0021] Reference to the wild-type polypeptide is understood to include the mature form of the polypeptide. A "mature" polypeptide or variant, thereof, is one in which a signal sequence is absent, for example, cleaved from an immature form of the polypeptide during or following expression of the polypeptide.

[0022] The term "variant," with respect to a polypeptide, refers to a polypeptide that differs from a specified wild-type, parental, or reference polypeptide in that it includes one or more naturally-occurring or man-made substitutions, insertions, or deletions of an amino acid. Similarly, the term "variant," with respect to a polynucleotide, refers to a polynucleotide that differs in nucleotide sequence from a specified wild-type, parental, or reference polynucleotide. The identity of the wild-type, parental, or reference polypeptide or polynucleotide will be apparent from context.

[0023] In the case of the present alpha-amylases, "activity" refers to alpha-amylase activity, which can be measured as described, herein.

[0024] The term "recombinant," when used in reference to a subject cell, nucleic acid, protein or vector, indicates that the subject has been modified from its native state. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell, or express native genes at different levels or under different conditions than found in nature. Recombinant nucleic acids differ from a native sequence by one or more nucleotides and/or are operably linked to heterologous sequences, *e.g.*, a heterologous promoter in an expression vector. Recombinant proteins may differ from a native sequence by one or more amino acids and/or are fused with heterologous sequences. A vector comprising a nucleic acid encoding an amylase is a recombinant vector.

[0025] The terms "recovered," "isolated," and "separated," refer to a compound, protein (polypeptides), cell, nucleic acid, amino acid, or other specified material or component that is removed from at least one other material or component with which it is naturally associated as found in nature. An "isolated" polypeptide, thereof, includes, but is not limited to, a culture broth containing secreted polypeptide expressed in a heterologous host cell.

[0026] The term "purified" refers to material (*e.g.*, an isolated polypeptide or polynucleotide) that is in a relatively pure state, *e.g.*, at least about 90% pure, at least about 95% pure, at least about 98% pure, or even at least about 99% pure.

[0027] The term "enriched" refers to material (*e.g.*, an isolated polypeptide or polynucleotide) that is about 50% pure, at least about 60% pure, at least about 70% pure, or even at least about 70% pure.

[0028] The terms "thermostable" and "thermostability," with reference to an enzyme, refer to the ability of the enzyme to retain activity after exposure to an elevated temperature. The thermostability of an enzyme, such as an amylase enzyme, is measured by its half-life ($t_{1/2}$) given in minutes, hours, or days, during which half the enzyme activity is lost under defined conditions. The half-life may be calculated by measuring residual alpha-amylase activity for example following exposure to (*i.e.*, challenge by) an elevated temperature.

[0029] A "pH range," with reference to an enzyme, refers to the range of pH values under which the enzyme exhibits catalytic activity.

[0030] The terms "pH stable" and "pH stability," with reference to an enzyme, relate to the ability of the enzyme to retain activity over a wide range of pH values for a predetermined period of time (*e.g.*, 15 min., 30 min., 1 hour).

[0031] The term "amino acid sequence" is synonymous with the terms "polypeptide," "protein," and "peptide," and are used interchangeably. Where such amino acid sequences exhibit activity, they may be referred to as an "enzyme." The conventional one-letter or three-letter codes for amino acid residues are used, with amino acid sequences being presented in the standard amino-to-carboxy terminal orientation (*i.e.*, N→C).

[0032] The term "nucleic acid" encompasses DNA, RNA, heteroduplexes, and synthetic molecules capable of encoding a polypeptide. Nucleic acids may be single stranded or double stranded, and may be chemically modified. The terms "nucleic acid" and "polynucleotide" are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences that encode a particular amino acid sequence. Unless otherwise indicated, nucleic acid sequences are presented in 5′-to-3′ orientation.

[0033] The term "hybridization" refers to the process by which one strand of nucleic acid forms a duplex with, *i.e.*, base pairs with, a complementary strand, as occurs during blot

hybridization techniques and PCR techniques. Stringent hybridization conditions are exemplified by hybridization under the following conditions: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0). Hybridized, duplex nucleic acids are characterized by a melting temperature (T_m), where one half of the hybridized nucleic acids are unpaired with the complementary strand. Mismatched nucleotides within the duplex lower the T_m.

[0034] A "synthetic" molecule is produced by *in vitro* chemical or enzymatic synthesis rather than by an organism.

[0035] The terms "transformed," "stably transformed," and "transgenic," used with reference to a cell means that the cell contains a non-native (*e.g.*, heterologous) nucleic acid sequence integrated into its genome or carried as an episome that is maintained through multiple generations.

[0036] The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", "transformation" or "transduction," as known in the art.

[0037] A "host strain" or "host cell" is an organism into which an expression vector, phage, virus, or other DNA construct, including a polynucleotide encoding a polypeptide of interest (*e.g.*, an amylase) has been introduced. Exemplary host strains are microorganism cells (*e.g.*, bacteria, filamentous fungi, and yeast) capable of expressing the polypeptide of interest and/or fermenting saccharides. The term "host cell" includes protoplasts created from cells.

[0038] The term "heterologous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell.

[0039] The term "endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

[0040] The term "expression" refers to the process by which a polypeptide is produced based on a nucleic acid sequence. The process includes both transcription and translation.

[0041] A "selective marker" or "selectable marker" refers to a gene capable of being expressed in a host to facilitate selection of host cells carrying the gene. Examples of selectable markers include but are not limited to antimicrobials (*e.g.*, hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage, such as a nutritional advantage on the host cell.

[0042] The term "vector" refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like.

[0043] An "expression vector" refers to a DNA construct comprising a DNA sequence encoding a polypeptide of interest, which coding sequence is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation.

[0044] The term "operably linked" means that specified components are in a relationship (including but not limited to juxtaposition) permitting them to function in an intended manner. For example, a regulatory sequence is operably linked to a coding sequence such that expression of the coding sequence is under control of the regulatory sequences.

[0045] A "signal sequence" is a sequence of amino acids attached to the N-terminal portion of a protein, which facilitates the secretion of the protein outside the cell. The mature form of an extracellular protein lacks the signal sequence, which is cleaved off during the secretion process.

[0046] "Biologically active" refer to a sequence having a specified biological activity, such an enzymatic activity.

[0047] The term "specific activity" refers to the number of moles of substrate that can be converted to product by an enzyme or enzyme preparation per unit time under specific conditions. Specific activity is generally expressed as units (U)/mg of protein.

[0048] "A cultured cell material comprising an amylase" or similar language, refers to a cell lysate or supernatant (including media) that includes an amylase as a component. The cell material may be from a heterologous host that is grown in culture for the purpose of producing the amylase.

[0049] "Percent sequence identity" means that a particular sequence has at least a certain percentage of amino acid residues identical to those in a specified reference sequence, when aligned using the CLUSTAL W algorithm with default parameters. *See* Thompson *et al.* (1994) *Nucleic Acids Res.* 22:4673-4680. Default parameters for the CLUSTAL W algorithm are:

Gap opening penalty:	10.0
Gap extension penalty:	0.05
Protein weight matrix:	BLOSUM series
DNA weight matrix:	IUB
Delay divergent sequences %:	40
Gap separation distance:	8
DNA transitions weight:	0.50
List hydrophilic residues:	GPSNDQEKR
Use negative matrix:	OFF
Toggle Residue specific penalties:	ON
Toggle hydrophilic penalties:	ON
Toggle end gap separation penalty	OFF.

[0050] Deletions are counted as non-identical residues, compared to a reference sequence. Deletions occurring at either termini are included. For example, a variant 500-amino acid residue polypeptide with a deletion of five amino acid residues from the C-terminus would have a percent sequence identity of 99% (495/500 identical residues × 100) relative to the parent polypeptide. Such a variant would be encompassed by the language, "a variant having at least 99% sequence identity to the parent."

[0051] "Fused" polypeptide sequences are connected, *i.e.*, operably linked, via a peptide bond between two subject polypeptide sequences.

[0052] The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina, particulary Pezizomycotina species.

[0053] The term "degree of polymerization" (DP) refers to the number (n) of anhydro-glucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides glucose and fructose. Examples of DP2 are the disaccharides maltose and sucrose. The term "DE," or "dextrose equivalent," is defined as the percentage of reducing sugar, *i.e.*, D-glucose, as a fraction of total carbohydrate in a syrup.

[0054] The term "dry solids content" (ds) refers to the total solids of a slurry in a dry weight percent basis. The term "slurry" refers to an aqueous mixture containing insoluble solids.

[0055] The phrase "simultaneous saccharification and fermentation (SSF)" refers to a process in the production of biochemicals in which a microbial organism, such as an ethanologenic

microorganism, and at least one enzyme, such as an amylase, are present during the same process step. SSF includes the contemporaneous hydrolysis of starch substrates (granular, liquefied, or solubilized) to saccharides, including glucose, and the fermentation of the saccharides into alcohol or other biochemical or biomaterial in the same reactor vessel.

[0056] An "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or other carbohydrates to ethanol.

[0057] The term "biochemicals" refers to a metabolite of a microorganism, such as citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta-lactone, sodium erythorbate, omega 3 fatty acid, butanol, iso-butanol, an amino acid, lysine, itaconic acid, other organic acids, 1,3-propanediol, vitamins, or isoprene or other biomaterial.

[0058] The term "fermented beverage" refers to any beverage produced by a method comprising a fermentation process, such as a microbial fermentation, *e.g.*, a bacterial and/or fungal fermentation. "Beer" is an example of such a fermented beverage, and the term "beer" is meant to comprise any fermented wort produced by fermentation/brewing of a starch-containing plant material. Often, beer is produced exclusively from malt or adjunct, or any combination of malt and adjunct.

[0059] The term "malt" refers to any malted cereal grain, such as malted barley or wheat.

[0060] The term "adjunct" refers to any starch and/or sugar containing plant material that is not malt, such as barley or wheat malt. Examples of adjuncts include common corn grits, refined corn grits, brewer's milled yeast, rice, sorghum, refined corn starch, barley, barley starch, dehusked barley, wheat, wheat starch, torrified cereal, cereal flakes, rye, oats, potato, tapioca, cassava and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like.

[0061] The term "mash" refers to an aqueous slurry of any starch and/or sugar containing plant material, such as grist, *e.g.*, comprising crushed barley malt, crushed barley, and/or other adjunct or a combination thereof, mixed with water later to be separated into wort and spent grains.

[0062] The term "wort" refers to the unfermented liquor run-off following extracting the grist during mashing.

[0063] "Iodine-positive starch" or "IPS" refers to (1) amylose that is not hydrolyzed after liquefaction and saccharification, or (2) a retrograded starch polymer. When saccharified starch or saccharide liquor is tested with iodine, the high DPn amylose or the retrograded starch polymer binds iodine and produces a characteristic blue color. The saccharide liquor is thus termed "iodine-positive saccharide," "blue saccharide," or "blue sac."

[0064] The terms "retrograded starch" or "starch retrogradation" refer to changes that occur spontaneously in a starch paste or gel on ageing.

[0065] The term "about" refers to ± 15% to the referenced value.

2. Alpha-amylase from *Bacillus* and Homologs

2.1 Alpha-amylase

[0066] Alpha-amylases constitute a group of enzymes present in microorganisms and tissues from animals and plants. They are capable of hydrolyzing alpha-1,4-glucosidic bonds of glycogen, starch, related polysaccharides, and some oligosaccharides. Although all alpha-amylases possess the same catalytic function, their amino acid sequences vary greatly. The sequence identity between different amylases can be virtually non-existent, e.g., falling below 25%. Despite considerable amino acid sequence variation, alpha-amylases share a common overall topological scheme that has been identified after the three-dimensional structures of alpha-amylases from different species have been determined. The common three-dimensional structure reveals three domains: (1) a "TIM" barrel known as domain A, (2) a long loop region known as domain B that is inserted within domain A, and (3) a region close to the C-terminus known as domain C that contains a characteristic beta-structure with a Greek-key motif. The TIM barrel of domain A consists of eight alpha-helices and eight parallel beta- strands, *i.e.*, (β/α)8, that alternate along the peptide backbone. This structure, named after a conserved glycolytic enzyme triosephosphate isomerase, has been known to be common among conserved protein folds. Domain B is a loop region inserted between βA3 and αA3 (the third β-strand and α-helix in domain A). Both domain A and domain B are directly involved in the catalytic function of an alpha-amylase, because the three-dimensional structure indicates that domain A flanks the active site and domain B overlays the active site. Furthermore, domain A is considered the catalytic domain, as amino acid residues of the active site are located in loops that link beta-strands to the adjacent alpha-helices. Domain B is believed to determine the specificity of the enzyme by affecting substrate binding. MacGregor et al., Biochim. Biophys. Acta. 1546:1-20 (2001).

2.2 Alpha-amylase from *Bacillus*

[0067] The present disclosure relates to isolated, derived or derivable (synthetic or engineered) polypeptides from *Bacillus* and homologs having alpha-amylase activity and compositions comprising such polypeptides or active fragments. The present disclosure further relates to polynucleotides encoding such polypeptides, engineered nucleic acid constructs, vectors and host cells comprising genes encoding such polypeptides, which may also enable the production of such polypeptides. Moreover, the disclosure relates to methods of recombinantly producing such polypeptides or such compositions, as well as methods of using or applying the polypeptides or compositions thus produced in industrial settings, for example, for starch liquefaction and saccharification, removing starchy stains, textile desizing, and food or beverage preparation.

[0068] "Termamyl-like" alpha-amylases refer to a group of alpha-amylases widely used in the starch-processing industry. The *B. licheniformis* alpha-amylase having an amino acid sequence of SEQ ID NO: 2 of U.S. Patent No. 6,440,716 is commercially available as Termamyl®. Termamyl-like alpha-amylases commonly refer to a group of highly homologous alpha-amylases produced by *Bacillus spp.* Other members of the group include the alpha- amylases from *Geobacillus stearothermophilus* (previously known as *Bacillus stearothermophilus*; both names are used interchangeably in the present disclosure) and *B. amyloliquefaciens*, and those derived from *Bacillus sp.* NCIB 12289, NCIB 12512, NCIB 12513, and DSM 9375, all of which are described in detail in U.S. Patent No. 6,440,716 and WO 95/26397, and incorporated herein by reference.

[0069] Although alpha-amylases universally contain the three domains discussed above, the three-dimensional structures of some alpha-amylases, such as AmyE from *B. subtilis*, differ significantly from Termamyl-like alpha-amylases. These enzymes are collectively referred as non-Termamyl-like alpha-amylases.

2.2.1 Polypeptides of alpha-amylases

[0070] An aspect of the present disclosure relates to polypeptides having alpha-amylase activity, selected from the group consisting of:

a) a polypeptide comprising an amino acid sequence that is at least about 85% identical to that of SEQ ID NO: 10, at least about 92% identical to that of SEQ ID

NO: 12, at least about 83% identical to that of SEQ ID NO: 14, or at least about 94% identical to that of SEQ ID NO: 16; and

b) a polypeptide comprising an amino acid sequence that is at least about 87% identical to that of amino acids 1-440 of SEQ ID NO: 10; at least 94% identical to that of amino acids 1-440 of SEQ ID NO: 12; at least 87% identical to that of amino acids 1-441 of SEQ ID NO: 14; or at least 94% identical to that of amino acids 1-433 of SEQ ID NO: 16; and

c) (i) a polypeptide encoded by a polynucleotide that hybridizes under at least medium-high stringency conditions with the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (ii) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (iii) a full-length complementary strand of (i) or (ii); or under at least high stringency conditions with (iv) the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (v) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (vi) a full-length complementary strand of (iv) or (v); and

d) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least about 85%, at least about 90%, at least about 95% identical to the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; and

e) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the polypeptide of SEQ ID NO: 10, 12, 14, or 16; and

f) the polypeptide of (a), (b), (c), (d), (e) or (f) having alpha-amylase activity but lacking a signal sequence or a carbohydrate binding module; and

g) an active fragment of the polypeptide of (a), (b), (c), (d), (e), (f) or (g) having alpha-amylase activity.

[0071] The sequence of amino acids in positions 27-666 of SEQ ID NO: 2 is a mature alpha-amylase sequence set forth as SEQ ID NO: 10. The sequence of amino acids in positions 1-26 of SEQ ID NO: 2 is the signal peptide. It is believed that the subsequence of amino acids in positions 1-440 of SEQ ID NO: 10 is the catalytic domain of the alpha-amylase and that the mature enzyme additionally comprises a linker region and at least one starch binding domain in positions 539-640. Since the object of the present invention is to obtain a polypeptide which exhibits alpha-amylase activity, the present invention relates to any alpha-amylase

enzyme comprising the sequence of amino acids nos. 1-440 of SEQ ID NO: 10, ie a catalytic domain, optionally operably linked, either N-terminally or C-terminally, to one or two or more than two other domains of a different functionality.

[0072] The sequence of amino acids in positions 27-659 of SEQ ID NO: 4 is a mature alpha-amylase sequence set forth as SEQ ID NO: 12. The sequence of amino acids in positions 1-26 of SEQ ID NO: 4 is the signal peptide. It is believed that the subsequence of amino acids in positions 1-440 of SEQ ID NO: 12 is the catalytic domain of the alpha-amylase and that the mature enzyme additionally comprises a linker region and at least one starch binding domain in positions 538-633. Since the object of the present invention is to obtain a polypeptide which exhibits alpha-amylase activity, the present invention relates to any alpha-amylase enzyme comprising the sequence of amino acids nos. 1-440 of SEQ ID NO: 12, ie a catalytic domain, optionally operably linked, either N-terminally or C-terminally, to one or two or more than two other domains of a different functionality.

[0073] The sequence of amino acids in positions 27-667 of SEQ ID NO: 6 is a mature alpha-amylase sequence set forth as SEQ ID NO: 14. The sequence of amino acids in positions 1-26 of SEQ ID NO: 6 is the signal peptide. It is believed that the subsequence of amino acids in positions 1-441 of SEQ ID NO: 14 is the catalytic domain of the alpha-amylase and that the mature enzyme additionally comprises a linker region and at least one starch binding domain in positions 540-641. Since the object of the present invention is to obtain a polypeptide which exhibits alpha-amylase activity, the present invention relates to any alpha-amylase enzyme comprising the sequence of amino acids nos. 1-441 of SEQ ID NO: 14, ie a catalytic domain, optionally operably linked, either N-terminally or C-terminally, to one or two or more than two other domains of a different functionality.

[0074] The sequence of amino acids in positions 34-659 of SEQ ID NO: 8 is a mature alpha-amylase sequence set forth as SEQ ID NO: 16. The sequence of amino acids in positions 1-33 of SEQ ID NO: 8 is the signal peptide. It is believed that the subsequence of amino acids in positions 1-433 of SEQ ID NO: 16 is the catalytic domain of the alpha-amylase and that the mature enzyme additionally comprises a linker region and at least one starch binding domain in positions 531-626. Since the object of the present invention is to obtain a polypeptide which exhibits alpha-amylase activity, the present invention relates to any alpha-amylase enzyme comprising the sequence of amino acids nos. 1-433 of SEQ ID NO: 16, ie a catalytic

domain, optionally operably linked, either N-terminally or C-terminally, to one or two or more than two other domains of a different functionality.

[0075] In some embodiments, the present invention also relates to carbohydrate binding domains having a sequence identity to amino acids 539 to 640 of SEQ ID NO: 10 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0076] In some embodiments, the present invention also relates to carbohydrate binding domains having a sequence identity to amino acids 538 to 633 of SEQ ID NO: 12 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0077] In some embodiments, the present invention also relates to carbohydrate binding domains having a sequence identity to amino acids 540 to 641 of SEQ ID NO: 14 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0078] In some embodiments, the present invention also relates to carbohydrate binding domains having a sequence identity to amino acids 531-626 of SEQ ID NO: 16 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0079] In some embodiments, the present alpha-amylases have a defined degree of amino acid sequences identity to SEQ ID NO: 10, 12, 14, or 16, for example, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity. In some embodiments, the present alpha-amylases are derived from a parental amylases having a defined degree of amino acid

sequence identity to SEQ ID NO: 10, 12, 14, or 16, for example, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity.

[0080] In some embodiments, the present alpha-amylases comprise conservative substitution of one or several amino acid residues relative to the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16. Exemplary conservative amino acid substitutions are listed in the Table I. Some conservative mutations can be produced by genetic manipulation, while others are produced by introducing synthetic amino acids into a polypeptide by other means.

Table 1. Conservative amino acid substitutions

For Amino Acid	*Code*	*Replace with any of*
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, b-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

[0081] In some embodiments, the present alpha-amylase comprises a deletion, substitution, insertion, or addition of one or a few amino acid residues relative to the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16. In some embodiments, the present alpha-amylases are

derived from the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16 by conservative substitution of one or several amino acid residues. In some embodiments, the present alpha-amylases are derived from the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16 by deletion, substitution, insertion, or addition of one or a few amino acid residues relative to the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16. In all cases, the expression "one or a few amino acid residues" refers to 10 or less, i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, amino acid residues.

[0082] In another embodiment, the present invention also relates to carbohydrate binding domain variants of SEQ ID NO: 10, 12, 14, or 16 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions.

[0083] In some embodiments, the present alpha-amylases are encoded by nucleic acids that hybridize under stringent conditions to nucleic acid sequences that are complementary to nucleic acids that encode SEQ ID NO: 2, 4, 6 or 8. An exemplary nucleic acid sequence that encodes SEQ ID NO: 2 is SEQ ID NO: 1. An exemplary nucleic acid sequence that encodes SEQ ID NO: 4 is SEQ ID NO: 3. An exemplary nucleic acid sequence that encodes SEQ ID NO: 6 is SEQ ID NO: 5. An exemplary nucleic acid sequence that encodes SEQ ID NO: 8 is SEQ ID NO: 7.

[0084] The present amylases may be "precursor," "immature," or "full-length," in which case they include a signal sequence, or "mature," in which case they lack a signal sequence. Mature forms of the polypeptides are generally the most useful. Unless otherwise noted, the amino acid residue numbering used herein refers to the mature forms of the respective amylase polypeptides. The present amylase polypeptides may also be truncated to remove the N or C- termini, so long as the resulting polypeptides retain amylase activity.

[0085] The present amylase may be a "chimeric" or "hybrid" polypeptide, in that it includes at least a portion from a first amylase, and at least a portion from a second amylase, glucoamylase, beta-amylase, alpha-glucosidase or other starch degrading enzymes, or even other glycosyl hydrolases, such as, without limitation, cellulases, hemicellulases, etc. (including such chimeric amylases that have recently been "rediscovered" as domain-swap amylases). The present amylases may further include heterologous signal sequence, an epitope to allow tracking or purification, or the like. Exemplary heterologous signal sequences are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces* CelA.

[0086] The catalytic domain of this invention operably linked to the carbohydrate binding domain may be from a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, e.g., an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase. The polynucleotide encoding the catalytic domain may be obtained from any prokaryotic, eukaryotic, or other source.

2.2.2 Polynucleotides of alpha-amylases

[0087] In another aspect, nucleic acids encoding polypeptides having alpha-amylase activity are provided. The nucleic acid may encode the amylase having the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16 an amylase having a specified degree of amino acid sequence identity to the amylase having the amino acid sequence of SEQ ID NO: 10, 12, 14, or 16. In some embodiments, the nucleic acid encodes an amylase having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity to SEQ ID NO: 10, 12, 14, or 16. It will be appreciated that due to the degeneracy of the genetic code, a plurality of nucleic acid may encode the same polypeptide.

[0088] In one embodiment, the nucleic acid hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions to a nucleic acid complementary to a nucleic acid encoding an amylase having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity to SEQ ID NO: 10, 12, 14, or 16. Such hybridization conditions are described herein but are also well known in the art.

[0089] In another embodiment, the present invention also relates to carbohydrate binding domains encoded by polynucleotides that hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with nucleotides

1693 to 1998 of SEQ ID NO: 1, 1690 to 1977 of SEQ ID NO: 3, 1696 to 2001 of SEQ ID NO: 5, 1690 to 1977 of SEQ ID NO: 7, or the full-length complement thereof (Sambrook et al., 1989, supra).

[0090] In yet another embodiment, the present invention also relates to carbohydrate binding domains encoded by polynucleotides having a sequence identity to nucleotides 1693 to 1998 of SEQ ID NO: 1 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0091] In another embodiment, the present invention also relates to carbohydrate binding domains encoded by polynucleotides having a sequence identity to nucleotides 1690 to 1977 of SEQ ID NO: 3 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0092] In another embodiment, the present invention also relates to carbohydrate binding domains encoded by polynucleotides having a sequence identity to nucleotides 1696 to 2001 of SEQ ID NO: 5 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0093] In another embodiment, the present invention also relates to carbohydrate binding domains encoded by polynucleotides having a sequence identity to nucleotides 1690 to 1977 of SEQ ID NO: 7 of at least 75%, e.g., at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

[0094] Nucleic acids may encode a "full-length" ("fl" or "FL") amylase, which includes a signal sequence, only the mature form of an amylase, which lacks the signal sequence, or a truncated form of an amylase, for example one which lacks the N or C-terminus of the mature form. Preferrably, the nucleic acid is of sufficient length to encode an active amylase enzyme.

[0095] A nucleic acid that encodes an alpha-amylase can be operably linked to various promoters and regulators in a vector suitable for expressing the alpha-amylase in host cells. Exemplary promoters are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces* CelA. Such a nucleic acid can also be linked to other coding sequences, *e.g.*, to encode a chimeric polypeptide.

3. Production of alpha-Amylases

[0096] The present alpha-amylases can be produced in host cells, for example, by secretion or intracellular expression. A cultured cell material (*e.g.*, a whole-cell broth) comprising an alpha- amylase can be obtained following secretion of the alpha-amylase into the cell medium. Optionally, the alpha-amylase can be isolated from the host cells, or even isolated from the cell broth, depending on the desired purity of the final alpha-amylase. A gene encoding an alpha-amylase can be cloned and expressed according to methods well known in the art. Suitable host cells include bacterial, fungal (including yeast and filamentous fungi), and plant cells (including algae). Particularly useful host cells include *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesi* or *Myceliopthora Thermophila*. Other host cells include bacterial cells, *e.g.*, *Bacillus subtilis* or *B. licheniformis*, as well as *Streptomyces*.

[0097] The host cell further may express a nucleic acid encoding homologous or heterologous glucoamylase, i.e., a glucoamylase that is not the same species as the host cell, or one or more enzymes. Additionally, the host may express one or more accessory enzymes, proteins, peptides. These may benefit liquefaction, saccharification, fermentation, SSF, and downstream processes. Furthermore, the host cell may produce ethanol and other biochemicals or biomaterials in addition to enzymes used to digest the various feedstock(s). Such host cells may be useful for fermentation or simultaneous saccharification and fermentation processes to reduce or eliminate the need to add enzymes.

