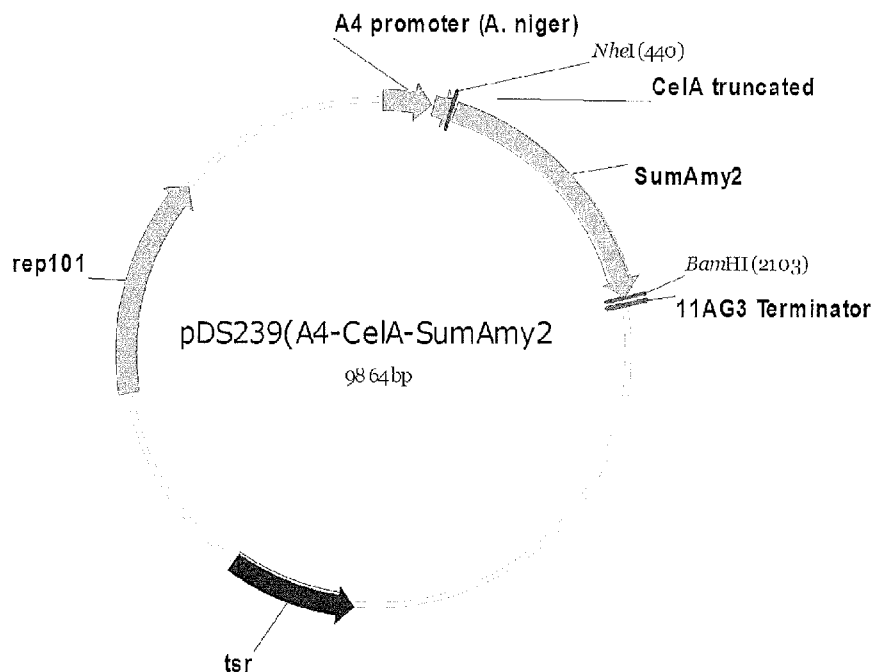




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[Continued on next page]

(54) **Title:** ALPHA-AMYLASE FROM *STREPTOMYCES UMBRINUS***Figure 1**

(57) **Abstract:** Disclosed are compositions and methods relating to an alpha-amylase from *Streptomyces umbrinus*. The compositions and methods are useful, for example, for 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.



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ALPHA-AMYLASE FROM *STREPTOMYCES UMBRINUS***5 CROSS REFERENCE TO RELATED APPLICATIONS**

[001] This application claims benefit of priority from international application PCT/CN2013/077134, filed 13 June 2013 and is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 [002] Disclosed are compositions and methods relating to an α -amylase enzyme from *Streptomyces umbrinus*. The α -amylases are useful, for example, for starch liquefaction and saccharification, cleaning starchy stains, textile desizing, baking, and brewing.

BACKGROUND

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

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

SUMMARY

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

1. In one aspect, a recombinant α -amylase is provided, having α -amylase activity and comprising an amino acid sequence having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 4.

2. In some embodiments, the α -amylase of paragraph 1 has at least 97% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 4.

3. In some embodiments, the α -amylase of any of the preceding numbered paragraphs further comprises conservative substitutions of one or several amino acid residues.

4. In some embodiments, the α -amylase of any of the preceding numbered paragraphs further comprises a deletion, substitution, insertion, or addition of one or a few amino acid residues.

5. In some embodiments, the α -amylase of any of the preceding numbered paragraphs is derived from the amino acid sequence of SEQ ID NO: 4 by conservative substitution of one or several amino acid residues.

6. In some embodiments, the α -amylase of any of the preceding numbered paragraphs is derived from the amino acid sequence of SEQ ID NO: 4 by deletion, substitution, insertion, or addition of one or a few amino acid residues.

7. In some embodiments, the α -amylase of any of the preceding numbered paragraphs is encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid that is complementary to a nucleic acid that encodes SEQ ID NO: 4.

8. In some embodiments, the α -amylase of any of the preceding numbered paragraphs is encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid that is complementary to the nucleic acid of SEQ ID NO: 1.

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

10. In some embodiments, the composition of the preceding numbered paragraph further comprises a surfactant.

11. In some embodiments, the composition of preceding numbered paragraphs 9 or 10 is a detergent composition.

12. In some embodiments, the composition of any of preceding numbered paragraphs 9-11 is a laundry detergent, a laundry detergent additive, or a manual or automatic dishwashing detergent.

13. In some embodiments, the composition of any of preceding numbered paragraphs 9-12 further comprises 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
5 lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, metalloproteinase, amadoriase, phytase, and an amylase other than the amylase of numbered paragraphs 1-8.

14. In some embodiments, the composition of preceding numbered paragraph 9 is
10 for saccharifying a composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

15. In some embodiments, the composition of preceding numbered paragraph 9 is for producing a fermented beverage or a baked food product.

16. In some embodiments, the composition of preceding numbered paragraphs 9 or
15 10 is for textile desizing.

17. In another aspect, a method for removing a starchy stain or soil from a surface is provided, comprising: contacting the surface with a composition comprising an effective amount of the α -amylase of any of numbered paragraphs 1-8; and allowing the α -amylase to hydrolyze starch components present in the starchy stain to produce smaller starch-derived
20 molecules that dissolve in aqueous solution; thereby removing the starchy stain from the surface.

18. In some embodiments of the method of numbered paragraph 17, the aqueous composition further comprises a surfactant.

19. In some embodiments of the method of numbered paragraphs 17 or 18, the surface is a textile surface or a surface on dishware.

20. In some embodiments of the method for any of numbered paragraphs 17-19, 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
25 lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, metalloproteinase, amadoriase, phytase, and an amylase other than the amylase of any one of numbered paragraphs 1-8.
30

21. In another aspect, a method for desizing a textile is provided, comprising: contacting a sized textile with an effective amount of the α -amylase of any of numbered paragraphs 1-8; and allowing the α -amylase to hydrolyze starch components in the size to produce smaller starch-derived molecules that dissolve in aqueous solution; thereby removing the size from the textile.

22. In another aspect, a method for saccharifying a composition comprising starch to produce a composition comprising glucose is provided, the method comprising: contacting the composition comprising starch with effective amount of the amylase of any of numbered paragraphs 1-8; and saccharifying the composition comprising starch to produce the composition comprising glucose; wherein the α -amylase catalyzes the saccharification of the starch solution to glucose.

23. In some embodiments of the method of numbered paragraph 22, the composition comprises starch comprises liquefied starch, gelatinized starch, or granular starch.

24. In another aspect, a method for preparing a foodstuff or beverage is provided, comprising, contacting a foodstuff or beverage comprising starch with an α -amylase of any of numbered paragraphs 1-8; and allowing the α -amylase to hydrolyze the starch to produce smaller starch-derived molecules.

25. In some embodiments, the method of numbered paragraph 24, further comprises contacting the foodstuff or beverage with 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, α -glucosidase, beta-glucosidase, phytase, or a combination thereof.

26. In some embodiments of the method of any one of numbered paragraphs 17–25, the α -amylase is expressed and secreted by a host cell.

27. In some embodiments of the method of numbered paragraph 26, the composition comprising starch is contacted with the host cell.

28. In some embodiments of the method of numbered paragraph 26 or 27, the host cell further expresses and secretes a glucoamylase or other enzyme.

29. In some embodiments of the method of any one of numbered paragraphs 26-28, the host cell is capable of fermenting the composition.

30. In another aspect, a composition comprising glucose produced by the method of any one of numbered paragraphs 22-29 is provided.

31. In another aspect, liquefied starch produced by the method of any one of numbered paragraphs 22-29 is provided.

5 32. In another aspect, a foodstuff or beverage produced by the method of any one of numbered paragraphs 24-29 is provided.

33. In another aspect, the use of the α -amylase of any of numbered paragraphs 1-8 in the production of a composition comprising glucose is provided.

10 34. In another aspect, the use of the α -amylase of any of numbered paragraphs 1-8 in the production of a liquefied starch is provided.

35. In another aspect, the use of the α -amylase of any of numbered paragraphs 1-8 in the production of a foodstuff or beverage is provided.

36. In another aspect, the use of the α -amylase of any of numbered paragraphs 1-8 in cleaning starchy stains.

15 37. In another aspect, the use of the α -amylase of any of numbered paragraphs 1-8 in textile desizing is provided.

38. In another aspect, a recombinant polynucleotide encoding a polypeptide of any of numbered paragraphs 1-8 is provided.

20 39. In some embodiments, the polynucleotide of numbered paragraph 38 has at least 80% nucleic acid sequence identity to the polynucleotide of SEQ ID NO: 1.

40. In some embodiments, the polynucleotide of numbered paragraphs 38 or 39 hybridizes under stringent conditions to a nucleic acid that is complementary to a nucleic acid encoding SEQ ID NO: 4.

25 41. In some embodiments, the polynucleotide of numbered paragraphs 38 or 39 hybridizes under stringent conditions to a nucleic acid that is complementary to the nucleic acid of SEQ ID NO: 1.

42. In another aspect, an expression vector comprising the polynucleotide of any of preceding numbered paragraphs 38-41 is provided.

30 43. In another aspect, a host cell comprising the expression vector of numbered paragraph 42 is provided.

[006] These and other aspects and embodiments of the compositions and methods will be apparent from the present description and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

- 5 [007] Figure 1 is a map of the expression plasmid made to express SumAmy2.
- [008] Figure 2 is a graph showing the pH profile of SumAmy2.
- [009] Figure 3 is a graph showing the temperature profile of SumAmy2.
- [0010] Figure 4A and 4B are graphs showing the dose response of SumAmy2 protein tested on CS28 microswatches at pH 8/ 25° (4A) and pH 10/ 32°C (4B).
- 10 [0011] Figure 5 is an amino acid sequence alignment of SumAmy2 (SEQ ID NO: 4) with AmyS (SEQ ID NO: 7) and AmyL (SEQ ID NO: 8) using CLUSTAL 2.1 multiple sequence alignment with default parameters.
- [0012] Figure 6A-C is an amino acid sequence alignment of SumAmy2 and homologs using CLUSTAL 2.1 multiple sequence alignment with default parameters.
- 15 [0013] Figure 7 is a phylogenetic tree for SumAmy2 and its homologs.

DETAILED DESCRIPTION

- [0014] Described are compositions and methods relating to an α -amylase enzyme from *Streptomyces umbrinus*, herein referred to as SumAmy2. Exemplary applications for the α -
- 20 amylase enzymes are for 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 and for baking and brewing. These and other aspects of the compositions and methods are described in detail, below.
- [0015] Prior to describing the various aspects and embodiments of the present compositions and
- 25 methods, the following definitions and abbreviations are described.

1. Definitions and Abbreviations

- [0016] In accordance with this detailed description, the following abbreviations and definitions apply. Note that the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an enzyme” includes a
- 30 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.

[0017] The present document is organized into a number of sections for ease of reading; however, the reader will appreciate that statements made in one section may apply to other sections. In this manner, the headings used for different sections of the disclosure should not be construed as limiting.

