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(54) Title: *PAENIBACILLUS CURDLANOLYTICUS* AMYLASE, AND METHODS OF USE, THEREOF

(57) Abstract: Disclosed are *Paenibacillus curdlanolyticus* alpha-amylase enzymes, nucleic acids encoding the same, and compositions and methods related to the production and use thereof. The alpha-amylases are useful, for example, for starch liquefaction and saccharification, for cleaning starchy stains in laundry or dishwashing, for textile processing (e.g., desizing), in animal feed for improving digestibility, and for baking and brewing.



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**PAENIBACILLUS CURDLANOLYTICUS AMYLASE, AND METHODS OF USE,
THEREOF**

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PRIORITY

[001] This application claims priority to international patent application no. PCT/CN2012/087135, filed December 21, 2012; the disclosure of which is hereby incorporated herein by reference in its entirety.

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TECHNICAL FIELD

[002] Disclosed are compositions and methods relating to *Paenibacillus curdlanolyticus* α -amylase enzymes. The α -amylases are useful, for example, for starch liquefaction and saccharification, cleaning starchy stains, textile desizing, baking, and brewing.

15

BACKGROUND

[003] α -Amylases hydrolyze starch, glycogen, and related polysaccharides by cleaving internal α -1,4-glucosidic bonds. α -Amylases, particularly from *Bacilli*, have been used for a variety of different purposes, including but not limited to 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 digestibility. These enzymes can also be used to remove starchy soils and stains during dishwashing and laundry washing. There is continued interest in the identification and/or engineering of α -amylases for industrial and commercial use.

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BRIEF SUMMARY

[004] The present disclosure provides, *inter alia*, *Paenibacillus curdlanolyticus* α -amylase enzymes, variant α -amylase enzymes, nucleic acids encoding the same, and compositions and methods related to the production and use thereof. Aspects of the present compositions and methods are described in the following numbered paragraphs:

30

1. In one aspect, a recombinant amylase polypeptide having at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3 is provided.

2. Some embodiments of the polypeptide of paragraph 1 further comprise a deletion of one or more amino acid residues corresponding to R177, G178, D179, and G180; using SEQ ID NO: 3 for numbering.

3. Some embodiments of the polypeptide of paragraph 1 further comprise a deletion of amino acid residues corresponding to R177 and G178, or D179 and G180, using SEQ ID NO: 3 for numbering.

4. Some embodiments of the polypeptide of paragraph 1 have at least 90% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3.

5. Some embodiments of the polypeptide of paragraph 1 have at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3.

6. In another aspect, a recombinant amylase polypeptide having at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 5 is provided.

7. Some embodiments of the polypeptide of paragraph 1 have at least 90%, or at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 5.

8. In another aspect, a composition comprising the α -amylase of any of the preceding paragraphs is provided.

9. In some embodiments of the composition of paragraph 8, the composition is effective for removing starchy stains from laundry, dishes, textiles, or hard surfaces.

10. Some embodiments of the composition of paragraph 8 further comprise a surfactant.

11. In some embodiments of the composition of paragraph 8, the composition is a detergent composition.

12. In some embodiments of the composition of paragraph 8, the composition is selected from the group consisting of a laundry detergent, a laundry detergent additive, a manual or automatic dishwashing detergent, or a dishwashing machine cleaning composition.

13. In some embodiments, the composition of paragraphs 8-12 is provided in unit dose format.

14. In some embodiments of the composition of paragraph 8-13, further comprise one or more additional enzymes selected from the group consisting of 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, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, and an amylase other than the amylase of any one of paragraphs 1-7.

15. In some embodiments of the composition of paragraph 8 is for liquifying starch.

5 16. In some embodiments, the composition of paragraph 15 is for saccharifying a composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

17. In some embodiments, the composition of paragraph 8 is for liquifying pullulan, or a composition comprising starch and pullulan.

10 18. In some embodiments, the composition of paragraphs 15 or 17 is for producing a fermented beverage.

19. In some embodiments, the composition of paragraph 15 or 17 is for producing a baked food product.

20. In some embodiments, the composition of paragraph 8 is for textile desizing.

15 21. In another aspect, a method for removing a starchy stain or soil from a surface is provided, comprising: contacting the surface in the presence of a aqueous composition comprising an effective amount of the variant amylase of any of the paragraphs 1-7, allowing the polypeptide to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in the aqueous composition, and rinsing the surface,
20 thereby removing the starchy stain from the surface.

22. In some embodiments of the method of paragraph 21, the aqueous composition further comprises a surfactant.

23. In some embodiments of the method of any of paragraphs 21-22, the surface is a textile surface.

25 24. In some embodiments of the method of any of paragraphs 21-22, the surface is on dishes or is the interior surface of a dishwashing machine.

25. In some embodiments of the method of any of paragraphs 21-22, the surface is a soiled hard surface.

26. In some embodiments of the method of any of paragraphs 21-25, the composition further comprises at least one additional enzymes selected from the group consisting of protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase.

27. In another aspect, a method of saccharifying a composition comprising starch to produce a composition comprising glucose is provided, wherein the method comprises: (i) contacting the solution comprising starch with effective amount of the variant amylase of any of the paragraphs 1-7; and (ii) saccharifying the solution comprising starch to produce the composition comprising glucose; wherein the variant amylase catalyzes the saccharification of the starch solution to glucose.

28. In some embodiments of the method of paragraph 27, the composition comprising starch comprises liquefied starch, gelatinized starch, or granular starch.

29. In some embodiments of the method of any one of paragraphs 27-28, saccharification is conducted at a temperature range of about 30°C to about 75°C.

30. In some embodiments of the method of paragraph 29, the temperature range is 47°C–74°C.

31. In some embodiments of the method of any one of paragraphs 27–30, saccharification is conducted over a pH range of pH 2.0-7.5.

32. In some embodiments of the method of paragraph 31, the pH range is pH 3.5–5.5.

33. In some embodiments of the method of paragraph 31, the pH range is pH 3.5–4.5.

34. Some embodiments of the method of any one of paragraphs 27-33 further comprise fermenting the glucose composition to produce an end of fermentation (EOF) product.

35. In some embodiments of the method of paragraph 34, the fermentation is a simultaneous saccharification and fermentation (SSF) reaction.

36. In some embodiments of the method of paragraph 35, the fermentation is conducted for 48–70 hours at pH 2–8 and in a temperature range of 25°C–70°C.

37. In some embodiments of the method of any one of paragraphs 35–36, the EOF product comprises ethanol.

5 38. In some embodiments of the method of paragraph 37, the EOF product comprises 8–18% (v/v) ethanol.

39. In some embodiments of the method of any one of paragraphs 34–38, the EOF product comprises a metabolite.

10 40. In some embodiments of the method of paragraph 39, the metabolite is 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, an amino acid, lysine, itaconic acid, 1,3-propanediol, or isoprene.

15 41. Some embodiments of the method of any one of paragraphs 27–40 further comprise adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, β amylase, α -amylase that is not the variant α -amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch solution.

20 42. In some embodiments of the method of paragraph 41, the glucoamylase is added to 0.1–2 glucoamylase units (GAU)/g ds.

43. In some embodiments of the method of any one of paragraphs 27–42, the amylase is expressed and secreted by a host cell.

44. In some embodiments of the method of paragraph 43, the composition comprising starch is contacted with the host cell.

25 45. In some embodiments of the method of paragraphs 43 or 44, the host cell further expresses and secretes a glucoamylase or other enzyme.

46. In some embodiments of the method of any one of paragraphs 43–45, the host cell is capable of fermenting the composition.

30 47. In some embodiments of the method of any one of paragraphs 27–46, the solution comprising starch further comprises pullulan, and the amylase hydrolyzes the pullulan.

48. In another aspect, a composition comprising glucose produced by the method of any one of paragraphs 27-47 is provided.

49. In another aspect, a liquefied starch produced by the method of any one of paragraphs 27-47 is provided.

5 50. In another aspect, a fermented beverage produced by the method of any one of paragraphs 27-47 is provided.

51. In another aspect, use of an amylase of any of paragraphs 1-7 in the production of a composition comprising glucose is provided.

10 52. In another aspect, use of an amylase of any of paragraphs 1-7 in the production of a liquefied starch is provided.

53. In another aspect, use of an amylase of any of paragraphs 1-7 in the production of a fermented beverage is provided.

54. In another aspect, use of an amylase of any of paragraphs 1-7 in cleaning starchy stains is provided.

15 55. In another aspect, use of an amylase of any of paragraphs 1-7 in textile desizing is provided.

56. In another aspect, a method of producing a food composition is provided, comprising: combining: (i) one or more food ingredients, and (ii) an α -amylase of any of paragraphs 1-7, wherein the variant α -amylase thereof catalyzes the hydrolysis of starch components present in the food ingredients to produce glucose.

57. In some embodiments of the method of paragraph 56, the food composition is selected from the group consisting of a food product, a baking composition, a food additive, an animal food product, a feed product, a feed additive, an oil, a meat, and a lard.

25 58. In some embodiments of the method of any one of paragraphs 56-57, the one or more food ingredients comprise a baking ingredient or an additive.

59. In some embodiments of the method of any one of paragraphs 56-58, the one or more food ingredients is selected from the group consisting of flour; an anti-staling amylase; a phospholipase; a phospholipid; a maltogenic alpha-amylase or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity; a bakery xylanase; and a lipase.

60. In another aspect, a method for hydrolyzing pullulan is provided, comprising contacting the pullulan with the α -amylase of any of paragraphs 1-7 for a sufficient time to hydrolyze the pullulan.

5 61. In some embodiments of the method of paragraph 60, the pullulan is present in a polysaccharide mixture further comprising starch.

62. Some embodiments of the method of paragraph 60 are performed in the presence of a reduced amount of an additional pullulanase enzyme compared to the amount of additional pullulanase that would be required using an α -amylase lacking pullulanase activity, such as a *Bacillus* α -amylase, instead of the α -amylase of paragraphs 1-7.

10 63. In some embodiments, the method of paragraph 60 is performed in the absence of a separate pullulanase enzyme.

64. In another aspect, a method of desizing a textile is provided, comprising contacting a desizing composition with a sized textile for a time sufficient to desize the textile, wherein the desizing composition comprises a variant α -amylase of any one of paragraphs 1-7.

15 65. In another aspect, a recombinant polynucleotide encoding a polypeptide of any of paragraphs 1-7 is provided.

66. In another aspect, an expression vector comprising the polynucleotide of paragraph 65 is provided.

20 67. In another aspect, a host cell comprising the expression vector of paragraph 66 is provided.

[005] These and other aspects and embodiments of the present compositions and methods are further described, below.

BRIEF DESCRIPTION OF THE DRAWINGS

25 [006] Figure 1 is a plasmid map of p2JM706.

[007] Figure 2 is a plasmid map of p2JM754

[008] Figure 3 is a graph showing the effect of pH on PcuAmy1 alpha amylase activity at 50°C in 10 min assays. The conditions were pH 3.0 to 10.0 in 25 mM sodium acetate/ 25 mM HEPES / 25 mM glycine buffer solution with 2 mM CaCl₂. The activity was reported as relative activity
30 where the activity at the pH optimum was set to 100%.

[009] Figure 4 is a graph showing the effect of temperature on PcuAmy1 alpha amylase activity. The assay was performed at various temperatures from 40°C to 99°C for 10 minutes using 1% (w/w) potato amylopectin as substrate in 50 mM sodium acetate buffer pH 5.0 with 2 mM CaCl₂. The activity was reported as relative activity where the activity at the temperature optimum was set to 100%.

[0010] Figure 5 is a graph showing the effect of temperature on the stability of PcuAmy1. The enzyme was incubated in 50 mM sodium acetate buffer pH 5.0 with 2 mM CaCl₂ at the desired temperature for 2 hours. The remaining alpha amylase activity was measured. The activity of the enzyme kept on ice was defined as 100% activity.

[0011] Figure 6 is a graph showing the effect of pH on PcuAmy1v1 alpha amylase activity. The effect was determined by assaying alpha amylase activity at 50 °C for 10 min over a pH range from pH 3.0 to 10.0 in 25 mM Sodium Acetate/ 25 mM HEPES / 25 mM Glycine buffer solution with 2 mM CaCl₂. The activity was reported as relative activity where the activity at the pH optimum was set to 100%.

[0012] Figure 7 is a graph showing the effect of temperature on PcuAmy1v1 alpha amylase activity. The assay was performed at various temperatures from 40°C to 99°C for 10 minutes using 1% (w/w) potato amylopectin as substrate in 50 mM sodium acetate buffer pH 5.0 with addition of 2 mM CaCl₂. The activity was reported as relative activity where the activity at the temperature optimum was set to 100%.

[0013] Figure 8 is a graph showing the effect of temperature on the stability of PcuAmy1v1. The enzyme was incubated in 50 mM sodium acetate buffer pH 5.0 with addition of 2 mM CaCl₂ at desired temperature for 2 hours. The remaining alpha amylase activity was measured. The activity of the enzyme kept on ice was defined as 100% activity.

[0014] Figure 9 is a graph showing the cleaning performance of PcuAmy1 and PcuAmy1v1 at pH 8, under conditions of high conductivity, and using α -amylase AA560 as a benchmark.

[0015] Figure 10 is a graph showing the cleaning performance of PcuAmy1 and PcuAmy1v1 at pH 8, under conditions of low conductivity, and using α -amylase AA560 as a benchmark.

[0016] Figure 11 is a graph showing viscosity reduction of corn flour slurries at enzyme doses ranging from 15 to 70 μ g.

[0017] Figure 12 is a graph showing the activation energy of inactivation of amylases as a function of pH. SPEZYME® Xtra (a modified *Geobacillus stearothermophilus* alpha amylase) is shown for comparison.

[0018] Figure 13 is a graph showing the activity of certain α -amylases on a pullulan substrate.

DETAILED DESCRIPTION

[0019] As summarized above, described herein are compositions and methods relating to

5 *Paenibacillus curdolanolyticus* α -amylase (PcuAmy1), and variants, thereof, which find use in numerous applications including starch liquefaction and saccharification, for cleaning starchy stains in laundry, dishwashing, and other applications, for textile processing (*e.g.*, desizing), in animal feed for improving digestibility, and for baking and brewing. These and other aspects of the compositions and methods are described in detail, below.

10 [0020] 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

[0021] 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
15 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.

[0022] 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
20 sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

[0023] 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. All references mentioned herein are hereby incorporated by reference. Abbreviations and definitions for certain terms are
25 set forth below.

1.1. Abbreviations and Acronyms

[0024] The following abbreviations/acronyms, when and if present, have the following meanings unless otherwise specified:

30	ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
	AE or AEO	alcohol ethoxylate
	AES or AEOS	alcohol ethoxysulfate
	AkAA	<i>Aspergillus kawachii</i> α -amylase
	AnGA	<i>Aspergillus niger</i> glucoamylase
	AOS	α -olefinsulfonate

	AS	alkyl sulfate
	cDNA	complementary DNA
	CMC	carboxymethylcellulose
	DE	dextrose equivalent
5	DNA	deoxyribonucleic acid
	DPn	degree of saccharide polymerization having n subunits
	ds or DS	dry solids
	DTMPA	diethylenetriaminepentaacetic acid
	EC	Enzyme Commission
10	EDTA	ethylenediaminetetraacetic acid
	EO	ethylene oxide (polymer fragment)
	EOF	End of Fermentation
	GA	glucoamylase
	GAU/g ds	glucoamylase activity unit/gram dry solids
15	HFCS	high fructose corn syrup
	HgGA	<i>Humicola grisea</i> glucoamylase
	IPTG	isopropyl β -D-thiogalactoside
	IRS	insoluble residual starch
	kDa	kiloDalton
20	LAS	linear alkylbenzenesulfonate
	LAT, BLA	<i>B. licheniformis</i> amylase
	MW	molecular weight
	MWU	modified Wohlgemuth unit; 1.6×10^{-5} mg/MWU = unit of activity
	NCBI	National Center for Biotechnology Information
25	NOBS	nonanoyloxybenzenesulfonate
	NTA	nitriloacetic acid
	OxAm	Purastar HPAM 5000L (Danisco US Inc.)
	PAHBAH	p-hydroxybenzoic acid hydrazide
	PEG	polyethyleneglycol
30	pI	isoelectric point
	PI	performance index
	ppm	parts per million, <i>e.g.</i> , μ g protein per gram dry solid
	PVA	poly(vinyl alcohol)
	PVP	poly(vinylpyrrolidone)
35	RCF	relative centrifugal/centripetal force (<i>i.e.</i> , x gravity)
	RNA	ribonucleic acid
	SAS	alkanesulfonate
	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
	SSF	simultaneous saccharification and fermentation
40	SSU/g solid	soluble starch unit/gram dry solids
	sp.	species
	TAED	tetraacetylenehydrazide
	Tm	melting temperature
	TrGA	<i>Trichoderma reesei</i> glucoamylase
45	w/v	weight/volume
	w/w	weight/weight
	v/v	volume/volume
	wt%	weight percent
	°C	degrees Centigrade
50	H ₂ O	water
	dH ₂ O or DI	deionized water

	dH ₂ O	deionized water, Milli-Q filtration
	g or gm	grams
	μg	micrograms
	mg	milligrams
5	kg	kilograms
	μL and μl	microliters
	mL and ml	milliliters
	mm	millimeters
	μm	micrometer
10	M	molar
	mM	millimolar
	μM	micromolar
	U	units
	sec	seconds
15	min(s)	minute/minutes
	hr(s)	hour/hours
	DO	dissolved oxygen
	Ncm	Newton centimeter
	ETOH	ethanol
20	eq.	equivalents
	N	normal
	uPWA	variant α-amylase derived from <i>Pyrococcus woesei</i>
	PWA	α-amylase from <i>Pyrococcus woesei</i>
	MWCO	molecular weight cut-off
25	SSRL	Stanford Synchrotron Radiation Lightsource
	PDB	Protein Database
	CAZy	Carbohydrate-Active Enzymes database
	Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
30		

1.2. Definitions

[0025] The terms “amylase” or “amylolytic enzyme” refer to an enzyme that is, among other things, capable of catalyzing the degradation of starch. α-Amylases are hydrolases that cleave the α-D-(1→4) O-glycosidic linkages in starch. Generally, α-amylases (EC 3.2.1.1; α-D-(1→4)-glucan glucanohydrolase) are defined as endo-acting enzymes cleaving α-D-(1→4) O-glycosidic linkages within the starch molecule in a random fashion yielding polysaccharides containing three or more (1-4)-α-linked D-glucose units. In contrast, the exo-acting amylolytic enzymes, such as β-amylases (EC 3.2.1.2; α-D-(1→4)-glucan maltohydrolase) and some product-specific amylases like maltogenic α-amylase (EC 3.2.1.133) cleave the polysaccharide/starch molecule from the non-reducing end of the substrate. β-amylases, α-glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; α-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.