3.1. Vectors

[0098] A DNA construct comprising a nucleic acid encoding alpha-amylases can be constructed to be expressed in a host cell. Because of the well-known degeneracy in the genetic code, different polynucleotides that encode an identical amino acid sequence can be designed and made with routine skill. It is also well-known in the art to optimize codon use for a particular host cell. Nucleic acids encoding alpha-amylases can be incorporated into a

vector. Vectors can be transferred to a host cell using well-known transformation techniques, such as those disclosed below.

[0099] The vector may be any vector that can be transformed into and replicated within a host cell. For example, a vector comprising a nucleic acid encoding an alpha-amylase can be transformed and replicated in a bacterial host cell. A vector comprising a nucleic acid encoding an alpha-amylase can also be transformed and conveniently integrated into the chromosome (in one or more copies) of a bacterial host cell, and the integration is generally considered to be an advantage, as the DNA sequence is more likely to be stably maintained in the cell. A representative useful vector is p2JM103BBI (Vogtentanz, Protein Expr Purif, 55:40-52, 2007), which can be modified with routine skill and integrated into the chromosome of host cell such that they comprise other DNA fragments to improve the expression alpha-amylases of the invention.

[00100] Host cells that serve as expression hosts can include filamentous fungi, for example. The Fungal Genetics Stock Center (FGSC) Catalogue of Strains lists suitable vectors for expression in fungal host cells. See FGSC, Catalogue of Strains, University of Missouri, at www.fgsc.net (last modified January 17, 2007). A representative useful vector is pTrex3gM (see, Published US Patent Application 20130323798) and pTTT (see, Published US Patent Application 20110020899), which can be inserted into genome of host. The vectors pTrex3gM and pTTT can both be modified with routine skill such that they comprise and express a polynucleotide encoding an alpha-amylase polypeptide of the invention.

[00101] A nucleic acid encoding an alpha-amylase can be operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Exemplary promoters for directing the transcription of the DNA sequence encoding an alpha-amylase, especially in a bacterial host, are the promoters derived from the lac operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* or *celA*, the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the *Bacillus subtilis xylA* and *xylB* genes *etc.* For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger*

glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, or *A. nidulans* acetamidase. When a gene encoding an amylase is expressed in a bacterial species such as *E. coli*, a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter. Examples of suitable promoters for the expression in a yeast species include but are not limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the *Pichia pastoris* AOX1 or AOX2 promoters. Examples of suitable promoter for the expression in filamentous fungi include but are not limited to *cbh1*, an endogenous, inducible promoter from *T. reesei*. *See* Liu *et al.* (2008) "Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (cbh1) promoter optimization," *Acta Biochim. Biophys. Sin (Shanghai)* 40(2): 158-65.

[00102] The coding sequence can be operably linked to a signal sequence. The DNA encoding the signal sequence may be the DNA sequence naturally associated with the amylase gene to be expressed or from a different genus or species. A DNA construct or vector comprising a signal sequence and a promoter sequence can be introduced into a fungal host cell and can be derived from the same source. For example, the signal sequence is the *cbh1* signal sequence that is operably linked to a *cbh1* promoter.

[00103] An expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably linked to the DNA sequence encoding an alpha-amylase. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

[00104] The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1, and pIJ702.

[00105] The vector may also comprise a selectable marker, *e.g.*, a product of a gene complements a defect in the isolated host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or a gene that confers antibiotic resistance such as, *e.g.*, ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and xx*sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, such as known in the art. *See e.g.*, International PCT Application WO 91/17243.

[00106] Intracellular expression may be advantageous in some respects, *e.g.*, when using certain bacteria or fungi as host cells to produce large amounts of amylase for subsequent enrichment or purification. Extracellular secretion of amylase into the culture medium can also be used to make a cultured cell material comprising the isolated amylase.

[00107] The expression vector typically includes the components of a cloning vector, such as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences such as a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a sequence coding for an amino acid sequence capable of targeting the amylase to a host cell organelle such as a peroxisome, or to a particular host cell compartment. Such a targeting sequence includes but is not limited to the sequence, SKL. For expression under the direction of control sequences, the nucleic acid sequence of the amylase is operably linked to the control sequences in proper manner with respect to expression.

[00108] The procedures used to ligate the DNA construct encoding an amylase, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (*see, e.g.,* Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., Cold Spring Harbor, 1989, and 3rd ed., 2001).

3.2. Transformation and Culture of Host Cells

[00109] An isolated cell, either comprising a DNA construct or an expression vector, is advantageously used as a host cell in the recombinant production of an amylase. The cell may be transformed with the DNA construct encoding the enzyme, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage, as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.*, by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

[00110] Examples of suitable bacterial host organisms are Gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus,*

Bacillus brevis, Geobacillus (formerly *Bacillus*) *stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus lautus, Bacillus megaterium,* and *Bacillus thuringiensis*; *Streptomyces* species such as *Streptomyces murinus*; lactic acid bacterial species including *Lactococcus* sp. such as *Lactococcus lactis*; *Lactobacillus* sp. including *Lactobacillus reuteri*; *Leuconostoc* sp.; *Pediococcus* sp.; and *Streptococcus* sp. Alternatively, strains of a Gram negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, or to *Pseudomonadaceae* can be selected as the host organism.

[00111] A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp., or *Kluyveromyces*, *Yarrowinia, Schizosaccharomyces* species or a species of *Saccharomyces,* including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces* such as, for example, *S. pombe* species. A strain of the methylotrophic yeast species, *Pichia pastoris*, can be used as the host organism. Alternatively, the host organism can be a *Hansenula* species.

[00112] Suitable host organisms among filamentous fungi include species of *Aspergillus, e.g., Aspergillus niger, Aspergillus oryzae, Aspergillus tubigensis, Aspergillus awamori*, or *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, *e.g., Fusarium oxysporum* or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species. In addition, *Trichoderma sp.* can be used as a host. A suitable procedure for transformation of *Aspergillus* host cells includes, for example, that described in EP 238023. An amylase expressed by a fungal host cell can be glycosylated, *i.e.*, will comprise a glycosyl moiety. The glycosylation pattern can be the same or different as present in the wild-type amylase. The type and/or degree of glycosylation may impart changes in enzymatic and/or biochemical properties.

[00113] It is advantageous to delete genes from expression hosts, where the gene deficiency can be cured by the transformed expression vector. Known methods may be used to obtain a fungal host cell having one or more inactivated genes. Gene inactivation may be accomplished by complete or partial deletion, by insertional inactivation or by any other means that renders a gene nonfunctional for its intended purpose, such that the gene is prevented from expression of a functional protein. Any gene from a *Trichoderma* sp. or other filamentous fungal host that has been cloned can be deleted, for example, *cbh1*, *cbh2*, *egl1*,

and *egl2* genes. Gene deletion may be accomplished by inserting a form of the desired gene to be inactivated into a plasmid by methods known in the art.

[00114] The suitable host cell may be the ethanologenic microbial cell, which may express one or more of the AmyE homologs (and AmyE itself, described in US9040279) described herein, and/or other Bacillus amylases (including from *B. licheniformis*, *B. stearothermophilus*, *B. substilis*, and other *Bacillus* species), and/or amylases from other sources. These may further express a homologous or heterologous starch degrading enzymes, such as glucoamylase, i.e., a glucoamylase that is not the same species as the host cell. Additionally, the host may express one or more accessory enzymes, proteins, and/or peptides. These may benefit pretreatment, liquefaction, saccharification, fermentation, SSF, stillage, condensed distillers solubles or syrup, etc processes. Furthermore, the host cell may produce ethanol and other biochemicals or biomaterials in addition to enzymes used to digest the various feedstock(s). Such host cells may be useful for fermentation or simultaneous saccharification and fermentation processes to reduce or eliminate the need to add enzymes.

[00115] Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, *e.g.*, lipofection mediated and DEAE-Dextrin mediated transfection; incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art. *See, e.g.*, Sambrook *et al.* (2001), *supra*. The expression of heterologous protein in *Trichoderma* is described, for example, in U.S. Patent No. 6,022,725. Reference is also made to Cao *et al.* (2000) *Science* 9:991-1001 for transformation of *Aspergillus* strains. Genetically stable transformants can be constructed with vector systems whereby the nucleic acid encoding an amylase is stably integrated into a host cell chromosome. Transformants are then selected and purified by known techniques.

[00116] The preparation of *Trichoderma* sp. for transformation, for example, may involve the preparation of protoplasts from fungal mycelia. *See* Campbell *et al.* (1989) *Curr. Genet.* 16: 53-56. The mycelia can be obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall, resulting in protoplasts. The protoplasts are protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like.

Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M, *e.g.*, a 1.2 M of sorbitol can be used in the suspension medium.

[00117] Uptake of DNA into the host *Trichoderma* sp. strain depends upon the calcium ion concentration. Generally, between about 10-50 mM $CaCl_2$ is used in an uptake solution. Additional suitable compounds include a buffering system, such as TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 and polyethylene glycol. The polyethylene glycol is believed to fuse the cell membranes, thus permitting the contents of the medium to be delivered into the cytoplasm of the *Trichoderma* sp. strain. This fusion frequently leaves multiple copies of the plasmid DNA integrated into the host chromosome.

[00118] Usually transformation of *Trichoderma* sp. uses protoplasts or cells that have been subjected to a permeability treatment, typically at a density of 10^5 to 10^7/mL, particularly $2x10^6$/mL. A volume of 100 µL of these protoplasts or cells in an appropriate solution (*e.g.*, 1.2 M sorbitol and 50 mM $CaCl_2$) may be mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension; however, it is useful to add about 0.25 volumes to the protoplast suspension. Additives, such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like, may also be added to the uptake solution to facilitate transformation. Similar procedures are available for other fungal host cells. *See, e.g.*, U.S. Patent No. 6,022,725.

3.3. Expression

[00119] A method of producing an amylase may comprise cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium.

[00120] The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell and obtaining expression of an amylase. Suitable media and media components are available from commercial suppliers or may be prepared according to published recipes (*e.g.*, as described in catalogues of the American Type Culture Collection).

[00121] An enzyme secreted from the host cells can be used in a whole broth preparation. In the present methods, the preparation of a spent whole fermentation broth of a recombinant microorganism can be achieved using any cultivation method known in the art resulting in the expression of an alpha-amylase. Fermentation may, therefore, be understood as comprising

shake flask cultivation, small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the amylase to be expressed or isolated. The term "spent whole fermentation broth" is defined herein as unfractionated contents of fermentation material that includes culture medium, extracellular proteins (*e.g.*, enzymes), and cellular biomass. It is understood that the term "spent whole fermentation broth" also encompasses cellular biomass that has been lysed or permeabilized using methods well known in the art.

[00122] An enzyme secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

[00123] The polynucleotide encoding an amylase in a vector can be operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, *i.e.* the vector is an expression vector. The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. The control sequences may in particular comprise promoters.

[00124] Host cells may be cultured under suitable conditions that allow expression of an amylase. Expression of the enzymes may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone, IPTG or Sophorose. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TNT™ (Promega) rabbit reticulocyte system.

[00125] An expression host also can be cultured in the appropriate medium for the host, under aerobic conditions. Shaking or a combination of agitation and aeration can be provided, with production occurring at the appropriate temperature for that host, *e.g.*, from about 25°C to about 75°C (*e.g.*, 30°C to 45°C), depending on the needs of the host and production of the desired alpha-amylase. Culturing can occur from about 12 to about 100 hours or greater (and any hour value there between, *e.g.*, from 24 to 72 hours). Typically, the

culture broth is at a pH of about 3.0 to about 8.0, again depending on the culture conditions needed for the host relative to production of an amylase.

3.4. Identification of Amylase Activity

[00126] To evaluate the expression of an amylase in a host cell, assays can measure the expressed protein, corresponding mRNA, or alpha-amylase activity. For example, suitable assays include Northern blotting, reverse transcriptase polymerase chain reaction, and *in situ* hybridization, using an appropriately labeled hybridizing probe. Suitable assays also include measuring amylase activity in a sample, for example, by assays directly measuring reducing sugars such as glucose in the culture media. For example, glucose concentration may be determined using glucose reagent kit No. 15-UV (Sigma Chemical Co.) or an instrument, such as Technicon Autoanalyzer. Alpha-Amylase activity also may be measured by any known method, such as the PAHBAH or ABTS assays, described below.

3.5. Methods for Enriching and Purifying alpha-Amylases

[00127] Fermentation, separation, and concentration techniques are well known in the art and conventional methods can be used in order to prepare a concentrated alpha-amylase polypeptide-containing solution.

[00128] After fermentation, a fermentation broth is obtained, and the microbial cells and various suspended solids, including residual raw fermentation materials, are removed by conventional separation techniques in order to obtain an amylase solution. Filtration, centrifugation, microfiltration, rotary vacuum drum filtration, ultrafiltration, centrifugation followed by ultra-filtration, extraction, or chromatography, or the like, are generally used.

[00129] It is desirable to concentrate an alpha-amylase polypeptide-containing solution in order to optimize recovery. The enzyme containing solution is concentrated using conventional concentrated techniques until the desired enzyme concentration is obtained. Concentration of the enzyme containing solution may be achieved by any of the techniques discussed herein. Exemplary methods of enrichment and purification include but are not limited to rotary vacuum filtration and/or ultrafiltration. The enzyme solution is concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated alpha-amylase polypeptide-containing solution is at a desired level.

[00130] Concentration may be performed using, *e.g.*, a precipitation agent, such as a metal halide precipitation agent. Metal halide precipitation agents include but are not limited to

alkali metal chlorides, alkali metal bromides and blends of two or more of these metal halides. Exemplary metal halides include sodium chloride, potassium chloride, sodium bromide, potassium bromide and blends of two or more of these metal halides. The metal halide precipitation agent, sodium chloride, can also be used as a preservative.

[00131] The metal halide precipitation agent is used in an amount effective to precipitate an amylase. The selection of at least an effective amount and an optimum amount of metal halide effective to cause precipitation of the enzyme, as well as the conditions of the precipitation for maximum recovery including incubation time, pH, temperature and concentration of enzyme, will be readily apparent to one of ordinary skill in the art, after routine testing.

[00132] Generally, at least about 5% w/v (weight/volume) to about 25% w/v of metal halide is added to the concentrated enzyme solution, and usually at least 8% w/v. Generally, no more than about 25% w/v of metal halide is added to the concentrated enzyme solution and usually no more than about 20% w/v. The optimal concentration of the metal halide precipitation agent will depend, among others, on the nature of the specific alpha-amylase polypeptide and on its concentration in the concentrated enzyme solution.

[00133] Another alternative way to precipitate the enzyme is to use organic compounds. Exemplary organic compound precipitating agents include: 4-hydroxybenzoic acid, alkali metal salts of 4-hydroxybenzoic acid, alkyl esters of 4-hydroxybenzoic acid, and blends of two or more of these organic compounds. The addition of the organic compound precipitation agents can take place prior to, simultaneously with or subsequent to the addition of the metal halide precipitation agent, and the addition of both precipitation agents, organic compound and metal halide, may be carried out sequentially or simultaneously.

[00134] Generally, the organic compound precipitation agents can be, for example, linear or branched alkyl esters of 4-hydroxybenzoic acid, wherein the alkyl group contains from 1 to 10 carbon atoms, and blends of two or more of these organic compounds. Exemplary organic compounds are linear alkyl esters of 4-hydroxybenzoic acid, wherein the alkyl group contains from 1 to 6 carbon atoms, and blends of two or more of these organic compounds. Methyl esters of 4-hydroxybenzoic acid, propyl esters of 4-hydroxybenzoic acid, butyl ester of 4-hydroxybenzoic acid, ethyl ester of 4-hydroxybenzoic acid and blends of two or more of these organic compounds can also be used. Additional organic compounds also include but are not limited to 4-hydroxybenzoic acid methyl ester (named methyl PARABEN), 4-

hydroxybenzoic acid propyl ester (named propyl PARABEN), which also are amylase preservative agents. For further descriptions, *see*, *e.g.*, U.S. Patent No. 5,281,526.

[00135] Addition of the organic compound precipitation agent provides the advantage of high flexibility of the precipitation conditions with respect to pH, temperature, alpha-amylase concentration, precipitation agent concentration, and time of incubation.

[00136] The organic compound precipitation agent is used in an amount effective to improve precipitation of the enzyme by means of the metal halide precipitation agent. The selection of at least an effective amount and an optimum amount of organic compound precipitation agent, as well as the conditions of the precipitation for maximum recovery including incubation time, pH, temperature and concentration of enzyme, will be readily apparent to one of ordinary skill in the art, in light of the present disclosure, after routine testing.

[00137] Generally, at least about 0.01% w/v of organic compound precipitation agent is added to the concentrated enzyme solution and usually at least about 0.02% w/v. Generally, no more than about 0.3% w/v of organic compound precipitation agent is added to the concentrated enzyme solution and usually no more than about 0.2% w/v.

[00138] The concentrated polypeptide solution, containing the metal halide precipitation agent, and the organic compound precipitation agent, can be adjusted to a pH, which will, of necessity, depend on the enzyme to be enriched or purified. Generally, the pH is adjusted at a level near the isoelectric point of the amylase. The pH can be adjusted at a pH in a range from about 2.5 pH units below the isoelectric point (pI) up to about 2.5 pH units above the isoelectric point.

[00139] The incubation time necessary to obtain an enriched or purified enzyme precipitate depends on the nature of the specific enzyme, the concentration of enzyme, and the specific precipitation agent(s) and its (their) concentration. Generally, the time effective to precipitate the enzyme is between about 1 to about 30 hours; usually it does not exceed about 25 hours. In the presence of the organic compound precipitation agent, the time of incubation can still be reduced to less about 10 hours and in most cases even about 6 hours.

[00140] Generally, the temperature during incubation is between about 4°C and about 50°C. Usually, the method is carried out at a temperature between about 10°C and about 45°C (*e.g.*, between about 20°C and about 40°C). The optimal temperature for inducing

precipitation varies according to the solution conditions and the enzyme or precipitation agent(s) used.

[00141] The overall recovery of enriched or purified enzyme precipitate, and the efficiency with which the process is conducted, is improved by agitating the solution comprising the enzyme, the added metal halide and the added organic compound. The agitation step is done both during addition of the metal halide and the organic compound, and during the subsequent incubation period. Suitable agitation methods include mechanical stirring or shaking, vigorous aeration, or any similar technique.

[00142] After the incubation period, the enriched or purified enzyme is then separated from the dissociated pigment and other impurities and collected by conventional separation techniques, such as filtration, centrifugation, microfiltration, rotary vacuum filtration, ultrafiltration, press filtration, cross membrane microfiltration, cross flow membrane microfiltration, or the like. Further enrichment or purification of the enzyme precipitate can be obtained by washing the precipitate with water. For example, the enriched or purified enzyme precipitate is washed with water containing the metal halide precipitation agent, or with water containing the metal halide and the organic compound precipitation agents.

[00143] During fermentation, an alpha-amylase polypeptide accumulates in the culture broth. For the isolation, enrichment, or purification of the desired alpha-amylase, the culture broth is centrifuged or filtered to eliminate cells, and the resulting cell-free liquid is used for enzyme enrichment or purification. In one embodiment, the cell-free broth is subjected to salting out using ammonium sulfate at about 70% saturation; the 70% saturation-precipitation fraction is then dissolved in a buffer and applied to a column such as a Sephadex G-100 column, and eluted to recover the enzyme-active fraction. For further enrichment or purification, a conventional procedure such as ion exchange chromatography may be used.

[00144] A more specific example of enrichment or purification, is described in Sumitani *et al.* (2000) "New type of starch-binding domain: the direct repeat motif in the C-terminal region of *Bacillus* sp. 195 alpha-amylase contributes to starch binding and raw starch degrading," *Biochem. J.* 350: 477-484, and is briefly summarized here. The enzyme obtained from 4 liters of a *Streptomyces lividans* TK24 culture supernatant was treated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered by centrifugation at 10,000 × g (20 min. and 4°C) and re-dissolved in 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. The solubilized precipitate was then dialyzed against the same buffer. The dialyzed

sample was then applied to a Sephacryl S-200 column, which had previously been equilibrated with 20 mM Tris/HCl buffer, (pH 7.0), 5 mM $CaCl_2$, and eluted at a linear flow rate of 7 mL/hr with the same buffer. Fractions from the column were collected and assessed for activity as judged by enzyme assay and SDS-PAGE. The protein was further purified as follows. A Toyopearl HW55 column (Tosoh Bioscience, Montgomeryville, PA; Cat. No. 19812) was equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 1.5 to 0 M $(NH_4)_2SO_4$ in 20 mM Tris/HCL buffer, pH 7.0 containing 5 mM $CaCl_2$. The active fractions were collected, and the enzyme precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered, re-dissolved, and dialyzed as described above. The dialyzed sample was then applied to a Mono Q HR5/5 column (Amersham Pharmacia; Cat. No. 17-5167-01) previously equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$, at a flow rate of 60 mL/hour. The active fractions are collected and added to a 1.5 M $(NH_4)_2SO_4$ solution. The active enzyme fractions were re-chromatographed on a Toyopearl HW55 column, as before, to yield a homogeneous enzyme as determined by SDS-PAGE. *See* Sumitani *et al.* (2000) *Biochem. J.* 350: 477-484, for general discussion of the method and variations thereon.

[00145] For production scale recovery, alpha-amylase polypeptides can be enriched or partially purified as generally described above by removing cells via flocculation with polymers. Alternatively, the enzyme can be enriched or purified by microfiltration followed by concentration by ultrafiltration using available membranes and equipment. However, for some applications, the enzyme does not need to be enriched or purified, and whole broth culture can be lysed and used without further treatment. The enzyme can then be processed, for example, into granules.

4. Compositions and Methods for Starch Degradation

[00146] The present alpha-amylases are useful for a variety of industrial applications. For example, alpha-amylases are useful in a starch degradation processes, particularly in liquefaction of gelatinized starch, simultaneous liquefaction and saccharification, saccharification, fermentation, and/or simultaneous saccharification and fermentation (SSF).

[00147] The present alpha-amylases can also be used in a granular or raw starch hydrolysis (RSH) or granular starch hydrolysis (GSH) process for producing desired sugars and fermentation products. The term "granular starch" means raw uncooked starch, i. e., starch in

its natural form found in cereal, tubers or grains. Starch is formed within plant cells as tiny granules insoluble in water. When put in cold water, the starch granules may absorb a small amount of the liquid and swell. Raw or granular starch hydrolysis processes can be carried out at temperatures at or below the gelatinization temperature of the starch. The desired end-product from the different processes may be any product that may be produced by the enzymatic conversion of the starch substrate. For example, the desired product may be a syrup rich in glucose, maltose, oligosaccharides and/or polysaccharidesps, which can be used in other processes, such as the preparation of HFCS, or which can be converted into a number of other useful products, such as ascorbic acid intermediates (*e.g.*, gluconate; 2-keto-L-gulonic acid; 5-keto-gluconate; and 2,5-diketogluconate); 1,3-propanediol; aromatic amino acids (*e.g.*, tyrosine, phenylalanine and tryptophan); organic acids (*e.g.*, lactate, pyruvate, succinate, isocitrate, and oxaloacetate); amino acids (*e.g.*, lysine, serine, threonine, tyrosine and glycine); citric acid, antibiotics; antimicrobials; enzymes; vitamins; and hormones.

[00148] The starch conversion process may be a precursor to, or simultaneous with, a fermentation process designed to produce alcohol for fuel or drinking (*i.e.*, potable alcohol), or other Biochemicals or Biomaterials. One skilled in the art is aware of various fermentation conditions that may be used in the production of these end-products. These various uses of alpha-amylases are described in more detail below.

4.1. Preparation of Starch Substrates

[00149] Those of general skill in the art are well aware of available methods that may be used to prepare starch substrates for use in the processes disclosed herein. For example, a useful starch substrate may be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically, the granular starch may be obtained from corn, cobs, wheat, barley, rye, triticale, milo, sago, millet, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. Corn contains about 60-68% starch; barley contains about 55-65% starch; millet contains about 75-80% starch; wheat contains about 60-65% starch; and polished rice contains 70-72% starch. Specifically contemplated starch substrates are corn starch and wheat starch. The starch from a grain may be ground or whole and includes corn solids, such as kernels, bran and/or cobs. The starch may also be highly refined raw starch or feedstock from starch refinery processes. Various starches also are commercially available. For example, corn starch is available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starch is available from Sigma; sweet potato starch is available from Wako

Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical Pharmaceutical Co. (Japan).

[00150] The starch substrate can be a crude starch from milled whole grain, which contains non-starch fractions, *e.g.*, germ residues and fibers. Milling may comprise wet milling or dry milling or grinding. In wet milling, whole grain is soaked in water or dilute acid to separate the grain into its component parts, *e.g.*, starch, protein, germ, oil, kernel fibers. Wet milling efficiently separates the germ and meal (*i.e.*, starch granules and protein) and is especially suitable for production of syrups. Starch to be processed may be a highly refined starch quality, for example, at least 90%, at least 95%, at least 97%, or at least 99.5% pure. In dry milling or grinding, whole kernels are ground into a fine powder and often processed without fractionating the grain into its component parts. In some cases, oils and/or fiber from the kernels are recovered. Dry ground grain thus will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch. Dry grinding of the starch substrate can be used for production of ethanol and other biochemical and biomaterials.

4.2. Gelatinization and Liquefaction of Starch

[00151] As used herein, the term "liquefaction" or "liquefy" means a process by which gelatinized starch is converted to less viscous liquid containing shorter chain soluble dextrins, liquefaction-inducing and/or saccharifying enzymes optionally may be added. In some embodiments, the starch substrate prepared as described above is slurried with water. The starch slurry may contain starch as a weight percent of dry solids of about 10-55%, about 20-45%, about 30-45%, about 30-40%, or about 30-35%. alpha-Amylase (EC 3.2.1.1) may be added to the slurry, with a metering pump, for example. The alpha-amylase typically used for this application is a thermal stable, bacterial alpha-amylase, such as a *Geobacillus stearothermophilus* alpha-amylase, *Cytophaga*_alpha-amylase, etc, for example Spezyme® RSL (DuPont product), Spezyme AA (DuPont product), Spezyme® Fred (DuPont product), Clearflow AA (DuPont product), Spezyme Alpha PF (DuPont product), Spezyme Powerliq (DuPont product) can be used here. The alpha-amylase may be supplied, for example, at about 1500 units per kg dry matter of starch. To optimize alpha-amylase stability and activity, the pH of the slurry typically is adjusted to about pH 5.5-6.5 or a pH most suitable for the amylase to be added and about 1 mM of calcium (about 40 ppm free calcium ions) can also be added. *Geobacillus stearothermophilus* variants or other alpha-amylases may require

different conditions. Bacterial alpha-amylase remaining in the slurry following liquefaction may be deactivated via a number of methods, including lowering the pH in a subsequent reaction step or by removing calcium from the slurry in cases where the enzyme is dependent upon calcium.