5 [0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

1.1. Abbreviations and Acronyms

[0019] The following abbreviations/acronyms have the following meanings unless otherwise
10 specified:

	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
15	AnGA	<i>Aspergillus niger</i> glucoamylase
	AOS	α -olefinsulfonate
	AS	alkyl sulfate
	cDNA	complementary DNA
	CMC	carboxymethylcellulose
20	DE	dextrose equivalent
	DNA	deoxyribonucleic acid
	DPn	degree of saccharide polymerization having n subunits
	ds or DS	dry solids
	DTMPA	diethylenetriaminepentaacetic acid
25	EC	Enzyme Commission
	EDTA	ethylenediaminetetraacetic acid
	EO	ethylene oxide (polymer fragment)
	EOF	End of Fermentation
	GA	glucoamylase
30	GAU/g ds	glucoamylase activity unit/gram dry solids
	HFCS	high fructose corn syrup
	HgGA	<i>Humicola grisea</i> glucoamylase
	IPTG	isopropyl β -D-thiogalactoside
	IRS	insoluble residual starch
35	kDa	kiloDalton
	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
40	NCBI	National Center for Biotechnology Information
	NOBS	nonanoyloxybenzenesulfonate
	NTA	nitriloacetic acid
	OxAm	Purastar HPAM 5000L (Danisco US Inc.)
	PAHBAH	p-hydroxybenzoic acid hydrazide
45	PEG	polyethyleneglycol

	pI	isoelectric point
	PI	performance index
	ppm	parts per million, <i>e.g.</i> , µg protein per gram dry solid
	PVA	poly(vinyl alcohol)
5	PVP	poly(vinylpyrrolidone)
	RCF	relative centrifugal/centripetal force (<i>i.e.</i> , x gravity)
	RNA	ribonucleic acid
	SAS	alkanesulfonate
	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
10	SSF	simultaneous saccharification and fermentation
	SSU/g solid	soluble starch unit/gram dry solids
	sp.	species
	TAED	tetraacetythylenediamine
	Tm	melting temperature
15	TrGA	<i>Trichoderma reesei</i> glucoamylase
	w/v	weight/volume
	w/w	weight/weight
	v/v	volume/volume
	wt%	weight percent
20	°C	degrees Centigrade
	H ₂ O	water
	dH ₂ O or DI	deionized water
	dIH ₂ O	deionized water, Milli-Q filtration
	g or gm	grams
25	µg	micrograms
	mg	milligrams
	kg	kilograms
	µL and µl	microliters
	mL and ml	milliliters
30	mm	millimeters
	µm	micrometer
	M	molar
	mM	millimolar
	µM	micromolar
35	U	units
	sec	seconds
	min(s)	minute/minutes
	hr(s)	hour/hours
	DO	dissolved oxygen
40	Ncm	Newton centimeter
	ETOH	ethanol
	eq.	equivalents
	N	normal
	MWCO	molecular weight cut-off
45	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
50		

1.2. Definitions

[0020] 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 $\rightarrow$ 4) O-glycosidic linkages in starch. Generally, α -amylases (EC 3.2.1.1; α -D-(1 $\rightarrow$ 4)-glucan glucanohydrolase) are defined as endo-acting enzymes cleaving α -D-(1 $\rightarrow$ 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 $\rightarrow$ 4)-glucan maltohydrolase) and some product-specific amylases like maltogenic α -amylase (EC 3.2.1.133) cleave the polysaccharide 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 $\rightarrow$ 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.

[0021] “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 of glucose per minute from soluble starch substrate (4% DS) at pH 4.5, 50°C. DS refers to “dry solids.”

[0022] The term “starch” refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein X can be any number. The term includes plant-based materials such as grains, cereal, grasses, tubers and roots, and more specifically materials obtained from wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, milo, potato, sweet potato, and tapioca. The term “starch” includes granular starch. The term “granular starch” refers to raw, *i.e.*, uncooked starch, *e.g.*, starch that has not been subject to gelatinization.

[0023] The terms, “wild-type,” “parental,” or “reference,” with respect to a polypeptide, refer to a naturally-occurring polypeptide that does not include a man-made substitution, insertion, or deletion at one or more amino acid positions. Similarly, the terms “wild-type,” “parental,” or “reference,” with respect to a polynucleotide, refer to a naturally-occurring polynucleotide that does not include a man-made nucleoside change. However, note that a polynucleotide encoding a wild-type, parental, or reference polypeptide is not limited to a naturally-occurring

polynucleotide, and encompasses any polynucleotide encoding the wild-type, parental, or reference polypeptide.

[0024] 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.

[0025] The term “variant,” with respect to a polypeptide, refers to a polypeptide that differs from a specified wild-type, parental, or reference polypeptide in that it includes one or more naturally-occurring or man-made substitutions, insertions, or deletions of an amino acid.

Similarly, the term “variant,” with respect to a polynucleotide, refers to a polynucleotide that differs in nucleotide sequence from a specified wild-type, parental, or reference polynucleotide. The identity of the wild-type, parental, or reference polypeptide or polynucleotide will be apparent from context.

[0026] In the case of the present α -amylases, “activity” refers to α -amylase activity, which can be measured as described, herein.

[0027] 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.

[0028] The terms “recovered,” “isolated,” and “separated,” refer to a compound, protein (polypeptides), cell, nucleic acid, amino acid, or other specified material or component that is removed from at least one other material or component with which it is naturally associated as found in nature. An “isolated” polypeptides, thereof, includes, but is not limited to, a culture broth containing secreted polypeptide expressed in a heterologous host cell.

[0029] The term “purified” refers to material (*e.g.*, an isolated polypeptide or polynucleotide) that is in a relatively pure state, *e.g.*, at least about 90% pure, at least about 95% pure, at least about 98% pure, or even at least about 99% pure.

[0030] The term “enriched” refers to material (*e.g.*, an isolated polypeptide or polynucleotide) that is in about 50% pure, at least about 60% pure, at least about 70% pure, or even at least about 70% pure.

[0031] 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.

[0032] A “pH range,” with reference to an enzyme, refers to the range of pH values under which the enzyme exhibits catalytic activity.

[0033] 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).

[0034] 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).

[0035] 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.

[0036] “Hybridization” refers to the process by which one strand of nucleic acid forms a duplex with, *i.e.*, base pairs with, a complementary strand, as occurs during blot hybridization techniques and PCR techniques. Stringent hybridization conditions are exemplified by hybridization under the following conditions: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M Na₃ 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 .

[0037] A “synthetic” molecule is produced by *in vitro* chemical or enzymatic synthesis rather than by an organism.

[0038] 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.

[0039] 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.

[0040] A “host strain” or “host cell” is an organism into which an expression vector, phage, virus, or other DNA construct, including a polynucleotide encoding a polypeptide of interest (*e.g.*, an amylase) has been introduced. Exemplary host strains are microorganism cells (*e.g.*, bacteria, filamentous fungi, and yeast) capable of expressing the polypeptide of interest and/or fermenting saccharides. The term “host cell” includes protoplasts created from cells.

[0041] 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.

[0042] The term “endogenous” with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

[0043] 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.

[0044] 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.

[0045] 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.

[0046] An “expression vector” refers to a DNA construct comprising a DNA sequence encoding a polypeptide of interest, which coding sequence is operably linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation.

[0047] The term “operably linked” means that specified components are in a relationship (including but not limited to juxtaposition) permitting them to function in an intended manner. For example, a regulatory sequence is operably linked to a coding sequence such that expression of the coding sequence is under control of the regulatory sequences.

- 5 [0048] A “signal sequence” is a sequence of amino acids attached to the N-terminal portion of a protein, which facilitates the secretion of the protein outside the cell. The mature form of an extracellular protein lacks the signal sequence, which is cleaved off during the secretion process.

[0049] “Biologically active” refer to a sequence having a specified biological activity, such an enzymatic activity.

- 10 [0050] The term “specific activity” refers to the number of moles of substrate that can be converted to product by an enzyme or enzyme preparation per unit time under specific conditions. Specific activity is generally expressed as units (U)/mg of protein.

[0051] As used herein, “water hardness” is a measure of the minerals (*e.g.*, calcium and magnesium) present in water.

- 15 [0052] A “swatch” is a piece of material such as a fabric that has a stain applied thereto. The 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,
20 pigment, oil, or mixtures of these compounds.

- [0053] 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
25 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
30 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.

[0054] “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.

[0055] “Percent sequence identity” means that a particular sequence has at least a certain

percentage of amino acid residues identical to those in a specified reference sequence, when

aligned using the CLUSTAL W algorithm with default parameters. *See* Thompson *et al.* (1994)

Nucleic Acids Res. 22:4673-4680. Default parameters for the CLUSTAL W algorithm are:

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

[0056] Deletions are counted as non-identical residues, compared to a reference sequence.

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

[0057] “Fused” polypeptide sequences are connected, *i.e.*, operably linked, via a peptide bond between two subject polypeptide sequences.

[0058] The term “filamentous fungi” refers to all filamentous forms of the subdivision Eumycotina, particularly Pezizomycotina species.

[0059] The term “degree of polymerization” (DP) refers to the number (n) of anhydro-

glucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides glucose and fructose. Examples of DP2 are the disaccharides maltose and sucrose. The term “DE,” or

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

[0060] 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.

5 [0061] The phrase “simultaneous saccharification and fermentation (SSF)” refers to a process in the production of biochemicals in which a microbial organism, such as an ethanologenic microorganism, and at least one enzyme, such as an amylase, are present during the same process step. SSF includes the contemporaneous hydrolysis of starch substrates (granular, liquefied, or solubilized) to saccharides, including glucose, and the fermentation of the
10 saccharides into alcohol or other biochemical or biomaterial in the same reactor vessel.

[0062] An “ethanologenic microorganism” refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

[0063] 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
15 fermentation. “Beer” is an example of such a fermented beverage, and the term “beer” is meant to comprise any fermented wort produced by fermentation/brewing of a starch-containing plant material. Often, beer is produced exclusively from malt or adjunct, or any combination of malt and adjunct.

[0064] The term “malt” refers to any malted cereal grain, such as malted barley or wheat.

20 [0065] 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, cassava and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or
25 wheat syrups, and the like.

[0066] 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.

[0067] The term “wort” refers to the unfermented liquor run-off following extracting the grist
30 during mashing.

[0068] “Iodine-positive starch” or “IPS” refers to (1) amylose that is not hydrolyzed after liquefaction and saccharification, or (2) a retrograded starch polymer. When saccharified starch

or saccharide liquor is tested with iodine, the high DPn amylose or the retrograded starch polymer binds iodine and produces a characteristic blue color. The saccharide liquor is thus termed “iodine-positive saccharide,” “blue saccharide,” or “blue sac.”

[0069] The terms “retrograded starch” or “starch retrogradation” refer to changes that occur spontaneously in a starch paste or gel on ageing.

[0070] The term “about” refers to $\pm 15\%$ to the referenced value.