[0026] By "PcuAmy1 α -amylase" is meant an enzyme having at least 60% amino acid sequence identity to SEQ ID NO: 3 and having amylase activity (as described above). For example, a PcuAmy1 α -amylase having amylase activity can have at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% or even at least 99% amino acid sequence identity to SEQ ID NO: 3. In certain embodiments, the PcuAmy1 α -amylase is 100% identical to SEQ ID NO: 3.

[0027] "Pullulanases" (EC 3.2.1.41), also known as α -dextrin 6-glucanohydrolase, pullulan 6-glucanohydrolase, limit dextrinase, and amylopectin 6-glucanohydrolase are enzymes that hydrolyse α -1,6 linkages in branched polysaccharides, such as pullulan.

[0028] By "nucleic acid encoding a PcuAmy1 α -amylase" (or variants thereof) is meant a nucleic acid (or polynucleotide) having at least 40% nucleic acid sequence identity to SEQ ID NO: 1 in which the encoded PcuAmy1 α -amylase has amylase activity. For example, a nucleic acid encoding a PcuAmy1 α -amylase can have at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% or even at least 99% amino acid sequence identity to SEQ ID NO: 1. In certain embodiments, the nucleic acid encoding a PcuAmy1 α -amylase is 100% identical to SEQ ID NO: 1. In additional embodiments, a nucleic acid encoding a PcuAmy1 α -amylase hybridizes under stringent conditions to a nucleic acid having a sequence complementary to SEQ ID NO: 1.

[0029] By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed element(s) is required or mandatory, but that one or more other elements are optional and may or may not be present. For example, the phrase "an enzyme comprising amino acid sequence X" means an enzyme that has amino acid sequence X but that may also include additional sequences, *e.g.*, an amino acid or amino acid sequence that is N terminal (or C terminal) to amino acid sequence X. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed element(s) is required or mandatory, and that no other element(s) may be present. For example, the phrase "an enzyme consisting of amino acid sequence X" means an enzyme that has amino acid sequence X and that does not

include any additional amino acid sequences. By "consisting essentially of" is meant including any element(s) listed after the phrase, and limited to other element(s) that do not interfere with or contribute to the activity or action specified in the disclosure for the listed element(s). Thus, the phrase "consisting essentially of" indicates that the listed elements are required or

5 mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[0030] "Enzyme units" herein refer to the amount of product formed per time under the specified conditions of the assay. For example, a "glucoamylase activity unit" (GAU) is defined as the amount of enzyme that produces 1 g of glucose per hour from soluble starch substrate (4% DS) at 60°C, pH 4.2. A "soluble starch unit" (SSU) is the amount of enzyme that produces 1 mg
10 of glucose per minute from soluble starch substrate (4% DS) at pH 4.5, 50°C. DS refers to "dry solids." As another example, maltogenic amylase activity can be measured in degrees Diastatic Power (DP°) Units. This assay is based on a 30-min hydrolysis of a starch substrate at pH 4.6 and 20°C. The reducing sugar groups produced on hydrolysis are measured in a titrimetric
15 procedure using alkaline ferricyanide. One unit of diastase activity, expressed as degrees DP (DP°), is defined as the amount of enzyme, contained in 0.1 ml of a 5% solution of the sample enzyme preparation, that will produce sufficient reducing sugars to reduce 5 mL of Fehling's solution when the sample is incubated with 100 mL of the substrate for 1 hour at 20°C.

[0031] In the case of the present α -amylases, "activity" refers to α -amylase activity, which can
20 be measured as described herein.

[0032] 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,
25 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.

[0033] The terms "wild-type" with respect to a polypeptide refers to a naturally-occurring polypeptide that does not include a man-made substitution, insertion, or deletion at one or more
30 amino acid positions. Similarly, the term "wild-type" with respect to a polynucleotide refers to a naturally-occurring polynucleotide that does not include a man-made nucleoside change. However, note that a polynucleotide encoding a wild-type polypeptide is not limited to a

naturally-occurring polynucleotide, and encompasses any polynucleotide encoding the wild-type polypeptide.

[0034] The term “parental” or “reference” with respect to a polypeptide or polynucleotide sequence means a polypeptide or polynucleotide sequence that serves as the template sequence used for generating altered (or variant) forms of the polypeptide or polynucleotide.

[0035] 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.

[0036] The term “variant” with respect to a polypeptide refers to a polypeptide that differs from a specified parental or reference polypeptide (*e.g.*, a wild-type polypeptide) in that it includes one or more naturally-occurring or man-made changes including but not limited to substitutions, insertions, or deletions (*e.g.*, truncation) of one or more amino acids. Similarly, the term “variant,” with respect to a polynucleotide, refers to a polynucleotide that differs in nucleotide sequence from a specified parental or reference polynucleotide. The identity of the parental or reference polypeptide or polynucleotide will be apparent from context. A variant can include one or more specific substitutions, insertions, and/or deletions as well as having a % sequence identity to the parental sequence.

[0037] By “alteration” with respect to a polypeptide or polynucleotide sequence is meant that the polypeptide or polynucleotide is different with respect to a parental or reference sequence. An alteration may be a variation in the amino acid or polynucleotide sequence (as described above) or it may be a modification that does not impact the sequence, *e.g.*, a chemical or enzymatic modification of the polypeptide or polynucleotide.

[0038] 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.

[0039] The term “isolated” refers to a compound, polypeptide, cell, nucleic acid, amino acid, or other specified material or component that is present in a form that is not found in nature. For example, an isolated component is one that has been removed from or is present at a substantially different relative concentration with respect to at least one other material or component with which it is naturally associated as found in nature. Thus, an isolated polypeptide (or enzyme) includes, but is not limited to, a culture supernatant (or broth) containing the polypeptide that is obtained from host cells that express and secrete the polypeptide.

[0040] The term “enriched” refers to a compound, polypeptide, cell, nucleic acid, amino acid, or other specified material or component that is present in a composition at a relative or absolute concentration that is higher than a starting composition.

[0041] The term “purified” refers to a compound, polypeptide, cell, nucleic acid, amino acid, or other specified material or component that is present in a relatively pure state as compared to a starting composition. Purity in some instances may be defined in percentages, where in certain embodiments a component in a composition may be at least 10% pure (*e.g.*, on the basis of weight, on a molecular basis, etc.), at least 20% pure, at least 30% pure, at least 40% pure at least 50% pure, at least 60% pure, at least 70% pure, at least 80% pure, at least 90% pure, at least 95% pure, at least 98% pure, or even at least 99% pure.

[0042] 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 α -amylase activity following exposure to (*i.e.*, challenge by) an elevated temperature.

[0043] A “pH range,” with reference to an enzyme, refers to the range of pH values under which the enzyme exhibits catalytic activity.

[0044] 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).

[0045] 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).

[0046] 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 chemical modifications. 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.

[0047] By “homologue” shall mean an entity having a specified degree of identity with the subject amino acid sequences and the subject nucleotide sequences. A homologous sequence is taken to include an amino acid sequence that is at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% identical to the subject sequence, using conventional sequence alignment tools (*e.g.*, Clustal, BLAST, and the like).

Typically, homologues will include the same active site residues as the subject amino acid sequence, unless otherwise specified.

[0048] The term “plurality” refers to two or more.

[0049] “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. A nucleic acid sequence is considered to be “selectively hybridizable” to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate to high stringency hybridization and wash conditions. Hybridization conditions are based on the melting temperature (T_m) of the nucleic acid binding complex or probe. For example, “maximum stringency” typically occurs at about $T_m-5^\circ\text{C}$ (5° below the T_m of the probe); “high stringency” at about $5-10^\circ\text{C}$ below the T_m ; “intermediate stringency” at about $10-20^\circ\text{C}$ below the T_m of the probe; and “low stringency” at about $20-25^\circ\text{C}$ below the T_m . Functionally, maximum stringency conditions may be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

[0050] Moderate and high stringency hybridization conditions are well known in the art. 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 . Very stringent hybridization conditions involve 68°C and 0.1X SSC. A nucleic acid encoding a variant α -amylase may have a T_m reduced by 1°C – 3°C or more compared to a duplex formed between the nucleotide of SEQ ID NO: 1 and its identical complement.

5 [0051] Another example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C. An example of moderate stringent conditions include an overnight
10 incubation at 37°C in a solution comprising 20% formamide, 5 x SSC (150mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1x SSC at about 37 - 50°C. Those of skill in the art know how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

[0052] A “synthetic” molecule is produced by *in vitro* chemical or enzymatic synthesis rather
15 than by an organism.

[0053] 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.

20 [0054] 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.

[0055] 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.*,
25 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.

[0056] 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.

[0057] The term “endogenous” with reference to a polynucleotide or protein refers to a
30 polynucleotide or protein that occurs naturally in the host cell.

[0058] 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.

[0059] 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.

5 [0060] A “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.

[0061] 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
10 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.

[0062] The term “operably linked” means that specified components are in a relationship
15 (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.

[0063] 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
20 extracellular protein lacks the signal sequence, which is cleaved off during the secretion process.

[0064] “Biologically active” refers to a sequence having a specified biological activity, such as enzymatic activity.

[0065] The term “specific activity” refers to the quantity of substrate that can be converted to product by a given amount of enzyme or enzyme preparation, typically in a given amount of
25 time under specified conditions. Specific activity is generally expressed as units (U)/mg of enzyme.

[0066] As used herein, “water hardness” is a measure of the minerals (*e.g.*, calcium and magnesium) present in water.

[0067] A “swatch” is a piece of material such as a fabric that has a stain applied thereto. The
30 material can be, for example, fabrics made of cotton, polyester or mixtures of natural and synthetic fibers. The swatch can further be paper, such as filter paper or nitrocellulose, or a piece of a hard material such as ceramic, metal, or glass. For amylases, the stain is starch based,

but can include blood, milk, ink, grass, tea, wine, spinach, gravy, chocolate, egg, cheese, clay, pigment, oil, or mixtures of these compounds.

[0068] A “smaller swatch” is a section of the swatch that has been cut with a single hole punch device, or has been cut with a custom manufactured 96-hole punch device, where the pattern of the multi-hole punch is matched to standard 96-well microtiter plates, or the section has been otherwise removed from the swatch. The swatch can be of textile, paper, metal, or other suitable material. The smaller swatch can have the stain affixed either before or after it is placed into the well of a 24-, 48- or 96-well microtiter plate. The smaller swatch can also be made by applying a stain to a small piece of material. For example, the smaller swatch can be a stained piece of fabric 5/8" or 0.25" in diameter. The custom manufactured punch is designed in such a manner that it delivers 96 swatches simultaneously to all wells of a 96-well plate. The device allows delivery of more than one swatch per well by simply loading the same 96-well plate multiple times. Multi-hole punch devices can be conceived of to deliver simultaneously swatches to any format plate, including but not limited to 24-well, 48-well, and 96-well plates. In another conceivable method, the soiled test platform can be a bead made of metal, plastic, glass, ceramic, or another suitable material that is coated with the soil substrate. The one or more coated beads are then placed into wells of 96-, 48-, or 24-well plates or larger formats, containing suitable buffer and enzyme.

[0069] “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.

[0070] "Percent sequence identity," "percent amino acid sequence identity," "percent gene sequence identity," and/or "percent nucleic acid/polynucleotide sequence identity," with respect to two amino acid, polynucleotide and/or gene sequences (as appropriate), refer to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. Thus, “80% amino acid sequence identity” means that 80% of the amino acids in two optimally aligned polypeptide sequences are identical.

[0071] The phrase "substantially identical" in the context of comparing a nucleic acid or a polypeptide sequence to a reference sequence refers to a polynucleotide or polypeptide that has at least 70% sequence identity to the reference sequence, including at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97% , at least 98%, and at least 99% sequence identity, using a sequence alignment program or algorithm (*e.g.*, BLAST, ALIGN, CLUSTAL)

under standard parameters. One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions (*e.g.*, within a range of medium to high stringency).

[0072] Percent Sequence identity can be determined by known methods of sequence alignment. A commonly used alignment method is BLAST, although there are other methods that also find use in aligning sequences. An example of a useful algorithm for aligning two or more sequences is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pair-wise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (Feng and Doolittle, *J Mol Evol*, 35:351-360, 1987).

The method is similar to that described by Higgins and Sharp (Higgins and Sharp, *CABIOS* 5:151-153, 1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

[0073] Another example of a useful algorithm is the BLAST algorithm, described by Altschul *et al.*, (Altschul *et al.*, *J Mol Biol*, 215:403-410, 1990; and Karlin *et al.*, *Proc Natl Acad Sci USA*, 90:5873-5787, 1993). A particularly useful BLAST program is the WU-BLAST-2 program (See, Altschul *et al.*, *Meth Enzymol*, 266:460-480, 1996). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. However, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

[0074] To illustrate, the percent (%) nucleic acid sequence identity would be defined as the percentage of nucleotide residues in a candidate nucleic acid sequence that are identical to the

nucleotide residues of the starting nucleic acid sequence (*i.e.*, the sequence of interest). When using the BLASTN module of WU-BLAST-2 it is set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

[0075] The NCBI BLAST2 algorithm finds the most relevant sequences in terms of biological similarity but is not recommended for query sequences of less than 20 nucleotides, for example. Example default BLAST parameters for a nucleotide sequence are:

- Word size : 11
- E-value cutoff : 10
- Scoring Matrix : NUC.3.1 (match = 1, mismatch = -3)
- Gap Opening : 5
- Gap Extension : 2

and the following parameters for protein searches:

- Word size : 3
- E-value cutoff : 10
- Scoring Matrix : BLOSUM62
- Gap Opening : 11
- Gap extension : 1

[0076] The CLUSTAL W algorithm is another example of a sequence alignment algorithm. *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.

[0077] Deletions are counted as non-identical residues, compared to a reference sequence.

Deletions occurring at either termini are included. For example, a variant with five amino acid deletions of the C-terminus of the mature PcuAmy1 polypeptide of SEQ ID NO:3 would have a percent sequence identity of 99% ($475 / 480$ identical residues $\times 100$, rounded to the nearest whole number) relative to the mature polypeptide. Such a variant would be encompassed by a variant having “at least 99% sequence identity” to a mature amylase polypeptide. This can be

described as percent identity over the alignment length where the query sequence is 480 amino acids in length and the mature polypeptide (subject) is 475 amino acids in length.

[0078] “Fused” polypeptide sequences are connected, *i.e.*, operably linked, via a peptide bond between two subject polypeptide sequences.

- 5 [0079] The term “filamentous fungi” refers to all filamentous forms of the subdivision Eumycotina, particularly Pezizomycotina species.

[0080] 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
10 “dextrose equivalent,” is defined as the percentage of reducing sugar, *i.e.*, D-glucose, as a fraction of total carbohydrate in a syrup.

[0081] 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.

[0082] The phrase “simultaneous saccharification and fermentation (SSF)” refers to a process in
15 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.

20 [0083] An “ethanologenic microorganism” refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

[0084] 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
25 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. Examples of beers include: full malted beer, beer brewed under the “Reinheitsgebot,” ale, India pale ale, lager, pilsner, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock, dopplebock,
30 stout, porter, 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.

[0085] The term “malt” refers to any malted cereal grain, such as malted barley or wheat.

5 [0086] The term “maltogenic amylase” refers to enzymes that are capable of producing significant amounts of maltose from starch or hydrolyzed starch.

[0087] 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, torrefied cereal, cereal flakes, rye, oats, potato, tapioca, 10 cassava and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like.

[0088] 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.

15 [0089] The term “wort” refers to the unfermented liquor run-off following extracting the grist during mashing.

[0090] “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 20 polymer binds iodine and produces a characteristic blue color. The saccharide liquor is thus termed “iodine-positive saccharide,” “blue saccharide,” or “blue sac.”

[0091] The terms “retrograded starch” or “starch retrogradation” refer to changes that occur spontaneously in a starch paste or gel on ageing.

[0092] The term “about” refers to $\pm 5\%$ to the referenced value.

25 [0093] “Combinatorial variants” are variants comprising two or more substitutions, deletions, and/or insertions, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, etc., or more.

2. α -Amylase Polypeptides and Polynucleotides

[0094] In one aspect, an α -amylase from *Paenibacillus curdlanolyticus* (herein, "PcuAmy1") is provided, or a variant or derivative, thereof. The amino acid sequence of the mature of

5 PcuAmy1 polypeptide is shown, below (SEQ ID NO: 3):

ADNGTIMQYFEWYLPNDGAHWNRLNNDQAQNLKNVGITAVWIPPAYKGGSSADVGYGVYD
 TYDLGEFNQKGTVRTKYGTKSELISAVNNLHAKGIAVYGDVVLNHRMNADATELVDAVE
 VDPNNRNVETTSTYQIQAWTQYDFPGRGNTYSSFKWRWYHFDGVDWDQSRGLNRIYKLR
GDGKDWDWEVDSEYGN DYLMGADLDFNHPDVVNETKTWGKWFVNTVNLDGVRLDVAVKH
 10 IKFDFMRDWVNNVRSTTGKNLFAVGEYWHYDVNKLNSYITKTNGTMSLFDVPLHFRFYD
 ASNGGGGYDMRNLNNTLMSSNPMKAVTFVENHDTQPTQALQSTVQSWFKPLAYATILT
 REQGYPCVFYGDYYGTSDGKISSYKPIMDKLLNARKVYAYGTQRDYFDHPDIVGWTREG
 DAAHAGSGLATLITDGPGGSKWMYVGTSKAGQVWTDKTGNRSGTVTIDANGWGNFWVNG
 GSVSVWAK

15 [0095] In some embodiments, the present amylase is a variant of PcuAmy1 α -amylase having a defined degree of amino acid sequence homology/identity to SEQ ID NO: 3, for example, at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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
 20 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 homology/identity.

[0096] In some embodiments, the present amylase is a variant of PcuAmy1 α -amylase having a mutation at one of more of amino acid residues 177, 178, 179, and/or 180. These residues are shown underlined in SEQ ID NO: 3, above. In particular embodiments, the present amylase are
 25 variants of PcuAmy1 having deletions of residues R-177 and G-178 or deletions of residues D-179 and G-180.