[00152] The slurry of starch plus the alpha-amylase may be pumped continuously through a jet cooker, which is steam heated to 80-110°C, depending upon the source of the starch containing feedstock. Gelatinization occurs rapidly under these conditions, and the enzymatic activity, combined with the significant shear forces, begins the hydrolysis of the starch substrate. The residence time in the jet cooker is brief. The partially gelatinized starch may then be passed into a series of holding tubes maintained at 105-110°C and held for 5-8 min. to complete the gelatinization process ("primary liquefaction"). Hydrolysis to the required DE is completed in holding tanks at 85-95°C or higher temperatures for about 1 to 2 hours ("secondary liquefaction"). These tanks may contain baffles to discourage back mixing. As used herein, the term "minutes of secondary liquefaction" refers to the time that has elapsed from the start of secondary liquefaction to the time that the Dextrose Equivalent (DE) is measured. The slurry is then allowed to cool to room temperature. This cooling step can be 30 minutes to 180 minutes, *e.g.* 90 minutes to 120 minutes. The liquefied starch typically is in the form of a slurry having a dry solids content (w/w) of about 10-50%; about 10-45%; about 15-40%; about 20-40%; about 25-40%; or about 25-35%.

[00153] Liquefaction with alpha-amylases advantageously can be conducted at low pH, eliminating the requirement to adjust the pH to about pH 5.5-6.5. Alpha-amylases can be used for liquefaction at a pH range of 2 to 7, *e.g.*, pH 3.0 – 7.5, pH 4.0 – 6.0, or pH 4.5 – 5.8. Alpha-amylases can maintain liquefying activity at a temperature range of about 70°C – 140°C, *e.g.*, 85°C, 90°C, or 95°C. For example, liquefaction can be conducted with 800 µg an amylase in a solution of 25% DS corn starch for 10 min at pH 5.8 and 85°C, or pH 4.5 and 95°C. Liquefying activity can be assayed using any of a number of known viscosity assay methods in the art.

[00154] In particular embodiments using the present alpha-amylases, startch liquifaction is performed at a temperature range of 90-115°C, for the purpose of producing high-purity glucose syrups, HFCS, maltodextrins, etc.

4.3. Saccharification

[00155] The liquefied starch may be saccharified into a syrup rich in lower DP (*e.g.*, DP1 + DP2) saccharides, using alpha-amylases, optionally in the presence of another enzyme(s). The exact composition of the products of saccharification depends on the combination of enzymes used, as well as the type of starch processed. Advantageously, the syrup obtainable using the provided alpha-amylases may contain a weight percent of DP2 of the total oligosaccharides in the saccharified starch exceeding 30%, *e.g.*, 45% – 65% or 55% – 65%. The weight percent of (DP1 + DP2) in the saccharified starch may exceed about 70%, *e.g.*, 75% – 85% or 80% – 85%. The present amylases also produce a relatively high yield of glucose, *e.g.*, DP1 > 20%, in the syrup product.

[00156] Whereas liquefaction is generally run as a continuous process, saccharification is often conducted as a batch process. Saccharification conditions are dependent upon the nature of the liquefact and type of enzymes available. In some cases, a saccharification process may involve temperatures of about 60-65°C and a pH of about 4.0-4.5, *e.g.*, pH 4.3. Saccharification may be performed, for example, at a temperature between about 40°C, about 50°C, or about 55°C to about 60°C or about 65°C, necessitating cooling of the Liquefact. The pH may also be adjusted as needed. Saccharification is normally conducted in stirred tanks, which may take several hours to fill or empty. Enzymes typically are added either at a fixed ratio to dried solids, as the tanks are filled, or added as a single dose at the commencement of the filling stage. A saccharification reaction to make a syrup typically is run over about 24-72 hours, for example, 24-48 hours. When a maximum or desired DE has been attained, the reaction is stopped by heating to 85°C for 5 min., for example. Further incubation will result in a lower DE, eventually to about 90 DE, as accumulated glucose re-polymerizes to isomaltose and/or other reversion products via an enzymatic reversion reaction and/or with the approach of thermodynamic equilibrium. Preferredly, saccharification optimally is conducted at a temperature range of about 30°C to about 75°C, *e.g.*, 45°C – 75°C or 47°C – 75°C. The saccharifying may be conducted over a pH range of about pH 3 to about pH 7, *e.g.*, pH 3.0 – pH 7.5, pH 3.5 – pH 5.5, pH 3.5, pH 3.8, or pH 4.5.

[00157] An amylase may be added to the slurry in the form of a composition. Amylase can be added to a slurry of a granular starch substrate. An amylase can be added as a whole broth, clarified, enriched, partially purified, or purified enzyme. The amylase also can be added as a whole broth product.

[00158] An amylase may be added to the slurry as an isolated enzyme solution. For example, an amylase can be added in the form of a cultured cell material produced by host cells expressing such amylase. An amylase may also be secreted by a host cell into the reaction medium during the fermentation or SSF process, such that the enzyme is provided continuously into the reaction. The host cell producing and secreting amylase may also express an additional enzyme, such as a glucoamylase. For example, U.S. Patent No. 5,422,267 discloses the use of a glucoamylase in yeast for production of alcoholic beverages. For example, a host cell, *e.g.*, *Trichoderma reesei* or *Aspergillus niger or Yeast*, may be engineered to co-express an amylase and a glucoamylase, *e.g.*, *Humicola* GA, *Trichoderma* GA, or variants of these, during saccharification. The host cell can be genetically modified so as not to express its endogenous glucoamylase and/or other enzymes, proteins or other materials. The host cell can be engineered to express a broad spectrum of various saccharolytic enzymes. For example, the recombinant yeast host cell can comprise nucleic acids encoding a glucoamylase, an alpha-glucosidase, beta-amylase, an enzyme that utilizes pentose sugar, an alpha-amylase, a pullulanase, an isoamylase, an isopullulanase, a phytase, a protease, and/or other enzymes. *See, e.g.*, WO 2011/153516 A2.

4.4. Raw starch hydrolysis

[00159] A "raw starch hydrolysis" process (RSH) differs from conventional starch treatment processes, including liquefying gelatinized starch at high temperature using typically a bacterial alpha-amylase, followed by simultaneous saccharification and fermentation carried out in the presence of a glucoamylase and a fermentation organism and possibly other enzymes. RSH process includes sequentially or simultaneously saccharifying and fermenting granular starch at or below the gelatinization temperature of the starch substrate typically in the presence of at least an amylase and/or glucoamylase. Starch heated in water begins to gelatinize between 50 °C and 75 °C (this temperature range seems a little low - can we check with Jay Shetty and others in the applications group?); the exact temperature of gelatinization depends on the specific starch. For example, the gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the gelatinization temperature of a given starch is the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein. S. and Lii. C., Starch/Starke, Vol. 44 (12) pp. 461-466 (1992).

[00160] AmyE homologs (and AmyE itself, described in US9040279) described herein expressed in bacterial, fungal, yeast or ethanologenic microbial cells can be used in raw starch hydrolysis process described herein.

[00161] In addition, alpha-amylase that is other than the alpha-amylase described in this invention, glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, α-glucosidase, beta-glucosidase, or a combination thereof can also be used in raw starch hydrolysis process described herein. The said enzymes can be co-expressed with alpha-amylase in this invention or directly added into the raw starch hydrolysis process.

4.5. Isomerization

[00162] The soluble starch hydrolysate produced by treatment with amylase can be converted into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS). This conversion can be achieved using a glucose isomerase, particularly a glucose isomerase immobilized on a solid support. The pH is increased to about 6.0 to about 8.0, *e.g.*, pH 7.5 (depending on the isomerase), and Ca^{2+} is removed by ion exchange. Suitable isomerases include SWEETZYME®, IT (Novozymes A/S); G-ZYME® IMGI, and G-ZYME® G993, KETOMAX®, G-ZYME® G993, G-ZYME® G993 liquid, and GENSWEET® IGI. Following isomerization, the mixture typically contains about 40-45% fructose, *e.g.*, 42% fructose.

4.6. Fermentation

[00163] The soluble starch hydrolysate, particularly a glucose rich syrup, can be fermented by contacting the starch hydrolysate with a fermenting organism typically at a temperature around 32°C, such as from 30°C to 35°C for alcohol-producing yeast. The temperature and pH of the fermentation will depend upon the fermenting organism. EOF products include metabolites, such as citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, amino acids, lysine and other amino acids, vitamins, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol, vitamins, and other biomaterials.

[00164] Ethanologenic microorganisms include yeast, such as *Saccharomyces cerevisiae* and bacteria, *e.g.*, *Zymomonas moblis*, expressing alcohol dehydrogenase and pyruvate decarboxylase. The ethanologenic microorganism can express xylose reductase and xylitol dehydrogenase, which convert xylose to xylulose. Improved strains of ethanologenic microorganisms, which can withstand higher temperatures, for example, are known in the art and can be used. *See* Liu *et al.* (2011) *Sheng Wu Gong Cheng Xue Bao* 27:1049-56. Commercial sources of yeast include ETHANOL RED® (LeSaffre); THERMOSACC® (Lallemand); RED STAR® (Red Star); FERMIOL® (DSM Specialties); and SUPERSTART® (Alltech). Microorganisms that produce other metabolites, such as citric acid and lactic acid, by fermentation are also known in the art. *See, e.g.*, Papagianni (2007) *Biotechnol. Adv.* 25:244-63; John *et al.* (2009) *Biotechnol. Adv.* 27:145-52.

[00165] The saccharification and fermentation processes may be carried out as an SSF process. Fermentation may comprise subsequent enrichment, purification, and recovery of ethanol, for example. During the fermentation, the ethanol content of the broth or "beer" may reach about 8-18% v/v, *e.g.*, 14-15% v/v. The broth may be distilled to produce enriched, *e.g.*, 96% pure, solutions of ethanol. Further, CO_2 generated by fermentation may be collected with a CO_2 scrubber, compressed, and marketed for other uses, *e.g.*, carbonating beverage or dry ice production. Solid waste from the fermentation process may be used as protein-rich products, *e.g.*, livestock feed.

[00166] As mentioned above, an SSF process can be conducted with fungal cells that express and secrete amylase continuously throughout SSF. The fungal cells expressing amylase also can be the fermenting microorganism, *e.g.*, an ethanologenic microorganism. Ethanol production thus can be carried out using a fungal cell that expresses sufficient amylase so that less or no enzyme has to be added exogenously. The fungal host cell can be from an appropriately engineered fungal strain. Fungal host cells that express and secrete other enzymes, in addition to amylase, also can be used. Such cells may express glucoamylase and/or a pullulanase, phytase, *alpha*-glucosidase, isoamylase, beta-amylase cellulase, xylanase, other hemicellulases, protease, *beta*-glucosidase, pectinase, esterase, redox enzymes, transferase, or other enzymes.

[00167] A variation on this process is a "fed-batch fermentation" system, where the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression may inhibit the metabolism of the cells and where it is desirable to

have limited amounts of substrate in the medium. The actual substrate concentration in fed-batch systems is estimated by the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases, such as CO_2. Batch and fed-batch fermentations are common and well known in the art.

[00168] Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation permits modulation of cell growth and/or product concentration. For example, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. Because growth is maintained at a steady state, cell loss due to medium being drawn off should be balanced against the cell growth rate in the fermentation. Methods of optimizing continuous fermentation processes and maximizing the rate of product formation are well known in the art of industrial microbiology.

4.7. Post fermentation and the products from post fermentation

[00169] Fermentation products, such as ethanol, are produced by first degrading starch-containing material into fermentable sugars by liquefaction and saccharification, or liquefaction followed by SSF, or saccharification followed by fermentation (raw starch process), and converting the sugars directly or indirectly into the desired fermentation product using a fermenting organism. Liquid fermentation products such as ethanol are recovered from the fermented mash (often referred to as "beer" or "beer mash"), e.g., by distillation, which separates the desired fermentation product from other liquids and/or solids. The remaining faction, referred to as "whole stillage", is separated into a solid and a liquid phase, e.g., by centrifugation. The solid phase is referred to as "wet cake" (or "wet grains" or "WDG") and the liquid phase (supernatant) is referred to as "thin stillage". Wet cake is dried to provide "Distillers Dried Grains" (DDG) used as nutrient in animal feed. Thin stillage is typically evaporated to provide condensate and syrup (or "thick stillage") or may alternatively be recycled directly to the slurry tank as "backset". Condensate may either be forwarded to a methanator before being discharged or may be recycled to the slurry tank. The syrup consisting mainly of limit dextrins and non-fermentable sugars may be blended into DDG or added to the wet cake before drying to produce DDGS (Distillers Dried Grain with Solubles).

[00170] It is known to commercially use the various byproducts and residues derived from the fermentation processes like the ethanol production process. Distillers residues or byproducts, as well as by-products of cereal and other food industry manufacturing, are known to have a certain value as sources of protein and energy for animal feed. Furthermore, the oil from the by-products like Whole Stillage, Wet Cake, Thin Stillage, DDG and/or DDGS can be recovered as a separate by-product for use in biodiesel production or other products

[00171] The by-products like DDG, DDGS or WDG comprises proteins, fibers, fat and unconverted starch. The Wet-Cake may be used in dairy feedlots. The dried DDGs may be used in live stock, e,g, dairy, beef and swine feeds and poultry feeds. While the protein content is high, the amino acid composition is not well suited for monogastric animals if used as animal feed Furthermore, the by-products contain significant levels of Crude Fibers (CF), which are structural carbohydrates consisting of cellulose, hemicellulose and indigestible materials like lignin. The proportion of cellulose and lignin in the crude fibers fraction also determines the digestibility of crude fibers and its solubility in the intestine. The soluble non-starch-polysaccharides (NSP) cannot be digested by monogastric animals like swine and poultry and can cause an increase in viscosity, due to their ability to bind water, This can result in moist, sticky droppings and wet litter. Another effect of NSP is the so-called "Nutrient Encapsulation". Essentially, the starch, protein, oil and other nutrients are encapsulated within the plant cell which is an impermeable barrier preventing full utilization of the nutrients within the cell.

[00172] Furthermore, the soluble NSP can cause an increase in viscosity during fermentation and can influence separation and drying conditions of fermentation by-products like DDGS in the production process.

[00173] Therefore, a number of specific processes or treatment methods have been used and are being investigated for improving the quality of the by-products from fermentation processes. For example, adding enzymes to the liquefaction, saccharification, fermentation or SSF, whole stillage, Wet-Cake, and/or Thin Stillage, etc, in the ethanol production process has been used to improve the solid-liquid separation in the process, and/or to alter or improve the yield and/or quality of the by-products. Addition of enzymes to the liquefaction, saccharification, fermentation or SSF, whole stillage, Wet-Cake, and/or Thin Stillage, etc. is also being used or investigated as a route to access residual starch, and in some cases, to

access the cellulosic and/or hemicellulosic sugars associated with the corn fiber. These sugars can then be utilized by appropriate hosts to produce fermentation products, including ethanol. The present amylases may be used in these processes, as well as other starch degrading enzymes, such as alpha-amylase that is other than the alpha-amylase described in this invention, glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, α-glucosidase, beta-glucosidase, or a combination thereof, even hemicellulases, cellulases. The enzymes can be added at any step in the process.

4.8. Compositions Comprising Alpha-Amylases

[00174] In some embodiments, a polypeptide comprising an amino acid sequence that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, identical to that of SEQ ID NO: 2, 4, 6 or 8 can be used in the enzyme composition.

[00175] Alpha-amylases may be combined with a glucoamylase (EC 3.2.1.3), *e.g.*, a *Trichoderma* glucoamylase or variant thereof. An exemplary glucoamylase is *Trichoderma reesei* glucoamylase (TrGA) and variants thereof that possess superior specific activity and thermal stability. *See* U.S. Published Applications Nos. 2006/0094080, 2007/0004018, and 2007/0015266 (Danisco US Inc.). Suitable variants of TrGA include those with glucoamylase activity and at least 80%, at least 90%, or at least 95% sequence identity to wild-type TrGA. Alpha-amylases advantageously increase the yield of glucose produced in a saccharification process catalyzed by TrGA.

[00176] Alternatively, the glucoamylase may be another glucoamylase derived from plants (including algae), fungi, or bacteria. For example, the glucoamylases may be *Aspergillus niger* G1 or G2 glucoamylase or its variants (*e.g.*, Boel *et al.* (1984) *EMBO J.* 3:1097-1102; WO 92/00381; WO 00/04136 (Novo Nordisk A/S)); and *A. awamori* glucoamylase (*e.g.*, WO 84/02921 (Cetus Corp.)). Other contemplated *Aspergillus* glucoamylase include variants with enhanced thermal stability, *e.g.*, G137A and G139A (Chen *et al.* (1996) *Prot. Eng.* 9:499-505); D257E and D293E/Q (Chen *et al.* (1995) *Prot. Eng.* 8:575-582); N182 (Chen *et al.* (1994) *Biochem. J.* 301:275-281); A246C (Fierobe *et al.* (1996) *Biochemistry*, 35: 8698-8704); and variants with Pro residues in positions A435 and S436 (Li *et al.* (1997) *Protein Eng.* 10:1199-1204). Other contemplated glucoamylases include *Talaromyces*

glucoamylases, in particular derived from *T. emersonii* (*e.g.*, WO 99/28448 (Novo Nordisk A/S), *T. leycettanus* (*e.g.*, U.S. Patent No. RE 32,153 (CPC International, Inc.)), *T. duponti*, or *T. thermophilus* (*e.g.*, U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (*e.g.*, EP 135138 (CPC International, Inc.) and *C. thermohydrosulfuricum* (*e.g.*, WO 86/01831 (Michigan Biotechnology Institute)). Suitable glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase shown in SEQ ID NO:2 in WO 00/04136 (Novo Nordisk A/S). Also suitable are commercial glucoamylases, such as AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (Novozymes); OPTIDEX® 300 and OPTIDEX L-400 (Danisco US Inc.); AMIGASE™ and AMIGASE™ PLUS (DSM); G-ZYME® G900 (Enzyme Bio-Systems); and G-ZYME® G990 ZR (*A. niger* glucoamylase with a low protease content). Still other suitable glucoamylases include *Aspergillus fumigatus* glucoamylase, *Talaromyces* glucoamylase, *Thielavia* glucoamylase, *Trametes* glucoamylase, *Thermomyces* glucoamylase, *Athelia* glucoamylase, *Pycnoporus* glucoamylase, *Penicillim* glucoamylases or *Humicola* glucoamylase (*e.g.*, HgGA). Glucoamylases typically are added in an amount of about 0.1 – 2 glucoamylase units (GAU)/g ds, *e.g.*, about 0.16 GAU/g ds, 0.23 GAU/g ds, or 0.33 GAU/g ds. Other suitable enzymes that can be used with amylase include a phytase, protease, pullulanase, beta-amylase, isoamylase, a different alpha-amylase, alpha-glucosidase, cellulase, xylanase, other hemicellulases, beta-glucosidase, transferase, pectinase, lipase, cutinase, esterase, redox enzymes, or a combination thereof. For example, a debranching enzyme, such as an isoamylase (EC 3.2.1.68), may be added in effective amounts well known to the person skilled in the art. A pullulanase (EC 3.2.1.41), *e.g.*, PROMOZYME®, is also suitable. Further suitable enzymes include proteases, such as fungal and bacterial proteases. Fungal proteases include those obtained from *Aspergillus*, such as *A. niger*, *A. awamori*, *A. oryzae*; *Mucor* (*e.g.*, *M. miehei*); *Rhizopus*; and *Trichoderma*.

[00177] Beta-Amylases (EC 3.2.1.2) are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages into amylopectin and related glucose polymers, thereby releasing maltose. Beta-Amylases have been isolated from various plants and microorganisms. *See* Fogarty *et al.* (1979) in PROGRESS IN INDUSTRIAL MICROBIOLOGY, Vol. 15, pp. 112-115. These beta-Amylases have optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from about 4.5 to about 7.0. Contemplated beta-amylases include, but are not limited to, beta-amylases from barley SPEZYME® BBA 1500,

SPEZYME® DBA, OPTIMALT™ ME, OPTIMALT™ BBA (Danisco US Inc.); and NOVOZYM™ WBA (Novozymes A/S).

[00178] Compositions comprising the present amylases may be aqueous or non-aqueous formulations, granules, powders, gels, slurries, pastes, etc., which may further comprise any one or more of the additional enzymes listed, herein, along with buffers, salts, preservatives, water, co-solvents, surfactants, and the like. Such compositions may work in combination with endogenous enzymes or other ingredients already present in a slurry, water bath, washing machine, food or drink product, etc, for example, endogenous plant (including algal) enzymes, residual enzymes from a prior processing step, and the like.

5. Compositions and Methods for Cleaning

[00179] An aspect of the present compositions and methods is a cleaning composition that includes an amylase as a component. An amylase polypeptide can be used as a component in detergent compositions for hand washing, laundry washing, dishwashing, and other hard-surface cleaning.

5.1. Overview

[00180] Preferably, an amylase is incorporated into detergents at or near a concentration conventionally used for amylase in detergents. For example, an amylase polypeptide may be added in amount corresponding to 0.00001 – 1 mg (calculated as pure enzyme protein) of amylase per liter of wash/dishwash liquor.

[00181] An amylase polypeptide may be a component of a detergent composition, as the only enzyme or with other enzymes including other amylolytic enzymes. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, *e.g.*, as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1,000 to 20,000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in, for example, GB 1483591. Liquid enzyme preparations may, for instance, be

stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are known in the art. Protected enzymes may be prepared according to the method disclosed in for example EP 238 216. Polyols have long been recognized as stabilizers of proteins, as well as improving protein solubility.

[00182] The detergent composition may be in any useful form, *e.g.,* as powders, granules, pastes, or liquid. A liquid detergent may be aqueous, typically containing up to about 70% of water and 0% to about 30% of organic solvent. It may also be in the form of a compact gel type containing only about 30% water.

[00183] The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0% to about 50% of anionic surfactant, such as linear alkylbenzenesulfonate (LAS); alpha-olefinsulfonate (AOS); alkyl sulfate (fatty alcohol sulfate) (AS); alcohol ethoxysulfate (AEOS or AES); secondary alkanesulfonates (SAS); alpha-sulfo fatty acid methyl esters; alkyl- or alkenylsuccinic acid; or soap. The composition may also contain 0% to about 40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (as described for example in WO 92/06154).

[00184] The detergent composition may additionally comprise one or more other enzymes, such as proteases, another amylolytic enzyme, cutinase, lipase, cellulase, pectate lyase, perhydrolase, xylanase, peroxidase, and/or laccase in any combination.

[00185] The detergent may contain about 1% to about 65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (*e.g.,* SKS-6 from Hoechst). The detergent may also be unbuilt, *i.e.* essentially free of detergent builder. The enzymes can be used in any composition compatible with the stability of the enzyme. Enzymes generally can be protected against deleterious components by known forms of encapsulation, for example, by granulation or sequestration in hydro gels. Enzymes, and specifically amylases, either with or without starch binding domains, can be used in a variety of compositions including laundry and

dishwashing applications, surface cleaners, as well as in compositions for ethanol production from starch or biomass.

[00186] The detergent may comprise one or more polymers. Examples include carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

[00187] The detergent may contain a bleaching system, which may comprise a H_2O_2 source such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids (*e.g.,* the amide, imide, or sulfone type peroxyacids). The bleaching system can also be an enzymatic bleaching system, for example, perhydrolase, such as that described in International PCT Application WO 2005/056783.

[00188] The enzymes of the detergent composition may be stabilized using conventional stabilizing agents, *e.g.,* a polyol such as propylene glycol or glycerol; a sugar or sugar alcohol; lactic acid; boric acid or a boric acid derivative such as, *e.g.,* an aromatic borate ester; and the composition may be formulated as described in, *e.g.,* WO 92/19709 and WO 92/19708.

[00189] The detergent may also contain other conventional detergent ingredients such as *e.g.*, fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, tarnish inhibiters, optical brighteners, or perfumes.

[00190] The pH (measured in aqueous solution at use concentration) is usually neutral or alkaline, *e.g.*, pH about 7.0 to about 11.0.

[00191] Particular forms of detergent compositions for inclusion of the present alpha-amylase are described, below.

5.2. Heavy Duty Liquid (HDL) laundry detergent composition

[00192] Exemplary HDL laundry detergent compositions includes a detersive surfactant (10%-40% wt/wt), including an anionic detersive surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates, and/or

mixtures thereof), and optionally non-ionic surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl alkoxylated alcohol, for example a C_8-C_{18} alkyl ethoxylated alcohol and/or C_6-C_{12} alkyl phenol alkoxylates), wherein the weight ratio of anionic detersive surfactant (with a hydrophilic index (HIc) of from 6.0 to 9) to non-ionic detersive surfactant is greater than 1: 1. Suitable detersive surfactants also include cationic detersive surfactants (selected from a group of alkyl pyridinium compounds, alkyl quarternary ammonium compounds, alkyl quarternary phosphonium compounds, alkyl ternary sulphonium compounds, and/or mixtures thereof); zwitterionic and/or amphoteric detersive surfactants (selected from a group of alkanolamine sulpho-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof.

[00193] The composition may optionally include, a surfactancy boosting polymer consisting of amphiphilic alkoxylated grease cleaning polymers (selected from a group of alkoxylated polymers having branched hydrophilic and hydrophobic properties, such as alkoxylated polyalkylenimines in the range of 0.05wt%-10wt%) and/or random graft polymers (typically comprising of hydrophilic backbone comprising monomers selected from the group consisting of: unsaturated C_1-C_6 carboxylic acids, ethers, alcohols, aldehydes, ketones, esters, sugar units, alkoxy units, maleic anhydride, saturated polyalcohols such as glycerol, and mixtures thereof; and hydrophobic side chain(s) selected from the group consisting of: C_4-C_{25} alkyl group, polypropylene, polybutylene, vinyl ester of a saturated C_1-C_6 mono-carboxylic acid, C_1-C_6 alkyl ester of acrylic or methacrylic acid, and mixtures thereof.

[00194] The composition may include additional polymers such as soil release polymers (include anionically end-capped polyesters, for example SRP1, polymers comprising at least one monomer unit selected from saccharide, dicarboxylic acid, polyol and combinations thereof, in random or block configuration, ethylene terephthalate-based polymers and co-polymers thereof in random or block configuration, for example Repel-o-tex SF, SF-2 and SRP6, Texcare SRA100, SRA300, SRN100, SRN170, SRN240, SRN300 and SRN325, Marloquest SL), anti-redeposition polymers (0.1 wt% to 10wt%, include carboxylate polymers, such as polymers comprising at least one monomer selected from acrylic acid, maleic acid (or maleic anhydride), fumaric acid, itaconic acid, aconitic acid, mesaconic acid, citraconic acid, methylenemalonic acid, and any mixture thereof, vinylpyrrolidone homopolymer, and/or polyethylene glycol, molecular weight in the range of from 500 to

100,000 Da); cellulosic polymer (including those selected from alkyl cellulose, alkyl alkoxyalkyl cellulose, carboxyalkyl cellulose, alkyl carboxyalkyl cellulose examples of which include carboxymethyl cellulose, methyl cellulose, methyl hydroxyethyl cellulose, methyl carboxymethyl cellulose, and mixures thereof) and polymeric carboxylate (such as maleate/acrylate random copolymer or polyacrylate homopolymer).