2. α -Amylase from *Streptomyces umbrinus*

[0071] An aspect of the present compositions and methods relates to an α -amylase enzyme from *Streptomyces umbrinus*, herein referred to as SumAmy2. As described in detail in the appended Examples, the amino acid sequence of SumAmy2 (*i.e.*, SEQ ID NO: 4) shares very low amino acid sequence identity (*i.e.*, about 20%) with the amino acid sequences of *Geobacillus* (formerly *Bacillus*) *stearothermophilus* α -amylase (AmyS; SEQ ID NO: 7) and *Bacillus licheniformis* α -amylase (AmyL; SEQ ID NO: 8) using a CLUSTAL 2.1 multiple sequence alignment. These latter two enzymes are typical of those currently used in commercial applications; therefore, the present α -amylases represent a significant departure from these previously described enzymes in terms of sequence space. SumAmy2 is most similar to an α -amylase from *Streptomyces griseus*, with which it shares 94% amino acid sequence identity. Amino acid sequence alignments are shown in Figures 5 and 6 and a phylogenetic tree is shown in Figure 7. Percent amino acid sequence identities to homologs are shown in Table 3.

[0072] In some embodiments, the present α -amylases have a defined degree of amino acid sequence identity to SEQ ID NO: 4, for example, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity. In some embodiments, the present α -amylase are derived from a parental amylase having a defined degree of amino acid sequence identity to SEQ ID NO: 4, for example, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99%, amino acid sequence identity.

[0073] In some embodiments, the present α -amylases comprise conservative substitution of one or several amino acid residues relative to the amino acid sequence of SEQ ID NO: 4.

Exemplary conservative amino acid substitutions are listed in the Table 1. Some conservative mutations can be produced by genetic manipulation, while others are produced by introducing synthetic amino acids into a polypeptide other means.

Table 1. Conservative amino acid substitutions

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

[0074] In some embodiments, the present α -amylases comprises a deletion, substitution,

insertion, or addition of one or a few amino acid residues relative to the amino acid sequence of SEQ ID NO: 4. In some embodiments, the present α -amylases are derived from the amino acid sequence of SEQ ID NO: 4 by conservative substitution of one or several amino acid residues.

In some embodiments, the present α -amylases are derived from the amino acid sequence of SEQ ID NO: 4 by deletion, substitution, insertion, or addition of one or a few amino acid residues

relative to the amino acid sequence of SEQ ID NO: 4. In all cases, the expression “one or a few amino acid residues” refers to 10 or less, *i.e.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, amino acid residues.

[0075] In some embodiments, the present α -amylases are encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid sequence that is complementary to a nucleic acid that encodes SEQ ID NO: 4. An exemplary nucleic acid sequence that encodes

SEQ ID NO: 4 is SEQ ID NO: 1.

[0076] The present amylases may be “precursor,” “immature,” or “full-length,” in which case they include a signal sequence, or “mature,” in which case they lack a signal sequence. Mature forms of the polypeptides are generally the most useful. Unless otherwise noted, the amino acid residue numbering used herein refers to the mature forms of the respective amylase

5 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.

[0077] The present amylase may be a “chimeric” or “hybrid” polypeptide, in that it includes 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

10 amylases). The present amylases may further include heterologous signal sequence, an epitope to allow tracking or purification, or the like. Exemplary heterologous signal sequences are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces* CelA.

[0078] In another aspect, nucleic acids encoding an α -amylase polypeptide are provided. The nucleic acid may encode the amylase having the amino acid sequence of SEQ ID NO: 4, or an

15 amylase having a specified degree of amino acid sequence identity to the amylase having the amino acid sequence of SEQ ID NO: 4. In some embodiments, the nucleic acid encodes an amylase having 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: 4. It will be appreciated that due to the degeneracy of the genetic code, a plurality of nucleic acids may encode the same polypeptide.

20 [0079] In another example, the nucleic acid hybridizes under stringent or very stringent conditions to a nucleic acid complementary to a nucleic acid encoding an amylase having 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: 4. Such hybridization conditions are described herein but are also well known in the art. In some embodiments, the nucleic acid has at least 80%, at least 85%, at least

25 90%, at least 95%, or even at least 98% amino acid sequence identity to SEQ ID NO: 1

[0080] 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. Preferably, the nucleic acid are of sufficient length to encode an active amylase enzyme.

30 [0081] 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 α -Amylases

[0082] The present α -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 α -amylase can be obtained following secretion of the α -amylase into the cell medium. Optionally, the α -amylase can be isolated from the host cells, or even isolated from the cell broth, depending on the desired purity of the final α -amylase. A gene encoding an α -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*.

[0083] 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

[0084] A DNA construct comprising a nucleic acid encoding α -amylases can be constructed to be expressed in a host cell. Representative nucleic acids that encode α -amylases include SEQ ID NO: 1. Because of the well-known degeneracy in the genetic code, different polynucleotides that encode an identical amino acid sequence can be designed and made with routine skill. It is also well-known in the art to optimize codon use for a particular host cell. Nucleic acids encoding α -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.

[0085] The vector may be any vector that can be transformed into and replicated within a host cell. For example, a vector comprising a nucleic acid encoding an α -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.

[0086] A nucleic acid encoding an α -amylase can be operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Exemplary promoters for directing the transcription of the DNA sequence encoding an α -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.

[0087] 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.

[0088] An expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably linked to the DNA sequence encoding an α -amylase. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

- 5 [0089] 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.

[0090] 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.*

- 10 *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.

- 15 [0091] 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.

- [0092] The expression vector typically includes the components of a cloning vector, such as, for
20 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
25 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.

- 30 [0093] The procedures used to ligate the DNA construct encoding an amylase, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (*see, e.g.*,

Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., Cold Spring Harbor, 1989, and 3rd ed., 2001).

3.2. Transformation and Culture of Host Cells

[0094] 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.

[0095] Examples of suitable bacterial host organisms are Gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Geobacillus* (formerly *Bacillus*) *stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus megaterium*, and *Bacillus thuringiensis*; *Streptomyces* species such as *Streptomyces murinus*; lactic acid bacterial species including *Lactococcus* sp. such as *Lactococcus lactis*; *Lactobacillus* sp. including *Lactobacillus reuteri*; *Leuconostoc* sp.; *Pediococcus* sp.; and *Streptococcus* sp. Alternatively, strains of a Gram negative bacterial species belonging to *Enterobacteriaceae* including *E. coli*, or to *Pseudomonadaceae* can be selected as the host organism.

[0096] A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp., or *Kluyveromyces*, *Yarrowinia*, *Schizosaccharomyces* species or a species of *Saccharomyces*, including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces* such as, for example, *S. pombe* species. A strain of the methylotrophic yeast species, *Pichia pastoris*, can be used as the host organism. Alternatively, the host organism can be a *Hansenula* species. Suitable host organisms among filamentous fungi include species of *Aspergillus*, *e.g.*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus tubigensis*, *Aspergillus awamori*, or *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, *e.g.*, *Fusarium oxysporum* or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species. In addition, *Trichoderma* sp. can be used as a host. A suitable procedure for transformation of *Aspergillus* host cells includes, for

example, that described in EP 238023. An amylase expressed by a fungal host cell can be glycosylated, *i.e.*, will comprise a glycosyl moiety. The glycosylation pattern can be the same or different as present in the wild-type amylase. The type and/or degree of glycosylation may impart changes in enzymatic and/or biochemical properties.

5 [0097] It is advantageous to delete genes from expression hosts, where the gene deficiency can be cured by the transformed expression vector. Known methods may be used to obtain a fungal host cell having one or more inactivated genes. Gene inactivation may be accomplished by complete or partial deletion, by insertional inactivation or by any other means that renders a gene nonfunctional for its intended purpose, such that the gene is prevented from expression of a
10 functional protein. Any gene from a *Trichoderma* sp. or other filamentous fungal host that has been cloned can be deleted, for example, *cbh1*, *cbh2*, *egl1*, and *egl2* genes. Gene deletion may be accomplished by inserting a form of the desired gene to be inactivated into a plasmid by methods known in the art.

[0098] Introduction of a DNA construct or vector into a host cell includes techniques such as
15 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
20 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.

25 [0099] 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
30 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.

[00100] Uptake of DNA into the host *Trichoderma* sp. strain depends upon the calcium ion concentration. Generally, between about 10-50 mM CaCl₂ 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.

[00101] Usually transformation of *Trichoderma* sp. uses protoplasts or cells that have been subjected to a permeability treatment, typically at a density of 10⁵ to 10⁷/mL, particularly 2x10⁶/mL. A volume of 100 µL of these protoplasts or cells in an appropriate solution (*e.g.*, 1.2 M sorbitol and 50 mM CaCl₂) may be mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension; however, it is useful to add about 0.25 volumes to the protoplast suspension. Additives, such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like, may also be added to the uptake solution to facilitate transformation. Similar procedures are available for other fungal host cells. *See, e.g.*, U.S. Patent No. 6,022,725.

3.3. Expression

[00102] 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.

[00103] 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).

[00104] 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.

[00105] 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.

[00106] 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.

[00107] Host cells may be cultured under suitable conditions that allow expression of an amylase. Expression of the enzymes may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG or Sophorose. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TNTTM (Promega) rabbit reticulocyte system.

[00108] 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 α -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 4.0 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

[00109] To evaluate the expression of an amylase in a host cell, assays can measure the expressed protein, 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.

3.5. Methods for Enriching and Purifying α -Amylases

[00110] Fermentation, separation, and concentration techniques are well known in the art and conventional methods can be used in order to prepare a concentrated α -amylase polypeptide-containing solution.

[00111] After fermentation, a fermentation broth is obtained, the microbial cells and various suspended solids, including residual raw fermentation materials, are removed by conventional separation techniques in order to obtain an amylase solution. Filtration, centrifugation, microfiltration, rotary vacuum drum filtration, ultrafiltration, centrifugation followed by ultrafiltration, extraction, or chromatography, or the like, are generally used.

[00112] It is desirable to concentrate an α -amylase polypeptide-containing solution in order to optimize recovery. Use of unconcentrated solutions requires increased incubation time in order to collect the enriched or purified enzyme precipitate.

[00113] The enzyme containing solution is 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.

[00114] The enzyme solution is concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated α -amylase polypeptide-containing solution is at a desired level.

[00115] 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.

[00116] 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.

[00117] 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 α -amylase polypeptide and on its concentration in the concentrated enzyme solution.

[00118] 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.

[00119] 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.

[00120] Addition of the organic compound precipitation agent provides the advantage of high flexibility of the precipitation conditions with respect to pH, temperature, α -amylase concentration, precipitation agent concentration, and time of incubation.

[00121] 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.

[00122] 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.

[00123] 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.

[00124] 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.

[00125] 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.

[00126] 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.

[00127] 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.

[00128] During fermentation, an α -amylase polypeptide accumulates in the culture broth. For the isolation, enrichment, or purification of the desired 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.

[00129] 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).

[00130] 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. *See* Sumitani *et al.* (2000) *Biochem. J.* 350: 477-484, for general discussion of the method and variations thereon.

[00131] For production scale recovery, 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 available membranes and equipment. However, for some applications, the enzyme does not need to be enriched or purified, and whole broth culture can be lysed and used without further treatment. The enzyme can then be processed, for example, into granules.