[0097] The amino acid sequence of the mature form of PcuAmy1v1, having deletions of residues R-177 and G-178, is shown, below (SEQ ID NO: 5):

ADNGTIMQYFEWYLPNDGAHWNRLNNDQAQNLKNVGITAVWIPPAYKGGSSADV
 30 YGVYD TYDLGEFNQKGTVRTKYGTKSELISAVNNLHAKGIAVYGDVVLNHRMNA
 DATELVDAVEVDPNNRNVETTSTYQIQAWTQYDFPGRGNTYSSFKWRWYHFDGV
 DWDQSRGLNRIYKLDGKDWDWEVDSEYGN DYLMGADLDFNHPDVVNETKTWGK
 WFNVTVNLDGVR

SYITKTNGTMSLFVDVPLHFRFYDASNGGGGYDMRNLLNNTLMSSNPMKAVTFVE
 NHDTQPTQALQSTVQSWFKPLAYATILTREQGYPCVFYGDYYGTSDGKISSYKP
 IMDKLLNARKVYAYGTQRDYFDHPDIVGWTRREGDAAHAGSGLATLITDGPGGSK
 WMYVGTSKAGQVWTDKTGNRSGTIVTIDANGWGNFWVNGGSVSVWAK

5 [0098] In some embodiments, the present α -amylases additionally have at least one mutation known to produce a performance, stability, or solubility benefit in other microbial α -amylases, including but not limited to those having a similar fold and/or having 60% or greater amino acid sequence identity to PcuAmy1 (SEQ ID NO: 3) or any of the well-known *Bacillus* amylases (e.g., from *B. licheniformis*, *B. stearothermophilus*, and *B. amyloliquifaciens*), Carbohydrate-
 10 Active Enzymes database (CAZy) Family 13 amylases, or any amylase that has heretofore been referred to by the descriptive term, "Termamyl-like." Amino acid sequence identity can be determined using Clustal W with default parameters.

[0099] Furthermore, the present amylases may include any number of conservative or non-natural or chemically modified amino acid substitutions. The reader will appreciate that some
 15 of the above mentioned conservative mutations can be produced by genetic manipulation, while others are produced by introducing synthetic amino acids into a polypeptide by genetic or other means.

[00101] The present amylase 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
 20 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.

[00102] The present amylase may be a "chimeric" or "hybrid" polypeptide, in that it includes
 25 at least a portion of a first amylase polypeptide, and at least a portion of a second amylase polypeptide (such chimeric amylases 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* Cella.

30 [00103] In another aspect, nucleic acids encoding PcuAmy1 α -amylase, or a variant, thereof, are provided. The nucleic acid may encode a particular amylase polypeptide, or an amylase having a specified degree of amino acid sequence identity to the particular amylase.

[00104] In one example, the nucleic acid encodes an amylase having at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% or even at least 99%

homology/identity to SEQ ID NO: 3 (excluding the portion of the nucleic acid that encodes the signal sequence). It will be appreciated that due to the degeneracy of the genetic code, a plurality of nucleic acids may encode the same polypeptide.

[00105] In another example, the nucleic acid hybridizes under stringent or very stringent conditions to a nucleic acid encoding (or complementary to a nucleic acid encoding) an amylase having at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, 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% or even at least 99% homology/identity to SEQ ID NO: 1 (excluding the portion of the nucleic acid that encodes the signal sequence). Such hybridization conditions are described herein.

[00106] 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, which lacks the N or C-terminus of the mature form.

[00107] A nucleic acid that encodes an α -amylase can be operably linked to various promoters and regulators in a vector suitable for expressing the α -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 Amylases and Variants Thereof

[00108] The present amylases and variants thereof (referred to below as “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 amylase can be obtained following secretion of the amylase into the cell medium. Optionally, the amylase can be isolated from the host cells, or even isolated from the cell broth, depending on the desired purity of the final amylase. A gene encoding an 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* or *Trichoderma reesei*. Other host cells include bacterial cells, *e.g.*, *Bacillus subtilis* or *B. licheniformis*, as well as *Streptomyces*.

[00109] The host cell further may express a nucleic acid encoding a homologous or heterologous glucoamylase, *i.e.*, a glucoamylase that is not the same species as the host cell, or one or more other enzymes. The glucoamylase may be a variant glucoamylase, such as one of the glucoamylase variants disclosed in U.S. Patent No. 8,058,033 (Danisco US Inc.), for example. Additionally, the host may express one or more accessory enzymes, proteins, peptides. These may benefit liquefaction, saccharification, fermentation, SSF, etc., processes. Furthermore, the host cell may produce biochemicals 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

[00110] A DNA construct comprising a nucleic acid encoding an amylase or variant thereof as described herein can be constructed such that the amylase (or variant) is expressed in a host cell. One representative nucleic acid that encodes an amylase is SEQ ID NO: 1. Because of the well-known degeneracy in the genetic code, variant 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 variant 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.

[00111] 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 a variant amylase can be transformed and replicated in a bacterial host cell as a means of propagating and amplifying the vector. The vector also may be transformed into an expression host, so that the encoding nucleic acids can be expressed as a functional amylase. 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 vector is pJG153, a promoterless Cre expression vector that can be replicated in a bacterial host. *See* Harrison *et al.* (June 2011) *Applied Environ. Microbiol.* 77: 3916-22. pJG153 can be modified with routine skill to comprise and express a nucleic acid encoding an amylase variant.

[00112] A nucleic acid encoding a variant 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 a variant amylase, especially in a bacterial host, are the promoter of the lac operon of *E. coli*, the *Streptomyces coelicolor* agarase gene dagA or celA promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (amyL), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (amyM), the promoters of the *Bacillus amyloliquefaciens* α -amylase (amyQ), the promoters of 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 α -amylase, *A. niger* acid stable α -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. *cbh1* is 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.

[00113] 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 signal sequence and a promoter sequence comprising a DNA construct or vector 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.

[00114] An expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably linked to the DNA sequence encoding a variant amylase. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

[00115] 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.

[00116] The vector may also comprise a selectable marker, *e.g.*, a gene the product of which 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 *xxsC*, 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.

[00117] 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.

[00118] 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.

[00119] 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; 3rd ed., 2001; and 4th ed., 2012).

3.2. Transformation and Culture of Host Cells

[00120] 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.

5 [00121] The present invention also relates to a recombinant host cell comprising the aforementioned nucleic acid construct. The recombinant host cell can be advantageously used in the recombinant production of the disclosed polypeptides. The host cell can have a vector comprising the nucleotide sequence which is introduced into the host cell. The induced vector can be maintained in the host cell as a chromosomal integrant or as a self-replicating extra-
10 chromosomal vector.

[00122] The host cell can be any convenient cell, including a fungal cell (*e.g.*, a filamentous fungal cell), a bacterial cell, a yeast cell, a plant cell, a seaweed or an algal cell, etc.

[00123] Examples of suitable bacterial host cells (or organisms) include Gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus*
15 *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.
20 Alternatively, strains of a Gram negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, *Pseudomonas* sp. or to *Pseudomonadaceae* can be selected as the host organism.

[00124] The host cell can be a eukaryote, such as a mammalian, insect, plant, or fungal cell. In certain embodiments, the host cell is a fungal cell. The phyla *Ascomycota*, *Basidiomycota*,
25 *Chytridiomycota*, *Zygomycota* and *Oomycota* and all mitosporic fungi are included herein when referring to “fungi”. In another embodiment, the fungal host cell is a yeast cell. As used herein examples of “yeast” include ascosporeogenous yeast (*Endomycetales*), basidiosporeogenous yeast, and yeast belonging to the Fungi Imperfecti (*Blastomycetes*). (See “Biology and Activities of Yeast”, Skinner, F. A., Passmore, S. M., and Davenport, R. R., eds, Soc. App. Bacteriol.
30 Symposium Series No. 9, 1980A; and Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK for a description of yeast and fungi, respectively).

[00125] A suitable yeast host cell can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp., *Candida* sp., or *Kluyveromyces* sp., *Yarrowinia* sp., *Schizosaccharomyces* species or a species of *Saccharomyces*, including *Saccharomyces cerevisiae* or a species belonging to

5 *Schizosaccharomyces* such as, for example, *S. pombe* species. In certain embodiments, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, *Kluyveromyces lactis*, or a *Yarrowia lipolytica* cell. A strain of the methylotrophic yeast species, *Pichia pastoris*, can also be used as the host organism.

10 [00126] In certain embodiments, the host cell is a filamentous fungal host cell is, e.g., a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma*. As such, suitable host organisms among filamentous fungi include species of *Aspergillus*, e.g.,

Aspergillus niger, *Aspergillus oryzae*, *Aspergillus tubigenensis*, *Aspergillus awamori*, or

15 *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, e.g., *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium*

20 *trichothecioides*, or *Fusarium venenatum* cell, or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species. The filamentous fungal host cell can be a *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*,

25 *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

[00127] 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

30 impart changes in enzymatic and/or biochemical properties.

[00128] It can be advantageous to delete genes from expression hosts, where the gene deficiency can be cured by the transformed expression vector. Known methods can be used to obtain a fungal host cell having one or more inactivated genes. Gene inactivation can 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. For example, genes from a *Trichoderma* sp. or other filamentous fungal host that have been cloned can be deleted, for example, *cbh1*, *cbh2*, *egl1*, and *egl2* genes. Gene deletion can be accomplished by inserting a form of the desired gene to be inactivated into a plasmid by methods known in the art.

[00129] 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.

[00130] 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 solution of sorbitol can be used in the suspension medium.

[00131] Uptake of DNA into a 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.

[00132] Transformation of *Trichoderma* sp. can use protoplasts or cells that have been subjected to a permeability treatment, typically at a density of 10^5 to 10^7 /mL, particularly 2×10^6 /mL. A volume of 100 μ L 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.

[00133] In another embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

3.3. Expression

[00134] 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.

[00135] The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question 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).

[00136] 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 α -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.

[00137] 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.

[00138] 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.

[00139] 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, enzyme production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG or Sophorose. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TNT™ (Promega) rabbit reticulocyte system.

[00140] 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 variant 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 2.0 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

[00141] To evaluate the expression of an amylase in a host cell, assays can measure the expressed enzyme, corresponding mRNA, or α -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. α -Amylase activity also may be measured by any known method, such as the PAHBAH or ABTS assays, described below. Amylase assays are described in detail in the Examples, particularly Example 1.

3.5. Methods for Enriching or Purifying Variants Amylases

[00142] Fermentation, separation, and concentration techniques are well known in the art and can be used to prepare variant α -amylase polypeptide-containing solutions of desired purity and concentration.

[00143] After fermentation, a fermentation broth is obtained. The microbial cells and various suspended solids, including residual raw fermentation materials, can be 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, can be used.

[00144] It is often desirable to concentrate a variant α -amylase polypeptide-containing solution in order to optimize recovery. Use of unconcentrated solutions can result in increased incubation time in order to collect the enriched or purified enzyme precipitate.

[00145] The enzyme containing solution can be concentrated using conventional concentration techniques until the desired enzyme level 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.

[00146] The enzyme solution can be concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated variant α -amylase polypeptide-containing solution is at a desired level.

[00147] 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.

[00148] 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.

[00149] 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 variant α -amylase polypeptide and on its concentration in the concentrated enzyme solution.

[00150] 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.

[00151] Generally, the organic precipitation agents are selected from the group consisting of alkali metal salts of 4-hydroxybenzoic acid, such as sodium or potassium salts, and linear or branched alkyl esters of 4-hydroxybenzoic acid, wherein the alkyl group contains from 1 to 12 carbon atoms, and blends of two or more of these organic compounds. 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 both amylase preservative agents. For further descriptions, *see*, *e.g.*, U.S. Patent No. 5,281,526.

[00152] Addition of the organic compound precipitation agent provides the advantage of high flexibility of the precipitation conditions with respect to pH, temperature, variant amylase concentration, precipitation agent concentration, and time of incubation.

[00153] 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.

[00154] 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.

[00155] 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.

[00156] 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.

[00157] 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.

[00158] 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.

[00159] 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.

[00160] During fermentation, a variant α -amylase polypeptide accumulates in the culture broth. For the isolation, enrichment, or purification of the desired variant α -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.

[00161] Enriched or purified enzymes are useful for laundry and cleaning applications. For example, they can be used in laundry detergents and spot removers. They can be made into a final product that is either liquid (solution, slurry) or solid (granular, powder).

[00162] 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 α -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 $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation. The precipitate was recovered by centrifugation at $10,000 \times g$ (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 $(\text{NH}_4)_2\text{SO}_4$. The enzyme was eluted with a linear gradient of 1.5 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in 20 mM Tris/HCL buffer, pH 7.0 containing 5 mM CaCl_2 . The active fractions were collected, and the enzyme precipitated with $(\text{NH}_4)_2\text{SO}_4$ 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 $(\text{NH}_4)_2\text{SO}_4$ 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.

[00163] For production scale recovery, variant α -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 conventional 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 Uses of Variant Amylases

[00164] Variants amylases are useful for a variety of industrial applications. For example, variant amylases are useful in a starch conversion process, particularly in a saccharification process of a starch that has undergone liquefaction. The desired end-product 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 and maltose, 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.*, serine and glycine); antibiotics; antimicrobials; enzymes; vitamins; and hormones.

[00165] 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). One skilled in the art is aware of various fermentation conditions that may be used in the production of these end-products. Variant amylases are also useful in compositions and

methods of food preparation. These various uses of variant amylases are described in more detail below.

4.1. Preparation of Starch Substrates

[00166] 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).

[00167] 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 either 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. 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 from the kernels are recovered. Dry ground grain thus can comprise significant amounts of non-starch carbohydrate compounds, in addition to starch.

4.2. Gelatinization and Liquefaction of Starch

[00168] As used herein, the term “liquefaction” or “liquefy” means a process by which starch is converted to less viscous and shorter chain dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of an α -amylase, although additional liquefaction-inducing 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%. α -Amylase (EC 3.2.1.1) may be added to the slurry, with a metering pump, for example. The α -amylase typically used for this application is a thermally stable, bacterial α -amylase, such as a *Geobacillus stearothermophilus* α -amylase.

The α -amylase is usually supplied, for example, at about 1500 units per kg dry matter of starch.

- 5 To optimize α -amylase stability and activity, the pH of the slurry typically is adjusted to about pH 5.5-6.5 and about 1 mM of calcium (about 40 ppm free calcium ions) can also be added.

Geobacillus stearothermophilus variants or other α -amylases may require different conditions.

- Bacterial α -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
10 calcium from the slurry in cases where the enzyme is dependent upon calcium.

- [00169] The slurry of starch plus the α -amylase may be pumped continuously through a jet cooker, which is steam heated to 105°C. 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 partly gelatinized starch
15 may 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
20 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%.

- 25 [00170] Liquefaction with variant amylases advantageously can be conducted at low pH, eliminating the requirement to adjust the pH to about pH 5.5-6.5. Variant 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. Variant amylases can maintain liquefying activity at a temperature range of about 85°C – 95°C, *e.g.*, 85°C, 90°C, or 95°C. For example, liquefaction can be conducted with 800 μ g an amylase in a
30 solution of 25% DS corn starch for 10 min at pH 5.8 and 85°C, or pH 4.5 and 95°C, for example. Liquefying activity can be assayed using any of a number of known viscosity assays in the art.

[00171] Because the present α -amylases demonstrate hydrolytic activity on a pullulan substrate, they can be used in a liquefaction to hydrolyze pullulan, optionally in the absence of a separate (*i.e.*, additional to the α -amylase enzyme) pullulanase enzyme, or in the presence of a reduced amount of separate pullulanase enzyme compared to an equivalent liquefaction in which a traditional α -amylase lacking pullulanase activity is used.

4.3. Saccharification

[00172] The liquefied starch can be saccharified into a syrup rich in lower DP (*e.g.*, DP1 + DP2) saccharides, using variant 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 granular starch processed. Advantageously, the syrup obtainable using the provided variant 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.

[00173] Whereas liquefaction is generally run as a continuous process, saccharification is often conducted as a batch process. Saccharification typically is most effective at temperatures of about 60-65°C and a pH of about 4.0-4.5, *e.g.*, pH 4.3, necessitating cooling and adjusting the pH of the liquefied starch. 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. 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. When using an amylase, 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 – 74°C. The saccharification 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.

[00174] 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 in an amount of about 0.6 – 10 ppm ds, *e.g.*, 2 ppm ds. An amylase can be added as a whole broth, clarified, enriched, partially purified, or purified enzyme. The specific activity of the amylase may be about 300 U/mg of enzyme, for example, measured with the PAHBAH assay. The amylase also can be added as a whole broth product.

[00175] 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 an 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*, may be engineered to co-express PcuAmy1, or a variant thereof, and a glucoamylase, *e.g.*, a variant GA, *Aspergillus niger* GA, *Aspergillus niger* GA variant, HgGA, HgGA variant, TrGA, or a TrGA variant, 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, an enzyme that utilizes pentose sugar, an α -amylase, a pullulanase, an isoamylase, and/or an isopullulanase. *See, e.g.*, WO 2011/153516 A2.

[00176] Because the present α -amylases demonstrate hydrolytic activity on a pullulan substrate, they can be used in a saccharification to hydrolyze pullulan, optionally in the absence of a separate (*i.e.*, additional to the α -amylase enzyme) pullulanase enzyme, or in the presence of a reduced amount of separate pullulanase enzyme compared to an equivalent saccharification in which a traditional α -amylase lacking pullulanase activity is used.

4.4. Isomerization

[00177] 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.

4.5. Fermentation

[00178] 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. Fermentation products include metabolites, including but not limited to organic acids, amino acids, biofuels, and other biochemical, including, but not limited to, ethanol, citric acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol, and biodiesel.

[00179] Ethanologenic microorganisms include yeast, such as *Saccharomyces cerevisiae* and bacteria, *e.g.*, *Zymomonas mobilis*, 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(7): 1049-56.*

Commercial sources of yeast include ETHANOL RED® (LeSaffre); Thermosacc® (Lallemand); RED STAR® (Red Star); FERMIOL® (DSM Specialties); and SUPERSTART® (Alltech). Butanologenic microorganisms may include, for example, butanologenic *Clostridia*, such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharobutylacetonicum*. *See, e.g., Ezeji et al. (2007) "Bioproduction of butanol from biomass: from genes to bioreactors," Curr. Opin. Biotechnol. 18(3): 220-27.* 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) "Advances in citric acid fermentation by Aspergillus niger: biochemical aspects, membrane transport and modeling," Biotechnol. Adv. 25(3): 244-63; John et al. (2009) "Direct lactic acid fermentation: focus on simultaneous saccharification and lactic acid production," Biotechnol. Adv. 27(2): 145-52.*

[00180] 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, CO₂ generated by fermentation may be collected with a CO₂ 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.