[00195] The composition may further include saturated or unsaturated fatty acid, preferably saturated or unsaturated C_{12}-C_{24} fatty acid (0 wt% to 10 wt%); deposition aids (examples for which include polysaccharides, preferably cellulosic polymers, poly diallyl dimethyl ammonium halides (DADMAC), and co-polymers of DAD MAC with vinyl pyrrolidone, acrylamides, imidazoles, imidazolinium halides, and mixtures thereof, in random or block configuration, cationic guar gum, cationic cellulose such as cationic hydoxyethyl cellulose, cationic starch, cationic polyacylamides, and mixtures thereof).

[00196] The composition may further include dye transfer inhibiting agents, examples of which include manganese phthalocyanine, peroxidases, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinyloxazolidones and polyvinylimidazoles and/or mixtures thereof; chelating agents, examples of which include ethylene-diamine-tetraacetic acid (EDTA), diethylene triamine penta methylene phosphonic acid (DTPMP), hydroxy-ethane diphosphonic acid (HEDP), ethylenediamine N,N'-disuccinic acid (EDDS), methyl glycine diacetic acid (MGDA), diethylene triamine penta acetic acid (DTPA), propylene diamine tetracetic acid (PDT A), 2-hydroxypyridine-N-oxide (HPNO), or methyl glycine diacetic acid (MGDA), glutamic acid N,N-diacetic acid (N,N-dicarboxymethyl glutamic acid tetrasodium salt (GLDA), nitrilotriacetic acid (NTA), 4,5-dihydroxy-m-benzenedisulfonic acid, citric acid and any salts thereof, N-hydroxyethylethylenediaminetri-acetic acid (HEDTA), triethylenetetraaminehexaacetic acid (TTHA), N-hydroxyethyliminodiacetic acid (HEIDA), dihydroxyethylglycine (DHEG), ethylenediaminetetrapropionic acid (EDTP), and derivatives thereof.

[00197] The composition preferably included enzymes (generally about 0.01 wt% active enzyme to 0.03wt% active enzyme) selected from proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferases, perhydrolases, arylesterases, and any mixture thereof. The composition may include an enzyme stabilizer (examples of which

include polyols such as propylene glycol or glycerol, sugar or sugar alcohol, lactic acid, reversible protease inhibitor, boric acid, or a boric acid derivative, *e.g.*, an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid).

[00198] The composition optionally include silicone or fatty-acid based suds suppressors; heuing dyes, calcium and magnesium cations, visual signaling ingredients, anti-foam (0.001 wt% to about 4.0wt%), and/or structurant/thickener (0.01 wt% to 5wt%, selected from the group consisting of diglycerides and triglycerides, ethylene glycol distearate, microcrystalline cellulose, cellulose based materials, microfiber cellulose, biopolymers, xanthan gum, gellan gum, and mixtures thereof).

[00199] The composition can be any liquid form, for example a liquid or gel form, or any combination thereof. The composition may be in any unit dose form, for example a pouch.

5.3. Heavy Duty Dry/Solid (HDD) laundry detergent composition

[00200] Exemplary HDD laundry detergent compositions includes a detersive surfactant, including anionic detersive surfactants (*e.g.*, linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates and/or mixtures thereof), non-ionic detersive surfactant (*e.g.*, linear or branched or random chain, substituted or unsubstituted C_8-C_{18} alkyl ethoxylates, and/or C_6-C_{12} alkyl phenol alkoxylates), cationic detersive surfactants (*e.g.*, alkyl pyridinium compounds, alkyl quaternary ammonium compounds, alkyl quaternary phosphonium compounds, alkyl ternary sulphonium compounds, and mixtures thereof), zwitterionic and/or amphoteric detersive surfactants (*e.g.*, alkanolamine sulpho-betaines), ampholytic surfactants, semi-polar non-ionic surfactants, and mixtures thereof; builders including phosphate free builders (for example zeolite builders examples which include zeolite A, zeolite X, zeolite P and zeolite MAP in the range of 0wt% to less than 10wt%), phosphate builders (for example sodium tri-polyphosphate in the range of 0wt% to less than 10wt%), citric acid, citrate salts and nitrilotriacetic acid, silicate salt (*e.g.*, sodium or potassium silicate or sodium meta-silicate in the range of 0wt% to less than 10wt%, or layered silicate (SKS-6)); carbonate salt (*e.g.*, sodium carbonate and/or sodium bicarbonate in the range of 0 wt% to less than 80 wt%); and bleaching agents including photobleaches (*e.g.*, sulfonated zinc phthalocyanines, sulfonated aluminum phthalocyanines, xanthenes dyes, and mixtures thereof) hydrophobic or hydrophilic bleach activators (*e.g.*, dodecanoyl oxybenzene sulfonate, decanoyl oxybenzene sulfonate, decanoyl oxybenzoic acid or salts thereof, 3,5,5-

trimethy hexanoyl oxybenzene sulfonate, tetraacetyl ethylene diamine-TAED, nonanoyloxybenzene sulfonate-NOBS, nitrile quats, and mixtures thereof), sources of hydrogen peroxide (*e.g.*, inorganic perhydrate salts examples of which include mono or tetra hydrate sodium salt of perborate, percarbonate, persulfate, perphosphate, or persilicate), preformed hydrophilic and/or hydrophobic peracids (*e.g.*, percarboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts, and mixtures thereof), and/or bleach catalysts (*e.g.*, imine bleach boosters (examples of which include iminium cations and polyions), iminium zwitterions, modified amines, modified amine oxides, N-sulphonyl imines, N-phosphonyl imines, N-acyl imines, thiadiazole dioxides, perfluoroimines, cyclic sugar ketones, and mixtures thereof, and metal-containing bleach catalysts (*e.g.*, copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations along with an auxiliary metal cations such as zinc or aluminum and a sequestrate such as ethylenediaminetetraacetic acid, ethylenediaminetetra(methylenephosphonic acid), and water-soluble salts thereof).

[00201] The composition preferably includes enzymes, *e.g.*, proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferase, perhydrolase, arylesterase, and any mixture thereof.

[00202] The composition may optionally include additional detergent ingredients including perfume microcapsules, starch encapsulated perfume accord, hueing agents, additional polymers, including fabric integrity and cationic polymers, dye-lock ingredients, fabric-softening agents, brighteners (for example C.I. Fluorescent brighteners), flocculating agents, chelating agents, alkoxylated polyamines, fabric deposition aids, and/or cyclodextrin.

5.4. Automatic dishwashing (ADW) detergent composition

[00203] Exemplary ADW detergent composition includes non-ionic surfactants, including ethoxylated non-ionic surfactants, alcohol alkoxylated surfactants, epoxy-capped poly(oxyalkylated) alcohols, or amine oxide surfactants present in amounts from 0 to 10% by weight; builders in the range of 5-60% including phosphate builders (*e.g.*, mono-phosphates, di-phosphates, tri-polyphosphates, other oligomeric-poylphosphates, sodium tripolyphosphate-STPP) and phosphate-free builders (*e.g.*, amino acid-based compounds including methyl-glycine-diacetic acid (MGDA) and salts and derivatives thereof, glutamic-N,N-diacetic acid (GLDA) and salts and derivatives thereof, iminodisuccinic acid (IDS) and

salts and derivatives thereof, carboxy methyl inulin and salts and derivatives thereof, nitrilotriacetic acid (NTA), diethylene triamine penta acetic acid (DTPA), B-alaninediacetic acid (B-ADA) and their salts, homopolymers and copolymers of poly-carboxylic acids and their partially or completely neutralized salts, monomeric polycarboxylic acids and hydroxycarboxylic acids and their salts in the range of 0.5% to 50% by weight; sulfonated/carboxylated polymers in the range of about 0.1 % to about 50% by weight to to provide dimensional stability; drying aids in the range of about 0.1 % to about 10% by weight (*e.g.*, polyesters, especially anionic polyesters, optionally together with further monomers with 3 to 6 functionalities - typically acid, alcohol or ester functionalities which are conducive to polycondensation, polycarbonate-, polyurethane- and/or polyurea-polyorganosiloxane compounds or precursor compounds, thereof, particularly of the reactive cyclic carbonate and urea type); silicates in the range from about 1 % to about 20% by weight (including sodium or potassium silicates for example sodium disilicate, sodium meta-silicate and crystalline phyllosilicates); inorganic bleach (*e.g.*, perhydrate salts such as perborate, percarbonate, perphosphate, persulfate and persilicate salts) and organic bleach (*e.g.*, organic peroxyacids, including diacyl and tetraacylperoxides, especially diperoxydodecanedioc acid, diperoxytetradecanedioc acid, and diperoxyhexadecanedioc acid); bleach activators (*i.e.*, organic peracid precursors in the range from about 0.1 % to about 10% by weight); bleach catalysts (*e.g.*, manganese triazacyclononane and related complexes, Co, Cu, Mn, and Fe bispyridylamine and related complexes, and pentamine acetate cobalt(III) and related complexes); metal care agents in the range from about 0.1% to 5% by weight (*e.g.*, benzatriazoles, metal salts and complexes, and/or silicates); enzymes in the range from about 0.01 to 5.0 mg of active enzyme per gram of automatic dishwashing detergent composition (*e.g.*, proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferase, perhydrolase, arylesterase, and mixtures thereof); and enzyme stabilizer components (*e.g.*, oligosaccharides, polysaccharides, and inorganic divalent metal salts).

5.5. Additional detergent compositions

[00204] Additional exemplary detergent formulations to which the present amylase can be added are described, below, in the numbered paragraphs.

[00205] 1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 7% to about

12%; alcohol ethoxysulfate (*e.g.*, C_{12-18} alcohol, 1-2 ethylene oxide (EO)) or alkyl sulfate (*e.g.*, C_{16-18}) about 1% to about 4%; alcohol ethoxylate (*e.g.*, C_{14-15} alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na_2CO_3) about 14% to about 20%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 2 to about 6%; zeolite (*e.g.*, $NaA1SiO_4$) about 15% to about 22%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 6%; sodium citrate/citric acid (*e.g.*, $C_6H_5Na_3O_7/C_6H_8O_7$) about 0% to about 15%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 11% to about 18%; TAED about 2% to about 6%; carboxymethylcellulose (CMC) and 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid, copolymer, PVP, PEG) 0-3%; enzymes (calculated as pure enzyme) 0.0001-0.1% protein; and minor ingredients (*e.g.*, suds suppressors, perfumes, optical brightener, photobleach) 0-5%.

[00206] 2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 6% to about 11%; alcohol ethoxysulfate (*e.g.*, C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (*e.g.*, C_{16-18}) about 1% to about 3%; alcohol ethoxylate (*e.g.*, C_{14-15} alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na_2CO_3) about 15% to about 21%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 24% to about 34%; sodium sulfate (*e.g.*. Na_2SO_4) about 4% to about 10%; sodium citrate/citric acid (*e.g.*, $C_6H_5Na_3O_7$/ $C_6H_8O_7$) 0% to about 15%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 1-6%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00207] 3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 5% to about 9%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO) about 7% to about 14%; Soap as fatty acid (*e.g.*, C_{16-22} fatty acid) about 1 to about 3%; sodium carbonate (as Na_2CO_3) about 10% to about 17%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 3% to about 9%; zeolite (as $NaA1SiO_4$) about 23% to about 33%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 4%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 8% to about 16%; TAED about 2% to about 8%; phosphonate (*e.g.*, EDTMPA) 0% to about 1%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 0-3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, suds suppressors, perfume, optical brightener) 0-5%.

[00208] 4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 8% to about 12%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO) about 10% to about 25%; sodium carbonate (as Na_2CO_3) about 14% to about 22%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 5%; zeolite (*e.g.*, $NaA1SiO_4$) about 25% to about 35%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 10%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 1-3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00209] 5) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO) about 12% to about 18%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 13%; alkenylsuccinic acid (C_{12-14}) 0% to about 13%; aminoethanol about 8% to about 18%; citric acid about 2% to about 8%; phosphonate 0% to about 3%; polymers (*e.g.*, PVP, PEG) 0% to about 3%; borate (*e.g.*, B_4O_7) 0% to about 2%; ethanol 0% to about 3%; propylene glycol about 8% to about 14%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, dispersants, suds suppressors, perfume, optical brightener) 0-5%.

[00210] 6) An aqueous structured liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) 3-9%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 10%; zeolite (as $NaA1SiO_4$) about 14% to about 22%; potassium citrate about 9% to about 18%; borate (*e.g.*, B_4O_7) 0% to about 2%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, PEG, PVP) 0% to about 3%; anchoring polymers such as, *e.g.*, lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1, MW 3800) 0% to about 3%;glycerol 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, dispersants, suds suppressors, perfume, optical brighteners) 0-5%.

[00211] 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising fatty alcohol sulfate about 5% to about 10%; ethoxylated fatty acid monoethanolamide about 3% to about 9%; soap as fatty acid 0-3%; sodium carbonate (*e.g.*, Na_2CO_3) about 5% to about 10%; Soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 20% to about 40%; Sodium sulfate (*e.g.*, Na_2SO_4) about 2% to about 8%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 12% to about 18%; TAED about 2% to

about 7%; polymers (*e.g.*, maleic/acrylic acid copolymer, PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, suds suppressors, perfume) 0-5%.

[00212] 8) A detergent composition formulated as a granulate comprising linear alkylbenzenesulfonate (calculated as acid) about 8% to about 14%; ethoxylated fatty acid monoethanolamide about 5% to about 11%; soap as fatty acid 0% to about 3%; sodium carbonate (*e.g.*, Na_2CO_3) about 4% to about 10%; soluble silicate (Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 30% to about 50%; sodium sulfate (*e.g.*, Na_2SO_4) about 3% to about 11%; sodium citrate (*e.g.*, $C_6H_5Na_3O_7$) about 5% to about 12%; polymers (*e.g.*, PVP, maleic/acrylic acid copolymer, PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00213] 9) A detergent composition formulated as a granulate comprising linear alkylbenzenesulfonate (calculated as acid) about 6% to about 12%; nonionic surfactant about 1% to about 4%; soap as fatty acid about 2% to about 6%; sodium carbonate (*e.g.*, Na_2CO_3) about 14% to about 22%; zeolite (*e.g.*, $NaA1SiO_4$) about 18% to about 32%; sodium sulfate (*e.g.*, Na_2SO_4) about 5% to about 20%; sodium citrate (*e.g.*, $C_6H_5Na_3O_7$) about 3% to about 8%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 4% to about 9%; bleach activator (*e.g.*, NOBS or TAED) about 1% to about 5%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, polycarboxylate or PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, perfume) 0-5%.

[00214] 10) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 23%; alcohol ethoxysulfate (*e.g.*, C_{12-15} alcohol, 2-3 EO) about 8% to about 15%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) about 3% to about 9%; soap as fatty acid (*e.g.*, lauric acid) 0% to about 3%; aminoethanol about 1% to about 5%; sodium citrate about 5% to about 10%; hydrotrope (*e.g.*, sodium toluensulfonate) about 2% to about 6%; borate (*e.g.*, B_4O_7) 0% to about 2%; carboxymethylcellulose 0% to about 1%; ethanol about 1% to about 3%; propylene glycol about 2% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, polymers, dispersants, perfume, optical brighteners) 0-5%.

[00215] 11) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 20% to about 32%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) 6-12%; aminoethanol about 2% to about 6%; citric acid about 8% to about 14%; borate (*e.g.*, B_4O_7) about 1% to about 3%; polymer (*e.g.*, maleic/acrylic acid copolymer, anchoring polymer such as, *e.g.*, lauryl methacrylate/acrylic acid copolymer) 0% to about 3%; glycerol about 3% to about 8%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, hydrotropes, dispersants, perfume, optical brighteners) 0-5%.

[00216] 12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap) about 25% to about 40%; nonionic surfactant (*e.g.*, alcohol ethoxylate) about 1% to about 10%; sodium carbonate (*e.g.*, Na_2CO_3) about 8% to about 25%; soluble silicates (*e.g.*, Na_2O, $2SiO_2$) about 5% to about 15%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 5%; zeolite ($NaA1SiO_4$) about 15% to about 28%; sodium perborate (*e.g.*, $NaBO_3{\cdot}4H_2O$) 0% to about 20%; bleach activator (TAED or NOBS) about 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, perfume, optical brighteners) 0-3%.

[00217] 13) Detergent compositions as described in compositions 1)-12) *supra*, wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

[00218] 14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C_{12}-C_{18}) alkyl sulfate about 9% to about 15%; alcohol ethoxylate about 3% to about 6%; polyhydroxy alkyl fatty acid amide about 1% to about 5%; zeolite (*e.g.*, $NaA1SiO_4$) about 10% to about 20%; layered disilicate (*e.g.*, SK56 from Hoechst) about 10% to about 20%; sodium carbonate (*e.g.*, Na_2CO_3) about 3% to about 12%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) 0% to about 6%; sodium citrate about 4% to about 8%; sodium percarbonate about 13% to about 22%; TAED about 3% to about 8%; polymers (*e.g.*, polycarboxylates and PVP) 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, photobleach, perfume, suds suppressors) 0-5%.

[00219] 15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C_{12}-C_{18}) alkyl sulfate about 4% to about 8%; alcohol ethoxylate about 11% to about 15%; soap about 1% to about 4%; zeolite MAP or zeolite A about 35% to

about 45%; sodium carbonate (as Na_2CO_3) about 2% to about 8%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) 0% to about 4%; sodium percarbonate about 13% to about 22%; TAED 1-8%; carboxymethylcellulose (CMC) 0% to about 3%; polymers (*e.g.*, polycarboxylates and PVP) 0% to about 3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, phosphonate, perfume) 0-3%.

[00220] 16) Detergent formulations as described in 1)-15) supra, which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

[00221] 17) Detergent compositions as described supra in 1), 3), 7), 9), and 12), wherein perborate is replaced by percarbonate.

[00222] 18) Detergent compositions as described supra in 1), 3), 7), 9), 12), 14), and 15), which additionally contain a manganese catalyst. The manganese catalyst for example is one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching," *Nature* 369: 637-639 (1994).

[00223] 19) Detergent composition formulated as a non-aqueous detergent liquid comprising a liquid nonionic surfactant such as, *e.g.*, linear alkoxylated primary alcohol, a builder system (*e.g.*, phosphate), an enzyme(s), and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

[00224] As above, the present amylase polypeptide may be incorporated at a concentration conventionally employed in detergents. It is at present contemplated that, in the detergent composition, the enzyme may be added in an amount corresponding to 0.00001-1.0 mg (calculated as pure enzyme protein) of amylase polypeptide per liter of wash liquor.

[00225] The detergent composition may also contain other conventional detergent ingredients, *e.g.*, deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners, and perfumes.

[00226] The detergent composition may be formulated as a hand (manual) or machine (automatic) laundry detergent composition, including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for manual or automatic dishwashing operations.

[00227] Any of the cleaning compositions described, herein, may include any number of additional enzymes. In general the enzyme(s) should be compatible with the selected detergent, (*e.g.*, with respect to pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, and the like), and the enzyme(s) should be present in effective amounts. The following enzymes are provided as examples.

[00228] *Proteases*: Suitable proteases include those of animal, vegetable or microbial origin. Chemically modified or protein engineered mutants are included, as well as naturally processed proteins. The protease may be a serine protease or a metalloprotease, an alkaline microbial protease, a trypsin-like protease, or a chymotrypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, *e.g.*, subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147, and subtilisin 168 (*see, e.g.,* WO 89/06279). Examples of trypsin-like proteases are trypsin (*e.g.,* of porcine or bovine origin), and *Fusarium* proteases (*see, e.g.,* WO 89/06270 and WO 94/25583). Examples of useful proteases also include but are not limited to the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946. Commercially available protease enzymes include but are not limited to: ALCALASE®, SAVINASE®, PRIMASE™, DURALASE™, ESPERASE®, KANNASE™, and BLAZE™ (Novo Nordisk A/S and Novozymes A/S); MAXATASE®, MAXACAL™, MAXAPEM™, PROPERASE®, PURAFECT®, PURAFECT OXP™, FN2™, and FN3™ (Danisco US Inc.). Other exemplary proteases include NprE from *Bacillus amyloliquifaciens* and ASP from *Cellulomonas* sp. strain 69B4.

[00229] *Lipases*: Suitable lipases include those of bacterial or fungal origin. Chemically modified, proteolytically modified, or protein engineered mutants are included. Examples of useful lipases include but are not limited to lipases from *Humicola* (synonym *Thermomyces*), *e.g.*, from *H. lanuginosa* (*T. lanuginosus*) (*see e.g.,* EP 258068 and EP 305216), from *H. insolens* (*see e.g.,* WO 96/13580); a *Pseudomonas* lipase (*e.g.*, from *P. alcaligenes* or *P. pseudoalcaligenes*; *see, e.g.,* EP 218 272), *P. cepacia* (*see e.g.,* EP 331 376), *P. stutzeri* (*see e.g.,* GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (*see e.g.,* WO 95/06720 and WO 96/27002), *P. wisconsinensis* (*see e.g.,* WO 96/12012); a *Bacillus* lipase (*e.g.,* from *B. subtilis*; *see e.g.,* Dartois *et al. Biochemica et Biophysica Acta*, 1131: 253-360 (1993)), *B. stearothermophilus* (*see e.g.,* JP 64/744992), or *B. pumilus* (*see e.g.,* WO 91/16422). Additional lipase variants contemplated for use in the formulations include those described for example in: WO 92/05249, WO 94/01541, WO 95/35381, WO 96/00292, WO 95/30744,

WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079, WO 97/07202, EP 407225, and EP 260105. Some commercially available lipase enzymes include LIPOLASE® and LIPOLASE ULTRA™ (Novo Nordisk A/S and Novozymes A/S).

[00230] *Polyesterases*: Suitable polyesterases can be included in the composition, such as those described in, for example, WO 01/34899, WO 01/14629, and US6933140.

[00231] *Amylases*: The compositions can be combined with other amylases, such as non-production enhanced amylase. These can include commercially available amylases, such as but not limited to STAINZYME®, NATALASE®, DURAMYL®, TERMAMYL®, FUNGAMYL® and BAN™ (Novo Nordisk A/S and Novozymes A/S); RAPIDASE®, POWERASE®, and PURASTAR® (from Danisco US Inc.).

[00232] *Cellulases*: Cellulases can be added to the compositions. Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*, *Acremonium*, *e.g.*, the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed for example in U.S. Patent Nos. 4,435,307; 5,648,263; 5,691,178; 5,776,757; and WO 89/09259. Exemplary cellulases contemplated for use are those having color care benefit for the textile. Examples of such cellulases are cellulases described in for example EP 0495257, EP 0531372, WO 96/11262, WO 96/29397, and WO 98/08940. Other examples are cellulase variants, such as those described in WO 94/07998; WO 98/12307; WO 95/24471; PCT/DK98/00299; EP 531315; U.S. Patent Nos. 5,457,046; 5,686,593; and 5,763,254. Commercially available cellulases include CELLUZYME® and CAREZYME® (Novo Nordisk A/S and Novozymes A/S); CLAZINASE® and PURADAX HA® (Danisco US Inc.); and KAC-500(B)™ (Kao Corporation).

[00233] *Peroxidases/Oxidases*: Suitable peroxidases/oxidases contemplated for use in the compositions include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, *e.g.*, from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257. Commercially available peroxidases include for example GUARDZYME™ (Novo Nordisk A/S and Novozymes A/S).

[00234] The detergent composition can also comprise 2,6-beta-D-fructan hydrolase, which is effective for removal/cleaning of biofilm present on household and/or industrial textile/laundry.

[00235] The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive, *i.e.* a separate additive or a combined additive, can be formulated *e.g.*, as a granulate, a liquid, a slurry, and the like. Exemplary detergent additive formulations include but are not limited to granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids or slurries.

[00236] Non-dusting granulates may be produced, *e.g.*, as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (*e.g.*, polyethyleneglycol, PEG) with mean molar weights of 1,000 to 20,000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in, for example, GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

[00237] The detergent composition may be in any convenient form, *e.g.*, a bar, a tablet, a powder, a granule, a paste, or a liquid. A liquid detergent may be aqueous, typically containing up to about 70% water, and 0% to about 30% organic solvent. Compact detergent gels containing about 30% or less water are also contemplated. The detergent composition can optionally comprise one or more surfactants, which may be non-ionic, including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants can be present in a wide range, from about 0.1% to about 60% by weight.

[00238] When included therein the detergent will typically contain from about 1% to about 40% of an anionic surfactant, such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid, or soap.

[00239] When included therein, the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl-N-alkyl derivatives of glucosamine ("glucamides").

[00240] The detergent may contain 0% to about 65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (*e.g.*,SKS-6 from Hoechst).

[00241] The detergent may comprise one or more polymers. Exemplary polymers include carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates *e.g.,* polyacrylates, maleic/acrylic acid copolymers), and lauryl methacrylate/acrylic acid copolymers.

[00242] The enzyme(s) of the detergent composition may be stabilized using conventional stabilizing agents, *e.g.*, as polyol (*e.g.,* propylene glycol or glycerol), a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative (*e.g.*, an aromatic borate ester), or a phenyl boronic acid derivative (*e.g.,* 4-formylphenyl boronic acid). The composition may be formulated as described in WO 92/19709 and WO 92/19708.

[00243] It is contemplated that in the detergent compositions, in particular the enzyme variants, may be added in an amount corresponding to about 0.01 to about 100 mg of enzyme protein per liter of wash liquor (*e.g.*, about 0.05 to about 5.0 mg of enzyme protein per liter of wash liquor or 0.1 to about 1.0 mg of enzyme protein per liter of wash liquor).