4. Compositions and Uses of α -amylases

[00132] α -amylases are useful for a variety of industrial applications. For example, α -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.

[00133] 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. α -amylases are also useful in compositions and methods of food preparation. These various uses of α -amylases are described in more detail below.

4.1. Preparation of Starch Substrates

- 5 [00134] 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.
- 10 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.
- 15 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).
- [00135] The starch substrate can be a crude starch from milled whole grain, which contains
- 20 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
- 25 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 will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch. Dry grinding of the starch substrate can be used for production of ethanol and other biochemicals. The starch to be
- 30 processed may be a highly refined starch quality, for example, at least 90%, at least 95%, at least 97%, or at least 99.5% pure.

4.2. Gelatinization and Liquefaction of Starch

[00136] 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. 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 calcium from the slurry in cases where the enzyme is dependent upon calcium.

[00137] 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 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 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%.

[00138] Liquefaction with α -amylases advantageously can be conducted at low pH, eliminating the requirement to adjust the pH to about pH 5.5-6.5. α -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. α -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 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.

[00139] In particular embodiments using the present α -amylases, starch liquifaction is performed at a temperature range of 90-115°C, for the purpose of producing high-purity glucose syrups, HFCS, maltodextrins, etc.

4.3. Saccharification

[00140] The liquefied starch can be saccharified into a syrup rich in lower DP (*e.g.*, DP1 + DP2) saccharides, using α -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 α -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.

[00141] 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 saccharifying may be conducted over a pH range of about pH 3 to about pH 7, *e.g.*, pH 3.0 – pH 7.5, pH 3.5 – pH 5.5, pH 3.5, pH 3.8, or pH 4.5.

[00142] 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.

5 [00143] 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
10 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 an amylase and a glucoamylase, *e.g.*, HgGA, 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,
15 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.

20 4.4. Isomerization

[00144] 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
25 (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

30 [00145] The soluble starch hydrolysate, particularly a glucose rich syrup, can be fermented by contacting the starch hydrolysate with a fermenting organism typically at a temperature around 32°C, such as from 30°C to 35°C for alcohol-producing yeast. The temperature and pH of the fermentation will depend upon the fermenting organism. EOF products include metabolites,

such as citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine and other amino acids, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol and other biomaterials.

5 [00146] 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
10 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). Microorganisms that produce other metabolites, such as citric acid and lactic acid, by
15 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.

[00147] The saccharification and fermentation processes may be carried out as an SSF process. Fermentation may comprise subsequent enrichment, purification, and recovery of
20 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,
25 *e.g.*, livestock feed.

[00148] 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
30 enzyme has to be added exogenously. The fungal host cell can be from an appropriately engineered fungal strain. Fungal host cells that express and secrete other enzymes, in addition to amylase, also can be used. Such cells may express glucoamylase and/or a pullulanase, phytase,

alpha-glucosidase, isoamylase, beta-amylase cellulase, xylanase, other hemicellulases, protease, *beta*-glucosidase, pectinase, esterase, redox enzymes, transferase, or other enzyme.

[00149] 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.

[00150] 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 α -Amylases

[00151] α -amylases may be combined with a glucoamylase (EC 3.2.1.3), *e.g.*, a *Trichoderma* glucoamylase or variant thereof. An exemplary glucoamylase is *Trichoderma reesei* glucoamylase (TrGA) and variants thereof that possess superior specific activity and thermal stability. See U.S. Published Applications Nos. 2006/0094080, 2007/0004018, and 2007/0015266 (Danisco US Inc.). Suitable variants of TrGA include those with glucoamylase activity and at least 80%, at least 90%, or at least 95% sequence identity to wild-type TrGA. α -amylases advantageously increase the yield of glucose produced in a saccharification process catalyzed by TrGA.

[00152] Alternatively, the glucoamylase may be another glucoamylase derived from plants (including algae), fungi, or bacteria. For example, the glucoamylases may be *Aspergillus niger* G1 or G2 glucoamylase or its variants (*e.g.*, Boel *et al.* (1984) *EMBO J.* 3: 1097-1102; WO 92/00381; WO 00/04136 (Novo Nordisk A/S)); and *A. awamori* glucoamylase (*e.g.*, WO 84/02921 (Cetus Corp.)). Other contemplated *Aspergillus* glucoamylase include variants with

enhanced thermal stability, *e.g.*, G137A and G139A (Chen *et al.* (1996) *Prot. Eng.* 9: 499-505); D257E and D293E/Q (Chen *et al.* (1995) *Prot. Eng.* 8: 575-582); N182 (Chen *et al.* (1994) *Biochem. J.* 301: 275-281); A246C (Fierobe *et al.* (1996) *Biochemistry*, 35: 8698-8704); and variants with Pro residues in positions A435 and S436 (Li *et al.* (1997) *Protein Eng.* 10: 1199-1204). Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *T. emersonii* (*e.g.*, WO 99/28448 (Novo Nordisk A/S), *T. leycettanus* (*e.g.*, U.S. Patent No. RE 32,153 (CPC International, Inc.)), *T. duponti*, or *T. thermophilus* (*e.g.*, U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (*e.g.*, EP 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 (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.

[00153] Other suitable enzymes that can be used with amylase include a 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 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*.

[00154] 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 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).

- 5 [00155] Compositions comprising the present amylases may be aqueous or non-aqueous formulations, granules, powders, gels, slurries, pastes, etc., which may further comprise any one or more of the additional enzymes listed, herein, along with buffers, salts, preservatives, water, co-solvents, surfactants, and the like. Such compositions may work in combination with endogenous enzymes or other ingredients already present in a slurry, water bath, washing
10 machine, food or drink product, etc, for example, endogenous plant (including algal) enzymes, residual enzymes from a prior processing step, and the like.

5. Compositions and Methods for Baking and Food Preparation

- [00156] 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
15 for preparing such a food composition comprising mixing α -amylase with one or more food ingredients, or uses thereof.

- [00157] 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
20 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.

- [00158] 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
25 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,
30 ground rice, enriched flower, and custard powder.

- [00159] 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.

- 5 [00160] 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
10 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.*,
15 from plant sources, such as soy bean, or from microbial sources, such as *Bacillus*.

- [00161] 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
20 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.

- 25 [00162] 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
30 μ mol butyric acid per minute at 30°C, pH 7.0, with gum arabic as emulsifier and tributyrin as substrate.

- [00163] 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*.

[00164] 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.

[00165] 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.

[00166] 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, an amadoriase, a metalloproteinase, a pyranose oxidase, a lipooxygenase, an L-amino acid oxidase or a carbohydrate oxidase. The additional enzyme(s) may be of any origin, including mammalian and plant, and particularly of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

[00167] 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®.

[00168] 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.

[00169] 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.

[00170] 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.

[00171] 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.

[00172] 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.

[00173] 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.

[00174] 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 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.

[00175] 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

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.

[00176] 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 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, 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.

[00177] 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.

[00178] 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.

[00179] 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.

[00180] In a further aspect of the invention, the food composition is an animal feed composition, animal feed additive and/or pet food comprising an amylase and variants thereof. The present invention further relates to a method for preparing such an animal feed composition, animal feed additive composition and/or pet food comprising mixing an amylase and variants thereof with one or more animal feed ingredients and/or animal feed additive ingredients and/or pet food ingredients. Furthermore, the present invention relates to the use of an amylase in the preparation of an animal feed composition and/or animal feed additive composition and/or pet food.

[00181] The term “animal” includes all non-ruminant and ruminant animals. In a particular embodiment, the animal is a non-ruminant animal, such as a horse and a mono-gastric animal. Examples of mono-gastric animals include, but are not limited to, pigs and swine, such as piglets, growing pigs, sows; poultry such as turkeys, ducks, chicken, broiler chicks, layers; fish such as salmon, trout, tilapia, catfish and carps; and crustaceans such as shrimps and prawns. In a further embodiment the animal is a ruminant animal including, but not limited to, cattle, young calves, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn and nilgai.

[00182] In the present context, it is intended that the term “pet food” is understood to mean a food for a household animal such as, but not limited to dogs, cats, gerbils, hamsters, chinchillas,

fancy rats, guinea pigs; avian pets, such as canaries, parakeets, and parrots; reptile pets, such as turtles, lizards and snakes; and aquatic pets, such as tropical fish and frogs.

[00183] The terms “animal feed composition,” “feedstuff” and “fodder” are used interchangeably and may comprise one or more feed materials selected from the group

5 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,
10 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.

6. Textile Desizing Compositions and Use

[00184] Also contemplated are compositions and methods of treating fabrics (*e.g.*, to desize a
15 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.

[00185] An amylase can be applied during or after the weaving of a textile, or during the
20 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
25 can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

[00186] 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
30 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.

7. Cleaning Compositions

[00187] 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.

7.1. Overview

[00188] Preferably, an amylase is incorporated into detergents at or near a concentration conventionally used for amylase in detergents. For example, an amylase polypeptide may be added in amount corresponding to 0.00001 – 1 mg (calculated as pure enzyme protein) of amylase per liter of wash/dishwash liquor. Exemplary formulations are provided herein, as exemplified by the following:

[00189] 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.

[00190] 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.

- 5 [00191] 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.
- 10 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).
- 15 [00192] 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.

- [00193] 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
- 20 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
- 25 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.

- [00194] The detergent may comprise one or more polymers. Examples include
- 30 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.

[00195] 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 tetraacetythylenediamine (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.

[00196] 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.

[00197] 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.

[00198] The pH (measured in aqueous solution at use concentration) is usually neutral or alkaline, *e.g.*, pH about 7.0 to about 11.0.

[00199] Particular forms of detergent compositions for inclusion of the present α -amylase are described, below.

7.2. Heavy Duty Liquid (HDL) laundry detergent composition

[00200] 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 sulfo-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof.

[00201] 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.

[00202] 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 mixtures thereof) and polymeric carboxylate (such as maleate/acrylate random copolymer or polyacrylate homopolymer).

[00203] The composition may further include saturated or unsaturated fatty acid, preferably saturated or unsaturated C₁₂-C₂₄ fatty acid (0 wt% to 10 wt%); deposition aids (examples for which include polysaccharides, preferably cellulosic polymers, poly diallyl dimethyl ammonium halides (DADMAC), and co-polymers of DAD MAC with vinyl pyrrolidone, acrylamides, imidazoles, imidazolium 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.

[00204] 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.

[00205] The composition preferably included enzymes (generally about 0.01 wt% active enzyme to 0.03wt% active enzyme) selected from proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferases, perhydrolases, arylesterases, and any mixture thereof. The composition may include an enzyme stabilizer (examples of which include polyols such as propylene glycol or glycerol, sugar or sugar alcohol, lactic acid, reversible protease inhibitor, boric acid, or a boric acid derivative, *e.g.*, an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid).

[00206] The composition optionally include silicone or fatty-acid based suds suppressors; hueing 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).

[00207] 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.

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

[00208] 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).

[00209] The composition preferably includes enzymes, *e.g.*, proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferase, perhydrolase, arylesterase, and any mixture thereof.