[00181] 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 PcuAmy1, or a variant, thereof, can also be used. Such cells may express glucoamylase and/or a trehalase, isoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, pullulanase, phytase, *beta*-amylase, *alpha*-amylase that is not PcuAmy1, or a variant, thereof, *alpha*-glucosidase, isoamylase, *beta*-amylase cellulase, xylanase, other hemicellulases, protease, cellulase, lipase, cutinase, *beta*-glucosidase, pectinase, esterase, redox enzymes, transferase, branching enzyme, lyase or other hydrolases, or other enzyme.

[00182] 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 CO₂. Batch and fed-batch fermentations are common and well known in the art.

[00183] 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.

4.6. Compositions Comprising Amylases or Variant Amylases

[00184] Amylases or variant amylases as described herein may be combined with a
 5 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%
 10 sequence identity to wild-type TrGA. Variant amylases advantageously increase the yield of glucose produced in a saccharification process catalyzed by TrGA.

[00185] 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
 15 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
 20 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
 25 genus *Clostridium*, in particular *C. thermoamylolyticum* (*e.g.*, EP 135,138 (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; SANTM SUPER and AMGTM E
 30 (Novozymes); OPTIDEX[®] 300 and OPTIDEX L-400 (Danisco US Inc.); AMIGASETM and AMIGASETM 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, 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.

- 5 [00186] Other suitable enzymes that can be used in combinations with amylase include phytase, protease, pullulanase, β -amylase, isoamylase, a different α -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
- 10 the person skilled in the art. A pullulanase (EC 3.2.1.41), *e.g.*, PROMOZYME®, is also suitable. Pullulanase typically is added at 100 U/kg ds. 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*.
- 15 [00187] β -Amylases (EC 3.2.1.2) are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4- α -glucosidic linkages into amylopectin and related glucose polymers, thereby releasing maltose. β -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 β -Amylases have optimum temperatures in the range from 40°C to 65°C and optimum pH in the
- 20 range from about 4.5 to about 7.0. Contemplated β -amylases include, but are not limited to, β -amylases from barley SPEZYME® BBA 1500, SPEZYME® DBA, OPTIMALT™ ME, OPTIMALT™ BBA (Danisco US Inc.); and NOVOZYM™ WBA (Novozymes A/S).

- [00188] Compositions comprising the present amylases may be aqueous or non-aqueous formulations, granules, powders, gels, slurries, pastes, etc., which may further comprise any one
- 25 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.

30 4.7. Compositions and Methods for Baking and Food Preparation

[00189] 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 variant amylase with one or more food ingredients, or uses thereof.

[00190] 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.

[00191] 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.

[00192] For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of α -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 α -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 α -amylase(s), may be added to the flour to augment the level of endogenous α -amylase activity in flour.

[00193] 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-staling 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-staling 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 α -amylase (EC 3.2.1.133), *e.g.*, from *Bacillus*. NOVAMYL® is an exemplary maltogenic α -amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen *et al.* (1997) *Starch* 50: 39-45. Other examples of anti-staling endo-amylases include bacterial α -amylases derived from *Bacillus*, such as *B. licheniformis* or *B.*

amyloliquefaciens. The anti-staling amylase may be an exo-amylase, such as β -amylase, *e.g.*, from plant sources, such as soy bean, or from microbial sources, such as *Bacillus*.

[00194] 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 A₁ or A₂ 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.

[00195] 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 μ mol butyric acid per minute at 30°C, pH 7.0, with gum arabic as emulsifier and tributyrin as substrate.

[00196] 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*.

[00197] 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.

[00198] 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.

[00199] Optionally, an additional enzyme may be used together with the anti-staling amylase and the phospholipase. The additional enzyme may be a second amylase, such as an amyloglucosidase, a β -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- α -glucan branching enzyme), a 4- α -glucanotransferase (dextrin glycosyltransferase) or an oxidoreductase, *e.g.*, a peroxidase, a laccase, a glucose oxidase, 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.

[00200] 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®.

[00201] 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.

[00202] An amylase may be used in a pre-mix, comprising flour together with an anti-staling 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-staling amylase and a phospholipase, for use as a baking additive.

[00203] 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.

[00204] Enveloped particles, *i.e.*, α -amylase particles, can comprise an amylase. To prepare enveloped α -amylase particles, the enzyme is contacted with a food grade lipid in sufficient quantity to suspend all of the α -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.

[00205] The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the α -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 α -amylase particles. Thus, each α -amylase particle is individually enveloped in a lipid. For example, all or substantially all of the α -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 slurring the α -amylase particles so that the lipid thoroughly wets the surface of each α -amylase particle.

After a short period of stirring, the enveloped α -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 α -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.

5 **[00206]** 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 α -amylase. However, the packaged mix may further contain additional ingredients as required by the manufacturer or baker. After the enveloped α -amylase has been incorporated into the dough, the baker continues through the normal production process for that product.

10 **[00207]** The advantages of enveloping the α -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 α -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
15 vehicle also provides a sustained release of the active enzyme into the baked good. That is, following the baking process, active α -amylase is continually released from the protective coating at a rate that counteracts, and therefore reduces the rate of, staling mechanisms.

[00208] In general, the amount of lipid applied to the α -amylase particles can vary from a few percent of the total weight of the α -amylase to many times that weight, depending upon the
20 nature of the lipid, the manner in which it is applied to the α -amylase particles, the composition of the dough mixture to be treated, and the severity of the dough-mixing operation involved.

[00209] 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 α -amylase, prepared as discussed
25 above, that will be required to achieve the desired anti-staling effect. The amount of the enveloped α -amylase required is calculated based on the concentration of enzyme enveloped and on the proportion of α -amylase to flour specified. A wide range of concentrations has been found to be effective, although, as has been discussed, observable improvements in anti-staling do not correspond linearly with the α -amylase concentration, but above certain minimal levels,
30 large increases in α -amylase concentration produce little additional improvement. The α -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-staling effect the baker wishes to achieve.

[00210] A method of preparing a baked good may comprise: a) preparing lipid-coated α -amylase particles, where substantially all of the α -amylase particles are coated; b) mixing a dough containing flour; c) adding the lipid-coated α -amylase to the dough before the mixing is complete and terminating the mixing before the lipid coating is removed from the α -amylase; d) proofing the dough; and e) baking the dough to provide the baked good, where the α -amylase is inactive during the mixing, proofing and baking stages and is active in the baked good.

[00211] The enveloped α -amylase can be added to the dough during the mix cycle, *e.g.*, near the end of the mix cycle. The enveloped α -amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped α -amylase throughout the dough; however, the mixing stage is terminated before the protective coating becomes stripped from the α -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 α -amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped α -amylase should have a total volume sufficient to allow the enveloped α -amylase to be spread throughout the dough mix. If the preparation of enveloped α -amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped α -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 α -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 α -amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped α -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 α -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 α -amylase particles.

[00212] 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 a oil/meat/lard composition and/or additive ingredients.

[00213] 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.

5 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
10 food.

[00214] 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;
15 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.

[00215] In the present context, it is intended that the term “pet food” is understood to
20 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.

[00216] The terms “animal feed composition,” “feedstuff” and “fodder” are used interchangeably and may comprise one or more feed materials selected from the group
25 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,
30 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.

4.8. Textile Desizing Compositions and Use

[00217] Also contemplated are 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.

[00218] 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.

[00219] 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.

4.9. Cleaning Compositions

[00220] 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. Such compositions include heavy duty liquid (HDL), heavy duty dry (HDD), and hand (maual) laundry detergent compositions, including unit dose format laundry detergent

compositions, and automatic dishwashing (ADW) and hand (manual) dishwashing compositions, including unit dose format dishwashing compositions.

4.9.1. Overview

[00221] In certain embodiments, 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. Exemplary formulations are provided herein, as exemplified by the following:

[00222] 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.

[00223] 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.

[00224] 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); α -olefinsulfonate (AOS); alkyl sulfate (fatty alcohol sulfate) (AS); alcohol ethoxysulfate (AEOS or AES); secondary alkanesulfonates (SAS); α -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).

[00225] 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.

[00226] 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.

[00227] 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.

[00228] The detergent may contain a bleaching system, which may comprise a H₂O₂ source such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator such as tetraacetylenediamine (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.

[00229] 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.

[00230] 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 inhibitors, optical brighteners, or perfumes.

[00231] The pH (measured in aqueous solution at use concentration) is usually neutral or alkaline, *e.g.*, pH about 7.0 to about 11.0.

[00232] Particular forms of detergent compositions for inclusion of the present α -amylase are described, below. Many of these composition can be provided in unit dose format for ease of use. Unit dose formulations and packaging are described in, for example, US20090209445A1, US20100081598A1, US7001878B2, EP1504994B1, WO2001085888A2, WO2003089562A1, WO2009098659A1, WO2009098660A1, WO2009112992A1, WO2009124160A1, WO2009152031A1, WO2010059483A1, WO2010088112A1, WO2010090915A1, WO2010135238A1, WO2011094687A1, WO2011094690A1, WO2011127102A1, WO2011163428A1, WO2008000567A1, WO2006045391A1, WO2006007911A1, WO2012027404A1, EP1740690B1, WO2012059336A1, US6730646B1, WO2008087426A1, WO2010116139A1, and WO2012104613A1.

4.9.2. Heavy Duty Liquid (HDL) laundry detergent composition

[00233] Exemplary HDL laundry detergent compositions includes a deterative surfactant (10%-40% wt/wt), including an anionic deterative surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxyated 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 alkoxyated alcohol, for example a C₈-C₁₈ alkyl ethoxyated alcohol and/or C₆-C₁₂ alkyl phenol alkoxyates), wherein the weight ratio of anionic deterative surfactant (with a hydrophilic index (Hic) of from 6.0 to 9) to non-ionic deterative surfactant is greater than 1: 1. Suitable deterative surfactants also include cationic deterative 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 deterative surfactants (selected from a group of alkanolamine sulpho-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof.

[00234] The composition may optionally include, a surfactancy boosting polymer consisting of amphiphilic alkoxyated grease cleaning polymers (selected from a group of alkoxyated polymers having branched hydrophilic and hydrophobic properties, such as alkoxyated 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 C₁-C₆ 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: C₄-C₂₅ alkyl group, polypropylene, polybutylene, vinyl ester of a saturated C₁-C₆ mono-carboxylic acid, C₁-C₆ alkyl ester of acrylic or methacrylic acid, and mixtures thereof.

[00235] 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, methylenemalononic 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).

[00236] The composition may further include saturated or unsaturated fatty acid, *e.g.*, saturated or unsaturated C₁₂-C₂₄ fatty acid (0 wt% to 10 wt%); deposition aids (examples for which include polysaccharides, *e.g.*, 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 hydroxyethyl cellulose, cationic starch, cationic polyacrylamides, and mixtures thereof.

[00237] 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, polyvinylloxazolidones 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.

[00238] The composition can further include 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).

[00239] The composition optionally includes 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).

[00240] 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.

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

[00241] Exemplary HDD laundry detergent compositions includes a deterative surfactant, including anionic deterative surfactants (*e.g.*, linear or branched or random chain, substituted or

unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxyated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates and/or mixtures thereof), non-ionic deterative surfactant (*e.g.*, linear or branched or random chain, substituted or unsubstituted C₈-C₁₈ alkyl ethoxylates, and/or C₆-C₁₂ alkyl phenol alkoxyates), cationic deterative 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 deterative surfactants (*e.g.*, alkanolamine sulfo-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 sequester such as ethylenediaminetetraacetic acid, ethylenediaminetetra(methylenephosphonic acid), and water-soluble salts thereof).

[00242] The composition can include 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.

[00243] 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, alkoxyated polyamines, fabric deposition aids, and/or cyclodextrin.

4.9.4. Automatic dishwashing (ADW) detergent composition

[00244] Exemplary ADW detergent composition includes non-ionic surfactants, including ethoxylated non-ionic surfactants, alcohol alkoxyated 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-polyphosphates, 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 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 metasilicate 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 diperoxydodecanedioic acid, diperoxytetradecanedioic acid, and diperoxyhexadecanedioic 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.*, benzotriazoles, 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).

4.9.5. Additional detergent compositions

[00245] Additional exemplary detergent formulations to which the present amylase can be added are described, below, in the numbered paragraphs.

[00246] 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.*, C₁₂₋₁₈ alcohol, 1-2 ethylene oxide (EO)) or alkyl sulfate (*e.g.*, C₁₆₋₁₈) about 1% to about 4%; alcohol ethoxylate (*e.g.*, C₁₄₋₁₅ alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na₂CO₃) about 14% to about 20%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) about 2 to about 6%; zeolite (*e.g.*, NaAlSiO₄) about 15% to about 22%; sodium sulfate (*e.g.*, Na₂SO₄) 0% to about 6%; sodium citrate/citric acid (*e.g.*, C₆H₅Na₃O₇/C₆H₈O₇) about 0% to about 15%; sodium perborate (*e.g.*, NaBO₃H₂O) 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%.

[00247] 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.*, C₁₂₋₁₈ alcohol, 1-2 EO) or alkyl sulfate (*e.g.*, C₁₆₋₁₈) about 1% to about 3%; alcohol ethoxylate (*e.g.*, C₁₄₋₁₅ alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na₂CO₃) about 15% to about 21%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) about 1% to about 4%; zeolite (*e.g.*, NaAlSiO₄) about 24% to about 34%; sodium sulfate (*e.g.*, Na₂SO₄) about 4% to about 10%; sodium citrate/citric acid (*e.g.*, C₆H₅Na₃O₇/C₆H₈O₇) 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%.

[00248] 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.*, C₁₂₋₁₅ alcohol, 7 EO) about 7% to about 14%; soap as fatty acid (*e.g.*, C₁₆₋₂₂ fatty acid) about 1 to about 3%; sodium carbonate (as Na₂CO₃) about 10% to about 17%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) about 3% to about 9%; zeolite (as NaAlSiO₄) about 23% to about 33%; sodium sulfate (*e.g.*, Na₂SO₄) 0% to about 4%; sodium perborate (*e.g.*, NaBO₃H₂O) 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%.

[00249] 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.*, C₁₂₋₁₅ alcohol, 7 EO) about 10% to about 25%; sodium carbonate (as Na₂CO₃) about 14% to about 22%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) about 1% to about 5%; zeolite (*e.g.*, NaAlSiO₄) about 25% to about 35%; sodium sulfate (*e.g.*, Na₂SO₄) 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%.

[00250] 5) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C₁₂₋₁₅ alcohol, 7 EO or C₁₂₋₁₅ alcohol, 5 EO) about 12% to about 18%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 13%; alkenylsuccinic acid (C₁₂₋₁₄) 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.*, B₄O₇) 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%.

[00251] 6) An aqueous structured liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C₁₂₋₁₅ alcohol, 7 EO, or C₁₂₋₁₅ alcohol, 5 EO) 3-9%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 10%; zeolite (as NaAlSiO₄) about 14% to about 22%; potassium citrate about 9% to about 18%; borate (*e.g.*, B₄O₇) 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%.

[00252] 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.*, Na₂CO₃) about 5% to about 10%; Soluble silicate (*e.g.*, Na₂O, 2SiO₂) about 1% to about 4%; zeolite (*e.g.*, NaAlSiO₄) about 20% to about 40%; Sodium sulfate (*e.g.*, Na₂SO₄) about 2% to about 8%; sodium perborate (*e.g.*, NaBO₃H₂O) 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%.

[00253] 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.*, Na₂CO₃) about 4% to about 10%; soluble silicate (Na₂O, 2SiO₂) about 1% to about 4%; zeolite (*e.g.*, NaAlSiO₄) about 30% to about 50%; sodium sulfate (*e.g.*, Na₂SO₄) about 3% to about 11%; sodium citrate (*e.g.*, C₆H₅Na₃O₇) 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%.

[00254] 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.*, Na₂CO₃) about 14% to about 22%; zeolite (*e.g.*, NaAlSiO₄) about 18% to about 32%; sodium sulfate (*e.g.*, Na₂SO₄) about 5% to about 20%; sodium citrate (*e.g.*, C₆H₅Na₃O₇) about 3% to about 8%; sodium perborate (*e.g.*, NaBO₃H₂O) 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%.

[00255] 10) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 23%; alcohol ethoxysulfate (*e.g.*, C₁₂₋₁₅ alcohol, 2-3 EO) about 8% to about 15%; alcohol ethoxylate (*e.g.*, C₁₂₋₁₅ alcohol, 7 EO, or C₁₂₋₁₅ 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 toluenesulfonate) 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

5 ingredients (e.g., polymers, dispersants, perfume, optical brighteners) 0-5%.

[00256] 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%.

[00257] 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, α -olefinsulfonate, α -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 ($NaAlSiO_4$) 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%.

[00258] 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.

[00259] 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., $NaAlSiO_4$) 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%.

[00260] 15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C₁₂-C₁₈) 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 Na₂CO₃) about 2% to about 8%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) 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%.

[00261] 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.

[00262] 17) Detergent compositions as described supra in 1), 3), 7), 9), and 12), wherein perborate is replaced by percarbonate.

[00263] 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).

[00264] 19) Detergent composition formulated as a non-aqueous detergent liquid comprising a liquid nonionic surfactant such as, *e.g.*, linear alkoxyated 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.

[00265] 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.

[00266] 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.

[00267] 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.

[00268] 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.

[00269] *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). Additional examples include those mutant proteases described in U.S. Pat. Nos. RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, all of which are incorporated herein by reference. 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®, BLAZE™, POLARZYME®, OVOZYME®, KANNASE®, LIQUANASE®, NEUTRASE®, RELASE®, and ESPERASE® (Novo Nordisk A/S and Novozymes A/S), MAXATASE®, MAXACAL™, MAXAPEM™, PROPERASE®, PURAFECT®, PURAFECT OXP™, PURAFECT PRIME™, FNA™, FN2™, FN3™, OPTICLEAN®, OPTIMASE®, PURAMAX™, EXCELLASE™, and PURAFAST™ (Danisco US, Inc./DuPont Industrial Biosciences, Palo Alto, California, USA), BLAP™ and BLAP™ variants (Henkel Kommanditgesellschaft auf Aktien, Duesseldorf, Germany), and KAP (B. alkalophilus subtilisin; Kao Corp., Tokyo, Japan). Another exemplary proteases NprE from *Bacillus amyloliquifaciens* and ASP from *Cellulomonas* sp. strain 69B4 (Danisco US, Inc./DuPont Industrial Biosciences, Palo Alto, California, USA). Various proteases are described in WO95/23221, WO 92/21760, WO 09/149200, WO 09/149144, WO 09/149145, WO 11/072099, WO 10/056640, WO 10/056653, WO 11/140364, WO 12/151534, U.S. Pat. Publ. No. 2008/0090747, and U.S. Pat. Nos. 5,801,039, 5,340,735, 5,500,364, 5,855,625, US RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, and various other patents. In some further

embodiments, metalloproteases find use in the present invention, including but not limited to the neutral metalloprotease described in WO 07/044993. Suitable proteases include naturally occurring proteases or engineered variants specifically selected or engineered to work at relatively low temperatures.