[00244] Yet additional exemplary detergent formulations to which the present amylase can be added are described in, *e.g.*, WO2010065455, WO2011072099, WO2011130222, WO2011140364, WO2011156297, WO2011156298, WO2011130076, WO2011133381, WO2011156297, WO2011156298, EP1794295B1, US20110195481, US20110212876, US20110257063, WO2010039958, WO2011072117, WO2011098531, WO2011100410, WO2011130076, WO2011133381, WO2011140316, US20070215184, US20070251545, US20090075857, US20090137444, US20090143271, US20100011513, US20100093588, US20110201536, US20110232004, US20110237482, US20110312868, US20120003326,

US20120004155, WO2011131585, EP707628B1, US5719115, EP736084B1, US5783545, EP767830B1, US5972668, EP746599B1, US5798328, EP662117B1, US5898025, US6380140, EP898613B1, US3975280, US6191092, US6329333, US6530386, EP1307547B1, US7153818, EP1421169B1, US6979669, EP1529101B1, US7375070, EP1385943B1, US7888104, EP1414977B1, US5855625, EP1921147B1, EP1921148B1, EP701605B1, EP1633469B1, EP1633470B1, EP1794293B1, EP171007B1, US4692260, US7569226, EP1165737B1, US6391838, US6060441, US2009017074, US7320887, EP1737952B1, US7691618, US20070256251, US20050261156, US20050261158, US20100234267, US20110136720, US20110201536, US7811076, US5929017, US5156773, EP2343310A1, WO2011083114, EP214761B1, US4876024, EP675944B1, US5763383, EP517761B1, US6624129, EP1054956B1, US6939702, US6964944, EP832174B1, US20060205628, US20070179076, US20080023031, US20110015110, US20110028372, US4973417, US5447649, US5840677, US5965503, US5972873, US5998344, US6071356, WO9009428, EP1661978A1, EP1698689A1, EP1726636A1, EP1867707A1, EP1876226A1, EP1876227A1, EP0205208A2, EP0206390A2, EP0271152, EP0271154, EP0341999, EP0346136, EP2135934, US20120208734, WO2011127102, WO2012142087, WO2012145062, EP1790713B1, US8066818B2, US8163686B2, US8283300B2, US8354366B2, US20120125374, US3929678, and US5898025.

5.6. Methods of Assessing Amylase Activity in Detergent Compositions

[00245] Numerous alpha-amylase cleaning assays are known in the art, including swatch and micro-swatch assays.

[00246] The principle of swatch and micro-swatch assay is the liberation of an orange-dye due to the hydrolysis of rice starch incorporated in a cotton swatch or microswatch. The absorbance at 488 nm of the wash liquid was measured and related to the level of amylase activity in the sample analyzed at the desired conditions (pH, temperature, and buffer). The equipment used for this assay included a Biomek FX Robot (Beckman Coulter), a SpectraMAX MTP Reader (type 340-Moleeular Devices) and iEMS incubator/shaker (Thermo Scientific).

6. Compositions and Methods for Baking and Food Preparation

[00247] The present invention also relates to a "food composition," including but not limited to a food product, animal feed and/or food/feed additives, comprising an amylase, and

methods for preparing such a food composition comprising mixing alpha-amylase with one or more food ingredients, or uses thereof.

[00248] Furthermore, the present invention relates to the use of an amylase in the preparation of a food composition, wherein the food composition is baked subsequent to the addition of the polypeptide of the invention. As used herein the term "baking composition" means any composition and/or additive prepared in the process of providing a baked food product, including but not limited to bakers flour, a dough, a baking additive and/or a baked product. The food composition or additive may be liquid or solid.

[00249] As used herein, the term "flour" means milled or ground cereal grain. The term "flour" also may mean Sago or tuber products that have been ground or mashed. In some embodiments, flour may also contain components in addition to the milled or mashed cereal or plant matter. An example of an additional component, although not intended to be limiting, is a leavening agent. Cereal grains include wheat, oat, rye, and barley. Tuber products include tapioca flour, cassava flour, and custard powder. The term "flour" also includes ground corn flour, maize-meal, rice flour, whole-meal flour, self-rising flour, tapioca flour, cassava flour, ground rice, enriched flower, and custard powder.

[00250] For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of alpha-amylase activity in the flour. A level of activity that is too high may result in a product that is sticky and/or doughy and therefore unmarketable. Flour with insufficient alpha-amylase activity may not contain enough sugar for proper yeast function, resulting in dry, crumbly bread, or baked products. Accordingly, an amylase, by itself or in combination with another alpha-amylase(s), may be added to the flour to augment the level of endogenous alpha-amylase activity in flour.

[00251] An amylase can further be added alone or in a combination with other amylases to prevent or retard staling, *i.e.*, crumb firming of baked products. The amount of anti-stalling amylase will typically be in the range of 0.01-10 mg of enzyme protein per kg of flour, *e.g.*, 0.5 mg/kg ds. Additional anti-stalling amylases that can be used in combination with an amylase include an endo-amylase, *e.g.*, a bacterial endo-amylase from *Bacillus*. The additional amylase can be another maltogenic alpha-amylase (EC 3.2.1.133), *e.g.*, from *Bacillus*. NOVAMYL® is an exemplary maltogenic alpha-amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen *et al.* (1997) *Starch* 50:39-45. Other examples of anti-stalling endo-amylases include bacterial alpha-

amylases derived from *Bacillus*, such as *B. licheniformis* or *B. amyloliquefaciens*. The anti-stalling amylase may be an exo-amylase, such as beta-amylase, *e.g.*, from plant sources, such as soy bean, or from microbial sources, such as *Bacillus*.

[00252] The baking composition comprising an amylase further can comprise a phospholipase or enzyme with phospholipase activity. An enzyme with phospholipase activity has an activity that can be measured in Lipase Units (LU). The phospholipase may have A_1 or A_2 activity to remove fatty acid from the phospholipids, forming a lysophospholipid. It may or may not have lipase activity, *i.e.*, activity on triglyceride substrates. The phospholipase typically has a temperature optimum in the range of 30-90°C., *e.g.*, 30-70°C. The added phospholipases can be of animal origin, for example, from pancreas, *e.g.*, bovine or porcine pancreas, snake venom or bee venom. Alternatively, the phospholipase may be of microbial origin, *e.g.*, from filamentous fungi, yeast or bacteria, for example.

[00253] The phospholipase is added in an amount that improves the softness of the bread during the initial period after baking, particularly the first 24 hours. The amount of phospholipase will typically be in the range of 0.01-10 mg of enzyme protein per kg of flour, *e.g.*, 0.1-5 mg/kg. That is, phospholipase activity generally will be in the range of 20-1000 LU/kg of flour, where a Lipase Unit is defined as the amount of enzyme required to release 1 µmol butyric acid per minute at 30°C, pH 7.0, with gum arabic as emulsifier and tributyrin as substrate.

[00254] Compositions of dough generally comprise wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, cornstarch, rye meal, rye flour, oat flour, oatmeal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch. The dough may be fresh, frozen or par-baked. The dough can be a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, *e.g.*, sodium bicarbonate or by adding a leaven, *i.e.*, fermenting dough. Dough also may be leavened by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), *e.g.*, a commercially available strain of *S. cerevisiae*.

[00255] The dough may also comprise other conventional dough ingredients, *e.g.*, proteins, such as milk powder, gluten, and soy; eggs (*e.g.*, whole eggs, egg yolks or egg whites); an oxidant, such as ascorbic acid, potassium bromate, potassium iodate,

azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; or a salt, such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough further may comprise fat, *e.g.*, triglyceride, such as granulated fat or shortening. The dough further may comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin. In particular, the dough can be made without addition of emulsifiers.

[00256] The dough product may be any processed dough product, including fried, deep fried, roasted, baked, steamed and boiled doughs, such as steamed bread and rice cakes. In one embodiment, the food product is a bakery product. Typical bakery (baked) products include bread - such as loaves, rolls, buns, bagels, pizza bases etc. pastry, pretzels, tortillas, cakes, cookies, biscuits, crackers etc.

[00257] Optionally, an additional enzyme may be used together with the anti-stalling amylase and the phospholipase. The additional enzyme may be a second amylase, such as an amyloglucosidase, a beta-amylase, a cyclodextrin glucanotransferase, or the additional enzyme may be a peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a cellulase, a xylanase, a protease, a protein disulfide isomerase, *e.g.*, a protein disulfide isomerase as disclosed in WO 95/00636, for example, a glycosyltransferase, a branching enzyme (1,4-alpha-glucan branching enzyme), a 4-alpha-glucanotransferase (dextrin glycosyltransferase) or an oxidoreductase, *e.g.*, a peroxidase, a laccase, a glucose oxidase, an amadoriase, a metalloproteinase, a pyranose oxidase, a lipooxygenase, an L-amino acid oxidase or a carbohydrate oxidase. The additional enzyme(s) may be of any origin, including mammalian and plant, and particularly of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

[00258] The xylanase is typically of microbial origin, *e.g.*, derived from a bacterium or fungus, such as a strain of *Aspergillus*. Xylanases include PENTOPAN® and NOVOZYM 384®, for example, which are commercially available xylanase preparations produced from *Trichoderma reesei*. The amyloglucosidase may be an *A. niger* amyloglucosidase (such as AMG®). Other useful amylase products include GRINDAMYL® A 1000 or A 5000 (Grindsted Products, Denmark) and AMYLASE H™ or AMYLASE P™ (DSM). The

glucose oxidase may be a fungal glucose oxidase, in particular an *Aspergillus niger* glucose oxidase (such as GLUZYME®). An exemplary protease is NEUTRASE®.

[00259] The process may be used for any kind of baked product prepared from dough, either of a soft or a crisp character, either of a white, light or dark type. Examples are bread, particularly white, whole-meal or rye bread, typically in the form of loaves or rolls, such as, but not limited to, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

[00260] An amylase may be used in a pre-mix, comprising flour together with an anti-stalling amylase, a phospholipase, and/or a phospholipid. The pre-mix may contain other dough-improving and/or bread-improving additives, *e.g.*, any of the additives, including enzymes, mentioned above. An amylase can be a component of an enzyme preparation comprising an anti-stalling amylase and a phospholipase, for use as a baking additive.

[00261] The enzyme preparation is optionally in the form of a granulate or agglomerated powder. The preparation can have a narrow particle size distribution with more than 95% (by weight) of the particles in the range from 25 to 500 μm. Granulates and agglomerated powders may be prepared by conventional methods, *e.g.*, by spraying an amylase onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, *e.g.*, a salt (such as NaCl or sodium sulfate), a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

[00262] Enveloped particles, *i.e.*, alpha-amylase particles, can comprise an amylase. To prepare enveloped alpha-amylase particles, the enzyme is contacted with a food grade lipid in sufficient quantity to suspend all of the alpha-amylase particles. Food grade lipids, as used herein, may be any naturally organic compound that is insoluble in water but is soluble in non-polar organic solvents such as hydrocarbon or diethyl ether. Suitable food grade lipids include, but are not limited to, triglycerides either in the form of fats or oils that are either saturated or unsaturated. Examples of fatty acids and combinations thereof which make up the saturated triglycerides include, but are not limited to, butyric (derived from milk fat), palmitic (derived from animal and plant fat), and/or stearic (derived from animal and plant fat). Examples of fatty acids and combinations thereof which make up the unsaturated triglycerides include, but are not limited to, palmitoleic (derived from animal and plant fat), oleic (derived from animal and plant fat), linoleic (derived from plant oils), and/or linolenic

(derived from linseed oil). Other suitable food grade lipids include, but are not limited to, monoglycerides and diglycerides derived from the triglycerides discussed above, phospholipids and glycolipids.

[00263] The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the alpha-amylase particles in such a fashion that the lipid material covers at least a portion of the surface of at least a majority, *e.g.*, 100% of the alpha-amylase particles. Thus, each alpha-amylase particle is individually enveloped in a lipid. For example, all or substantially all of the alpha-amylase particles are provided with a thin, continuous, enveloping film of lipid. This can be accomplished by first pouring a quantity of lipid into a container, and then slurrying the alpha-amylase particles so that the lipid thoroughly wets the surface of each alpha-amylase particle. After a short period of stirring, the enveloped alpha-amylase particles, carrying a substantial amount of the lipids on their surfaces, are recovered. The thickness of the coating so applied to the particles of alpha-amylase can be controlled by selection of the type of lipid used and by repeating the operation in order to build up a thicker film, when desired.

[00264] The storing, handling and incorporation of the loaded delivery vehicle can be accomplished by means of a packaged mix. The packaged mix can comprise the enveloped alpha-amylase. However, the packaged mix may further contain additional ingredients as required by the manufacturer or baker. After the enveloped alpha-amylase has been incorporated into the dough, the baker continues through the normal production process for that product.

[00265] The advantages of enveloping the alpha-amylase particles are two-fold. First, the food grade lipid protects the enzyme from thermal denaturation during the baking process for those enzymes that are heat labile. Consequently, while the alpha-amylase is stabilized and protected during the proving and baking stages, it is released from the protective coating in the final baked good product, where it hydrolyzes the glucosidic linkages in polyglucans. The loaded delivery vehicle also provides a sustained release of the active enzyme into the baked good. That is, following the baking process, active alpha-amylase is continually released from the protective coating at a rate that counteracts, and therefore reduces the rate of, staling mechanisms.

[00266] In general, the amount of lipid applied to the alpha-amylase particles can vary from a few percent of the total weight of the alpha-amylase to many times that weight,

depending upon the nature of the lipid, the manner in which it is applied to the alpha-amylase particles, the composition of the dough mixture to be treated, and the severity of the dough-mixing operation involved.

[00267] The loaded delivery vehicle, *i.e.*, the lipid-enveloped enzyme, is added to the ingredients used to prepare a baked good in an effective amount to extend the shelf-life of the baked good. The baker computes the amount of enveloped alpha-amylase, prepared as discussed above, that will be required to achieve the desired anti-stalling effect. The amount of the enveloped alpha-amylase required is calculated based on the concentration of enzyme enveloped and on the proportion of alpha-amylase to flour specified. A wide range of concentrations has been found to be effective, although, as has been discussed, observable improvements in anti-stalling do not correspond linearly with the alpha-amylase concentration, but above certain minimal levels, large increases in alpha-amylase concentration produce little additional improvement. The alpha-amylase concentration actually used in a particular bakery production could be much higher than the minimum necessary to provide the baker with some insurance against inadvertent under-measurement errors by the baker. The lower limit of enzyme concentration is determined by the minimum anti-stalling effect the baker wishes to achieve.

[00268] A method of preparing a baked good may comprise: a) preparing lipid-coated alpha-amylase particles, where substantially all of the alpha-amylase particles are coated; b) mixing a dough containing flour; c) adding the lipid-coated alpha-amylase to the dough before the mixing is complete and terminating the mixing before the lipid coating is removed from the alpha-amylase; d) proofing the dough; and e) baking the dough to provide the baked good, where the alpha-amylase is inactive during the mixing, proofing and baking stages and is active in the baked good.

[00269] The enveloped alpha-amylase can be added to the dough during the mix cycle, *e.g.*, near the end of the mix cycle. The enveloped alpha-amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped alpha-amylase throughout the dough; however, the mixing stage is terminated before the protective coating becomes stripped from the alpha-amylase particle(s). Depending on the type and volume of dough, and mixer action and speed, anywhere from one to six minutes or more might be required to mix the enveloped alpha-amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped

alpha-amylase should have a total volume sufficient to allow the enveloped alpha-amylase to be spread throughout the dough mix. If the preparation of enveloped alpha-amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped alpha-amylase is added to the dough. Recipes and production processes may require specific modifications; however, good results generally can be achieved when 25% of the oil specified in a bread dough formula is held out of the dough and is used as a carrier for a concentrated enveloped alpha-amylase when added near the end of the mix cycle. In bread or other baked goods, particularly those having a low fat content, *e.g.*, French-style breads, an enveloped alpha-amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped alpha-amylase properly with the dough. The range of suitable percentages is wide and depends on the formula, finished product, and production methodology requirements of the individual baker. Second, the enveloped alpha-amylase suspension should be added to the mix with sufficient time for complete mixture into the dough, but not for such a time that excessive mechanical action strips the protective lipid coating from the enveloped alpha-amylase particles.

[00270] In a further aspect of the invention, the food composition is an oil, meat, lard, composition comprising an amylase. In this context the term "oil, meat, lard, composition" means any composition, based on, made from and/or containing oil, meat or lard, respectively. Another aspect the invention relates to a method of preparing an oil or meat or lard composition and/or additive comprising an amylase, comprising mixing the polypeptide of the invention with an oil/meat/lard composition and/or additive ingredients.

[00271] In a further aspect of the invention, the food composition is an animal feed composition, animal feed additive and/or pet food comprising an amylase and variants thereof. The present invention further relates to a method for preparing such an animal feed composition, animal feed additive composition and/or pet food comprising mixing an amylase and variants thereof with one or more animal feed ingredients and/or animal feed additive ingredients and/or pet food ingredients. Furthermore, the present invention relates to the use of an amylase in the preparation of an animal feed composition and/or animal feed additive composition and/or pet food.

[00272] The term "animal" includes all non-ruminant and ruminant animals. In a particular embodiment, the animal is a non-ruminant animal, such as a horse and a mono-gastric animal. Examples of mono-gastric animals include, but are not limited to, pigs and swine,

such as piglets, growing pigs, sows; poultry such as turkeys, ducks, chicken, broiler chicks, layers; fish such as salmon, trout, tilapia, catfish and carps; and crustaceans such as shrimps and prawns. In a further embodiment the animal is a ruminant animal including, but not limited to, cattle, young calves, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn and nilgai.

[00273] In the present context, it is intended that the term "pet food" is understood to mean a food for a household animal such as, but not limited to dogs, cats, gerbils, hamsters, chinchillas, fancy rats, guinea pigs; avian pets, such as canaries, parakeets, and parrots; reptile pets, such as turtles, lizards and snakes; and aquatic pets, such as tropical fish and frogs.

[00274] The terms "animal feed composition," "feedstuff" and "fodder" are used interchangeably and may comprise one or more feed materials selected from the group comprising a) cereals, such as small grains (*e.g.*, wheat, barley, rye, oats and combinations thereof) and/or large grains such as maize or sorghum; b) by products from cereals, such as corn gluten meal, Distillers Dried Grain Solubles (DDGS) (particularly corn based Distillers Dried Grain Solubles (cDDGS), wheat bran, wheat middlings, wheat shorts, rice bran, rice hulls, oat hulls, palm kernel, and citrus pulp; c) protein obtained from sources such as soya, sunflower, peanut, lupin, peas, fava beans, cotton, canola, fish meal, dried plasma protein, meat and bone meal, potato protein, whey, copra, sesame; d) oils and fats obtained from vegetable and animal sources; e) minerals and vitamins.

7. Compositions and Methods for Textile Desizing

[00275] The present invention also relates to compositions and methods of treating fabrics (*e.g.*, to desize a textile) using an amylase. Fabric-treating methods are well known in the art (*see*, *e.g.*, U.S. Patent No. 6,077,316). For example, the feel and appearance of a fabric can be improved by a method comprising contacting the fabric with an amylase in a solution. The fabric can be treated with the solution under pressure.

[00276] An amylase can be applied during or after the weaving of a textile, or during the desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives to increase their tensile strength and to prevent breaking. An amylase can be applied during or after the weaving to remove these sizing starch or starch derivatives. After weaving, an

amylase can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

[00277] An amylase can be used alone or with other desizing chemical reagents and/or desizing enzymes to desize fabrics, including cotton-containing fabrics, as detergent additives, *e.g.*, in aqueous compositions. An amylase also can be used in compositions and methods for producing a stonewashed look on indigo-dyed denim fabric and garments. For the manufacture of clothes, the fabric can be cut and sewn into clothes or garments, which are afterwards finished. In particular, for the manufacture of denim jeans, different enzymatic finishing methods have been developed. The finishing of denim garment normally is initiated with an enzymatic desizing step, during which garments are subjected to the action of amylolytic enzymes to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. An amylase can be used in methods of finishing denim garments (*e.g.*, a "bio-stoning process"), enzymatic desizing and providing softness to fabrics, and/or finishing process.

8. Compositions and Methods for Brewing

[00278] The present alpha-amylase may be a component of a brewing composition used in a process of brewing, *i.e.*, making a fermented malt beverage. Non-fermentable carbohydrates form the majority of the dissolved solids in the final beer. This residue remains because of the inability of malt amylases to hydrolyze the alpha-1,6-linkages of the starch. The non-fermentable carbohydrates contribute about 50 calories per 12 ounces of beer. An amylase, in combination with a glucoamylase and optionally a pullulanase and/or isoamylase, assist in converting the starch into dextrins and fermentable sugars, lowering the residual non-fermentable carbohydrates in the final beer.

[00279] The principal raw materials used in making these beverages are water, hops and malt. In addition, adjuncts such as common corn grits, refined corn grits, brewer's milled yeast, rice, sorghum, refined corn starch, barley, barley starch, dehusked barley, wheat, wheat starch, torrified cereal, cereal flakes, rye, oats, potato, tapioca, and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like may be used as a source of starch.

[00280] For a number of reasons, the malt, which is produced principally from selected varieties of barley, has the greatest effect on the overall character and quality of the beer. First, the malt is the primary flavoring agent in beer. Second, the malt provides the major

portion of the fermentable sugar. Third, the malt provides the proteins, which will contribute to the body and foam character of the beer. Fourth, the malt provides the necessary enzymatic activity during mashing. Hops also contribute significantly to beer quality, including flavoring. In particular, hops (or hops constituents) add desirable bittering substances to the beer. In addition, the hops act as protein precipitants, establish preservative agents and aid in foam formation and stabilization.

[00281] Grains, such as barley, oats, wheat, as well as plant components, such as corn, hops, and rice, are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, *i.e.*, partially germinated, resulting in an increase in the levels of enzymes, including alpha-amylase. For successful brewing, adequate levels of alpha-amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation. An amylase, by itself or in combination with another alpha-amylase(s), accordingly may be added to the components used for brewing.

[00282] As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed or coarsely ground forms. The methods described herein may be used to determine alpha-amylase activity levels in both flours and stock.

[00283] Processes for making beer are well known in the art. *See, e.g.*, Wolfgang Kunze (2004) "Technology Brewing and Malting," Research and Teaching Institute of Brewing, Berlin (VLB), 3rd edition. Briefly, the process involves: (a) preparing a mash, (b) filtering the mash to prepare a wort, and (c) fermenting the wort to obtain a fermented beverage, such as beer. Typically, milled or crushed malt is mixed with water and held for a period of time under controlled temperatures to permit the enzymes present in the malt to convert the starch present in the malt into fermentable sugars. The mash is then transferred to a mash filter where the liquid is separated from the grain residue. This sweet liquid is called "wort," and the left over grain residue is called "spent grain." The mash is typically subjected to an extraction, which involves adding water to the mash in order to recover the residual soluble extract from the spent grain. The wort is then boiled vigorously to sterilize the wort and help develop the color, flavor and odor. Hops are added at some point during the boiling. The wort is cooled and transferred to a fermentor.

[00284] The wort is then contacted in a fermentor with yeast. The fermentor may be chilled to stop fermentation. The yeast flocculates and is removed. Finally, the beer is cooled and stored for a period of time, during which the beer clarifies and its flavor develops, and any material that might impair the appearance, flavor and shelf life of the beer settles out. The beer usually contains from about 2% to about 10% v/v alcohol, although beer with a higher alcohol content, *e.g.*, 18% v/v, may be obtained. Prior to packaging, the beer is carbonated and, optionally, filtered and pasteurized.

[00285] The brewing composition comprising an amylase, in combination with a glucoamylase and optionally a pullulanase and/or isoamylase, may be added to the mash of step (a) above, *i.e.*, during the preparation of the mash. Alternatively, or in addition, the brewing composition may be added to the mash of step (b) above, *i.e.*, during the filtration of the mash. Alternatively, or in addition, the brewing composition may be added to the wort of step (c) above, *i.e.*, during the fermenting of the wort.

[00286] A fermented beverage, such as a beer, can be produced by one of the methods above. The fermented beverage can be a beer, such as full malted beer, beer brewed under the "Reinheitsgebot," ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, non-alcoholic malt liquor and the like, but also alternative cereal and malt beverages such as fruit flavored malt beverages, *e.g.*, citrus flavored, such as lemon-, orange-, lime-, or berry-flavored malt beverages, liquor flavored malt beverages, *e.g.*, vodka-, rum-, or tequila-flavored malt liquor, or coffee flavored malt beverages, such as caffeine-flavored malt liquor, and the like.

9. Reduction of Iodine-Positive Starch

[00287] Alpha-amylases may reduce the iodine-positive starch (IPS), when used in a method of liquefaction and/or saccharification. One source of IPS is from amylose that escapes hydrolysis and/or from retrograded starch polymer. Starch retrogradation occurs spontaneously in a starch paste, or gel on ageing, because of the tendency of starch molecules to bind to one another followed by an increase in crystallinity. Solutions of low concentration become increasingly cloudy due to the progressive association of starch molecules into larger articles. Spontaneous precipitation takes place and the precipitated starch appears to be reverting to its original condition of cold-water insolubility. Pastes of higher concentration on cooling set to a gel, which on ageing becomes steadily firmer due to

the increasing association of the starch molecules. This arises because of the strong tendency for hydrogen bond formation between hydroxy groups on adjacent starch molecules. *See* J.A. Radley, ed., STARCH AND ITS DERIVATIVES 194-201 (Chapman and Hall, London (1968)).

[00288] The presence of IPS in saccharide liquor negatively affects final product quality and represents a major issue with downstream processing. IPS plugs or slows filtration system, and fouls the carbon columns used for purification. When IPS reaches sufficiently high levels, it may leak through the carbon columns and decrease production efficiency. Additionally, it may result in hazy final product upon storage, which is unacceptable for final product quality. The amount of IPS can be reduced by isolating the saccharification tank and blending the contents back. IPS nevertheless will accumulate in carbon columns and filter systems, among other things. The use of alpha-amylases is expected to improve overall process performance by reducing the amount of IPS.

[00289] All references cited herein are herein incorporated by reference in their entirety for all purposes. In order to further illustrate the compositions and methods, and advantages thereof, the following specific examples are given with the understanding that they are illustrative rather than limiting.

EXAMPLES

EXAMPLE 1

The identification of amylases from *Bacillus* species

[00290] Bacterial strains including *Bacillus pumilus* strain S018, *Bacillus subtilis subsp. spizizenii* strain S015, *Bacillus sp* S026, and *Bacillus subtilis* SWT13 were selected as potential sources for different enzymes that are useful for various industrial applications.

[00291] For *Bacillus pumilus* strain S018, *Bacillus subtilis subsp. spizizenii* strain S015, and *Bacillus sp* S026, the genomic DNAs for sequencing were obtained by first growing these strains on LB agar plates (Difco) at 37 °C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The entire genome of these strains were sequenced by DuPont Pioneer (Johnston, Iowa, USA) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by DuPont Pioneer (Johnston, Iowa,

USA). One of the genes identified from each strain in this way encodes a protein that has homology to alpha-amylases of various other bacteria.

[00292] For *Bacillus subtilis* SWT13, the entire genome was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology and subsequently assembled by BaseClear. Contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified from the strain in this way encodes a protein that has homology to alpha-amylases of various other bacteria.