- 5 [00210] 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.

10 7.4. Automatic dishwashing (ADW) detergent composition

- [00211] 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-
15 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),
20 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
25 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
30 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).

7.5. Additional detergent compositions

[00212] Additional exemplary detergent formulations to which the present amylase can be added are described, below, in the numbered paragraphs.

[00213] 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%.

[00214] 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%.

[00215] 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%.

[00216] 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%.

[00217] 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%.

[00218] 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%.

- 5 [00219] 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising fatty alcohol sulfate about 5% to about 10%; ethoxylated fatty acid monoethanolamide about 3% to about 9%; soap as fatty acid 0-3%; sodium carbonate (*e.g.*, Na_2CO_3) about 5% to about 10%; Soluble silicate (*e.g.*, Na_2O , 2SiO_2) about 1% to about 4%; zeolite (*e.g.*, NaAlSiO_4) about 20% to about 40%; Sodium sulfate (*e.g.*, Na_2SO_4) about 2% to
10 about 8%; sodium perborate (*e.g.*, $\text{NaBO}_3\text{H}_2\text{O}$) 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%.

- [00220] 8) A detergent composition formulated as a granulate comprising linear
15 alkylbenzenesulfonate (calculated as acid) about 8% to about 14%; ethoxylated fatty acid monoethanolamide about 5% to about 11%; soap as fatty acid 0% to about 3%; sodium carbonate (*e.g.*, Na_2CO_3) about 4% to about 10%; soluble silicate (Na_2O , 2SiO_2) about 1% to about 4%; zeolite (*e.g.*, NaAlSiO_4) about 30% to about 50%; sodium sulfate (*e.g.*, Na_2SO_4) about 3% to about 11%; sodium citrate (*e.g.*, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$) about 5% to about 12%; polymers
20 (*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%.

- [00221] 9) A detergent composition formulated as a granulate comprising linear
25 alkylbenzenesulfonate (calculated as acid) about 6% to about 12%; nonionic surfactant about 1% to about 4%; soap as fatty acid about 2% to about 6%; sodium carbonate (*e.g.*, Na_2CO_3) about 14% to about 22%; zeolite (*e.g.*, NaAlSiO_4) about 18% to about 32%; sodium sulfate (*e.g.*, Na_2SO_4) about 5% to about 20%; sodium citrate (*e.g.*, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$) about 3% to about 8%; sodium perborate (*e.g.*, $\text{NaBO}_3\text{H}_2\text{O}$) 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.*,
30 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%.

[00222] 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.*, B₄O₇) 0% to about 2%;
 5 carboxymethylcellulose 0% to about 1%; ethanol about 1% to about 3%; propylene glycol about 2% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, polymers, dispersants, perfume, optical brighteners) 0-5%.

[00223] 11) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 20% to about 32%; alcohol ethoxylate (*e.g.*,
 10 C₁₂₋₁₅ alcohol, 7 EO, or C₁₂₋₁₅ alcohol, 5 EO) 6-12%; aminoethanol about 2% to about 6%; citric acid about 8% to about 14%; borate (*e.g.*, B₄O₇) 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,
 15 optical brighteners) 0-5%.

[00224] 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
 20 (*e.g.*, Na₂CO₃) about 8% to about 25%; soluble silicates (*e.g.*, Na₂O, 2SiO₂) about 5% to about 15%; sodium sulfate (*e.g.*, Na₂SO₄) 0% to about 5%; zeolite (NaA1SiO₄) about 15% to about 28%; sodium perborate (*e.g.*, NaBO₃·4H₂O) 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%.

25 **[00225]** 13) Detergent compositions as described in compositions 1)-12) *supra*, wherein all or part of the linear alkylbenzenesulfonate is replaced by (C₁₂-C₁₈) alkyl sulfate.

[00226] 14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C₁₂-C₁₈) 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.*,
 30 NaA1SiO₄) about 10% to about 20%; layered disilicate (*e.g.*, SK56 from Hoechst) about 10% to about 20%; sodium carbonate (*e.g.*, Na₂CO₃) about 3% to about 12%; soluble silicate (*e.g.*, Na₂O, 2SiO₂) 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%.

5 [00227] 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
10 (*e.g.*, optical brightener, phosphonate, perfume) 0-3%.

[00228] 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.

15 [00229] 17) Detergent compositions as described supra in 1), 3), 7), 9), and 12), wherein perborate is replaced by percarbonate.

[00230] 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).

20 [00231] 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.

[00232] As above, the present amylase polypeptide may be incorporated at a concentration
25 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.

[00233] The detergent composition may also contain other conventional detergent ingredients, *e.g.*, deflocculant material, filler material, foam depressors, anti-corrosion agents,
30 soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners, and perfumes.

[00234] 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.

[00235] 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.

[00236] *Proteases*: Suitable proteases include those of animal, vegetable or microbial origin. Chemically modified or protein engineered mutants are included, as well as naturally processed proteins. The protease may be a serine protease or a metalloprotease, an alkaline microbial protease, a trypsin-like protease, or a chymotrypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147, and subtilisin 168 (see, e.g., WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g., of porcine or bovine origin), and *Fusarium* proteases (see, e.g., WO 89/06270 and WO 94/25583). Examples of useful proteases also include but are not limited to the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946. Commercially available protease enzymes include but are not limited to: ALCALASE®, SAVINASE®, PRIMASE™, DURALASE™, ESPERASE®, KANNASE™, and BLAZE™ (Novo Nordisk A/S and Novozymes A/S); MAXATASE®, MAXACAL™, MAXAPEM™, PROPERASE®, PURAFECT®, PURAFECT OXP™, FN2™, and FN3™ (Danisco US Inc.). Other exemplary proteases include NprE from *Bacillus amyloliquifaciens* and ASP from *Cellulomonas* sp. strain 69B4.

[00237] *Lipases*: Suitable lipases include those of bacterial or fungal origin. Chemically modified, proteolytically modified, or protein engineered mutants are included. Examples of useful lipases include but are not limited to lipases from *Humicola* (synonym *Thermomyces*), e.g., from *H. lanuginosa* (*T. lanuginosus*) (see e.g., EP 258068 and EP 305216), from *H. insolens* (see e.g., WO 96/13580); a *Pseudomonas* lipase (e.g., from *P. alcaligenes* or *P. pseudoalcaligenes*; see, e.g., EP 218 272), *P. cepacia* (see e.g., EP 331 376), *P. stutzeri* (see e.g., GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (see e.g., WO 95/06720 and WO 96/27002), *P. wisconsinensis* (see e.g., WO 96/12012); a *Bacillus* lipase (e.g., from *B. subtilis*;

see e.g., Dartois *et al.* *Biochemica et Biophysica Acta*, 1131: 253-360 (1993)), *B. stearothermophilus* (see e.g., JP 64/744992), or *B. pumilus* (see e.g., WO 91/16422). Additional lipase variants contemplated for use in the formulations include those described for example in: WO 92/05249, WO 94/01541, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079, WO 97/07202, EP 407225, and EP 260105. Some commercially available lipase enzymes include LIPOLASE® and LIPOLASE ULTRA™ (Novo Nordisk A/S and Novozymes A/S).

[00238] *Polyesterases*: Suitable polyesterases can be included in the composition, such as those described in, for example, WO 01/34899, WO 01/14629, and US6933140.

[00239] *Amylases*: The compositions can be combined with other amylases, such as non-production enhanced amylase. These can include commercially available amylases, such as but not limited to STAINZYME®, NATALASE®, DURAMYL®, TERMAMYL®, FUNGAMYL® and BAN™ (Novo Nordisk A/S and Novozymes A/S); RAPIDASE®, POWERASE®, and PURASTAR® (from Danisco US Inc.).

[00240] *Cellulases*: Cellulases can be added to the compositions. Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*, *Acremonium*, e.g., the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed for example in U.S. Patent Nos. 4,435,307; 5,648,263; 5,691,178; 5,776,757; and WO 89/09259. Exemplary cellulases contemplated for use are those having color care benefit for the textile. Examples of such cellulases are cellulases described in for example EP 0495257, EP 0531372, WO 96/11262, WO 96/29397, and WO 98/08940. Other examples are cellulase variants, such as those described in WO 94/07998; WO 98/12307; WO 95/24471; PCT/DK98/00299; EP 531315; U.S. Patent Nos. 5,457,046; 5,686,593; and 5,763,254. Commercially available cellulases include CELLUZYME® and CAREZYME® (Novo Nordisk A/S and Novozymes A/S); CLAZINASE® and PURADAX HA® (Danisco US Inc.); and KAC-500(B)™ (Kao Corporation).

[00241] *Peroxidases/Oxidases*: Suitable peroxidases/oxidases contemplated for use in the compositions include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g., from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO

95/10602, and WO 98/15257. Commercially available peroxidases include for example GUARDZYME™ (Novo Nordisk A/S and Novozymes A/S).

[00242] The detergent composition can also comprise 2,6-β-D-fructan hydrolase, which is effective for removal/cleaning of biofilm present on household and/or industrial textile/laundry.

5 [00243] The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive, *i.e.* a separate additive or a combined additive, can be formulated *e.g.*, as a granulate, a liquid, a slurry, and the like. Exemplary detergent additive formulations include but are not limited to granulates, in particular non-
10 dusting granulates, liquids, in particular stabilized liquids or slurries.

[00244] Non-dusting granulates may be produced, *e.g.*, as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (*e.g.*, polyethyleneglycol, PEG) with mean molar weights of 1,000 to 20,000; ethoxylated nonylphenols having from 16 to 50
15 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
20 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.

[00245] 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
25 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.

[00246] When included therein the detergent will typically contain from about 1% to about
30 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.

[00247] When included therein, the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl-N-alkyl derivatives of glucosamine (“glucamides”).

[00248] 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).

[00249] 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.

[00250] 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.

[00251] 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).

[00252] Yet additional exemplary detergent formulations to which the present amylase can be added are described in, *e.g.*, WO2010065455, WO2011072099, WO2011130222, WO2011140364, WO2011156297, WO2011156298, WO2011130076, WO2011133381, WO2011156297, WO2011156298, EP1794295B1, US20110195481, US20110212876, US20110257063, WO2010039958, WO2011072117, WO2011098531, WO2011100410, WO2011130076, WO2011133381, WO2011140316, US20070215184, US20070251545, US20090075857, US20090137444, US20090143271, US20100011513, US20100093588, US20110201536, US20110232004, US20110237482, US20110312868, US20120003326, US20120004155, WO2011131585, EP707628B1, US5719115, EP736084B1, US5783545, EP767830B1, US5972668, EP746599B1, US5798328, EP662117B1, US5898025, US6380140,

EP898613B1, US3975280, US6191092, US6329333, US6530386, EP1307547B1, US7153818, EP1421169B1, US6979669, EP1529101B1, US7375070, EP1385943B1, US7888104, EP1414977B1, US5855625, EP1921147B1, EP1921148B1, EP701605B1, EP1633469B1, EP1633470B1, EP1794293B1, EP171007B1, US4692260, US7569226, EP1165737B1, 5 US6391838, US6060441, US2009017074, US7320887, EP1737952B1, US7691618, US20070256251, US20050261156, US20050261158, US20100234267, US20110136720, US20110201536, US7811076, US5929017, US5156773, EP2343310A1, WO2011083114, EP214761B1, US4876024, EP675944B1, US5763383, EP517761B1, US6624129, EP1054956B1, US6939702, US6964944, EP832174B1, US20060205628, US20070179076, 10 US20080023031, US20110015110, US20110028372, US4973417, US5447649, US5840677, US5965503, US5972873, US5998344, US6071356, WO9009428, EP1661978A1, EP1698689A1, EP1726636A1, EP1867707A1, EP1876226A1, EP1876227A1, EP0205208A2, EP0206390A2, EP0271152, EP0271154, EP0341999, EP0346136, EP2135934, US20120208734, WO2011127102, WO2012142087, WO2012145062, EP1790713B1, 15 US8066818B2, US8163686B2, US8283300B2, US8354366B2, US20120125374, US3929678, and US5898025.