5 [00270] *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
10 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).

20 [00271] *Polyesterases*: Suitable polyesterases can be included in the composition, such as those described in, for example, WO 01/34899, WO 01/14629, and US6933140.

[00272] *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®,
25 FUNGAMYL® and BAN™ (Novo Nordisk A/S and Novozymes A/S); RAPIDASE®, POWERASE®, and PURASTAR® (from Danisco US Inc.).

[00273] *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*,
30 *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
5 cellulases include CELLUZYME® and CAREZYME® (Novo Nordisk A/S and Novozymes A/S); CLAZINASE® and PURADAX HA® (Danisco US Inc.); and KAC-500(B)TM (Kao Corporation).

[00274] *Peroxidases/Oxidases*: Suitable peroxidases/oxidases contemplated for use in the compositions include those of plant, bacterial or fungal origin. Chemically modified or protein
10 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 GUARDZYMETM (Novo Nordisk A/S and Novozymes A/S).

[00275] The detergent composition can also comprise 2,6-β-D-fructan hydrolase, which is
15 effective for removal/cleaning of biofilm present on household and/or industrial textile/laundry.

[00276] 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
20 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.

[00277] 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)
25 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
30 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.

[00278] 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.

[00279] When included therein the detergent will typically contain from about 1% to about 40% of an anionic surfactant, such as linear alkylbenzenesulfonate, α -olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, α -sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid, or soap.

[00280] 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, alkyltrimethylammoniumoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl-N-alkyl derivatives of glucosamine ("glucamides").

[00281] 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).

[00282] 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.

[00283] 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.

[00284] 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).

[00285] Numerous exemplary detergent formulations to which the present amylases can be added (or is in some cases are identified as a component of) are described in WO2013063460.

- 5 These include commercially available unit dose detergent formulations/packages such as PUREX® UltraPacks (Henkel), FINISH® Quantum (Reckitt Benckiser), CLOROX™ 2 Packs (Clorox), OxiClean Max Force Power Paks (Church & Dwight), TIDE® Stain Release, CASCADE® ActionPacs, and TIDE® Pods™ (Procter & Gamble), PS.

4.9.6. Methods of Assessing Amylase Activity in Detergent Compositions

- 10 [00286] Numerous α -amylase cleaning assays are known in the art, including swatch and micro-swatch assays. The appended Examples describe only a few such assays.

[00287] 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.

15 4.10. Brewing Compositions

- [00288] A PcuAmy1 or variant thereof may be a component of a brewing composition used in a process of providing a fermented beverage, such as brewing. It is believed that 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 (about 340 grams) of beer. The PcuAmy1 or variant thereof, usually 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.

- 25 [00289] The principal raw materials used in making these beverages are water, hops and malt. In addition, but also exclusively, 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, torrifified 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.

[00290] For a number of reasons, the malt, which is produced principally from selected varieties of barley, has an important 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.

[00291] Cereals (grains), such as barley, oats, wheat, but also corn and rice are often used for brewing, both in industry and for home brewing, but also other plant components, such as hops are often added. 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 α -amylase. For successful brewing, adequate levels of α -amylase enzyme activity are necessary for formation of the appropriate levels of sugars for fermentation. PcuAmy1 amylase, or a variant thereof, by itself or in combination with another α -amylase(s), accordingly may be added to the components used for brewing.

[00292] 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 α -amylase activity levels in both flours and stock.

[00293] 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, malt and adjunct, or adjunct is mixed with water and held for a period of time under controlled temperatures to permit the enzymes present in the malt and/or adjunct 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 fermenter.

[00294] The wort is then contacted in a fermenter with yeast. The fermenter may be chilled to stop fermentation. The yeast, which may flocculate, 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.

[00295] The brewing composition comprising PcuAmy1, or a variant thereof, often but not necessarily in combination with one or more exogenous enzymes, such as glucoamylase(s), pullulanase(s) and/or isoamylase(s) and any combination thereof, may be added to the mash of step (a) above, such as 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, such as during the fermenting of the wort.

[00296] One aspect of the invention relates to the use of PcuAmy1, or variant thereof, in the production of a fermented beverage, such as a beer.

[00297] Another aspect concerns a method of providing a fermented beverage comprising the step of contacting a mash and/or a wort with PcuAmy1, or a variant thereof.

[00298] A further aspect relates to a method of providing a fermented beverage comprising the steps of: (a) preparing a mash, (b) filtering the mash to obtain a wort, and (c) fermenting the wort to obtain a fermented beverage, such as a beer, wherein PcuAmy1, or a variant, thereof is added to: (i) the mash of step (a) and/or (ii) the wort of step (b) and/or (iii) the wort of step (c).

[00299] According to yet another aspect, a fermented beverage, such as a beer, is produced or provided by a method comprising the step(s) of (1) contacting a mash and/or a wort with PcuAmy1, or a variant thereof; and/or (2) (a) preparing a mash, (b) filtering the mash to obtain a wort, and (c) fermenting the wort to obtain a fermented beverage, such as a beer, wherein PcuAmy1, or a variant thereof, is added to: (i) the mash of step (a) and/or (ii) the wort of step (b) and/or (iii) the wort of step (c).

[00300] Particular embodiments pertain to any of the above use, method or fermented beverage, wherein said fermented beverage is 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.

4.11. Reduction of Iodine-Positive Starch

[00301] PcuAmy1 amylase, or a variant, thereof 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)).

[00302] 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 results 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 variant amylases is expected to improve overall process performance by reducing the amount of IPS.

[00303] 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

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EXAMPLE 1

A. Cloning and expression of *Paenibacillus curdlanolyticus* Amylase (*PcuAmyI*)

[00304] *PcuAmyI* (NCBI accession No.: ZP_07385374.1) is an amylase from the bacterium strain: *Paenibacillus curdlanolyticus*. An expression plasmid was made to evaluate its expression in *Bacillus subtilis*. An expression construct was synthesized by GeneRay Biotech Co., Ltd (Shanghai, China), which 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 optimized sequence of *PcuAmyI* (SEQ ID NO: 1). The synthetic construct was digested with *XhoI* and *EcoRI*, and ligated into a p2JM-based vector digested with the same restriction enzymes to obtain the expression plasmid p2JM706 (Figure 1). The ligation mixture was used to transform chemically competent *E. coli* TOP10 cells (Invitrogen Corp.) following the manufacturer's protocol. The transformed cells were plated on Luria Agar plates supplemented with 50 ppm ampicillin and incubated overnight at 37°C. Three transformants were picked from the plate and inoculated into 5 ml Luria Broth supplemented with 50 ppm ampicillin. Cultures were grown overnight at 37°C. The plasmids were extracted and the sequence of the *PcuAmyI* gene was confirmed by DNA sequencing.

[00305] The p2JM706 plasmid was then transformed into *B. subtilis* cells (*degU^{Hy}32*, Δ *scoC*) and the transformed cells were spread on Luria Agar plates supplemented with 5ppm Chloramphenicol and 1% starch. Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250ml shake flask with Grant's II medium.

[00306] The codon-optimized nucleotide sequence of the *PcuAmyI* gene in plasmid p2JM706 is set forth as SEQ ID NO: 1:

GCCGACAACGGCACAATCATGCAGTATTTTCGAGTGGTACCTGCCGAACGACGGAGCGCACTGGA
ACAGACTTAATAACGACGCACAAAACCTGAAAAATGTGGGCATCACGGCAGTGTGGATTTCCTCC
GGCATAACAAGGGCGGCAGCTCAGCAGATGTTGGCTACGGAGTTTACGATAACATACGACCTGGGC
GAGTTCAATCAGAAAGGCACGGTCAGAACAAGTACGGAACGAAGAGCGAACTGATTTTCAGCGG
TCAACAATCTTCACGCAAAGGGCATTGCGGTTTACGGCGACGTGGTCCTGAACCATAGAATGAA

TCGGATGCAACGGAGCTTGTGGATGCGGTTGAGGTGGATCCGAACAACAGAAACGTCGAGACG
 ACAAGCACGTATCAGATCCAGGCATGGACGCAATACGATTTCCCGGGCAGAGGCAACACGTACA
 GCAGCTTTAAATGGAGATGGTATCACTTCGACGGCGTCGACTGGGACCAGAGCAGAGGCCTGAA
 CAGAATCTATAAGCTGAGAGGCGATGGCAAGGATTGGGACTGGGAGGTGACAGCGAGTACGGC
 5 AACTACGATTACCTGATGGGAGCGGACCTGGACTTCAACCACCCGGATGTGGTTAACGAAACAA
 AGACATGGGGCAAATGGTTTGTGAACACGGTGAACCTGGATGGCGTCGACTGGACGCGGTAA
 GCACATCAAGTTCGACTTCATGAGAGACTGGGTGAACAACGTGAGAAGCACGACGGGCAAGAAC
 CTTTTCGCAGTTGGCGAGTATTGGCACTACGACGTGAACAACTGAACAGCTACATCACGAAGA
 CGAATGGCACGATGAGCCTGTTTCGACGTGCCGCTGCACTTTAGATTTTATGATGCAAGCAACGG
 10 CGGAGGCGGCTACGACATGAGAAACCTGCTGAATAACACGCTGATGAGCAGCAACCCGATGAAG
 GCGGTTACATTCTGTTGAGAACCATGACACACAACCGACGCAGGCCCTGCAATCAACGGTCCAAA
 GCTGGTTTAAGCCGCTTTCGTATGCTACAATCCTGACGAGAGAGCAAGGCTACCCGTGCGTTTT
 CTACGGCGACTATTATGGAACAAGCGACGGCAAAATTAGCAGCTACAAGCCGATCATGGATAAG
 CTTCTTAACGCGAGAAAGGTGTACGCCTACGGCACGCAGAGAGATTACTTCGATCATCCGGACA
 15 TCGTTGGCTGGACAAGAGAAGGCGATGCAGCACATGCTGGCTCAGGACTGGCAACGCTTATCAC
 AGATGGCCCTGGCGGAAGCAAGTGGATGTATGTTGGAACGTCAAAGGCAGGCCAGGTCTGGACG
 GATAAAACAGGAAACAGAAGCGGAACGGTGACGATTGATGCCAATGGCTGGGGAACTTTTGGG
 TTAATGGCGGATCAGTTAGCGTTTGGGCAAAATAA

[00307] The amino acid sequence of the *PcuAmyI* precursor polypeptide is set forth as SEQ
 20 ID NO: 2. The signal peptide is shown in ***italics*** and bold.

MF***SIDFMKSRKRLISY******MVVF******AF******LAGLVFQPLGATKASA***ADNGT IMQYFEWYLPNDGAHWNRLNN
 DAQNLKNVGITAVWIPPAYKGGSSADVGYGVYDITYDLGEFNQKGTVRTKYGTKSELISAVNNLH
 AKGIAVYGDVVLNHRMNADATELVDAVEVDPNNRNVETTSTYQIQAWTQYDFPGRGNTYSSFKW
 RWHFDGVDWDQSRGLNRIYKLRGDGKDWDWEVDSEYGN DYLMGADLDFNHPDVVNETKTWKG
 25 W FVNTVNLDGVRRLDAVKHIKFD FMRDWNVNRSTTGKNLFAVGEYWHDVNKLNSYITKTNGTM
 SLFDVPLHFRFYDASNGGGGYDMRNLLNNTLMSSNPMKAVTFVENHDTQPTQALQSTVQSWFKP
 LAYATILTREQGYPCVFYGDYGTSDGKISSYKPIMDKLLNARKVYAYGTQRDYFDHPDIVGWT
 REGDAAHAGSGLATLITDGPGGSKWMYVGT SKAGQVWTDKTGNRSGTVTIDANGWGNFWVNGGS
 VSVWAK

30 [00308] The amino acid sequence of the mature form of *PcuAmyI* is set forth as SEQ ID
 NO: 3:

ADNGT IMQYFEWYLPNDGAHWNRLNNDAQNLKNVGITAVWIPPAYKGGSSADVGYGVYDITYDLG
 EFNQKGTVRTKYGTKSELISAVNNLHAKGIAVYGDVVLNHRMNADATELVDAVEVDPNNRNVET
 TSTYQIQAWTQYDFPGRGNTYSSFKWRWHFDGVDWDQSRGLNRIYKLRGDGKDWDWEVDSEYG
 35 NYDYLMGADLDFNHPDVVNETKTWKGW FVNTVNLDGVRRLDAVKHIKFD FMRDWNVNRSTTGKN

LEAVGEYWHYDVNKLNSYITKTNGTMSLFDVPLHFRFYDASNGGGGYDMRNLLNNTLMSSNPMK
 AVTFVENHDTQPTQALQSTVQSWFKPLAYATILTREQGYPCVFYGDYYGTSDGKISSYKPIMDK
 LLNARKVYAYGTQRDYFDHPDIVGWTREGDAAHAGSGLATLITDGPGGSKWMYVGTISKAGQVWT
 DKTGNRSGTVTIDANGWGNFWVNGGSVSVWAK

5

B. Cloning and expression of *PcuAmy1* variant *PcuAmy1v1*

[00309] A *PcuAmy1* variant (*PcuAmy1v1*) having deletions of Arg-177 and Gly-178 (i.e., Δ RG; Suzuki, Y. et al. (1989) *J. Biol. Chem.* 264:18933-38)) was constructed in an expression plasmid to evaluate its expression in *Bacillus subtilis*. Four primers were designed with

10 engineered restriction sites and overlapping regions based on SEQ ID NO: 1 (not shown). First, the former partial fragment before Arg-177 and Gly-178 and the latter partial sequence after Arg-177 and Gly-178 were obtained by PCR separately. Then, overlap extension PCR was performed by combining these two fragments to obtain a full length *PcuAmy1v1* gene.

[00310] The overlap extension PCR products were digested with *Bss*HII and *Xho*I and ligated
 15 into the p2JM vector which was digested with the same restriction enzymes to obtain the expression plasmid p2JM754 (Figure 2). The ligation mixture was used to transform *E. coli* TOP10 chemically competent cells (Invitrogen Corp.) following the manufacturer's protocol. The transformed cells were plated on Luria Agar plates supplemented with 50 ppm ampicillin and incubated overnight at 37°C. Three transformants were picked from the plate and
 20 inoculated into 5 ml Luria Broth supplemented with 50 ppm ampicillin. Cultures were grown overnight at 37°C, plasmid DNA extracted and the correct sequence of the *PcuAmy1v1* gene was confirmed by DNA sequencing.

[00311] The p2JM754 plasmid was then transformed into *B. subtilis* cells and the transformed cells were spread on Luria Agar plates supplemented with 5ppm Chloramphenicol and 1%
 25 starch. Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250ml shake flask with Grant's II medium.

[00312] The nucleotide sequence of the *PcuAmy1v1* gene of plasmid p2JM754 is set forth as SEQ ID NO: 4:

GCCGACAACGGCACAATCATGCAGTATTTTCGAGTGGTACCTGCCGAACGACGGAGCGCACTGGA
 30 ACAGACTTAATAACGACGCACAAAACCTGAAAAATGTGGGCATCACGGCAGTGTGGATTCTCTCC
 GGCATACAAGGGCGGCAGCTCAGCAGATGTTGGCTACGGAGTTTACGATACATACGACCTGGGC
 GAGTTCAATCAGAAAGGCACGGTCAGAACAAAGTACGGAACGAAGAGCGAACTGATTTTCAGCGG
 TCAACAATCTTCACGCAAAGGGCATTGCGGTTTACGGCGACGTGGTCCTGAACCATAGAATGAA
 TCGCGATGCAACGGAGCTTGTGGATGCGGTTGAGGTGGATCCGAACAACAGAAACGTCGAGACG

ACAAGCACGTATCAGATCCAGGCATGGACGCAATACGATTTCCCGGGCAGAGGCAACACGTACA
 GCAGCTTTAAATGGAGATGGTATCACTTCGACGGCGTCGACTGGGACCAGAGCAGAGGCCTGAA
 CAGAATCTATAAGCTGGATGGCAAGGATTGGGACTGGGAGGTCGACAGCGAGTACGGCAACTAC
 GATTACCTGATGGGAGCGGACCTGGACTTCAACCACCCGGATGTGGTTAACGAAACAAAGACAT
 5 GGGGCAAATGGTTTGTGAACACGGTGAACCTGGATGGCGTCAGACTGGACGCGGTTAAGCACAT
 CAAGTTCGACTTCATGAGAGACTGGGTGAACAACGTGAGAAGCACGACGGGCAAGAACCTTTTC
 GCAGTTGGCGAGTATTGGCACTACGACGTGAACAAACTGAACAGCTACATCACGAAGACGAATG
 GCACGATGAGCCTGTTTCGACGTGCCGCTGCACCTTTAGATTTTATGATGCAAGCAACGGCGGAGG
 CGGCTACGACATGAGAAACCTGCTGAATAACACGCTGATGAGCAGCAACCCGATGAAGGCGGTT
 10 ACATTCGTTGAGAACCATGACACACAACCGACGCAGGCCCTGCAATCAACGGTCCAAAGCTGGT
 TTAAGCCGCTTGCCTATGCTACAATCCTGACGAGAGAGCAAGGCTACCCGTGCGTTTTTCTACGG
 CGACTATTATGGAACAAGCGACGGCAAAATTAGCAGCTACAAGCCGATCATGGATAAGCTTCTT
 AACGCGAGAAAGGTGTACGCCTACGGCACGCAGAGAGATTACTTCGATCATCCGGACATCGTTG
 GCTGGACAAGAGAAGGCGATGCAGCACATGCTGGCTCAGGACTGGCAACGCTTATCACAGATGG
 15 CCCTGGCGGAAGCAAGTGGATGTATGTTGGAACGTCAAAGGCAGGCCAGGTCTGGACGGATAAA
 ACAGGAAACAGAAGCGGAACGGTGACGATTGATGCCAATGGCTGGGGAACTTTTGGGTAAATG
 GCGGATCAGTTAGCGTTTGGGCAAAATAA

[00313] The amino acid sequence of the mature form of PcuAmy1v1 is set forth as SEQ ID NO: 5:

20 ADNGTIMQYFEWYLPNDGAHWNRLNND AQNLKNVGITAVWIPPAYKGGSSADVGYGVYD TYDLG
 EFNQKGTVRTKYGTKSELISAVNNLHAKGIAVYGDVVLNHRMNADATELVDAVEVDPNNRNVET
 TSTYQIQAWTQYDFPGRGNTYSSFKWRWYHFDGVDWDQSRGLNRIYKLDGKDWDWEVDSEYGN Y
 DYLMGADLDFNHPDVVNETKTWGWKFVNTVNL DGVRLDAVKHIKFDFMRDWVNNVRSTTGKNLF
 AVGEYWHYDVNKLNSYITKTNGTMSLFDVPLHFRFYDASNGGGGYDMRNLNNTLMSSNPMKAV
 25 TFVENHDTQPTQALQSTVQSWFKPLAYATIL TREQGYPVIFYGDYYGTSDGKISSYKPI MDKLL
 NARKVYAYGTQRDYFDHPDIVGW TREGDAAHAGSGLATLITDGP GGSKWMYVGTSKAGQVWTDK
 TGNRSGTVTIDANGWGNFWVNGGSVSVWAK

EXAMPLE 2

30 Alpha Amylase Activity of PcuAmy1

[00314] The alpha amylase activity of PcuAmy1 was measured using 1% w/w potato amylopectin as substrate. The assay was performed both in 50 mM sodium acetate buffer pH 5.0 and in 50 mM HEPES buffer pH 8.0 at 50°C with addition of 2 mM CaCl₂ for 10 minutes.