- The amylase gene from *Bacillus pumilus* strain S018 named as *BpuAmy1*, is depicted in SEQ ID NO: 1. The corresponding protein encoded by the *BpuAmy1* gene is depicted in SEQ ID NO: 2.
- The amylase gene from *Bacillus subtilis subsp. spizizenii* strain S015 named as *BsuAmy3*, is depicted in SEQ ID NO: 3. The corresponding protein encoded by the *BsuAmy3* gene is depicted in SEQ ID NO: 4.
- The amylase gene fro*m Bacillus sp* S026 named as *BspAmy33*, is depicted in SEQ ID NO: 5. The corresponding protein encoded by the *BspAmy33* gene is depicted in SEQ ID NO: 6.
- The amylase gene fro*m Bacillus subtilis* SWT13 named as *BsuAmy4*, is depicted in SEQ ID NO: 7. The corresponding protein encoded by the *BsuAmy4* gene is depicted in SEQ ID NO: 8.

[00293] The nucleotide sequence of the *BpuAmy1* gene isolated from *Bacillus pumilus* strain S018 is set forth as SEQ ID NO: 1. The sequence encoding the predicted native signal peptide is shown in italics and bold:

ATGATTAAAAAACTATTAAATACCTCTTTACTGCCGTTATTCGCTGGATTATTATTGCTGTT TCATTTGATTCTGGCAGGTCCGGTGGCTTTAAATGCAGAGACCGCGAACCAATCAAATGAGC
ATACAACGCCATCAATAAAGAGCGGGACCATTCTTCATGCTTGGAATTGGTCGTTCAACACT
TTAAAAAATAATATGAAGGATATCCATGATGCAGGATATACTGCCATTCAGACGTCTCCTAT
TAATCAAGTAAAGGAAGGAAATAATGGAGATAAAAGCATGGGGAACTGGTACTGGCTTTACC
AGCCGACCTCTTACCAGATTGGCAACAGTTACTTGGGTTCCGAGGAAGAGTTTAAAGAAATG
TGTGCGACGGCTGAAGAATATGGTGTGAAGGTTATTGTCGATGCCGTCGTCAATCACACAAC
CAGTGACTATGCTGCGATTTCAAATGAAATTAAAAGCATTTCAAATTGGACGCATGGCAACA
CACAAATTAAAAATTGGTCTGATCGAAGGGATGTCACACAGAATTCATTGCTGGGGCTTTAT
GATTGGAATACGCAGAATACACAAGTACAGTCATATCTAAAAAATTTCCTGGAAAGAGTATT

GGATGACGGTGCCGACGGATTTAGGTATGATGCGGCCAAACATATAGAGCTTCCGGATGACA
GTGATTTTGGCAGTCATTTTTGGCCGAATATCACAGATACATCTGCAGAATTCCAATATGGA
GAAATATTACAAGACAGTACCTCCAGAGATGCTGCATATGCGAATTATATGAATGTCACAGC
ATCAAACTATGGGCATTCCATAAGATCTGCTTTAAAGAATCGCAATTTTAGCGCAGCGAATA
TCTCCAATTATGCATCTGATGTGTATGCGGACGATTTGGTCACATGGGTGGAATCGCATGAT
ACGTATGCCAATGATGATGAAGAATCAACATGGATGAGCGATGATGATATTCGTTTAGGCTG
GGCGGTTATTGCTTCTCGTTCAGGGAGTACACCTCTTTTTTTCTCCAGACCTGAGGGCGGCG
GAAATGGAGTAAGATTCCCTGGAAAAAGTCAAATAGGTGATCGCGGCAGTGCTCTATTTAAA
GATAAAGCCATCGAAGCGGTCAATACATTTCATAATGTAATGAGTGGACAGCCTGAGAAACT
CTCTAACCCGGATGGAAACAATCAGATATTCATGAATCAGCGCGGCTCTGATGGCGTAGTTT
TGGCAAATGCATCTTCATCATCAGTTTCCATTCATACTTCAACGGATTTACCTGATGGCAAC
TATGATAATAAAGCCGGGGATGGTTCGTTTAAAGTAGCGGATGGCAAACTGACAGGTATGAT
CAGCGGCAGATCTGTGGTTGTTCTTTATCATGACGATACTGCAAATGCACCTAATGTCTTCC
TCGAGAACTATAAAACAGGTGTAACACATTCATTCTATAATGATCAACTGACGGTAACTTTG
CGTGCAGATGCTAAAACAACAAACGCTGCTTATCAAATAAATAATGGTCATGAGACAGAGTA
TAAGGATGGAGCTCAATTAACGATCGGAAAAGGAGATCCATTCAACACAACATACAAAATCA
CTTTAACAGGAACGAACAGTGAGGGAGTAACCAGGACAAAAGAATACATTTTTATAAAAACT
GACCCATCTTCGGCGAAAATAATTGGTTATCAAAACCCAAATAATTGGAGTCAAGTTAATGC
ATATGTATATAAGGAGAATGGTGGTCAGGCAATAGAATTAACCGGATTTTGGCCAGGAAAAG
CAATGGCTAAGAATTCAGATGGAATTTACACTCTGACGTTACCCGCGGATACCGATACAAAA
AACACCAAAGTGATTTTTAATAATGGCAACACCCAAGTACCGGGACAGAATCAACCTGGCTT
TGATTATGTACAAAATGGGTTATATAATGACTCTGGCTTAAATGGTTCTCTGCCTCATTTAG
TCCGGATGTTCCGTTAA

[00294] The amino acid sequence of the BpuAmy1 precursor protein is set forth as SEQ ID NO: 2. the predicted native signal peptide is shown in italics and bold:

mikkllntsllplfaglllfhlilagpvalnaetanqsnehttpsiksgtilhawnwsfnt
lknnmkdihdagytaiqtspinqvkegnngdksmgnwywlyqptsyqignsylgseeefkem
cataeeygvkvivdavvnhttsdyaaisneiksisnwthgntqiknwsdrrdvtqnsllgly
dwntqntqvqsylknflervlddgadgfrydaakhielpddsdfgshfwpnitdtsaefqyg
eilqdstsrdaayanymnvtasnyghsirsalknrnfsaanisnyasdvyaddlvtwveshd
tyanddeestwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigdrgsalfk
dkaieavntfhnvmsgqpeklsnpdgnnqifmnqrgsdgvvlanassssvsihtstdlpdgn
ydnkagdgsfkvadgkltgmisgrsvvvlyhddtanapnvflenyktgvthsfyndqltvtl
radakttnaayqinngheteykdgaqltigkgdpfnttykitltgtnsegvtrtkeyifikt

dpssakiigyqnpnnwsqvnayvykenggqaieltgfwpgkamaknsdgiytltlpadtdtk
ntkvifnngntqvpgqnqpgfdyvqnglyndsglngslphlvrmfr

[00295] The nucleotide sequence of the *BsuAmy3* gene isolated from *Bacillus subtilis subsp. spizizenii* strain S015 is set forth as SEQ ID NO: 3. The sequence encoding the predicted native signal peptide is shown in italics and bold:

ATGTTTGAAAAACGATTCAAAACCTCTTTACTGCCGTTATTCGCTGGATTTTTTCTGCTGTT
TCATTTGGTTCTGGCAGAACCGGCGGCTGCGAATGCTGAAACAGCGGACAAATCGAATGAGC
TTACAGCGCCATCGATCAAAGACGGAACCATACTTCATGCTTGGAATTGGTCGTTCAATACG
TTAAAAAACAATATGAAGGATATTCATGATGCAGGATATACAGCCATTCAGACGTCTCCGAT
TAACCAAGTAAAGGAAGGGAACCAAGGAGACAAAAGCATGTCGAACTGGTACTGGCTGTATC
AGCCGACATCGTACCAAATTGGCAACCGTTACTTAGGTACTGAACAAGAATTTAAAGAAATG
TGCGCTGCTGCTGAAGAATATGGCGTAAAGGTCATTGTTGACGCAGTGATCAATCATACCAC
CAGTGACTATGCTGCGATTTCTAACGAGATTAAGAGTATTCCAAATTGGACTCATGGAAACA
CACAAATTAAAAACTGGTCCGATCGATGGGATGTCACGCAGAATTCATTGCTCGGGCTGTAT
GATTGGAATACACAAAATACACAAGTACAGTCCTATCTGAAACGTTTCTTAGAACGAGCATT
AAATGACGGCGCAGACGGATTTCGCTTTGACGCCGCCAAACATATAGAACTGCCGGATGACG
GGAATTACGGCAGTCCATTTTGGCCGAATCTCACAAATACATCTGCCGAGTTCCAATACGGA
GAAATCCTGCAAGATAGTGCCTCCAGAGATGCTGCATATGCGAATTATATGAACGTAACGGC
ATCTAACTATGGACATTCCATGAGGTCTGCTTTAAGGAACCGTAATCTGAGCGTGTCGAATA
TCTCCCATTATGTGTCTGATGTGTCTGCGGACAAGTTAGTCACATGGGTGGAATCGCATGAT
ACGTATGCCAATGATGAGGAAGAGTCAACATGGATGAGCGATGACGATATTCGTTTAGGCTG
GGCGGTGATTGCTTCCCGCTCAGGCAGTACGCCTCTCTTCTTTTCCAGACCCGAGGGCGGCG
GAAATGGGGTAAGATTCCCGGGGAAAAGCCAGATCGGCGATCGAGGGAGTGGTTTATTTGAA
GATCAGGCTATCGTTGCGGTCAATACATTTCACAATGTGATGGATGGACAGCCTGAGGAGCT
CTCGAATCCGAATGGAAACAATCAGATATTTATGAATCAGCGCGGCTCACATGGCCTTGTGC
TGGCAAATGCAGGTTCATCTTCTGTCACCATTAATACGTCAACAAAATTACCTGATGGCAGC
TATGATAATAAAGCTGGAGACGGTTCATTTCAAGTAACGAATGGTAAACTGACGGGCACGAT
CAATGCCAGATCTGTGGTTGTTATTTATCCTGACGATATCGCAAATGCGCCTCATGTCTTCC
TTGAGAATTATAAAACAGGGGTCACACATTCTTTCAATGATCAACTGGCGATCACCCTGCGT
GCAGATGCGAAAACAGCAAAAGCCGTGTATCAAATCAATAATGGGCAAGAGACAGTGTTTAA
GGATGGAGACCAATTAACAATCGGAAAAGGAGCTCCATTTGGCACAACATACACCATTACGT
TAAAAGGAACGAACAGTGATGGTGTAACGAGGACCCAAGAATACACGTTTGTCAAAAAAGAC
CCGTCTTCAGCCAAAATCATCGGCTATCAAAACCCAAATCATTGGGGCCAAGTAAATGCTTA

TATCTATAAAGATGATGGAGGCCGCGCAATAGAGTTGACCGGATCTTGGCCGGGAAAAGCAA
TGACCAGGAATGCGGATGGAATTTACACGCTGACGCTCCCTGCGGATACGGATACAACGAAC
GCCAAAGTGATTTTTAATAATGGCAGCGCCCAAGTGCCCGGCCAAAACCAGCCTGGCTTTGA
TTATGTGCAAAACGGTTTATATAACAACTCTGGGTTAAGCGGTTCTCTTCCTTATTGA

[00296] The amino acid sequence of the BsuAmy3 precursor protein is set forth as SEQ ID NO: 4. the predicted native signal peptide is shown in italics and bold:

Mfekrfktsllplfagfflfhlvlaepaaanaetadksneltapsikdgtilhawnwsfnt
lknnmkdihdagytaiqtspinqvkegnqgdksmsnwywlyqptsyqignrylgteqefkem
caaaeeygvkvivdavinhttsdyaaisneiksipnwthgntqiknwsdrwdvtqnsllgly
dwntqntqvqsylkrfleralndgadgfrfdaakhielpddgnygspfwpnltntsaefqyg
eilqdsasrdaayanymnvtasnyghsmrsalrnrnlsvsnishyvsdvsadklvtwveshd
tyandeeestwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigdrgsglfe
dqaivavntfhnvmdgqpeelsnpngnnqifmnqrgshglvlanagsssvtintstklpdgs
ydnkagdgsfqvtngkltgtinarsvvviypddianaphvflenyktgvthsfndqlaitlr
adaktakavyqinngqetvfkdgdqltigkgapfgttytitlkgtnsdgvtrtqeytfvkkd
pssakiigyqnpnhwgqvnayiykddggraieltgswpgkamtrnadgiytltlpadtdttn
akvifnngsaqvpgqnqpgfdyvqnglynnsglsgslpy

[00297] The nucleotide sequence of the *BspAmy33* gene isolated from *Bacillus sp.* is set forth as SEQ ID NO: 5. The sequence encoding the predicted native signal peptide is shown in italics and bold:

ATGTTTAAAAAACTATCGAACACCTCTTTACTTCCGATTTTCGCTGGATTATTGTTATTGTT
TCATTTGATTTTAGGAGGATCCGCCGCGGCAGCGAATGCCGGGTCAACGAACAAATCTGACG
AGTATACAGCGCCATCAATAGAAAGCGGAACCATTCTTCATGCTTGGAATTGGTCGTTTAAC
ACCTTAAAAAATCATATGAAGGATATTCATGATGCGGGATATACGGCGATTCAGACGTCTCC
GGTAAACCAAGTGAAGGAAGGAAATAATGGGGATAAAGGTATGGCGAACTGGTACTGGCTCT
ATCAGCCGACCTCTTACCAGATTGGCAACAGTTACTTAGGGACTGAAGAAGAATTCAAAGAA
ATGTGTGCCATGGCTGAAGAATATGGTGTGAAGGTCATTGTTGACGCAGTCATAAATCACAC
AACAAGTGACTATGCTGCAATATCGAGTGAAATTAAGAGTATTCCAAACTGGACGCATGGAA
ATACACAAATTAAAAATTGGTCCGATCGAAAAGATGTTACACAGAATTCATTGCTGGGGCTG
TATGATTGGAATACACAAAATACACAAGCACAGTCATATCTAAAACATTTCTTAGAGAGAGT
ATTGAATGATGGTGCCGCCGGGTTTAGGTTTGATGCGGCCAAACATATAGAGCTTCCGGATG
ATGGTGATTACGGCAGTGATTTTTGGCCGAATATGACAGATACATCTGCAGAGTTTCAATAT
GGAGAAATATTACAAGACAGTGCCTCCAGGGACACTGCATATGCGAATTATATGAATGTGAC

AGCATCTAACTATGGGCATACCATAAGATCTGCTTTAAAGAATCGTCATTTTAGTACGTCGA
ATATCTCCAATTATGCATCTGATGTGCCTGCAGATGATCTGGTCACATGGGTGGAATCACAT
GATACGTATGCCAATGATGATGAACAATCAACATGGATGAGCGATGATGATATTCGCTTGGG
CTGGGCGGTTATTGCTTCTCGTTCAGGGAGTACACCTCTCTTTTTCTCCAGACCTGAGGGCG
GCGGAAATGGCGTAAGATTCCCTGGAAAAAGTCAAATAGGTGATCGCGGAAGTGCCTTATTT
AAAGATCAAGCCATCACAGCGGTCAATAAATTTCATAATGTAATGACTGGAAAGCCTGAGAA
ACTCTCTAACCCGAATGGAAACAACCAGATATTCATGAATGAGCGCGGATCTGATGGCGTAG
TTTTGGCGAATGTAGGTTCATCATCGGTTTCTATTCATACTTCAACAGATTTACCTGATGGT
CATTATGATAATAAAGCCGGTGATGGTTCGTTTAAAGTAACGAATGGTAACCTTACAGGTAC
GATCGGCAGCAGAGCTGTGGTCGTTCTTTATCCTGACGATATCGCAAATGCACCTAATGTGT
TCATCGAGAACTATAAAACAGGTGTGACACATTCTTTTTATGATGATCAACTGACGGTCTCT
CTGCGTGCAGATGCTAAAATAACAAAAGCTGCTTATCAAATTAATAATGGTGATGAGGCAGA
GTTTAAGGATGGAGATCGATTAACGATTGGAAATGGAGATCCGTTTGACACAACATACAAGG
TCACTTTAACAGGAACGAACAGTAATGGAGTAAGCAGGACAAATGAATACACTTTTGTTAAA
ACAGATCCATCTTCCGTAAAAACAATTGGCTATCAAAATCCAAATAATTGGGGCCAAGTAAA
TGCATATATATATAAGGAGAATGGAGGTCAGGCAATAGAATTGACCGGATCTTGGCCAGGAA
AAGCAATGACTCAGAATGCAGATGGAATGTACACTCTGACGTTACCAGCTGATACTGATACA
ACCGGCGCCAAAGTGATTTTTAACAATGGCAGCAGCCAAGTACCCGGAATGAATCAGCCCGG
ATTTGAGTATGTGCGAAATGGTTTATATCATGACTCCGGCTTAACGGGGTATCTGCCTAATT
CAGGTCGGACATTCCGTTAA

[00298] The amino acid sequence of the BspAmy33 precursor protein is set forth as SEQ ID NO: 6. The predicted native signal peptide is shown in italics and bold:

mfkklsntsllpifaglllfhlilggsaaaanagstnksdeytapsiesgtilhawnwsfn
tlknhmkdihdagytaiqtspvnqvkegnngdkgmanwywlyqptsyqignsylgteeefke
mcamaeeygvkvivdavinhttsdyaaisseiksipnwthgntqiknwsdrkdvtqnsllgl
ydwntqntqaqsylkhflervlndgaagfrfdaakhielpddgdygsdfwpnmtdtsaefqy
geilqdsasrdtayanymnvtasnyghtirsalknrhfstsnisnyasdvpaddlvtwvesh
dtyanddeqstwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigdrgsalf
kdqaitavnkfhnvmtgkpeklsnpngnnqifmnergsdgvvlanvgsssvsihtstdlpdg
hydnkagdgsfkvtngnltgtigsravvvlypddianapnvfienyktgvthsfyddqltvs
lradakitkaayqinngdeaefkdgdrltignqdpfdttykvtltgtnsngvsrtneytfvk
tdpssvktigyqnpnnwgqvnayiykenggqaieltgswpgkamtqnadgmytltlpadtdt
tgakvifnngssqvpgmnqpgfeyvrnglyhdsgltgylpnsgrtfr

[00299] The nucleotide sequence of the *BsuAmy4* gene isolated from *Bacillus subtilis* SWT13 is set forth as SEQ ID NO: 7. The sequence encoding the predicted native signal peptide is shown in italics and bold:

ATGTTTAAAAAACGATTTAAAACCTCTTTACTGCCGTTATTCGCTGGATTTTTGTTGCTGTT
TCATATGGTTTTGGCAGGACCGGCGGCTGCAAACGCTGAAACTGCAAACAAATCGAATGAGG
TGACCGATTCATCGGTCAAAAACGGGACCATTCTTCATGCTTGGAATTGGTCATTCAATACG
TTAAAACACAATATGAAGGACATTCATGATGCAGGATATACAGCCATTCAGACGTCTCCGAT
TAACCAAGTAAAGGAAGGGAACCAAGGAAATAAAAGCATGCCGAACTGGTACTGGCTCTATC
AGCCGACATCGTACCAAATTGGCAACCACTACTTAGGCACTGAACAAGAATTTAAAGAAATG
TGTGCAGCCGCTGAAGAATATGGCGTAAAAGTCATAGTTGACGCGGTCATCAATCATACCAC
CAGTGACTATGCCGCCATTTCCAATGAGATTAAGAGTATTCCAAACTGGACACACGGAAACA
CACAAATTAAAAACTGGTCGGATCGATGGGATGTCACGCAGAATGCATTGCTAGGGTTGTAT
GATTGGAATACACAGAACACTGAGGTGCAAACCTATCTGAAACGTTTCTTAGAAAGAGCATT
GGATGACGGAGCAGACGGGTTCCGCTATGATGCCGCCAAGCATATAGAGCTTCCTGATGATG
GGAATTATGGCAGCCAGTTTTGGCCGAATATCACAAACACATCTGCAGAGTTTCAATACGGA
GAAATCCTTCAGGATAGTGCCTCCAGGGATGCTGCATATGCGAATTATATGAATGTGACAGC
GTCTAACTATGGGCATTCCATCAGATCCGCTTTAAAGAACCGCAATCTGAGTGTGTCGAATA
TCTCCCATTATGCATCTGACGTGTCTGCGGACAAGTTAGTCACATGGGTGGAATCACATGAT
ACGTATGCCAATGATGATGAAGAGTCCACATGGATGAGTGATGACGATATCCGTTTAGGCTG
GGCAGTGATTGGTTCGCGCTCAGGAAGCACGCCTCTTTTCTTTTCCAGGCCTGAGGGCGGAG
GAAATGGTGTGAGATTTCCAGGGAAAAGTCAAATAGGAGATCGCGGGAGCGCCTTATTTAAA
GATCAGGCGATTACTGCGGTCAATCAATTTCACAATGAAATGGCCGGGCAGCCTGAGGAACT
CTCAAATCCGAATGGGAACAATCAAATATTTATGAATCAGCGCGGCTCAAAAGGTGTTGTGC
TGGCAAATGCAGGTTCCTCTTCTGTCAGCATAAATGCTTCAACGAAATTACCTGATGGCAGC
TATGATAATAAAGCCGGGACCGGTTCATTTCAAGTCAGGGACGGTAAACTGACAGGCACGAT
CAATGCCAGATCTGTGGCTGTGCTTTATCTTGACGATATTGCAAATGCGCCTCAAGTTTTCC
TTGAGAATTACAAAGCAGGCGTAACACATTCTTTCAATGATCAACTGACGATTACCCTGCGT
GCAGATGCGAACACAACAAAAGCTGTTTATCAAATCAATAATGGACAAGAGACAGCGTTTAA
AGATGGAGATCAATTCACAATCGGAAAAGAAGATCCATTTGGCAAAACATACACCATCATGT
TAAAAGGAACGAACAGTGATGGTGTAACGAGGATCGAGGAGTACAGTTTTGTTAAAAGAGAT
CCAGCTTCGGCCAAAACCATCGGCTATCAAAATCCGAATCATTGGAGTCAGGTAAATGCTTA
TATCTATAAACAGGATGGGGGCGGGGCAATTGAATTGACCGGATCTTGGCCTGGAAAACCAA
TGACTAAAAATGCAGACGGAATTTACACGCTGACGCTGCCTGCGGATACGGATACAACCAAC

GCCAAAGTGATTTTTAATAATGGCAGCGCCCAAGTGCCTGGCCAGAATCAGCCTGGCTTTGA
TTACGTGCAAAATGGTTTATATAATGACGCGGGCTTAAGCGGTTCTCTTCCTTATTGA

[00300] The amino acid sequence of the BsuAmy4 precursor protein is set forth as SEQ ID NO: 8. The predicted native signal peptide is shown in italics and bold:

MFKKRFKTSLLPLFAGFLLLFHMVLAGPAAANAETANKSNEVTDSSVKNGTILHAWNWSFNT
LKHNMKDIHDAGYTAIQTSPINQVKEGNQGNKSMPNWYWLYQPTSYQIGNHYLGTEQEFKEM
CAAAEEYGVKVIVDAVINHTTSDYAAISNEIKSIPNWTHGNTQIKNWSDRWDVTQNALLGLY
DWNTQNTEVQTYLKRFLERALDDGADGFRYDAAKHIELPDDGNYGSQFWPNITNTSAEFQYG
EILQDSASRDAAYANYMNVTASNYGHSIRSALKNRNLSVSNISHYASDVSADKLVTWVESHD
TYANDDEESTWMSDDDIRLGWAVIGSRSGSTPLFFSRPEGGGNGVRFPGKSQIGDRGSALFK
DQAITAVNQFHNEMAGQPEELSNPNGNNQIFMNQRGSKGVVLANAGSSSVSINASTKLPDGS
YDNKAGTGSFQVRDGKLTGTINARSVAVLYLDDIANAPQVFLENYKAGVTHSFNDQLTITLR
ADANTTKAVYQINNGQETAFKDGDQFTIGKEDPFGKTYTIMLKGTNSDGVTRIEEYSFVKRD
PASAKTIGYQNPNHWSQVNAYIYKQDGGGAIELTGSWPGKPMTKNADGIYTLTLPADTDTTN
AKVIFNNGSAQVPGQNQPGFDYVQNGLYNDAGLSGSLPY

EXAMPLE 2

Expression of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

[00301] The DNA sequences of the mature proteins of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4 were optimized for protein expression in *B. subtilis*, then synthesized and inserted into the p2JM (Vogtentanz, Protein Expr Purif, 55:40-52, 2007) plasmid by GeneRay (Shanghai, China), resulting in BpuAmy1 (aprE-*BpuAmy1*) (Figure 1), BsuAmy3 (aprE-*BsuAmy3*) (Figure 2), BspAmy33 (aprE-*BspAmy33*) (Figure 3), and BsuAmy4 (aprE-*BsuAmy4*) (Figure 4).

[00302] Each plasmid contains an aprE promoter, an aprE signal sequence used to direct target protein secretion in *B. subtilis*, an oligonucleotide named AGK-proAprE that encodes peptide Ala-Gly-Lys to facilitate the secretion of the target protein, and the synthetic nucleotide sequence encoding the mature region of target genes (SEQ ID NO: 9, 11, 13, 15).

[00303] These plasmids were then transformed into *B. subtilis* cells and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1% corn starch. Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 mL shake flask with Grant's II medium.