7.6. Methods of Assessing Amylase Activity in Detergent Compositions

[00253] Numerous α -amylase cleaning assays are known in the art, including swatch and 20 micro-swatch assays. The appended Examples describe only a few such assays.

[00254] 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.

8. Brewing Compositions

25 [00255] The present α -amylase may be a component of a brewing composition used in a process of brewing, *i.e.*, making a fermented malt beverage. Non-fermentable carbohydrates form the majority of the dissolved solids in the final beer. This residue remains because of the inability of malt amylases to hydrolyze the alpha-1,6-linkages of the starch. The non-fermentable carbohydrates contribute about 50 calories per 12 ounces of beer. an amylase, in 30 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.

[00256] The principal raw materials used in making these beverages are water, hops and malt. In addition, adjuncts such as common corn grits, refined corn grits, brewer's milled yeast, rice, sorghum, refined corn starch, barley, barley starch, dehusked barley, wheat, wheat starch, torried cereal, cereal flakes, rye, oats, potato, tapioca, and syrups, such as corn syrup, sugar
5 cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like may be used as a source of starch.

[00257] For a number of reasons, the malt, which is produced principally from selected varieties of barley, has the greatest effect on the overall character and quality of the beer. First, the malt is the primary flavoring agent in beer. Second, the malt provides the major portion of
10 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
15 stabilization.

[00258] Grains, such as barley, oats, wheat, as well as plant components, such as corn, hops, and rice, also are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, *i.e.*, partially germinated, resulting in an increase in the levels of enzymes, including α -amylase. For successful brewing, adequate levels
20 of α -amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation. An amylase, by itself or in combination with another α -amylase(s), accordingly may be added to the components used for brewing.

[00259] 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
25 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.

[00260] Processes for making beer are well known in the art. *See, e.g.*, Wolfgang Kunze
30 (2004) "Technology Brewing and Malting," Research and Teaching Institute of Brewing, Berlin (VLB), 3rd edition. Briefly, the process involves: (a) preparing a mash, (b) filtering the mash to prepare a wort, and (c) fermenting the wort to obtain a fermented beverage, such as beer. Typically, milled or crushed malt is mixed with water and held for a period of time under

controlled temperatures to permit the enzymes present in the malt to convert the starch present in the malt into fermentable sugars. The mash is then transferred to a mash filter where the liquid is separated from the grain residue. This sweet liquid is called “wort,” and the left over grain residue is called “spent grain.” The mash is typically subjected to an extraction, which involves
5 adding water to the mash in order to recover the residual soluble extract from the spent grain. The wort is then boiled vigorously to sterilize the wort and help develop the color, flavor and odor. Hops are added at some point during the boiling. The wort is cooled and transferred to a fermentor.

[00261] The wort is then contacted in a fermentor with yeast. The fermentor may be chilled
10 to stop fermentation. The yeast flocculates and is removed. Finally, the beer is cooled and stored for a period of time, during which the beer clarifies and its flavor develops, and any material that might impair the appearance, flavor and shelf life of the beer settles out. The beer usually contains from about 2% to about 10% v/v alcohol, although beer with a higher alcohol content, *e.g.*, 18% v/v, may be obtained. Prior to packaging, the beer is carbonated and,
15 optionally, filtered and pasteurized.

[00262] The brewing composition comprising an amylase, in combination with a glucoamylase and optionally a pullulanase and/or isoamylase, may be added to the mash of step (a) above, *i.e.*, during the preparation of the mash. Alternatively, or in addition, the brewing composition may be added to the mash of step (b) above, *i.e.*, during the filtration of the mash.
20 Alternatively, or in addition, the brewing composition may be added to the wort of step (c) above, *i.e.*, during the fermenting of the wort.

[00263] A fermented beverage, such as a beer, can be produced by one of the methods above. The fermented beverage can be a beer, such as full malted beer, beer brewed under the “Reinheitsgebot,” ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer,
25 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
30 like.

9. Reduction of Iodine-Positive Starch

[00264] α -amylases may reduce the iodine-positive starch (IPS), when used in a method of liquefaction and/or saccharification. One source of IPS is from amylose that escapes hydrolysis

and/or from retrograded starch polymer. Starch retrogradation occurs spontaneously in a starch paste, or gel on ageing, because of the tendency of starch molecules to bind to one another followed by an increase in crystallinity. Solutions of low concentration become increasingly cloudy due to the progressive association of starch molecules into larger articles. Spontaneous precipitation takes place and the precipitated starch appears to be reverting to its original condition of cold-water insolubility. Pastes of higher concentration on cooling set to a gel, which on ageing becomes steadily firmer due to the increasing association of the starch molecules. This arises because of the strong tendency for hydrogen bond formation between hydroxy groups on adjacent starch molecules. *See* J.A. Radley, ed., STARCH AND ITS DERIVATIVES 194-201 (Chapman and Hall, London (1968)).

[00265] 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 α -amylases is expected to improve overall process performance by reducing the amount of IPS.

[00266] All references cited herein are herein incorporated by reference in their entirety for all purposes. In order to further illustrate the compositions and methods, and advantages thereof, the following specific examples are given with the understanding that they are illustrative rather than limiting.

EXAMPLES

Example 1

Cloning and expression of SumAmy2

[00267] The genome of the *Streptomyces umbrinus* strain was sequenced using Illumina sequencing technology. Genomic DNA was isolated as described in Kieser *et al.*, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, United Kingdom (2000) and used for genome sequencing and to amplify the *SumAmy2* gene for expression cloning. Genome sequencing and assembly of the sequence data was performed by BaseClear (Leiden, The

Netherlands). Contigs were annotated by BioXpr (Namur, Belgium). Based on the genome sequencing results, the full length *SumAmy2* gene was PCR amplified using primers listed below. The forward primer contains a *NheI* restriction site, and the reverse primer contains a *BamHI* restriction site.

5

239NheIFw (SEQ ID NO: 5):

5'-ACTGCGCTAGCCGGCCCCCGGCACA
GGCCGCCGCTCCGGGCGACAAGGA-3'

10

239BamHIRv (SEQ ID NO: 6):

5'-TCGCGGATCCAGGTCAGCCGCGCCAGACGTCGGC-3'

Cloning and expression of SumAmy2

[00268] The PCR product was digested with the restriction enzymes *NheI* and *BamHI* and
15 ligated into the pKB128 vector digested with same restriction enzymes to obtain the expression
plasmid pDS239 (Figure 1). The pKB128 plasmid is a derivative of the pKB105 plasmid
(described in U.S. Pat. Application Publication No. 2006/0154843) and is the source of the A4
promoter. The ligation mixture was then used to transform *E. coli* TOP10 chemically competent
cells (Invitrogen Corp.) following the manufacturer's protocol. The transformed cells were
20 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 plasmid
DNA was extracted from the cultures and the sequence of the *SumAmy2* gene was confirmed by
DNA sequencing. The pDS239 plasmid was then used to transform *S. lividans* TK23 derived
25 protoplasts, as described in U.S. Patent Application Publication No. 2006/0154843. The
transformation techniques used are described in Kieser *et al.* (*supra*). Transformed cells were
plated on R5 selection plates and incubated at 30°C for 3 days. One clone from the *Streptomyces*
transformation plate was inoculated in TSG medium in shake flasks at 28°C for 3 days. Cultures
were then transferred to a *Streptomyces* 2 Modified Medium (described in U.S. Patent
30 Application Publication No. 2006/0154843) and incubated for an additional 4 days at 28°C.

TSG medium: 16 g BD Difco tryptone, 4 g BD Bacto soytone, 20 g Sigma caseine
(hydrolysate), and 10 g potassium phosphate, dibasic, brought to 1 liter. After
autoclaving, 50% glucose was added to a final concentration of 1.5%.

R5 plates: 206 g sucrose, 0.5 g K₂SO₄, 20.24 g MgCl₂, 20 g glucose, 0.2 g Difco casamino acids, 10 g Difco yeast extracts, 11.46 g TES, 4 g L-Asp, 4 mL of trace elements, 44 g Difco agar, 20 mL 5% K₂HPO₄, 8 mL 5 M CaCl₂•2H₂O and 14 mL 1 N NaOH were added to a final volume of 1 liter after autoclaving. After 20 hours, a layer
5 of thiostrepton (50 µg/mL final concentration) was plated on the top of the plates.

[00269] The nucleotide sequence of the *SumAmy2* gene in the plasmid pDS239 (A4- *CelA-SumAmy2*) is set forth below as SEQ ID NO: 1:

GCCGCTCCGGGCGACAAGGACGTCACCGCCGTGATGTTTCGAGTGGAAGTTCGCCTCCATCGCCA
10 AAGCCTGCACGGACACCCTCGGTCCGGCGGGCTACGGCTACGTCCAGGTCTCGCCGCCCCAGGA
GCACATCCAGGGCGGCCAGTGGTGGACGTCCTACCAGCCCGTCAGCTACAGGATCGCCGGGCGG
CTCGGTGACCGCGCCGCCTTCTCCGCCATGGTCAACACGTGTCACTCGGCGGGCGTCAAGGTCTG
TCGCGGACGCCGTATCAACCACATGGCGGGCGGGCAACGGCACCGGCACCGGCGGGCTCCTCGTA
CACCAAGTACGACTACCCGGGCATCTACTCCGGCGCCGACATGGACGACTGCCGCGCCCAGATC
15 AGCAACTACAACGACCGCGGCAACGTCCAGAACTGCGAACTGGTCGGCCTGGCCGACCTGGACA
CCGGCGAGGACTACGTACGCGGCAGGATCGCCGGGTATCTCAACGACCTGCTCTCGCTCGGTGT
CGACGGCTTCCGCATCGACGCCGCCAAGCACATGCCCCGCCGGCGACCTCGCCAACATCAAGTCC
CGGCTGACCAACCCGAACGTCTACTGGAAGCAGGAGGCGATCTACGGGGCGGGTGAGGCCGTCT
CGCCCTCCGAATACCTCGGCACCGGCGACGTCCAGGAGTTCCGTTACGGGCGGAGCCTCAAGCA
20 GGTCTTCTCAACGAGAACCTCGCCACCTGAAGAACTTCGGCGAGGGCTGGGGCTTCATGGAG
TCCGGCAAGTCCGCCGTCTTCGTGACAAACCACGACACCGAGCGCGGCGGCGACACCCTGAACT
ACAAGAACGGCTCCGCCTACACCCTCGCCAACGTCTTCATGCTGGCCTACCCCTACGGCTCCCC
GGACGTCCACTCCGGCTACGAGTTTCAGCGACCACGACGCCGGACCGCCGAACGGCGGCCAGGTC
AACGCCTGCTACAGCGACGGCTGGAAGTGCCAGCACGCCTGGCGCGAGATCTCCTCCATGGTCG
25 GCCTCCGCAACGCGGCCCGCGGCCAGTCCGTCACCAACTGGTGGGACAACGGCGGCGACACAGAT
CGCCTTCGGCCGGGGCGACAAGGCGTACGTGCGCATCAACCACGAGGGCTCCGCGCTGAACCGG
ACCTTCCAGACCTCGCTGCCCCGCCGGTGACTACTGCGACGTCCAGTCCGGCAACGGCGTCAACG
TCAACGGCTCCGGCCAGTTCACCGCCACCGTCCCGGCCGGCACCGCCGTCGCCCTGCACGTGAA
CGCCCCGTACCTGCTCCGGAGGCGGCGGCACGAACCCCGACCCGGGCCCCGGTGACGGCTCCTCC
30 GGCGCCTCCTTCGGGGTGAACGCCACGACCCAGCCCGGCCAGAACATTTACGTCACCGGCGACC
AGGCGGCCCTCGGCAACTGGAACCCGGCGAACGCGCCGAAGCTCGACCCGGCCGCGTACCCCGT
GTGGAAGCTCGACGTGGCCCTGCCCCGCCGGGACCACGTTTCGCGTACAAGTACGTCCGCAAGGAC
GGCAGCGGCAACGTCACTTGGGAGAGCGGGGCGAACCGCACCGCGACCGTCCCGGCCTCCGGGA
AGGTCACGCTGACCGCCGACGTCTGGCGCGGCTGA

[00270] The amino acid sequence of the SumAmy2 protein with its native signal peptide is set forth below as SEQ ID NO: 2 (the predicted native signal peptide is shown in italics and bold):

5 ***MARRPLSVALALVAGAAALVIPTGFGAASPAQA***AAPGDKDVTAVMFEWKFA
 GYGYVQVSPPPQEHIQGGQWWTSYQPVSYRIAGRLGDRAAFSAMVNTCHSAGVKVVADAVINHMA
 AGNGTGTGGSSYTKYDYPGIYSGADMDDCRAQISNYNDRGNVQNCELVGLADLDTGEDYVRGRI
 AGYLNDLLSLGVDGFRIDAAKHMPAGDLANIKSRLTNPVYWKQEAIYGAGEAVSPSEYLGTD
 VQEFYGRSLKQVFLNENLAHLKNFGEWGFME
 10 SGKSAVFVDNHDTERGGDTLNYKNGSAYTLA
 NVFMLAYPYGSPDVHSGYEFSDHDAGPPNGGQVNACYS
 SDGWKCQHAWREISSMVGLRNAARGQS
 VTNWWNDGGDQIAFGRGDKAYVAINHEGSALNRTFQTS
 LPAGDYCDVQSGNGVTVNGSGQFTAT
 VPAGTAVALHVNARTCSGGGGTNPDPGPGDGSSGASFGV
 NATTPGQNIYVTGDQAALGNWNPA
 NAPKLDPAAYPVWKLDVALPAGTTFAYKYVRKDGSGNVT
 WESGANRTATVPASGKVTLTADVWR
 G

15 [00271] The amino acid sequence of the SumAmy2 protein with a CelA signal peptide is set forth, below, as SEQ ID NO: 3 (the CelA signal peptide is shown in italics and bold):

MGFGSAPIALCPLRTRRNALKRLLALLATGVSIVGLTALAGPPAQAAAPGDKDVTAVMFEWKFA
 SIAKACTDTLGPAGYGYVQVSPPPQEHIQGGQWWTSYQPVSYRIAGRLGDRAAFSAMVNTCHSAG
 20 VKVVADAVINHMAAGNGTGTGGSSYTKYDYPGIYSGADMDDCRAQISNYNDRGNVQNCELVGLA
 DLDTGEDYVRGRIAGYLNDLLSLGVDGFRIDAAKHMPAGDLANIKSRLTNPVYWKQEAIYGAG
 EAVSPSEYLGTDVQEFYGRSLKQVFLNENLAHLKNFGEWGFME
 TLNYKNGSAYTLANVFM
 25 LAYPYGSPDVHSGYEFSDHDAGPPNGGQVNACYS
 SDGWKCQHAWREISSMVGLRNAARGQSVTNWWNDGGDQIAFGRGDKAYVAINHEGSALNRTFQTS
 LPAGDYCDVQSGN
 GVTVNGSGQFTATVPAGTAVALHVNARTCSGGGGTNPDPGPGDGSSGASFGVNATTPGQNIYV
 TGDQAALGNWNPANAPKLDPAAYPVWKLDVALPAGTTFAYKYVRKDGSGNVTWESGANRTATVP
 ASGKVTLTADVWRG

30 [00272] The amino acid sequence of the predicted mature form of SumAmy2 protein is set forth, below, as SEQ ID NO: 4:

AAPGDKDVTAVMFEWKFA
 SIAKACTDTLGPAGYGYVQVSPPPQEHIQGGQWWTSYQPVSYRIAGR
 LGDRAAFSAMVNTCHSAGVKVVADAVINHMAAGNGTGTGGSSYTKYDYPGIYSGADMDDCRAQI
 SNYNDRGNVQNCELVGLADLDTGEDYVRGRIAGYLNDLLSLGVDGFRIDA
 AAKHMPAGDLANIKS
 RLTNPNVYWKQEAIYGAGEAVSPSEYLGTDVQEFYGRSLKQVFLNENLAHLKNFGEWGFME

SGKSAVFVDNHDTERGGDTLNYKNGSAYTLANVFMLAYPYGSPDVHSGYEFSDHDAGPPNGGQV
 NACYSDGWKCQHAWREISSMVGLRNAARGQSVTNWWDNNGDQIAFGRGDKAYVAINHEGSALNR
 TFQTSLPAGDYCDVQSGNGVTVNGSGQFTATVPAGTAVALHVNARTCSGGGGTNPDPGPGDGSS
 GASFGVNATTQPGQNIYVTGDQAALGNWNPANAPKLDPAAYPVWKLDVALPAGTTTFAYKYVRKD
 5 GSGNVTWESGANRTATVPASGKVTLTADVWRG

Example 2

Purification of SumAmy2 protein

[00273] SumAmy2 protein (SEQ ID NO. 4) was purified via two chromatography steps.

10 About 800 mL fermentation broth from the shake flask was concentrated to 50 mL, and then
 dialyzed against 20 mM Tris-HCl, pH 8.0 (buffer A) overnight. The solution was loaded onto a
 20 ml Hiprep Q FF column pre-equilibrated in buffer A. After sample loading, the column was
 washed with the same buffer for 2 column volumes, followed by a gradient elution of 0-50%
 buffer A with 1 M NaCl (buffer B) in 10 CV. Fractions were analyzed by SDS-PAGE gel and
 15 the target protein was found in the flow-through from the column. The solution was
 concentrated and loaded onto a HiLoad Superdex 75 gel filtration column in 20 mM sodium
 phosphate, pH 7.0 with 0.15 M NaCl (buffer C). The fractions containing target protein were
 pooled and concentrated using 10K Amicon Ultra-15 devices. The sample was above 95% pure
 and stored in 40% glycerol at -80°C until usage.

20

Example 3

Alpha-amylase Activity Assay of SumAmy2

[00274] Alpha-amylase activity of SumAmy2 protein (SEQ ID NO. 4) was assayed using a
 colorimetric assay to monitor the release of reducing sugars from a potato amylopectin substrate.

25 The activity is reported as equivalents of glucose released per minute. Potato amylopectin was
 used as substrate (AP, Fluka Cat. No. 10118) and a 2.5% solution was prepared by adding 1.25 g
 to 50 g water with 0.005% Tween followed by heating to ensure dissolution. Stock solutions of
 purified proteins were made by diluting samples to 0.4 mg/mL (400 ppm) in water with 0.005%
 Tween. A dilution buffer was prepared by mixing 5 mL of 0.5 M sodium acetate, pH 5.8, with
 30 2.5 mL 1 M NaCl, 0.2 mL 0.5 M CaCl₂, and 7.3 mL water/0.005% Tween. Serial dilutions of
 enzyme samples in dilution buffer were prepared in non-binding microtiter plates (MTP,
 Corning 3641), from 0 - 10 ppm. Then 15 µL of the dilution buffer, 25 µL of 2.5% AP, and 10
 µL of the enzyme serial dilution were added to a PCR plate. Thus the reaction mixture contains

1.25% AP, 0 – 2 ppm enzyme, in 50 mM sodium acetate, pH 5.8, with 50 mM NaCl and 2 mM CaCl₂. Reactions were carried out for 10 minutes at 50°C in a PCR machine, and aliquots of 0.5 N NaOH were added to each well to stop the reaction. Total reducing sugars present in each tube were assayed by a PAHBAH method (Lever, M. *et al.* (1973) 82:649-655.): 80 µL of 0.5 N NaOH was aliquoted into a microtiter plate followed by the addition of 20 µL of PAHBAH reagent (5% w/v 4-hydroxybenzoic acid hydrazide in 0.5 N HCl) and 10 µL of the reaction mixture. Plates were incubated at 95°C for 2 minutes, samples were transferred to polystyrene microtiter plates (Costar 9017) and absorbance was measured at 450 nm. Resulting absorbance values were plotted against enzyme concentration and linear regression was used to determine the slope of the line. Amylase activity of SumAmy2 amylase is 103 U/mg when calculated using the following equation:

$$\text{Specific Activity (U/mg)} = \text{Slope (enzyme)} / \text{slope (std)} * 100,$$

where 1 U = 1 µmol glucose equivalent/min

Example 4

Effect of pH on Amylase Activity of SumAmy2

[00275] The effect of pH on the α-amylase activity of SumAmy2 protein (SEQ ID NO. 4) was monitored using the PAHBAH assay protocol as described above, with a pH range from 3.0 to 10.0. Working buffers contain 2.5 mL of either 1 M sodium acetate (pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, or 6.5), 1 M HEPES (pH 7.0, 7.5, 8.0, 8.5, or 9.0), or 1 M CAPS (pH 10.0) with 2.5 mL of 1 M NaCl, 50 µL of 2 M CaCl₂, and 10 mL water/0.005% Tween. Enzyme stocks were prepared in water/0.005% Tween. Reactions were initiated by dispensing 10 µL of each enzyme stock to the PCR plate, mixing quickly by vortexing, and incubating for 10 minutes at 50 °C in a PCR machine. To terminate the reaction, 20 µL of 0.5 N NaOH was added to each well. Samples were transferred to polystyrene microtiter plates (Costar 9017) and absorbance was read at 450 nm. The absorbance from a buffer-only control was subtracted, and the resulting values were converted to percentages of relative activity, by defining the activity at the optimal pH as 100%. The pH optimum for SumAmy2 is approximately 7.0, exhibiting a broad pH range for ≥70% of activity, from approximately 5.3 to 8.2, under the conditions of this assay (Figure 2).