The glucose generated from amylopectin was quantified using PAHBAH (*p*-Hydroxy benzoic acid hydrazide) assay. The enzyme activity was calculated based on a glucose standard curve. In this assay, one alpha amylase unit is defined as the amount of enzyme required to generate 1 micromole of glucose per minute under the conditions of the assay. The specific activity of purified PcuAmy1 was determined to be 6722 units/mg at pH 5.0 and 2837 units/mg at pH 8.0 using the above method.

EXAMPLE 3

pH Profile of PcuAmy1

[00315] The pH profile of PcuAmy1 was determined by assaying alpha amylase activity at 50°C over a pH range from pH3.0 to 10.0 in 25 mM Sodium Acetate/ 25 mM HEPES / 25 mM Glycine buffer solution with addition of 2mM CaCl₂. Potato amylopectin (1% solution) dissolved in water was mixed with 250 mM buffer solution at a ratio of 9:1, and the substrate was equilibrated at 50°C before adding enzyme. After 10 minutes, the enzyme reaction was transferring 10 microliters to a new 96-well microtiter plate with 100 microliters of PAHBAH solution and read at 410 nm. Enzyme activity at each pH was reported as relative activity where the activity at the pH optimum was set to 100%. The pH profile of PcuAmy1 is shown in Figure 3. PcuAmy1 was found to have an optimum pH at about 5.4, and retain greater than 70% of maximum activity between pH 4.0 and 7.4.

EXAMPLE 4

Temperature Profile of PcuAmy1

[00316] The temperature optimum of purified PcuAmy1 was determined by measuring alpha amylase activity under various temperatures from 40°C to 99°C for 10 minutes in 50 mM sodium acetate buffer at pH 5.0 with addition of 2mM CaCl₂. The activity was reported as relative activity where the activity at the temperature optimum was set to 100%. The temperature profile of PcuAmy1 is shown in Figure 4. PcuAmy1 was found to have an optimum temperature of 62°C, and was found to retain greater than 70% of maximum activity between 40°C and 90°C.

EXAMPLE 5**Thermostability of PcuAmy1**

[00317] The enzyme was incubated in 50 mM sodium acetate buffer pH 5.0 with addition of 2 mM CaCl₂ at desired temperature for 2 hours in a PCR machine prior to addition into substrate. The remaining activity of the samples was measured as described in Example 2 using sodium acetate buffer pH 5.0 with addition of 2 mM CaCl₂. The activity of the sample kept on ice was defined as 100% activity. As shown in Figure 5, at temperatures lower than 56°C, PcuAmy1 retained over 50% activity during a 2-hour incubation period.

EXAMPLE 6**Alpha Amylase Activity of PcuAmy1v1**

[00318] The specific activity of purified PcuAmy1v1 was determined to be 6945 units/mg at pH5.0 and 3114 units/mg at pH8.0 using the method described in Example 2.

EXAMPLE 7**pH Profile of PcuAmy1v1**

[00319] The pH profile of PcuAmy1v1 was determined using the method described in Example 3. As shown in Figure 6, PcuAmy1v1 was found to have a similar pH profile as PcuAmy1 with optimum pH at about 5.5, and retain greater than 70% of maximum activity between pH 3.8 and 7.5.

EXAMPLE 8**Temperature Profile of PcuAmy1v1**

[00320] The temperature optimum of purified PcuAmy1v1 was determined using the method described in Example 4. The temperature profile of PcuAmy1v1 is shown in Figure 7. PcuAmy1v1 was found to have a similar temperature profile as PcuAmy1 with optimum temperature of 60°C, and was found to retain greater than 70% of maximum activity between 40°C and 92°C.

EXAMPLE 9**Thermostability of PcuAmy1v1**

[00321] The thermostability of purified PcuAmy1v1 was determined using the method described in Example 5. As shown in Figure 8, PcuAmy1v1 retained over 50% activity during a 2-hour incubation period at temperatures lower than 76 °C, which is significantly higher than that of wild type.

EXAMPLE 10**Cleaning performance of PcuAmy1 and PcuAmy1v1**

[00322] Purified PcuAmy1 and PcuAmy1v1 were analyzed in a 96-well CS28 rice starch micro-swatch applications cleaning assay. The principle of this amylase assay is the liberation of an orange dye due to the hydrolysis of rice starch incorporated in a cotton microswatch. The absorbance at 488 nm of the wash liquid is measured and this relates to the level of amylase activity in the sample analyzed at the desired conditions (pH, temperature, and buffer).

[00323] The equipment used for this set of assays includes a Biomek FX Robot (Beckman Coulter), a SpectraMAX MTP Reader (type 340-Molecular Devices) and iEMS incubator/shaker (Thermo Scientific). In this assay system, the reagent and solutions used are:

1) CS-28 Microswatches (rice starch, colored);

2) 10 mM HEPES, 2 mM CaCl₂, 0.005% TWEEN 80 buffer, pH 8.0, conductivity 1mS/cm;

3) 10 mM HEPES, 2 mM CaCl₂, 0.005% TWEEN 80 buffer, pH 8.0, conductivity 5mS/cm (adjusted with NaCl);

4) 10 mM NaCl, 0.1 mM CaCl₂, 0.005% TWEEN 80.

[00324] CS-28 Microswatches of 5.5 mm circular diameter are provided by the Center for Testmaterials (CFT, Vlaardingen, The Netherlands). Two microswatches are placed in each well of a 96-well Corning 9017 flat bottomed polystyrene MTP. The culture supernatants are diluted appropriately in 10 mM NaCl, 0.1 mM CaCl₂, 0.005% TWEEN[®]80 solution.

[00325] The incubator/shaker is set at 25°C and HEPES and enzyme (dilution series from 0-2 ppm final enzyme concentration) were added to each well, to a total volume of 180 µL/well. The MTP is sealed with a plate seal and placed in the iEMS incubator/shaker and incubated for 15 minutes at 1150 rpm at 25°C. Following incubation under the appropriate conditions, 100 µL of solution from each well is transferred to a new MTP, and the absorbance at 488 nm is

measured using a MTP-spectrophotometer. Controls containing two microswatches and buffer but no enzyme are included for subtraction of background cleaning performance. The absorbance at 488 nm is plotted against the enzyme concentration, and the data fitted to the following equation: $y=(ax)/(b+x)$

5 [00326] For additional information on the assay, *see* U.S. Patent No. 7,122,334.

[00327] PcuAmy1 was highly efficient in stain removal at pH 8.0, both in low and high conductivity conditions. PcuAmy1v1 performed better than PcuAmy and better than AA560. The results of the cleaning assay performed under high conductivity conditions are shown in Figure 9. The results of the cleaning assay performed under low conductivity conditions are
10 shown in Figure 10.

EXAMPLE 11

Viscosity reduction of corn flour starch slurry by PcuAmy1 and PcuAmy1v1

[00328] All viscosity experiments were performed using a Rapid Visco Analyser (Newport Scientific) with 38x68 plain washed cans (Part No. AA0384001) and a double skirted paddle (Part No. NS101783). Data acquisition and analysis was performed with Thermocline for Windows (version 3.11). Immediately before each run, 33g of 25% dry solids (ds) corn flour slurry was prepared in an RVA can as follows: 9.17 g corn flour (11.8% moisture content) was weighed out on an analytical balance and mixed with 23.83 g deionized water. Sample pH was
15 adjusted with 0.0828 ml 1 N sulfuric acid (for pH 5.7) or 0.285 ml 1 N sulfuric acid (for pH 5.2). Enzyme was added at the appropriate dose, and the can was placed in the viscometer. All runs were 10 minutes in length, with a temperature ramp to 85°C or 95°C over 80 seconds followed by a temperature hold at 85°C or 95°C for the remainder of the run.

[00329] Results of viscometer experiments are shown in Figure 11. PcuAmy1 shows
25 acceptable reduction in peak and final viscosity under these conditions. The engineered variant PcuAmyv1 shows improved performance in viscosity reduction, particularly at lower pH.

EXAMPLE 12

Effect of pH on thermal stability of PcuAmy1 and PcuAmy1v1

30 [00330] Thermal inactivation was assayed in 50 mM potassium acetate adjusted to the appropriate pH and supplemented with 1 mM CaCl₂ and 100 mM NaCl. Enzymes were used at 10 µg/ml. Thermal inactivation was carried out in PCR tube strips and temperature was maintained in a Tetrad PCR machine. At the indicated time points, strips were removed and

kept on ice until activity was assayed. Residual activity was measured using the Ceralpha amylase assay in microtiter plates. Ceralpha reactions were incubated for 5 minutes.

[00331] Residual activity as a function of time was fit to Equation 1 using Excel. Data were inspected manually and sets with fewer than two nonzero points were discarded. Similarly,

5 experiments where the activity decayed by less than 1/3 during the time course were discarded.

[00332] Thermal stability is reported here as the activation energy of enzyme inactivation.

Activation energy is a temperature-independent measure of stability, and can be used to predict the half-life of the enzyme at any temperature. Activation energy is obtained by measuring the

inactivation rate constant as a function of temperature and fitting to the Arrhenius equation. The

10 Arrhenius equation describes the dependence of a reaction rate constant on temperature. Alpha amylase inactivation is generally well described by the first order rate equation:

$$A(t) = A_0 e^{-kt} \quad (1)$$

[00333] And the half life of the enzyme is a function of the first order rate constant:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \quad (2)$$

15 [00334] The Arrhenius equation describes the temperature dependence of the rate constant k in terms of the activation energy E_a :

$$k = A e^{\frac{-E_a}{RT}} \quad (3)$$

[00335] Where R is the ideal gas constant, T is the absolute temperature (i.e. K, not C), and A

20 is the “preexponential factor.” A is a sort of speed limit for the reaction, the max speed it approaches as E_a approaches zero. In practice, Equation 3 is typically linearized by taking the natural log of both sides:

$$\ln(k) = \frac{-E_a}{R} \frac{1}{T} + \ln(A) \quad (4)$$

[00336] Thus a plot of $\ln(k)$ vs. $1/T$ has a slope proportional to E_a . Activation energy can be

25 [00337] Plots of activation energy of enzyme activity loss vs. pH are shown in Figure 12.

PcuAmy1 stability is strongly dependent on pH in the range of 4.0 to 6.2. PcuAmy-v1 is substantially more stable than the wild-type enzyme at all pH.

30 **EXAMPLE 13**

Activity of PcuAmy1 and PcuAmy1v1 on Pullulan substrate

[00338] Activity on pullulan was assessed in 50 mM KOAc pH 5.8, 0.25 mM CaCl_2 , 100 mM NaCl, 1% pullulan (Sigma). Different enzyme doses were incubated in buffer with substrate for 10 minutes at 50 °C. Activity was assessed by measuring reducing sugars present

35 after digestion using the PAHBAH method.

[00339] Pullulan activity is shown in Figure 13. Unlike other liquefaction alpha amylases such as FRED (*Bacillus licheniformis*), PcuAmy1 and PcuAmyv1 have significant activity on pullulan. Pullulan is a linear polymer of $\alpha 1 \rightarrow 6$ linked maltotriose. Thus, it contains both $\alpha 1 \rightarrow 6$ and $\alpha 1 \rightarrow 4$ linkages. Using ion exchange chromatography, the DP3 product of hydrolysis was determined to be panose, which indicated that α -1,4 glycosidic bonds in pullulan were cleaved. However, PcuAmy1 appears to be able to hydrolyze $\alpha 1 \rightarrow 4$ linkages very close to $\alpha 1 \rightarrow 6$ branches, thereby hydrolyzing pullulan to a much greater extent than typical α -amylases.

CLAIMS

What is claimed is:

- 5 1. A recombinant amylase polypeptide having at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3.
2. The amylase polypeptide of claim 1, further comprising a deletion of one or more amino acid residues corresponding to R177, G178, D179, and G180; using SEQ ID NO: 3 for
10 numbering.
3. The amylase polypeptide of claim 2, comprising a deletion of amino acid residues corresponding to R177 and G178, or D179 and G180, using SEQ ID NO: 3 for numbering.
- 15 4. The amylase polypeptide of any of the preceding claims, having at least 90% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3.
5. The amylase polypeptide of any of the preceding claims, having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 3.
20
6. A recombinant amylase polypeptide having at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 5.
7. The amylase polypeptide of claim 6, having at least 90%, or at least 95% amino acid
25 sequence identity to the amino acid sequence of SEQ ID NO: 5.
8. A composition comprising the α -amylase of any of the preceding claims.
9. The composition of claim 8, wherein the composition is effective for removing
30 starchy stains from laundry, dishes, textiles, or hard surfaces.
10. The composition of claim 8 or 9, further comprising a surfactant.
11. The composition of any of claims 8-10, wherein the composition is a detergent
35 composition.

12. The composition of any of claims 8-11, wherein the composition is selected from the group consisting of a laundry detergent, a laundry detergent additive, a manual or automatic dishwashing detergent, or a dishwashing machine cleaning composition.

5

13. The composition of any of claims 8-12, wherein the composition is provided in unit dose format.

14. The composition of any of claims 8-13, further comprising one or more additional
10 enzymes selected from the group consisting of protease, hemicellulase, cellulase, peroxidase, lipolytic enzyme, metallolipolytic enzyme, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, and an amylase other than the
15 amylase of any one of claims 1-7.

15. The composition of claim 8, wherein the composition is for liquifying starch.

16. The composition of claim 15, wherein the composition is for saccharifying a
20 composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

17. The composition of claim 8 wherein the composition is for liquifying pullulan, or a composition comprising starch and pullulan.

25

18. The composition of claim 15 or 17, wherein the composition is for producing a fermented beverage.

19. The composition of claim 15 or 17, wherein the composition is for producing a
30 baked food product.

20. The composition of claim 8, wherein the composition is for textile desizing.

21. A method for removing a starchy stain or soil from a surface, comprising:
contacting the surface in the presence of a aqueous composition comprising an
effective amount of the variant amylase of any of the claims 1-7,

5 allowing the polypeptide to hydrolyze starch components present in the starchy stain
to produce smaller starch-derived molecules that dissolve in the aqueous composition, and
rinsing the surface,
thereby removing the starchy stain from the surface.

10 22. The method of claim 21, wherein the aqueous composition further comprises a
surfactant.

23. The method of any of claims 21-22, wherein the surface is a textile surface.

15 24. The method of any of claims 21-22, wherein the surface is on dishes or is the interior
surface of a dishwashing machine.

25. The method of any of claims 21-22, wherein the surface is a soiled hard surface.

20 26. The method of any of claims 21-25, wherein the composition further comprises at
least one additional enzymes selected from the group consisting of 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,
25 malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase.

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

(i) contacting the solution comprising starch with effective amount of the
30 variant amylase of any of the claims 1-7; and

(ii) saccharifying the solution comprising starch to produce the composition
comprising glucose; wherein the variant amylase catalyzes the saccharification of the
starch solution to glucose.

28. The method of claim 27, wherein the composition comprising starch comprises liquefied starch, gelatinized starch, or granular starch.

29. The method of any one of claims 27-28, wherein saccharification is conducted at a
5 temperature range of about 30°C to about 75°C.

30. The method of claim 29, wherein the temperature range is 47°C–74°C.

31. The method of any one of claims 27–30, wherein saccharification is conducted over
10 a pH range of pH 2.0-7.5.

32. The method of claim 31, wherein the pH range is pH 3.5–5.5.

33. The method of claim 31, wherein the pH range is pH 3.5–4.5.
15

34. The method of any one of claims 27-33, further comprising fermenting the glucose composition to produce an end of fermentation (EOF) product.

35. The method of claim 34, wherein the fermentation is a simultaneous
20 saccharification and fermentation (SSF) reaction.

36. The method of claim 35, wherein the fermentation is conducted for 48–70 hours at pH 2–8 and in a temperature range of 25°C–70°C.

37. The method of any one of claims 35–36, wherein the EOF product comprises
25 ethanol.

38. The method of claim 37, wherein the EOF product comprises 8-18% (v/v) ethanol.

39. The method of any one of claims 34-38, wherein the EOF product comprises a
30 metabolite.

40. The method of claim 39, wherein the metabolite is 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, an amino acid, lysine, itaconic acid, 1,3-propanediol, or isoprene.

41. The method of any one of claims 27-40, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, pullulanase, β -amylase, α -amylase that is not the variant α -amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, or a combination thereof, to the starch solution.

42. The method of claim 41, wherein the glucoamylase is added to 0.1–2 glucoamylase units (GAU)/g ds.

43. The method of any one of claims 27–42, wherein the amylase is expressed and secreted by a host cell.

44. The method of claim 43, wherein the composition comprising starch is contacted with the host cell.

45. The method of claim 43 or 44, wherein the host cell further expresses and secretes a glucoamylase or other enzyme.

46. The method of any one of claims 43-45, wherein the host cell is capable of fermenting the composition.

47. The method of any one of claims 27-46, wherein the solution comprising starch further comprises pullulan, and wherein the amylase catalyzes the saccharification of the pullulan.

48. A composition comprising glucose produced by the method of any one of claims 27-47.

49. A liquefied starch produced by the method of any one of claims 27-47.

50. A fermented beverage produced by the method of any one of claims 27-47.

5 51. Use of an amylase of any of claims 1-7 in the production of a composition comprising glucose.