[00304] The nucleotide sequence of *BpuAmy1* gene in plasmid BpuAmy1 (aprE-*BpuAmy1*) is depicted in SEQ ID NO: 9. The predicted signal sequence is shown in italics and the AGK-proAprE oligonucleotide is shown in bold:

gtgagaagcaaaaaattgtggatcagcttgttgtttgcgttaacgttaatctttacgatggc
*gttcagcaacatgagcgcgcaggct***gctggaaaa**ggccctgttgctcttaatgcggaaacgg
caaaccagtcaaacgaacatacaacacctagcatcaagtcaggcacgattcttcacgcttgg
aattggtcattcaatacgcttaaaaacaatatgaaagatattcatgacgcaggctatacagc
aattcaaacatcaccgattaaccaagtcaaagagggaaataacggcgataaatcaatgggca
actggtattggctttatcaaccgacatcataccaaatcggaaacagctatctgggcagcgag
gaagagtttaaggaaatgtgtgccacagcggaggagtacggagttaaagtcatcgtggacgc
cgtcgttaatcacacaacatcagattatgccgcaatctcaaatgagattaagtcaattagca
attggacgcatggaaacacgcaaatcaaaaactggtcagatagaagagacgtgacacagaac
tcacttctgggcctgtatgattggaacacacaaaatacacaggtccagagctacctgaaaaa
cttccttgaaagagttctggacgacggagcagacggctttagatatgacgctgccaagcata
ttgagctgccggatgattcagattttggctcacatttttggccgaatatcacagatacatca
gccgagtttcagtacggcgaaatccttcaagatagcacgagcagagacgcagcctatgcgaa
ttacatgaatgttacggctagcaactatggacactcaatcagatcagcacttaagaacagaa
acttttcagcagcgaatatttcaaattatgcgtcagatgtctatgctgacgaccttgttaca
tgggttgagagccatgacacatacgccaatgatgacgaagagtcaacatggatgtcagacga
cgatattagactgggatgggctgtgattgcgtcaagatcaggatcaacaccgcttttttct
caagaccggagggaggcggcaatggcgttagatttccgggaaagtcacaaattggcgataga
ggaagcgctctgtttaaagacaaggctattgaggctgttaatacgtttcataatgttatgag
cggccaacctgagaagctgtcaaacccggatggcaacaatcaaatctttatgaatcagagag
gctcagacggagtcgttctggcaaatgcttcaagctcatcagtttcaattcatacaagcaca
gatcttccggacggaaactacgataacaaagcgggagatggctcatttaaggttgccgatgg
aaagctgacgggaatgattagcggcagaagcgttgttgtcctgtaccatgatgatacagcga
acgctccgaatgttttcctggaaaattacaagacaggagttacacactcattttacaatgat
cagcttacagtcacacttagagcagatgctaagacaacgaacgcagcataccagatcaataa
tggacatgagacagagtataaggatggcgcacaacttacaattggcaaaggagacccgtttta
acacaacgtataagattacacttacgggaacaaattcagaaggcgtcacaagaacgaaggaa
tatatcttcattaaaacagaccctagctcagcaaagattatcggatatcaaaatccgaataa
ttggagccaagttaatgcatatgtctacaaggagaacggaggacaagcaattgagctgacag
gattctggcctggcaaggcgatggcgaagaactcagatggaatctacacgctgacactgcct

gcagacacggatacaaaaaacacaaaggtcatctttaacaatggaaatacgcaggtgccggg
acagaaccagcctggatttgattatgtccaaaatggactgtataacgacagcggccttaacg
gcagcctgcctcatctggttagaatgttcagataa

[00305] The amino acid sequence of the BpuAmy1 precursor protein expressed from plasmid BpuAmy1 (aprE-*BpuAmy1*) is depicted in SEQ ID NO: 10. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold:

*mrskklwisllfaltliftmafsnmsaqa***agk**gpvalnaetanqsnehttpsiksgtilhaw
nwsfntlknnmkdihdagytaiqtspinqvkegnngdksmgnwywlyqptsyqignsylgse
eefkemcataeeygvkvivdavvnhttsdyaaisneiksisnwthgntqiknwsdrrdvtqn
sllglydwntqntqvqsylknflervlddgadgfrydaakhielpddsdfgshfwpnitdts
aefqygeilqdstsrdaayanymnvtasnyghsirsalknrnfsaanisnyasdvyaddlvt
wveshdtyanddeestwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigdr
gsalfkdkaieavntfhnvmsgqpeklsnpdgnnqifmnqrgsdgvvlanassssvsihtst
dlpdgnydnkagdgsfkvadgkltgmisgrsvvvlyhddtanapnvflenyktgvthsfynd
qltvtlradakttnaayqinngheteykdgaqltigkgdpfnttykitltgtnsegvtrtke
yifiktdpssakiigyqnpnnwsqvnayvykenggqaieltgfwpgkamaknsdgiytltlp
adtdtkntkvifnngntqvpgqnqpgfdyvqnglyndsglngslphlvrmfr

[00306] The nucleotide sequence of *BsuAmy3* gene in plasmid BsuAmy3 (aprE-*BsuAmy3*) is depicted in SEQ ID NO: 11. The predicted signal sequence is shown in italics and the AGK-proAprE oligonucleotide is shown in bold:

gtgagaagcaaaaaattgtggatcagcttgttgtttgcgttaacgttaatctttacgatggc
gttcagcaacatgagcgcgcaggct**gctggaaaa**gaaccggcagcggctaatgcggaaacgg
ccgataagagcaatgagcttacggcaccgagcatcaaggacggcacaatccttcacgcatgg
aactggtcatttaacacactgaaaaacaatatgaaagacattcacgatgcgggctacacggc
aatccaaacgtcacctattaatcaagtcaaggagggcaaccaaggcgataaatcaatgtcaa
attggtactggctgtaccaaccgacatcatatcagattggcaatagataccttggaacggaa
caagagtttaaggagatgtgtgcagcagccgaggaatacggcgtcaaagttattgttgacgc
tgttattaaccacacaacaagcgactatgcagcaatctcaaacgagattaaaagcattccga
attggacacacggaaacacgcaaattaagaattggtcagacagatgggatgtcacacagaat
agccttctgggactttacgattggaatacacaaaacacacaggtgcaaagctatcttaaaag
attccttgagagagcgctgaatgatggagccgatggattcagatttgacgcagcgaagcata
ttgaacttccggacgacggcaactatggcagccctttttggcctaatcttacgaacacaagc
gcagagtttcagtatggagaaattcttcaagactcagcgagcagagatgctgcttatgcaaa

ctatatgaacgtgacagcttcaaattatggacatagcatgagaagcgcactgagaaatagaa
atctttcagttagcaatatcagccattatgtttcagatgtctcagcggacaaactggttaca
tgggttgaatcacatgatacgtacgcgaacgatgaagaagaatcaacatggatgagcgatga
cgatattagactgggatgggcagttatcgcaagcagatcaggctcaacgccgctgtttttta
gcagacctgagggcggaggcaacggcgttagatttccgggcaagagccagatcggagataga
ggctcaggactgtttgaggatcaagcaatcgttgcagttaatacatttcataatgttatgga
tggccaaccggaagagctttcaaaccctaatggaaacaaccagatttttatgaatcaaagag
gaagccacggcctggttctggccaatgcaggatcatcaagcgtcacaatcaatacgagcacg
aaacttcctgatggaagctacgataacaaggccggagatggatcatttcaagttacaaatgg
caaacttacaggaacgattaatgcaagatcagtcgtggtgatttaccctgatgatatcgcca
acgcaccgcacgtgttcctggagaactataaaacaggcgttacacactcattcaacgaccag
cttgctattacgcttagagcggatgctaagacggctaaggctgtttaccagatcaataacgg
acaggagacagttttcaaagatggagatcagcttacaatcggaaagggcgcacctttggca
caacatatacgatcacgcttaaaggaacaaattcagacggagttacgagaacacaggagtac
acatttgttaagaaagatccgagcagcgcgaagatcattggctaccagaatcctaatcactg
gggccaagttaatgcctatatctataaggatgatggcggcagagctattgaactgacgggaa
gctggcctggaaaggccatgacaagaaatgcagatggcatttacacgctgacactgcctgct
gatacagatacaacaaacgcgaaggttatcttcaacaatggctcagctcaagtgcctggaca
aaatcaacctggatttgattacgtccaaaacggactgtataacaatagcggcctgtcaggca
gccttccgtactaa

[00307] The amino acid sequence of the BsuAmy3 precursor protein expressed from plasmid BsuAmy3 (aprE-*BsuAmy3*) is depicted in SEQ ID NO: 12. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold:

*Mrskklwisllfaltliftmafsnmsaqa***agk**epaaanaetadksneltapsikdgtilhaw
nwsfntlknnmkdihdagytaiqtspinqvkegnqgdksmsnwywlyqptsyqignrylgte
qefkemcaaaeeygvkvivdavinhttsdyaaisneiksipnwthgntqiknwsdrwdvtqn
sllglydwntqntqvqsylkrfleralndgadgfrfdaakhielpddgnygspfwpnltnts
aefqygeilqdsasrdaayanymnvtasnyghsmrsalrnrnlsvsnishyvsdvsadklvt
wveshdtyandeeestwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigdr
gsglfedqaivavntfhnvmdgqpeelsnpngnnqifmnqrgshglvlanagsssvtintst
klpdgsydnkagdgsfqvtngkltgtinarsvvviypddianaphvflenyktgvthsfndq
laitlradaktakavyqinngqetvfkdgdqltigkgapfgttytitlkgtnsdgvtrtqey

tfvkkdpssakiigyqnpnhwgqvnayiykddggraieltgswpgkamtrnadgiytltlpa
dtdttnakvifnngsaqvpgqnqpgfdyvqnglynnsglsgslpy

[00308] The nucleotide sequence of *BspAmy33* gene in plasmid BspAmy33 (aprE-*BspAmy33*) is depicted in SEQ ID NO: 13. The predicted signal sequence is shown in italics and the AGK-proAprE oligonucleotide is shown in bold:

gtgagaagcaaaaaattgtggatcagcttgttgtttgcgttaacgttaatctttacgatggc
gttcagcaacatgagcgcgcaggct**gctggaaaa**ggaagcgcagcagccgctaatgcgggct
caacgaacaagtcagatgagtatacagcaccgagcattgagtcaggcacgatcctgcacgca
tggaactggagctttaacacacttaaaaaccatatgaaagatattcacgatgccggctacac
agcgatccaaacgagccctgtcaaccaggtgaaagaaggcaacaacggagataaaggaatgg
caaactggtattggctgtaccaacctacgtcatatcaaatcggcaatagctaccttggaaca
gaggaagagttcaaagaaatgtgtgctatggctgaagagtatggagttaaggtgatcgtgga
tgctgtcattaatcatacaacatcagattatgccgccattagctcagaaattaagtcaatcc
ctaactggacacacggaaacacacaaatcaaaaattggtcagatagaaaggatgttacacaa
aattcactgctgggactgtatgactggaatacgcaaaatacacaggcccaatcatatctgaa
acattttctggagagagtcctgaatgatggagctgccggatttagatttgacgccgcgaaac
atattgaacttccggatgatggcgattatggaagcgacttctggcctaacatgacagataca
tcagcggagttccaatacggcgaaattctgcaagattcagcttcaagagacacggcatacgc
gaactacatgaatgttacggcaagcaactatggccacacaattagatcagcacttaagaata
gacacttctcaacgtcaaatatctcaaattatgcaagcgatgtccctgcagatgaccttgtt
acatgggttgaatcacatgatacatatgctaatgatgatgaacaatcaacatggatgagcga
tgacgatattagactgggatgggcagttatcgcgtcaagatcaggcagcacgccgcttttttt
tctcaagaccggagggcggaggcaatggagtgagattccctggcaaatcacaaattggagat
agaggcagcgcacttttcaaggatcaagctattacggctgttaataagtttcacaatgttat
gacgggcaaacctgaaaaactttcaaaccctaatggaaataaccaaatctttatgaacgaaa
gaggctcagatggcgttgtccttgcaaatgtgggaagctcaagcgtgagcattcacacatca
acagatcttcctgacggccactatgataataaggcgggagatggctcatttaaagtcacgaa
cggaaatctgacaggaacgattggctcaagagctgttgttgtgctttaccctgatgatattg
cgaatgcaccgaatgtctttattgagaactataaaacgggagtcacacactcattttatgat
gaccagctgacagttagccttagagccgacgccaaaattacaaaggcggcgtaccagattaa
caatggcgacgaggccgagtttaaggacggcgacagacttacaattggcaacggcgatcctt
ttgacacgacatataaagttacacttacgggaacgaactcaaacggagtttcaagaacaaat
gaatatacgttcgttaagacagatccttcatcagtgaaaacaatcggatatcagaacccgaa

taattggggacaagttaatgcctatatctacaaagagaatggaggacaagccattgagctta
caggatcatggccgggaaaagcaatgacacagaatgcggatggcatgtacacactgacactg
cctgcggatacggacacaacaggagcgaaagtcatcttcaataacggctcatcacaggttcc
tggcatgaatcagcctggatttgagtatgtcagaaacggcctgtatcacgatagcggactga
cgggatacctgccgaatagcggcagaacatttagataa

[00309] The amino acid sequence of the BspAmy33 precursor protein expressed from plasmid BspAmy33 (aprE-*BspAmy33*) is depicted in SEQ ID NO: 14. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold:

*Mrskklwisllfaltliftmafsnmsaqa***agk**gsaaaanagstnksdeytapsiesgtilha
wnwsfntlknhmkdihdagytaiqtspvnqvkegnngdkgmanwywlyqptsyqignsylgt
eeefkemcamaeeygvkvivdavinhttsdyaaisseiksipnwthgntqiknwsdrkdvtq
nsllglydwntqntqaqsylkhflervlndgaagfrfdaakhielpddgdygsdfwpnmtdt
saefqygeilqdsasrdtayanymnvtasnyghtirsalknrhfstsnisnyasdvpaddlv
twveshdtyanddeqstwmsdddirlgwaviasrsgstplffsrpegggngvrfpgksqigd
rgsalfkdqaitavnkfhnvmtgkpeklsnpngnnqifmnergsdgvvlanvgsssvsihts
tdlpdghydnkagdgsfkvtngnltgtigsravvvlypddianapnvfienyktgvthsfyd
dqltvslradakitkaayqinngdeaefkdgdrltignqdpfdttykvtltgtnsngvsrtn
eytfvktdpssvktigyqnpnnwgqvnayiykenggqaieltgswpgkamtqnadgmytltl
padtdttgakvifnngssqvpgmnqpgfeyvrnglyhdsgltgylpnsgrtfr

[00310] The nucleotide sequence of *BsuAmy4* gene in plasmid BsuAmy4 (aprE-*BsuAmy4*) is depicted in SEQ ID NO: 15. The predicted signal sequence is shown in italics and the AGK-proAprE oligonucleotide is shown in bold:

gtgagaagcaaaaaattgtggatcagcttgttgtttgcgttaacgttaatctttacgatggc
gttcagcaacatgagcgcgcaggct**gctggaaaa**gaaacagcgaacaaaagcaacgaagtta
cagatagctcagtcaaaaatggcacaattctgcatgcatggaattggagctttaacacgctg
aaacataacatgaaagacatccatgatgcaggctatacagcaattcaaacaagcccgattaa
tcaggtcaaagaaggcaatcaaggcaataaatcaatgccgaattggtattggctgtatcaac
cgacatcatatcagatcggcaatcattatctgggcacggaacaagaatttaaagaaatgtgc
gcagcagcggaagaatatggcgttaaagttattgttgatgcggtcatcaatcatacgacatc
agattatgcagcgatcagcaatgaaatcaaaagcattccgaattggacacatggcaacacac
aaatcaaaaattggagcgatagatgggatgtcacacaaaatgcactgctgggcctgtatgat
tggaatacacaaaatacagaagtccagacgtacctgaaaagatttctggaacgcgcactgga
tgatggcgcagatggctttagatatgatgcagcaaaacatattgaactgccggatgatggaa

attatggctcacaattttggccgaacattacaaatacaagcgcagaatttcagtacggcgaa
attcttcaagattcagcatcaagagatgcagcgtatgcgaattatatgaatgttacagcaag
caactacggccattcaattagaagcgcactgaaaaatagaaatctgagcgtcagcaacatta
gccattatgcatcagatgttagcgcagataaactggttacatgggttgaaagccatgataca
tatgcgaacgatgatgaagaatcaacgtggatgtcagatgatgatattcgcctgggctgggc
agttattggctcaagatcaggctcaacaccgctgtttttttcaagaccggaaggcggaggca
atggcgttagatttccgggaaaaagccaaattggcgatagaggctcagcactgtttaaagat
caagcaattacagcggtcaaccagtttcataatgaaatggcaggccaaccggaagaactgtc
aaatccgaatggcaataaccagatctttatgaatcaaagaggcagcaaaggcgttgttctgg
caaatgcaggctcatcatcagtttcaattaatgcgtcaacaaaacttccggatggcagctat
gataataaagcaggcacaggctcatttcaagttagagatggcaaactgacaggcacaattaa
cgcaagatcagttgcagttctgtatctggatgatattgcaaatgcaccgcaagtctttctgg
aaaactataaagctggcgtcacacatagctttaatgatcagctgacaattacactgagagcg
gatgcaaatacaacaaaagcggtctatcaaatcaacaacggccaagaaacagcatttaaaga
tggcgatcaatttacgatcggcaaagaagatccgtttggcaaaacgtatacaatcatgctga
aaggcacaaattcagatggcgtgacacgcattgaagaatacagctttgttaaaagagatccg
gcaagcgcaaaaacaattggctatcagaatccgaatcattggagccaagttaacgcgtatat
ctataaacaagatggcggaggcgcaattgaacttacaggcagctggcctggcaaaccgatga
caaaaaatgcagatggaatctatacactgacacttccggctgatacagatacaacgaatgcg
aaagtcatttttaacaatggctcagcgcaagttccgggacaaaatcaaccgggatttgatta
tgttcagaacggcctgtataatgatgctggcctgtcaggctcactgccgtat

[00311] The amino acid sequence of the BsuAmy4 precursor protein expressed from plasmid BsuAmy4 (aprE-*BsuAmy4*) is depicted in SEQ ID NO: 16. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold:

*Mrskklwisllfaltliftmafsnmsaqa***agk**ETANKSNEVTDSSVKNGTILHAWNWSFNTL
KHNMKDIHDAGYTAIQTSPINQVKEGNQGNKSMPNWYWLYQPTSYQIGNHYLGTEQEFKEMC
AAAEEYGVKVIVDAVINHTTSDYAAISNEIKSIPNWTHGNTQIKNWSDRWDVTQNALLGLYD
WNTQNTEVQTYLKRFLERALDDGADGFRYDAAKHIELPDDGNYGSQFWPNITNTSAEFQYGE
ILQDSASRDAAYANYMNVTASNYGHSIRSALKNRNLSVSNISHYASDVSADKLVTWVESHDT
YANDDEESTWMSDDDIRLGWAVIGSRSGSTPLFFSRPEGGGNGVRFPGKSQIGDRGSALFKD
QAITAVNQFHNEMAGQPEELSNPNGNNQIFMNQRGSKGVVLANAGSSSVSINASTKLPDGSY
DNKAGTGSFQVRDGKLTGTINARSVAVLYLDDIANAPQVFLENYKAGVTHSFNDQLTITLRA
DANTTKAVYQINNGQETAFKDGDQFTIGKEDPFGKTYTIMLKGTNSDGVTRIEEYSFVKRDP

ASAKTIGYQNPNHWSQVNAYIYKQDGGGAIELTGSWPGKPMTKNADGIYTLTLPADTDTTNA
KVIFNNGSAQVPGQNQPGFDYVQNGLYNDAGLSGSLPY

EXAMPLE 3

Purification of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

3.1 Purification of BpuAmy1

[00312] BpuAmy1 was purified via two chromatography steps: hydrophobic interaction and anion exchange chromatography. About 700 mL crude broth from shake flask was added ammonium sulphate to a final concentration of 1 M. This sample was then loaded onto a 60 mL phenyl-HP Sepharose column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate pH 7.5 with 1 M ammonium sulphate (buffer A). After washing with the same buffer for 2 column volumes (CVs), the column was eluted stepwise with 80%, 60%, 40%, 20%, and 0% buffer A in 2 CVs each, followed by 2 CVs of MilliQ H_2O. All fractions were analysed for amylase activity and SDS-PAGE. The target protein was mainly present in the 40% buffer A eluted fractions, which were pooled and dialyzed against 20 mM sodium phosphate pH 7.5 (buffer B). The desalted sample was then loaded onto a 30 mL Q-HP Sepharose column (GE Healthcare) in buffer B. The column was eluted stepwise with 0.1, 0.2, 0.3 and 1 M NaCl in buffer B in 2 CVs each. The target protein was present in the 0.3 M NaCl eluted fractions according to the activity assay and SDS-PAGE results. The fractions containing the target protein was concentrated and buffer-exchanged to 20 mM HEPES pH 8, 2 mM $CaCl_2$ using the 10 KDa Amicon Ultra-15 devices. The protein concentration was measured using Nanodrop 1000. The final product was stored in 40% glycerol at -20 °C until usage.

3.2 Purification of BsuAmy3

[00313] BsuAmy3 was purified via three chromatography steps: hydrophobic interaction, anion exchange and another hydrophobic interaction chromatography. About 700 mL crude broth from shake flask was added ammonium sulphate to a final concentration of 1 M. This sample was then loaded onto a 40 mL phenyl-HP Sepharose column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate pH 7.5 with 1 M ammonium sulphate (buffer A). After washing with the same buffer for 3 column volumes (CVs), the column was eluted

stepwise with 80%, 60%, 40% buffer A in 3 CVs each, followed by 4 CVs of MilliQ H_2O. All fractions were analysed for amylase activity and SDS-PAGE. The target protein was mainly present in the 60% and 40% buffer A eluted fractions, which were pooled and dialyzed against 20 mM sodium phosphate pH 7.5 (buffer B). The desalted sample was then loaded onto a 30 mL Q-HP Sepharose column (GE Healthcare) in buffer B. The column was eluted stepwise with 0.1, 0.15, 0.2 and 1 M NaCl in buffer B in 3 CVs each. The target protein was present in the 0.15 M NaCl eluted fractions according to the activity assay and SDS-PAGE results. The fractions containing the target protein were pooled and further polished by phenyl-HP column as in the first step. The purified protein was concentrated and buffer-exchanged to 20 mM HEPES pH 8, 2 mM $CaCl_2$ using the 10 KDa Amicon Ultra-15 devices. The protein concentration was measured using Nanodrop 1000. The final product was stored in 40% glycerol at -20 °C until usage.

3.3 Purification of BspAmy33

[00314] BspAmy33 was purified via three chromatography steps: hydrophobic interaction, anion exchange, and size exclusion chromatography. Ammonium sulphate was added to 700 mL crude broth from shake flask to a final concentration of 1 M. The solution was then loaded onto a 50 mL phenyl-HP Sepharose column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate pH 7.5 with 1 M ammonium sulphate (buffer A). After washing with the same buffer for 4 column volumes (CVs), the column was eluted stepwise with 20% and 0% buffer A in 4 CVs each, followed by 4 CVs of MilliQ H_2O. All fractions were analysed for amylase activity and SDS-PAGE. The target protein was mainly present in the 20% buffer A eluted fractions, which were pooled and dialyzed against 20 mM HEPES pH 7.0 (buffer B). The desalted sample was then loaded onto a 40 mL Q-FF Sepharose column (GE Healthcare) in buffer B. The column was eluted with a gradient of 0 – 0.5 M NaCl in buffer B in 20 CVs. The fractions containing the target protein was concentrated to less than 10 mL, and then loaded onto a Superdex 200 XK26/60 column in 20 mM HEPES pH 7.0 with 0.15 M NaCl. The fractions containing the target protein was concentrated and buffer-exchanged to 20 mM HEPES pH 8, 2 mM $CaCl_2$ using the 10 KDa Amicon Ultra-15 devices. The protein concentration was measured using Nanodrop 1000. The final product was stored in 40% glycerol at -20 °C until usage.

3.4 Purification of BsuAmy4

[00315] The 700 ml crude sample of BsuAmy4 from shake flask fermentation was concentrated, and ammonium sulfate was added to a final concentration of 1 M. The solution was loaded onto a HiPrep™ Phenyl FF column pre-equilibrated with 20 mM sodium acetate (pH 5.0) supplemented with additional 1 M ammonium sulfate. The target protein was eluted from the column with 20 mM sodium acetate (pH 5.0) buffer without ammonium sulfate. The corresponding active fractions were pooled, concentrated and buffer exchanged into 20 mM sodium phosphate (pH6.0) (Buffer A), using a VivaFlow 200 ultra-filtration device (Sartorius Stedim). The resulting solution was applied to a HiPrepTM Q FF 16/10 column pre-equilibrated with Buffer A. The target protein was eluted from the column with a linear salt gradient from 0 to 0.5 M NaCl in buffer A. The resulting active protein fractions were then pooled and concentrated via the 10K Amicon Ultra devices, and stored in 40% glycerol at -20 °C until usage.

EXAMPLE 4

Potato amylopectin-hydrolyzing activity of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

[00316] Alpha-amylase activity was determined using a colorimetric assay to monitor the release of reducing sugars from potato amylopectin for BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4, respectively. AmyE-FL and AmyE-Tr (described in US9040279) were included as controls. The activity was reported as equivalents of glucose released per minute. Substrate solutions were prepared by mixing 9 mL of 1% (w/w, in water) potato amylopectin (Sigma, Cat. No. 10118), 1 mL of 0.5 M buffer (pH 5.0 sodium acetate or pH 8.0 HEPES), and 40 μL of 0.5 M $CaCl_2$ into a 15-mL conical tube. Stock solutions of purified alpha-amylase samples were made by diluting original samples to 0.4 mg/mL (400 ppm) in water. Serial dilutions of enzyme samples and glucose standard were prepared in water in non-binding microtiter plates (MTP, Corning 3641). Then 90 μL of substrate solution (preincubated at 50 °C for 5 min at 600 rpm) and 10 μL of the enzyme serial dilution were added and mixed in non-binding microtiter plates (MTP, Corning 3641). All the incubations were done at 50 °C for 10 min at 600 rpm in a thermomixer (Eppendorf). After incubation, 50 μL of 0.5 N NaOH were added to each well to stop the reaction. Total reducing sugars

present in each well were measured using a PAHBAH method: 80 µL of 0.5 N NaOH was aliquoted into a microtiter plate, followed by the addition of 20 µL of PAHBAH reagent [5% w/v 4-hydroxybenzoic acid hydrazide in 0.5 N HCl] and 10 µL of each reaction mixture. Plates were incubated at 95 °C for 5 min and cooled down at 4 °C for 5 sec. Samples (80 µL) were then transferred to polystyrene microtiter plates [Costar 9017] and absorbance was read at 410 nm. Resulting absorbance values were plotted against enzyme concentration and linear regression was used to determine the slope of the linear region of the plot.

Specific Activity (U/mg) = Slope (enzyme) / slope (std) * 100

Define: 1 U = 1 µmol glucose equivalent/min

Table 2. Specific activities of BpuAmy1, BsuAmy3, BspAmy33, BsuAmy4, AmyE-FL, and AmyE-Tr

Enzyme Name	Spec. Activity (U/mg)	
	pH 5	pH 8
BpuAmy1	725.3	566.6
BsuAmy3	974.6	623.2
BspAmy33	688.6	393.0
BsuAmy4	752.0	699.0
AmyE-FL	799.8	547.8
AmyE-Tr	773.1	532.5

EXAMPLE 5

pH profiles of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

[00317] The effect of pH (from 3.0 to 10.0) on alpha-amylase activity was monitored using potato amylopectin (Sigma, Cat. No. 10118) (1% in water, w/w) as a substrate. Buffer working solutions consisted of the combination of glycine/sodium acetate/HEPES (250 mM), with pH varying from 3.0 to 10.0. Substrate solutions were prepared by mixing 896 µL of 1% (w/w, in water) potato amylopectin, 100 µL of 250 mM buffer working solution (pH from 3.0 to 10.0), and 4 µL of 0.5 M CaCl2. Enzyme working solutions were prepared in water at a certain dose (showing signal within the linear range as per dose response curve). All the incubations were done using the same protocol as described for alpha-amylase activity assay in Example 4. Enzyme activity at each pH was reported as relative activity compared to

enzyme activity at optimum pH. The pH profiles of these alpha-amylases were shown in Table 3.