52. Use of an amylase of any of claims 1-7 in the production of a liquefied starch.

10 53. Use of an amylase of any of claims 1-7 in the production of a fermented beverage.

54. Use of an amylase of any of claims 1-7 in cleaning starchy stains.

55. Use of an amylase of any of claims 1-7 in textile desizing.

15

56. A method of producing a food composition, comprising: combining

(i) one or more food ingredients, and

(ii) a variant α -amylase of any of claims 1-7,

wherein the variant α -amylase thereof catalyzes the hydrolysis of starch components

20 present in the food ingredients to produce glucose.

57. The method of claim 56, wherein the food composition is selected from the group consisting of a food product, a baking composition, a food additive, an animal food product, a feed product, a feed additive, an oil, a meat, and a lard.

25

58. The method of any one of claims 56-57, wherein the one or more food ingredients comprise a baking ingredient or an additive.

59. The method of any one of claims 56-58, wherein the one or more food ingredients
30 is selected from the group consisting of flour; an anti-staling amylase; a phospholipase; a phospholipid; a maltogenic alpha-amylase or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity; a bakery xylanase; and a lipase.

60. A method for hydrolyzing pullulan comprising contacting the pullulan with the α -amylase of any of claims 1-7 for a sufficient time to hydrolyze the pullulan.

5 61. The method of claim 60, wherein the pullulan is present in a polysaccharide mixture further comprising starch.

62. The method of claim 60, performed in the presence of a reduced amount of an additional pullulanase enzyme compared to the amount of additional pullulanase that would be
10 required using an α -amylase lacking pullulanase activity, such as a Bacillus α -amylase, instead of the α -amylase of paragraphs 1-7.

63. The method of claim 60, performed in the absence of a separate pullulanase enzyme.

15 64. A method of desizing a textile comprising contacting a desizing composition with a sized textile for a time sufficient to desize the textile, wherein the desizing composition comprises a variant α -amylase of any one of claims 1-7.

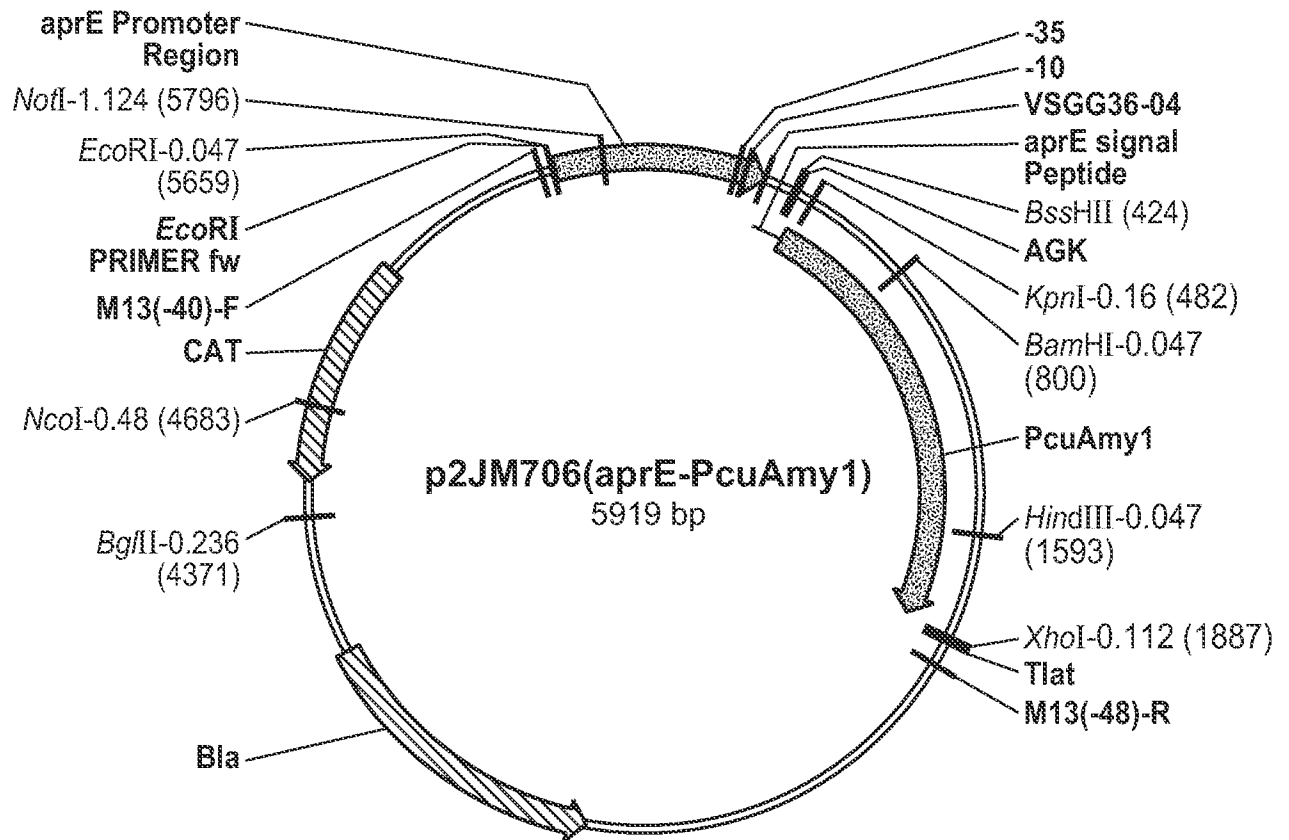
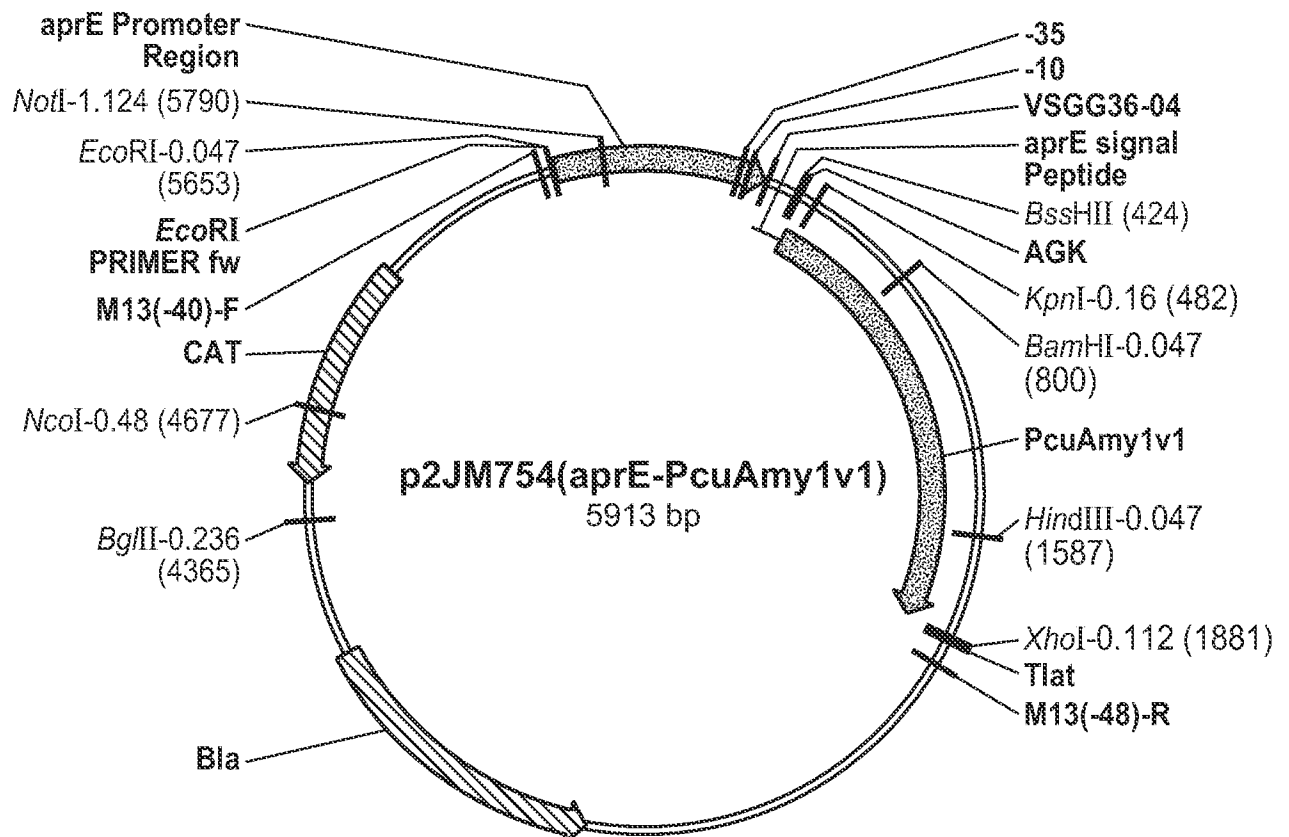
65. A recombinant polynucleotide encoding a polypeptide of any of claims 1-7.

20

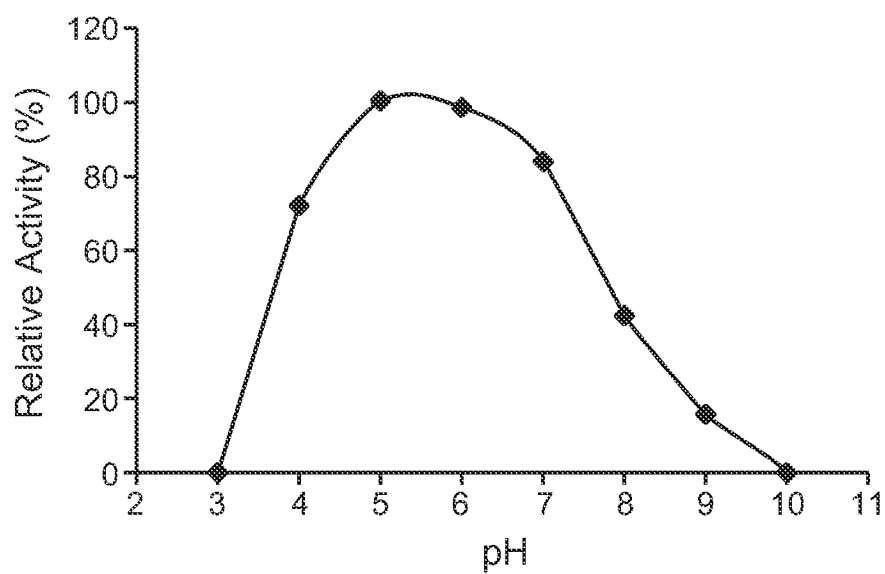
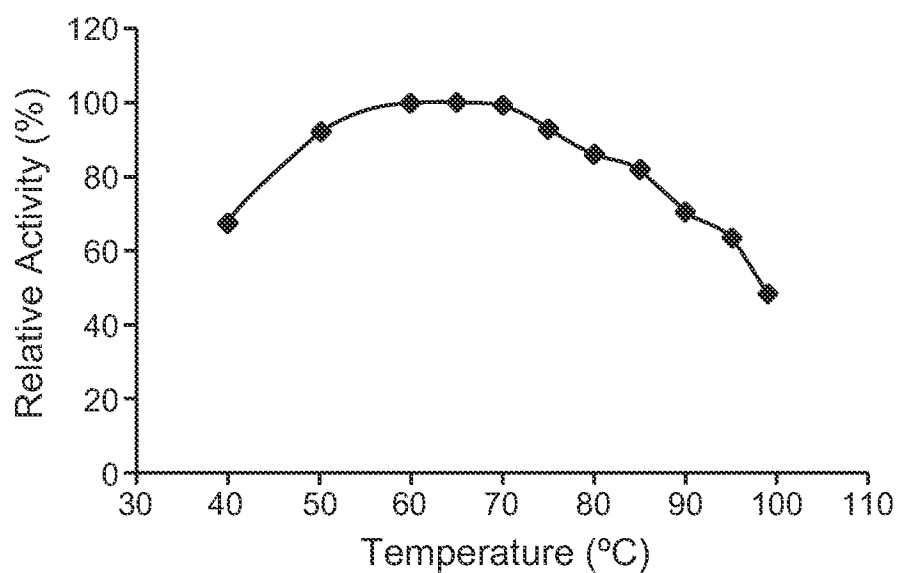
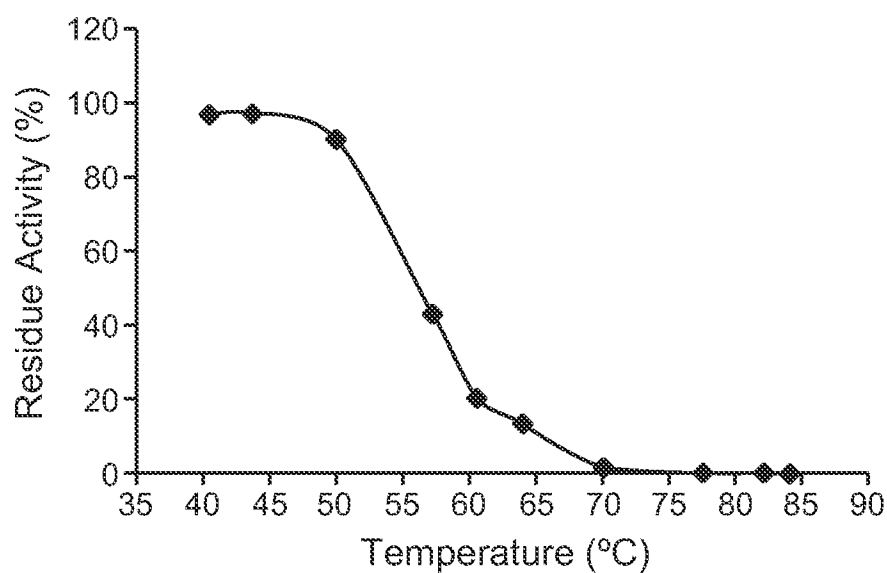
66. An expression vector comprising the polynucleotide of claim 65.

67. A host cell comprising the expression vector of claim 66.

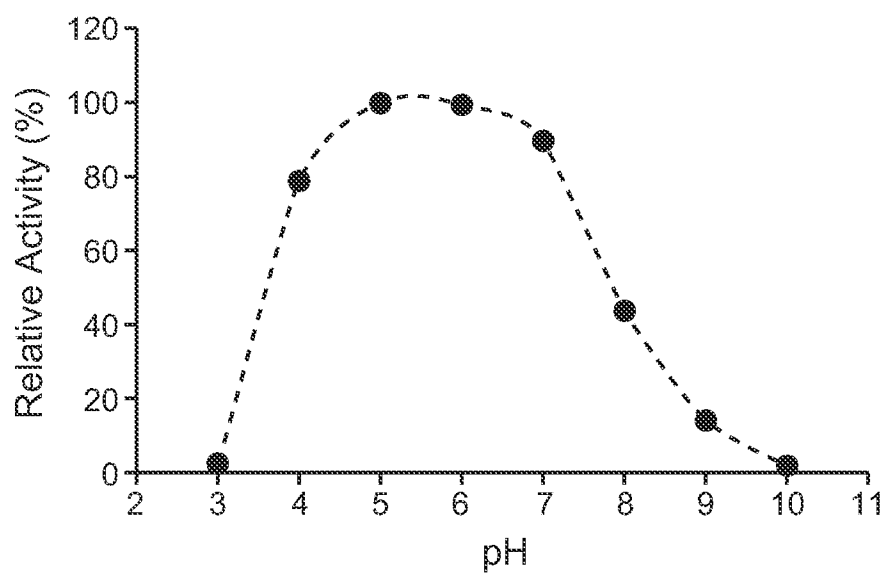
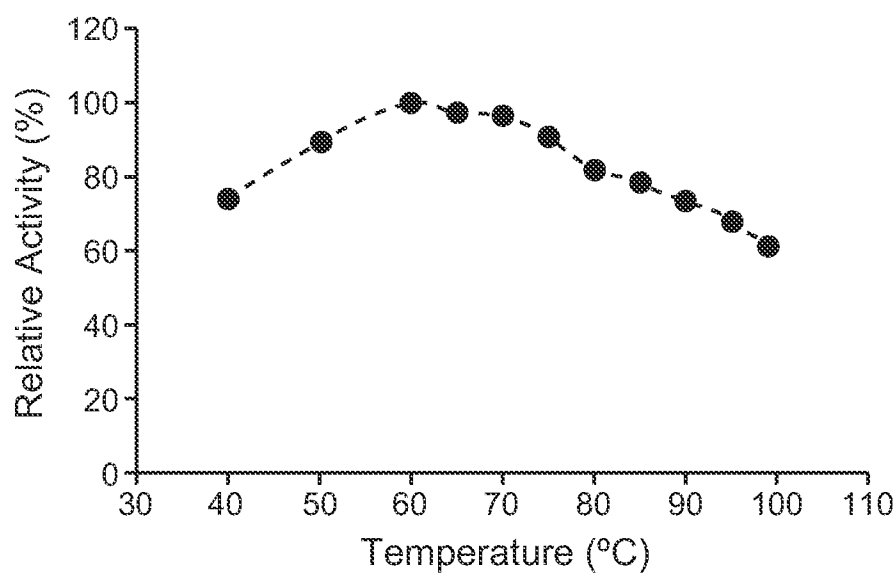
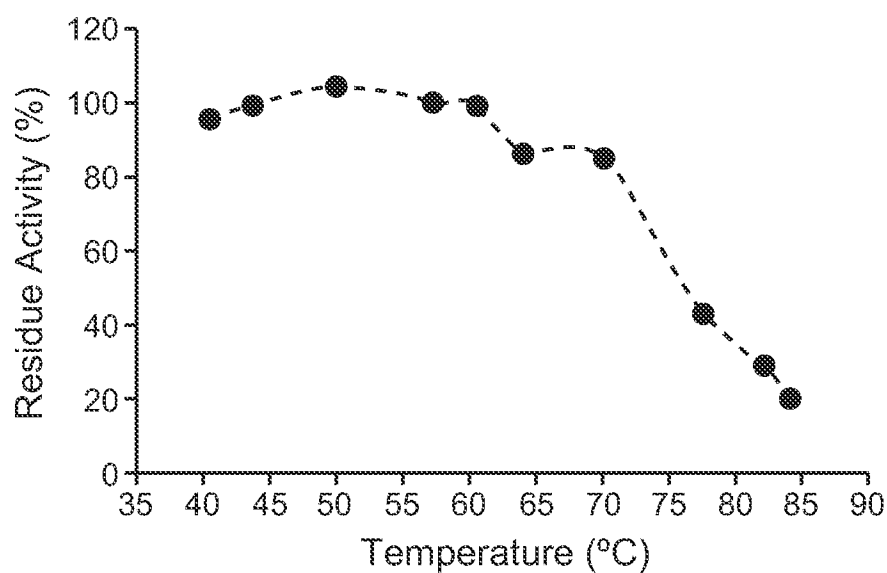
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**FIG. 1****FIG. 2**