Table 3. pH profiles of BpuAmy1, BsuAmy3, BspAmy33, BsuAmy4, AmyE-FL, and AmyE-Tr

pH	Relative Activity (%)					
	BpuAmy1	BsuAmy3	BspAmy33	BsuAmy4	AmyE-FL	AmyE-Tr
3	4	5	0	5	4	3
4	84	80	89	75	74	74
5	97	100	98	95	97	90
6	98	99	100	99	98	100
7	100	91	96	100	100	95
8	79	65	67	85	69	73
9	40	26	22	42	31	38
10	8	4	4	12	10	13

EXAMPLE 6

Temperature profile of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

[00318] The effect of temperature (from 30 to 80 °C) on alpha-amylase activity was monitored using potato amylopectin (Sigma, Cat. No. 10118) (1% in water, w/w) as a substrate.. Substrate solutions were prepared by mixing 3.6 mL of 1% (w/w, in water) potato amylopectin (Sigma, Cat. No. 10118), 0.4 mL of 0.5 M pH 5.0 sodium acetate buffer, and 16 μL of 0.5 M $CaCl_2$ into a 15-mL conical tube. Enzyme working solutions were prepared in water at a certain dose (showing signal within the linear range as per dose response curve). Incubations were done at temperatures from 30 to 80 °C, respectively, for 10 min in a PCR machine (T100 Thermal Cycler, Biorad). After incubation, samples were quenched and measured using the same protocol as described for alpha-amylase activity assay in Example 4. Activity at each temperature was reported as relative activity compared to enzyme activity

at optimum temperature. The temperature profiles of these alpha-amylases were shown in Table 4.

Table 4. Temperature profiles of BpuAmy1, BsuAmy3, BspAmy33, BsuAmy4, AmyE-FL, and AmyE-Tr

Temp. (°C)	Relative Activity (%)					
	BpuAmy1	**BsuAmy3**	**BspAmy33**	**BsuAmy4**	**AmyE-FL**	**AmyE-Tr**
30	2	3	3	5	13	9
40	34	33	33	35	51	56
45	51	52	54	55	65	76
50	68	77	68	73	84	100
60	100	100	100	100	100	91
70	57	87	61	94	88	66
75	24	54	31	54	57	32
80	1	13	2	11	18	8

EXAMPLE 7

Thermostability of BpuAmy1, BsuAmy3, BspAmy33, and BsuAmy4

[00319] Thermostability of alpha-amylases was determined by measuring the enzyme activity before and after enzyme samples pre-incubated at temperatures from 40 to 90 °C for 2 h. Enzymes were diluted to 10 ppm in 50 mM of sodium acetate buffer (pH 5.0) containing 2 mM of $CaCl_2$, and 40 µL was aliquot to PCR strip tubes. The tubes were transferred to a PCR machine at the desired temperature from 40 to 90 °C. After 2 hours preincubation, the enzymes were diluted to appropriate concentrations (showing signal within linear range as per dose response curve) and the residual activity of the enzyme after heat stress was assayed using the amylopectin/PAHBAH method as described in Example 4. The residual activities were converted to percentages of relative activity by defining the activity of the sample kept on ice as 100%. The results are shown in Table 5.

Table 5. Thermostability of BpuAmy1, BsuAmy3, BspAmy33, BsuAmy4, AmyE-FL, and AmyE-Tr

Temp. (°C)	Residual Activity (%)					
	BpuAmy1	BsuAmy3	BspAmy33	BsuAmy4	AmyE-FL	AmyE-Tr
40	88	93	87	76	72	81
45	73	87	79	49	64	68
50	36	61	42	27	44	39
55	7	24	7	12	6	11
60	0	2	2	0	2	1
65	0	0	1	0	1	0
70	0	1	1	0	0	0
75	0	1	0	0	0	0
80	0	0	0	0	0	0
85	0	0	0	0	0	0
90	0	1	0	0	0	0

EXAMPLE 8

Rapid Viscosity Analyzer (RVA) assay

[00320] The goal of Rapid Viscosity Analyzer (RVA) assay is to evaluate the liquefying performance of alpha-amylases by measuring their peak and final viscosities after incubation with corn flour. This is done by determining the peak and final viscosities of each sample at pH 5.8, 85 °C with the enzyme dosed at 70 µg.

Prior to each viscometer run, 33 g of 25% dry solids (ds) corn flour slurry was freshly prepared by mixing 9.16 g of corn flour (9.9% moisture content) and 23.84 g of Milli Q water.The pH of the slurry was adjusted to 5.8 with addition of 15 µL of 2 N sulfuric acid. Enzyme sample (70 µg) was then added to start the RVA run. A typical RVA run was 10 min total time. The temperature was held at 70 °C for 1 min, ramped to 85 °C for 1 min 20 sec, and then held at 85 °C for 7 min 40 sec. The results of RVA test on these alpha-amylases are summarized in Table 6.

Table 6. Summary of viscosities (peak and final) of RVA tests

Gene Name	Dosage (μg)	Peak Viscosity	Final Viscosity
BpuAmy1	70	16974	4669
BsuAmy3	70	11705	2595
BspAmy33	70	18846	6377
BsuAmy4	70	13394	3909
AmyE-FL	70	11983	2845
AmyE-Tr	70	9444	2774

EXAMPLE 9

Raw starch assay

[00321] The goal of raw starch assay is to evaluate alpha-amylase's capability of hydrolyzing raw starch against the benchmark *Aspergillus kawachi* amylase (AkAA) for granular starch hydrolyzing enzyme (GSHE) activity and direct starch to glucose/maltose activity (DSTG/DSTM). Alpha-amylase and glucoamylase were blended at a ratio of 1:6.6 in this assay. For alpha-amylase evaluation, TrGA was fixed as glucoamylase in the GA + AA blend. A Rezex™ RFQ-Fast Acid H+ (8%), LC Column (100 x 7.8 mm) (Phenomenex, Cat. No. 00D-0223-K0) was selected for sugar profile analysis after incubation and glucose (final product) amount was used to calculate PIs (Performance Indexes) of the candidates against AkAA.

[00322] To conduct the assay, firstly 150 μL of the substrate (1% corn starch in 50 mM pH 3.5/pH 4.5 sodium acetate buffer) was dispensed into Nunc U96PP 0.5 mL plates while stirring. Wide bore tips were required for transferring substrate. Then 10 μL of alpha-amylase and 10 μL of TrGA were added. The final dosages for alpha-amylase and TrGA were 10 ppm and 1.5 ppm, respectively. The incubations were done in iEMS (32 °C, 900 rpm) for 6, 20 and 28 h, respectively. To quench the reaction, 50 μL of 0.5 M NaOH was added and the starch plug was suspended by putting the plate on a shaker for 2 min. After that, the plate was sealed with a Biorad seal and centrifuged at 2500 rpm for 3 min. For HPLC analysis, the supernatant was diluted by a factor of 10 using 0.01 N H_2SO_4. The diluted supernatant was filtered and 10 μL of the solution was injected into an Agilent 1200 series HPLC equipped with a refractive index detector. The column used was a Phenomenex Rezex-RFQ Fast Fruit column (cat# 00D-0223-K0) with a Phenomenex Rezex ROA Organic Acid guard column (cat# 03B-0138-

K0). The mobile phase was 0.01 N H_2SO_4, and the flow rate was 1.0 mL/min at 80 °C. Table 7 showed the raw starch hydrolyzing capability of these alpha-amylases. Most of them exhibited better performance than AkAA at pH 4.5.

Table 7. Raw starch assay results

GA	AA	pH3.5			pH4.5		
		PI (AkAA+TrGA=1.0)			PI (AkAA+TrGA=1.0)		
		6 h	20 h	28 h	6 h	20 h	28 h
TrGA+	BpuAmy1	0.69	0.38	0.33	1.23	1.19	1.14
	BsuAmy3	0.99	0.56	0.51	1.46	1.36	1.27
	BspAmy33	0.50	0.29	0.26	1.23	1.13	1.10
	BsuAmy4	0.65	0.42	0.37	1.38	1.31	1.25
	AmyE-FL	0.94	0.58	0.50	1.58	1.42	1.31
	AmyE-Tr	0.60	0.34	0.30	0.93	0.89	0.90

CLAIMS

1. An isolated, synthetic, or recombinant polypeptide having alpha-amylase activity, selected from the group consisting of:

a) a polypeptide comprising an amino acid sequence that is at least about 85% identical to that of SEQ ID NO: 10, at least about 92% identical to that of SEQ ID NO: 12, at least about 83% identical to that of SEQ ID NO: 14, or at least about 94% identical to that of SEQ ID NO: 16; and

b) a polypeptide comprising an amino acid sequence that is at least about 87% identical to that of amino acids 1-440 of SEQ ID NO: 10; at least 94% identical to that of amino acids 1-440 of SEQ ID NO: 12; at least 87% identical to that of amino acids 1-441 of SEQ ID NO: 14; or at least 94% identical to that of amino acids 1-433 of SEQ ID NO: 16; and

c) a polypeptide encoded by a polynucleotide that hybridizes under at least medium-high stringency conditions with the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (ii) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (iii) a full-length complementary strand of (i) or (ii); or under at least high stringency conditions with (iv) the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; (v) a polypeptide encoded by a genomic DNA sequence comprising the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; or (vi) a full-length complementary strand of (iv) or (v); and

d) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least about 85%, at least about 90%, at least about 95% identical to the polypeptide coding sequence of SEQ ID NO: 1, 3, 5 or 7; and

e) a variant comprising a substitution, deletion, and/or insertion of one or more (several) amino acids of the polypeptide of SEQ ID NO: 10, 12, 14, or 16; and

f) the polypeptide of (a), (b), (c), (d), or (e) having alpha-amylase activity but lacking a signal sequence or a carbohydrate binding module; and

g) an active fragment of the polypeptide of (a), (b), (c), (d), (e), or (f) having alpha-amylase activity.

2. The polypeptide of claim 1, further comprising a heterologous amino acid sequence.

3. The polypeptide of claim 2, wherein the heterologous amino acid sequence comprises:

a) a heterologous signal sequence, a heterologous carbohydrate binding module, a heterologous catalytic domain (CD), or a combination thereof;

b) the sequence of a), wherein the heterologous signal sequence, carbohydrate binding module or heterologous catalytic domain (CD) is derived from a heterologous enzyme, a tag, an epitope, a targeting peptide, a cleavable sequence, a detectable moiety or an enzyme; or

c) the sequence of a), wherein the heterologous carbohydrate binding module (CBM) comprises is a xylan binding module, a cellulose binding module, a lignin binding module, a xylose binding module, a mannan binding module, a xyloglucan-specific module arabinofuranosidase binding module, or another carbohydrate binding module.

4. A polynucleotide encoding the polypeptide of any one of claims 1-3.

5. The polynucleotide of claim 4, wherein the polynucleotide hybridizes under stringent conditions to a nucleic acid that is complementary to a polynucleotide sequence of SEQ ID NO: 1, 3, 5 or 7.

6. The polynucleotide of claim 4 or 5, comprising a polynucleotide sequence that is at least about 85%, at least about 90%, at least about 95% identical to that of SEQ ID NO:1, 3, 5 and 7, together with at least one transcriptional or translational regulatory sequence that allows the polynucleotide sequence to be expressed by a host cell.

7. The polynucleotide of claim 6, wherein the transcriptional or translational regulatory sequence is one that is heterologous to the microorganism from which the polynucleotide sequence is derived.

8. A vector comprising the polynucleotide of any one of claims 4-7.

9. A host cell comprising the vector of claim 8.

10. The host cell comprising the polynucleotide of any one of claims 4-7.

11. The host cell of claim 9 or 10, which is a *Trichoderma, Aspergillus* or *Myceliopthora* cell.

12. The host cell of claim 9 or 10, which is an *E.coli, Bacillus, Streptomyces,* or *Pseudomonas* cell.

13. The host cell of claim 9 or 10, which is an ethanologenic microorganisms.

14. The host cell of any one of claims 9-13, which further expresses and secretes one or more starch degrading enzymes, and one or more additional enzymes selected from the group comprising protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, phytase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, transferrase.

15. The host cell of any one of claims 9-13, which can be fermented to produce ethanol, other biochemicals, or biomaterials.

16. A composition comprising the polypeptide having alpha-amylase activity of any one of claims 1-3.

17. The composition of claim 16, wherein the composition is for liquifying a composition comprising starch.

18. The composition of claim 17, wherein the liquefaction comprises a primary and/or secondary liquefaction step, further comprising adding additional substrate to the slurry in the primary and/or secondary liquefaction step.

19. The composition of claim 18, wherein the composition is for liquifying pullulan, or a composition comprising starch and pullulan.

20. The composition of any one of claims 17-19, wherein the composition is for producing a fermented beverage.

21. The composition of any one of claims 17-19, wherein the composition is for producing a baked food product.

22. The composition of claim 16, wherein the composition is for saccharifying a composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

23. The composition of claim 16, wherein the composition is for raw starch hydrolysis process.

24. The composition of claim 16, wherein the composition is for treating the fermented mash.

25. The composition of claim 24, wherein the fermented mash is derived from a process of producing a fermentation product utilizing starch-containing material as feedstock.

26. The composition of claim 24 or 25, wherein the fermented mash comprises beer well, whole stillage, thin stillage, wet cake, DDG or DDGS.

27. The composition of claim 16, wherein the composition can be used in dry milling or grinding, or wet milling.

28. The composition of claim 16, wherein the composition is effective for removing starchy stains from laundry, dishes, textiles, or hard surfaces.

29. The composition of claim 28, further comprising a surfactant.

30. The composition of claim 28 or 29, wherein the composition is a detergent composition.

31. The composition of any one of claims 28-30, wherein the composition is selected from the group comprising a laundry detergent, a laundry detergent additive, a manual or automatic dishwashing detergent, or a dishwashing machine cleaning composition.

32. The composition of any one of claims 28-31, further comprising one or more additional enzymes selected from the group comprising protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, phytase, hyaluronidase, chondroitinase, laccase, transferase and an alpha-amylase other than the alpha-amylase of any one of claims 1-3.

33. The composition of claim 16, wherein the composition is for textile desizing.

34. A method for liquefying a composition comprising starch comprising:

a) adding a polypeptide having alpha-amylase activity of any one of claims 1-3; and

b) liquefying the composition comprising starch to form a solubilized liquid or a slurry.

35. The method of claim 34, wherein the starch is obtained from plant material, corn, milo, rye, barley, wheat, sorghum, oats, rice, brans, cassava, millet, potato, sweet potato, or tapioca.

36. The method of claim 35, wherein the starch is granular starch from either a dry milling or grinding, or wet milling process.

37. The method of any one of claims 34-36, further comprising a primary and/or secondary liquefaction step, further comprising adding additional substrate to the slurry in the primary and/or secondary liquefaction step.

38. The method of any one of claims 34-37, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of claims 1-3, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch comprising solution.

39. Liquefied starch produced by the method of any one of claims 34-38.

40. A method of saccharifying a composition comprising starch to produce a composition comprising glucose, wherein the method comprises:

a) contacting the composition comprising starch with effective amount of the polypeptide having alpha-amylase activity of any one of claims 1-3; and

b) saccharifying the composition comprising starch to produce the composition comprising glucose; wherein the alpha-amylase catalyzes the saccharification of the starch to glucose.

41. The method of claim 40, wherein the composition comprising starch comprises liquefied starch, gelatinized starch, or granular starch.

42. The method of claim 40 or 41, further comprising fermenting the glucose composition to produce an end of fermentation (EOF) product.

43. The method of claim 42, wherein the fermentation is a simultaneous saccharification and fermentation (SSF) reaction.

44. The method of claim 42 or 43, wherein the EOF product comprises ethanol.

45. The method of claim 42 or 43, wherein the EOF product comprises a metabolite.

46. The method of claim 45, wherein the metabolite is citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, an organic acid, glucono delta-lactone, sodium erythorbate, omega 3 fatty acid, butanol, iso-butanol, an amino acid, lysine, tyrosine, threonine, glycine, itaconic acid, 1,3-propanediol, vitamins, or isoprene or other biochemicals or biomaterials.

47. The method of any one of claims 40-46, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of claims 1-3, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch comprising composition.

48. The method of any one of claims 40-47, wherein the composition comprising starch further comprises pullulan, and wherein the alpha-amylase catalyzes the saccharification of the pullulan.

49. The method of any one of claims 34-48, wherein the alpha-amylase is expressed and secreted by a host cell.

50. The method of claim 49, wherein the composition comprising starch is contacted with the host cell.

51. The method of claim 49 or 50, wherein the host cell further expresses and secretes a glucoamylase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of claims 1-3 or other enzymes.

52. The method of any one of claims 49-51, wherein the host cell is capable of fermenting the composition.

53. A method for removing a starchy stain or soil from a surface, comprising:

a) contacting the surface with a composition comprising an effective amount of the polypeptide having alpha-amylase activity of any one of claims 1-3; and

b) allowing the alpha-amylase to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in aqueous solution; thereby removing the starchy stain from the surface.

54. The method of claim 53, wherein the aqueous composition further comprises a surfactant.

55. The method of claim 53 or 54, wherein the surface is a textile surface or a surface on dishware.

56. The method for any of claims 53-55, wherein the composition further comprises at least one additional enzymes selected from protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, transferase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, beta-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, phytase, metalloproteinase, amadoriase, and an alpha-amylase other than the alpha-amylase of any one of claims 1-3.

57. A method for desizing a textile comprising:

a) contacting a sized textile with an effective amount of the polypeptide having alpha-amylase activity of any one of claims 1-3; and

b) allowing the alpha-amylase to hydrolyze starch components in the size to produce smaller starch-derived molecules that dissolve in aqueous solution; thereby removing the size from the textile.

58. A method for preparing a foodstuff or beverage comprising:

a) contacting a foodstuff or beverage comprising starch with an effective amount of the polypeptide having alpha-amylase activity of any one of claims 1-3; and

b) allowing the alpha-amylase to hydrolyze the starch to produce smaller starch-derived molecules.

59. The method of claim 58, further comprising contacting the foodstuff or beverage with glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, beta-amylase, alpha-amylase that is other than the alpha-amylase of any one of claims 1-3, protease, cellulase, hemicellulase, lipase,

cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, α-glucosidase, beta-glucosidase, or a combination thereof.

60. A foodstuff or beverage produced by the method of claim 58 or 59.

61. A method of making the polypeptide of any one of claims 1-3 or the composition of any one of claims 16-33 comprising cultivating the host cell of any one of claims 9-15.

62. The method of claim 61, further comprising a step during which the polypeptide is recovered, enriched and/or purified.

63. A whole broth formulation or cell culture composition comprising the polypeptide of any one of claims 1-3.

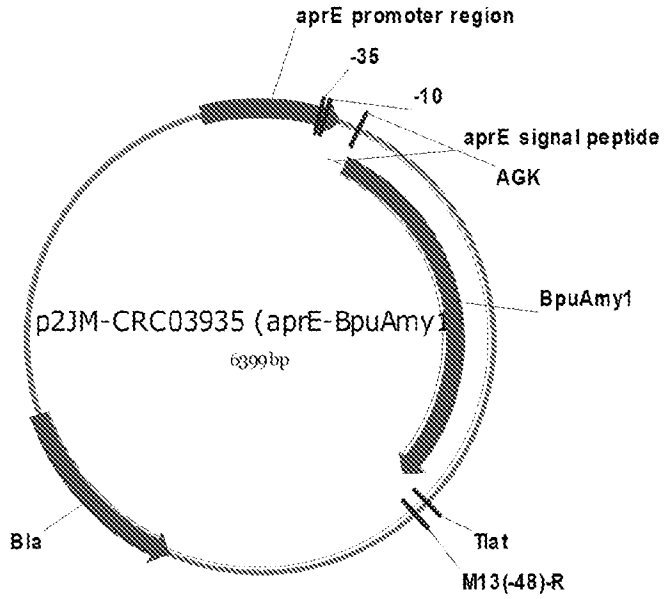

Figure 1. Plasmid map of p2JM-CRC03935 (aprE-*BpuAmy1*)

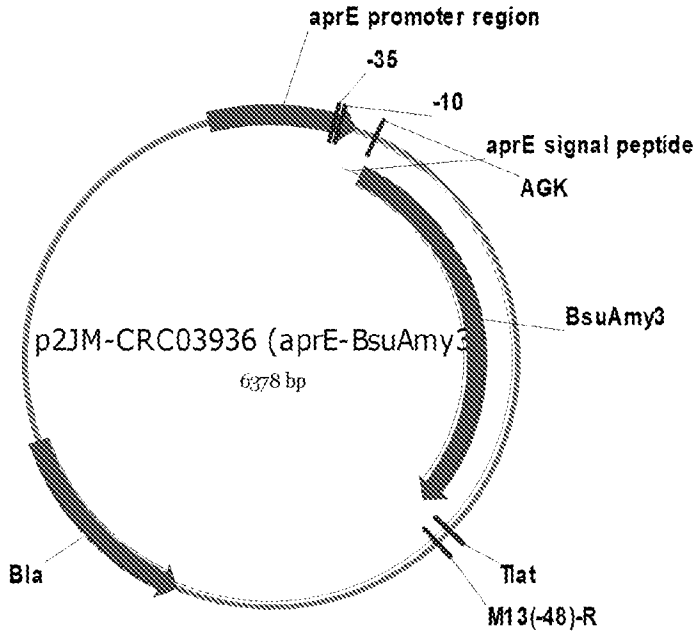

Figure 2. Plasmid map of p2JM-CRC03936 (aprE-*BsuAmy3*)

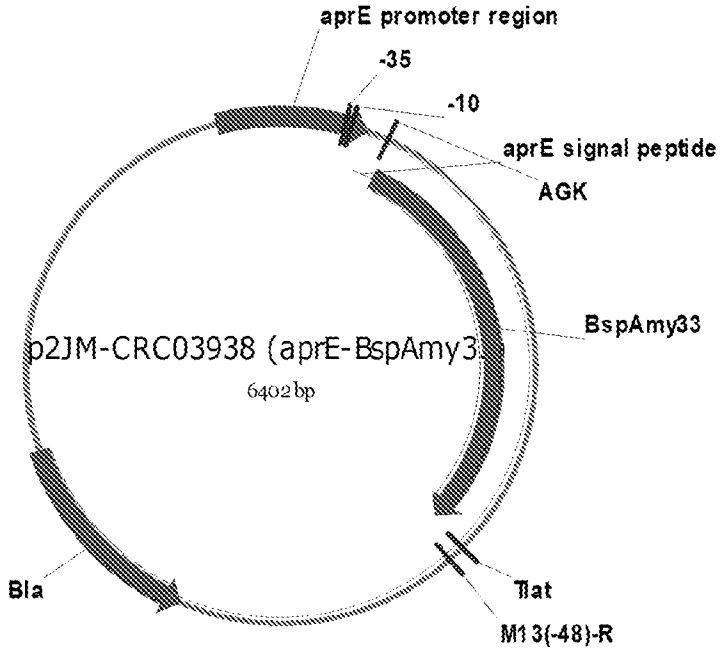

Figure 3. Plasmid map of p2JM-CRC03938 (aprE-*BspAmy33*)

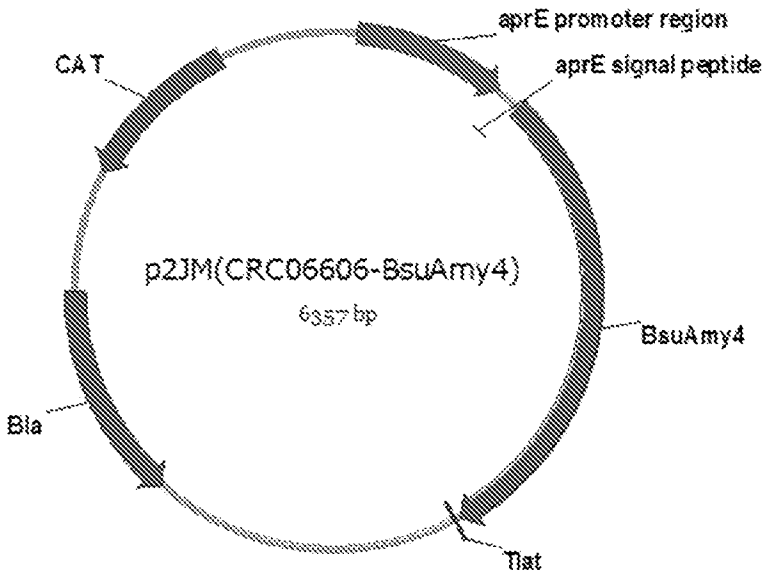

Figure 4. Plasmid map of p2JM-CRC06606 (aprE-*BsuAmy4*)

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13ter.1(c) and Rule 39)

Applicant's or agent's file reference NB40774WOPCT2	IMPORTANT DECLARATION	Date of mailing *(day/month/year)* 28 July 2017 (28-07-2017)
International application No. PCT/US2017/025217	International filing date *(day/month/year)* 30 March 2017 (30-03-2017)	(Earliest) Priority date *(day/month/year)* 1 April 2016 (01-04-2016)
International Patent Classification (IPC) or both national classification and IPC C12N9/2414; C12Y302/01001; A21D8/00		
Applicant DANISCO US INC		

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below

1. ☐ The subject matter of the international application relates to:
   - a. ☐ scientific theories.
   - b. ☐ mathematical theories
   - c. ☐ plant varieties.
   - d. ☐ animal varieties.
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
   - f. ☐ schemes, rules or methods of doing business.
   - g. ☐ schemes, rules or methods of performing purely mental acts.
   - h. ☐ schemes, rules or methods of playing games.
   - i. ☐ methods for treatment of the human body by surgery or therapy.
   - j. ☐ methods for treatment of the animal body by surgery or therapy.
   - k. ☐ diagnostic methods practised on the human or animal body.
   - l. ☐ mere presentations of information.
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.

2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
   ☐ the description ☐ the claims ☐ the drawings

3. ☒ The failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions prevents a meaningful search from being carried out:
   ☐ the written form has not been furnished or does not comply with the standard.
   ☒ the computer readable form has not been furnished or does not comply with the standard.

4. Further comments:

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 203

The claims 1-63 of the present application have, according to Rule 13eter. 1(d) PCT, not been searched since the sequence disclosed in the application does not comply with WIPO Standard ST.25 as prescribed under Rule 5.2 PCT. A Standard-compliant sequence listing has not been furnished in text (TEXT) format and the applicant has not remedied the deficiencies within the time limit fixed in the invitation pursuant to Rule13ter.1(a)PCT (see Guidelines for Search and Examination at the EPO as PCT Authority, B-VIII,3.2).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.