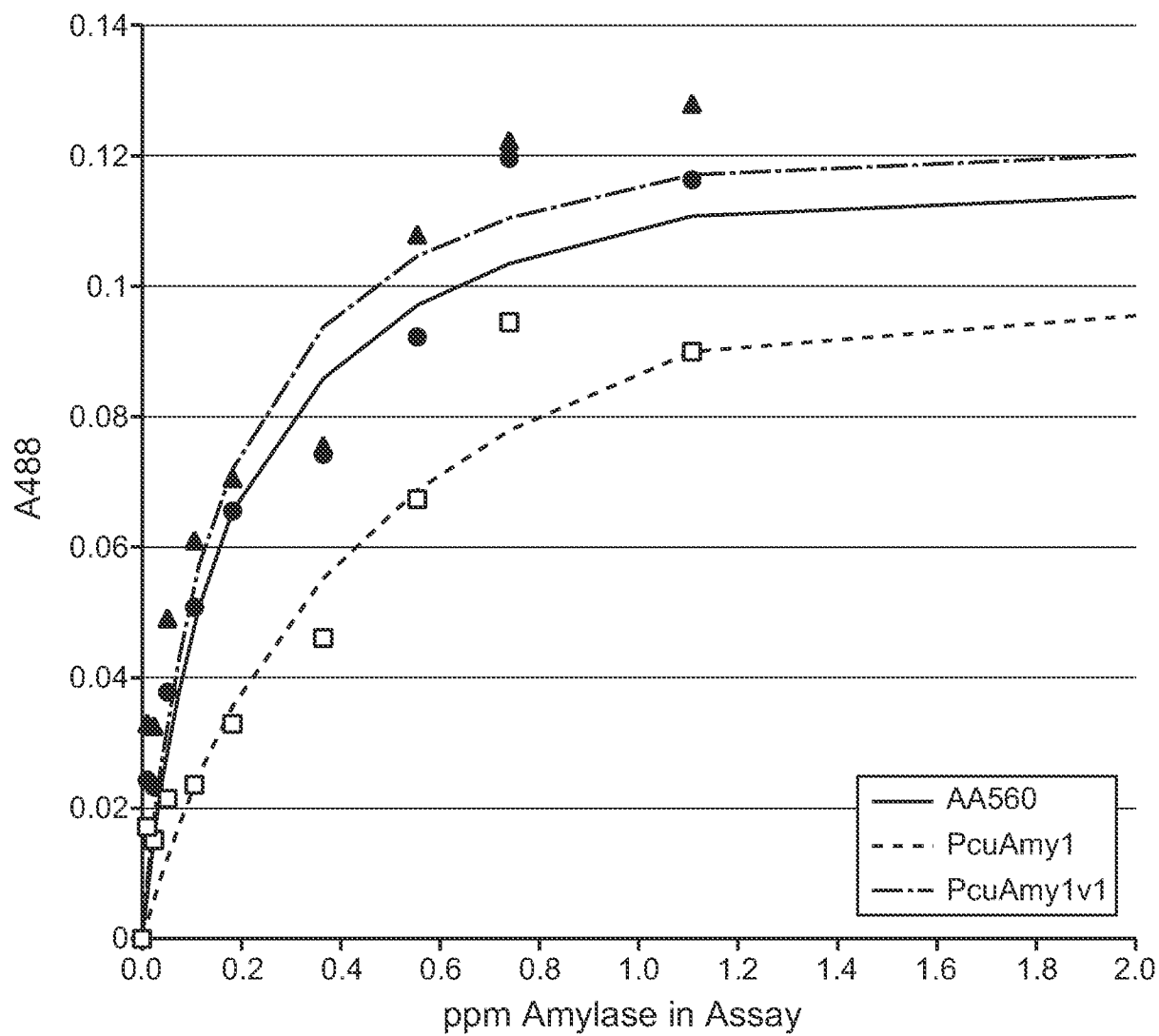
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FIG. 3**FIG. 4****FIG. 5**

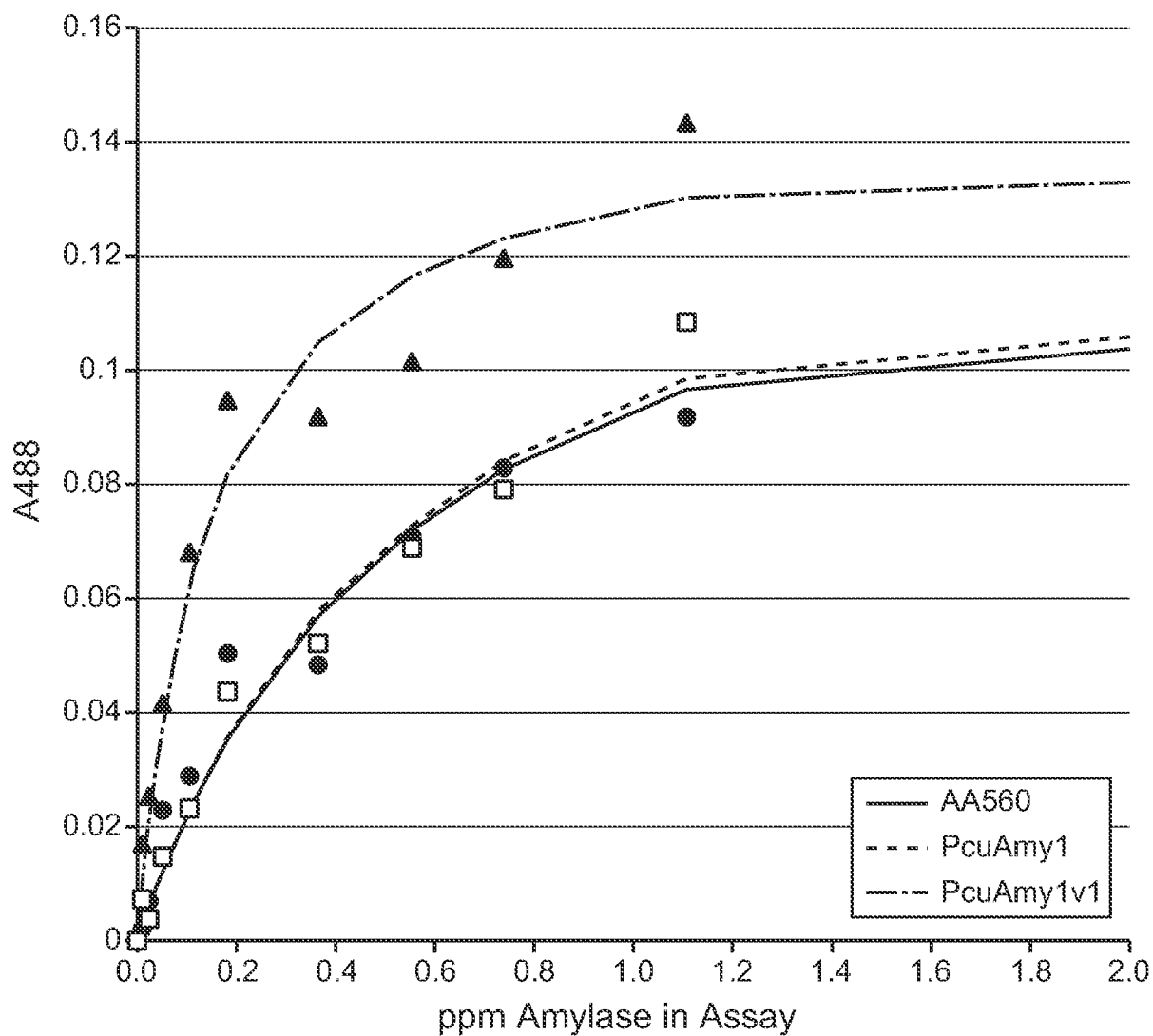
3 / 7

FIG. 6**FIG. 7****FIG. 8**

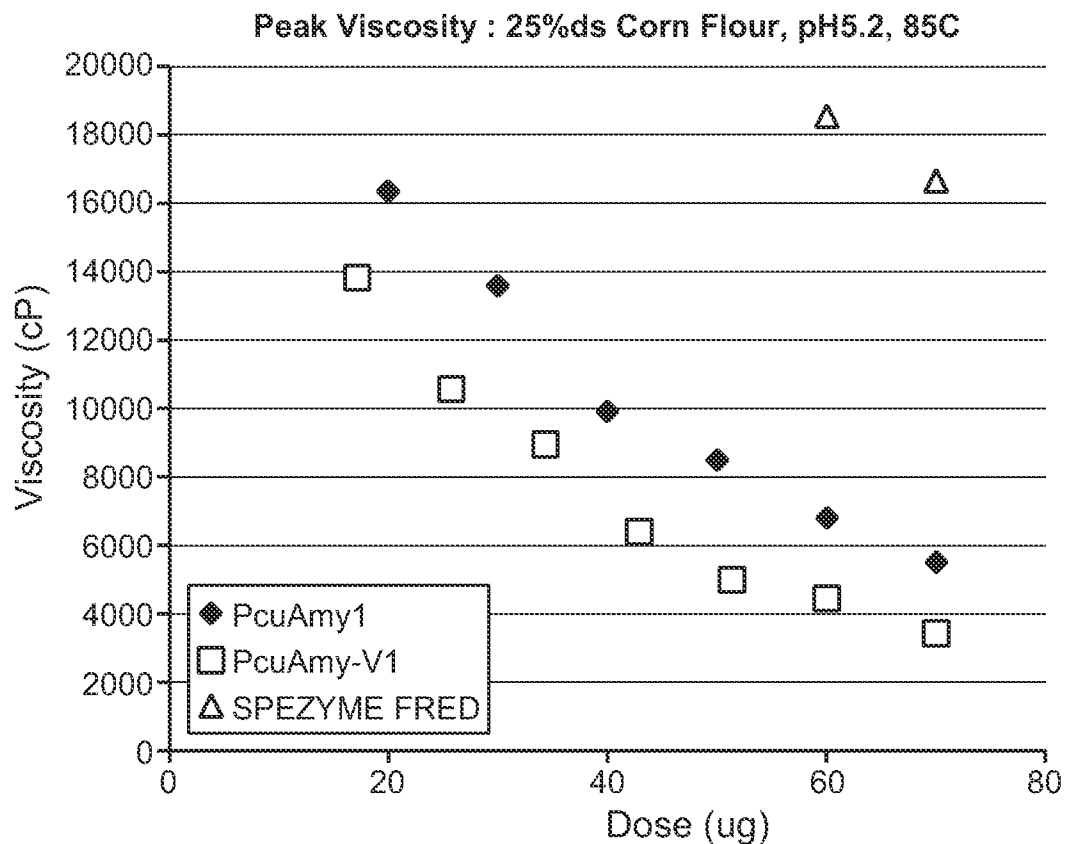
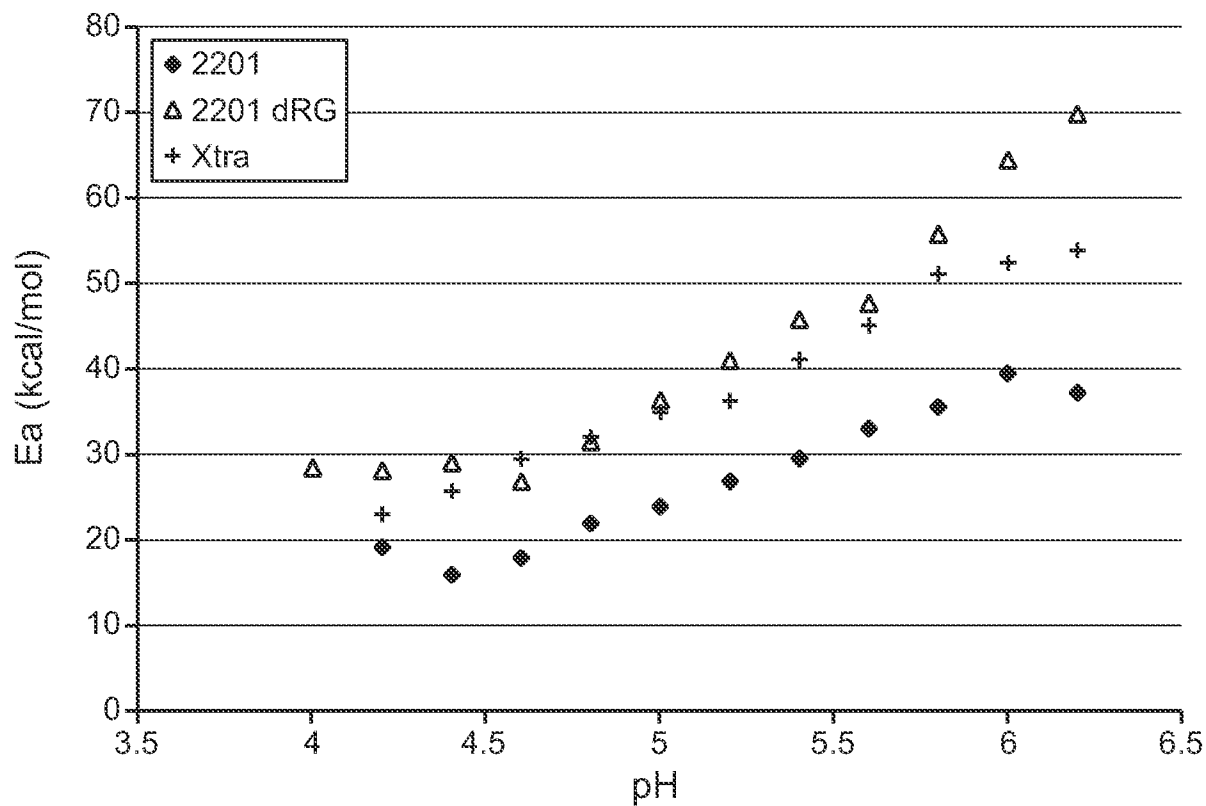
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**FIG. 9**

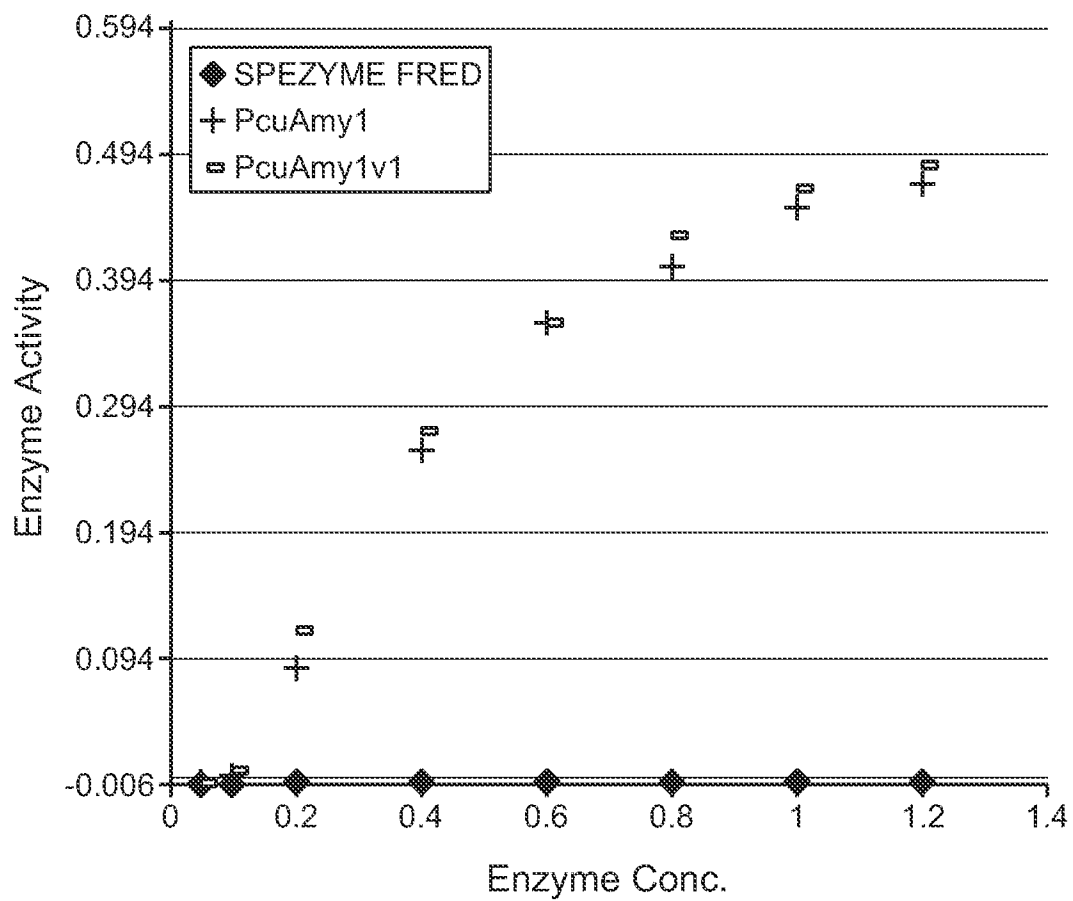
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**FIG. 10**

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**FIG. 11****FIG. 12**

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**FIG. 13**

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/074285

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/26
ADD.

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

B. FIELDS SEARCHED

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

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/017446 A2 (INTERNAT ADVANCED RES CT FOR POWDER METALLURG AND NEW MATERIALS ARCI []) 9 February 2012 (2012-02-09) whole document cf. esp to title and claim 1	48
X	----- CN 102 342 555 A (YUFEI WANG) 8 February 2012 (2012-02-08) whole document esp. abstract	50
X	----- DATABASE UniProt [Online] 2 November 2010 (2010-11-02), XP002723904, Database accession no. E014J1	1,4-7
Y	the whole document ----- -/--	1-67



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

13 May 2014

Date of mailing of the international search report

03/06/2014

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INTERNATIONAL SEARCH REPORT

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PCT/US2013/074285

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	whole document esp. pages 16, 18, 50 and seq id nos 4 and 2 -----	1-67
A	P. PASON ET AL: "Paenibacillus curdlanolyticus Strain B-6 Xylanolytic-Cellulolytic Enzyme System That Degrades Insoluble Polysaccharides", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 72, no. 4, 1 April 2006 (2006-04-01), pages 2483-2490, XP055106867, ISSN: 0099-2240, DOI: 10.1128/AEM.72.4.2483-2490.2006 the whole document -----	1-67

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International application No

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