Example 5

Effect of Temperature on Amylase Activity of SumAmy2

[00276] The effect of temperature on the α-amylase activity of SumAmy2 protein (SEQ ID NO. 4) was monitored using the PAHBAH assay protocol as described above, at temperatures

ranging from 15°C to 70°C. Reactions were initiated by dispensing 10 µL of enzyme to the PCR plate, mixing, and incubating for 10 minutes in a PCR machine at temperatures from 30 - 95°C, with increments every 5-10°C. To terminate the reaction, 20 µL of 0.5 N NaOH was added to each well. Samples were transferred to polystyrene microtiter plates (Costar 9017) and the absorbance was measured at 450 nm. The absorbance from a buffer-only control was subtracted, and the resulting values were converted to percentages of relative activity, by defining the activity at the optimal temperature at 100%. The temperature optimum for SumAmy2 is approximately 40°C, exhibiting a temperature range for ≥70% of activity, from approximately 20°C to 58°C, under the conditions of this assay (Figure 3).

Example 6

Cleaning Performance of SumAmy2 in Microswatch Assay

[00277] The cleaning performance of purified SumAmy2 protein (SEQ ID NO. 4) was analyzed in a microswatch assay. CFT CS-28 rice starch on cotton swatches (Center for Testmaterials BV, Vlaardingen, Netherlands) containing an indicator dye bound to the starch, were pre-punched by the manufacturer to form discs measuring 5.5 mm in diameter. Two discs were placed in each well of a 96-well corning 9017 flat bottomed polystyrene MTP.

[00278] The cleaning assay was carried out in either 10 mM HEPES, 2 mM CaCl₂, 0.005% TWEEN 80 buffer (pH 8.0, conductivity 5 mS/cm adjusted with 5 M NaCl) or 25 mM CAPS, 2 mM CaCl₂, 0.005% TWEEN 80 buffer (pH 10.0, conductivity 5 mS/cm adjusted with 5M NaCl). Enzymes were diluted in 10 mM NaCl, 0.1 mM CaCl₂, 0.005% TWEEN 80 solution to approximately 0, 0.011, 0.028, 0.056, 0.111, 0.222, 0.444, 0.667, 0.889, 1.111, and 5.556 ppm. iEMS incubator/shaker (Thermo Scientific) was set at the desired temperature of either 25°C or 32°C. 171 µL of either HEPES or CAPS buffer was added to each well of microswatch containing MTP and subsequently 9 µL of diluted enzyme was added to each well resulting in a total volume of 180 µL/well. The MTP was sealed with a plate seal and placed in the iEMS incubator/shaker and incubated for 15 minutes at 1150 rpm at 25 °C for cleaning at pH 8 and for 30 minutes at 1150 rpm at 32 °C for cleaning at pH 10. Following incubation under appropriate conditions, 100 µL of solution from each well was transferred to a new MTP, and the absorbance was measured at 488 nm using a MTP-spectrophotometer. Results are shown in Figure. 4A (pH 8 /25°) and Figure 4B (pH 10/32°C).

Example 7

Comparison of SumAmy2 protein sequence with other alpha-amylases

[00279] SumAmy2 protein (SEQ ID NO. 4) sequence was aligned with the sequences of *Geobacillus* (formerly *Bacillus*) *stearothermophilus* α -amylase (AmyS; SEQ ID NO: 7) and *Bacillus licheniformis* α -amylase (AmyL; SEQ ID NO: 8) using CLUSTAL 2.1 multiple sequence alignment. The sequence alignment is shown in Figure 5 and the percent identities to these proteins are shown in Table 2.

Table 2: Percent identity of SumAmy2 to AmyS and AmyL

	AmyS	AmyL	SumAmy2
AmyS	***	65.4	20.2
AmyL		***	20.1
SumAmy2			***

[00280] Homologs of SumAmy2 were identified by a BLAST search (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402, 1997) against the NCBI non-redundant protein database, nr, using the mature protein amino acid sequence (SEQ ID NO. 4). Percent identity (PID) is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Table 3 provides a list of sequences with the percent identity to SumAmy2.

Table 3. List of selected homologs of SumAmy2

Accession	Description	PID	SEQ ID NO
YP_001826792.1	<i>Streptomyces griseus</i> subsp. <i>griseus</i> NBRC 13350	94%	9
ZP_06908805.1	<i>Streptomyces pristinaespiralis</i> ATCC 25486	80%	10
CAB06622.1	<i>Streptomyces lividans</i>	79%	11
CAA73775.1	<i>Streptomyces</i> sp.	71%	12
BAE94180.1	<i>Brachybacterium</i> sp. LB25	53%	13
AAA82875.1	<i>Streptomyces</i> sp	61%	14
ABF13430.1	<i>Thermobifida fusca</i>	48%	15
EHK88249.1	<i>Saccharomonospora azurea</i> SZMC 14600	53%	16
XP_003033038.1	<i>Saccharomonospora azurea</i> SZMC 14600	51%	17
AEV86508.1	<i>Actinoplanes</i> sp. SE50/110	45%	18
1G94_A	<i>Pseudoalteromonas haloplantis</i>	50%	19

[00281] The amino acid sequence of mature SumAmy2 protein (SEQ ID NO: 4) was aligned to the sequences of representative homologs from Table 2 using CLUSTAL W multiple

sequence alignment program (default parameters). The alignment of the sequences is shown in Figure 6.

5 [00282] A phylogenetic tree for SumAmy2 protein (SEQ ID NO:4) was built using sequences of representative homologs from Table 3 and the Neighbor Joining method (NJ) of Saitou and Nei (Saitou, N. and Nei, M. (1987) *Mol. Biol. Evol.* 4:406-425). The NJ method works on a matrix of distances between all pairs of sequence to be analyzed. These distances are related to the degree of divergence between the sequences. Publically-available phylodendron-phylogenetic tree printer software was used to display the phylogenetic tree shown in Figure 7.

CLAIMS

What is claimed is:

- 5 1. A recombinant α -amylase having α -amylase activity and comprising an amino acid sequence having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 4.
- 10 2. The α -amylase of claim 1, having at least 97% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 4.
- 15 3. The α -amylase of any of the preceding claims, further comprising conservative substitutions of one or several amino acid residues.
4. The α -amylase any of the preceding claims, further comprising a deletion, substitution, insertion, or addition of one or a few amino acid residues.
- 20 5. The α -amylase of any of the preceding claims, derived from the amino acid sequence of SEQ ID NO: 4 by conservative substitution of one or several amino acid residues.
6. The α -amylase of any of the preceding claims, derived from the amino acid sequence of SEQ ID NO: 4 by deletion, substitution, insertion, or addition of one or a few amino acid residues.
- 25 7. The α -amylase of any of the preceding claims, encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid that is complementary to a nucleic acid that encodes SEQ ID NO: 4.
- 30 8. The α -amylase of any of the preceding claims, encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid that is complementary to the nucleic acid of SEQ ID NO: 1.
9. A composition comprising the α -amylase of any of the preceding claims.
- 35 10. The composition of claim 9, further comprising a surfactant.

11. The composition of claim 9 or 10, wherein the composition is a detergent composition.

12. The composition of any of claims 9-11, wherein the composition is a laundry detergent, a laundry detergent additive, or a manual or automatic dishwashing detergent.

13. The composition of any of claims 9-12, further comprising 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, lipoxxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, metalloproteinase, amadoriase and an amylase other than the amylase of claims 1-8.

14. The composition of claim 9, wherein the composition is for saccharifying a composition comprising starch, for SSF post liquefaction, or for direct SSF without prior liquefaction.

15. The composition of claim 9, wherein the composition is for producing a fermented beverage or a baked food product.

16. The composition of claim 9 or 10, wherein the composition is for textile desizing.

17. A method for removing a starchy stain or soil from a surface, comprising:
contacting the surface with a composition comprising an effective amount of the α -amylase of any of claims 1-8; and
allowing the α -amylase to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in aqueous solution;
thereby removing the starchy stain from the surface.

18. The method of claim 17, wherein the aqueous composition further comprises a surfactant.

19. The method of claim 17 or 18, wherein the surface is a textile surface or a surface on dishware.

20. The method for any of claims 17-19, 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, malanase, β -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, metalloproteinase, amadoriase, and an amylase other than the amylase of any one of claims 1-8.

21. A method for desizing a textile comprising:
contacting a sized textile with an effective amount of the α -amylase of any of claims 1-8;
and
allowing the α -amylase to hydrolyze starch components in the size to produce smaller starch-derived molecules that dissolve in aqueous solution;
thereby removing the size from the textile.

22. A method for saccharifying a composition comprising starch to produce a composition comprising glucose, the method comprising:
contacting the composition comprising starch with effective amount of the amylase of any of claims 1-8; and
saccharifying the composition comprising starch to produce the composition comprising glucose;
wherein the α -amylase catalyzes the saccharification of the starch solution to glucose.

23. The method of claim 22, wherein the composition comprising starch comprises liquefied starch, gelatinized starch, or granular starch.

24. A method for preparing a foodstuff or beverage comprising,
contacting a foodstuff or beverage comprising starch with an α -amylase of any of claims 1-8; and

allowing the α -amylase to hydrolyze the starch to produce smaller starch-derived molecules.

25. The method of claim 24, further comprising contacting the foodstuff or beverage with 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, α -glucosidase, beta-glucosidase, or a combination thereof.

26. The method of any one of claims 17–25, wherein the α -amylase is expressed and secreted by a host cell.

27. The method of claim 26, wherein the composition comprising starch is contacted with the host cell.

28. The method of claim 26 or 27, wherein the host cell further expresses and secretes a glucoamylase or other enzyme.

29. The method of any one of claims 26–28, wherein the host cell is capable of fermenting the composition.

30. A composition comprising glucose produced by the method of any one of claims 22–29.

31. Liquefied starch produced by the method of any one of claims 22–29.

32. A foodstuff or beverage produced by the method of any one of claims 24–29.

33. Use of the α -amylase of any of claims 1–8 in the production of a composition comprising glucose.

34. Use of the α -amylase of any of claims 1–8 in the production of a liquefied starch.

35. Use of the α -amylase of any of claims 1-8 in the production of a foodstuff or beverage.

36. Use of the α -amylase of any of claims 1-8 in cleaning starchy stains.

37. Use of the α -amylase of any of claims 1-8 in textile desizing.

38. A recombinant polynucleotide encoding a polypeptide of any of claims 1-8.

39. The polynucleotide of claim 38 having at least 80% nucleic acid sequence identity to the polynucleotide of SEQ ID NO: 1.

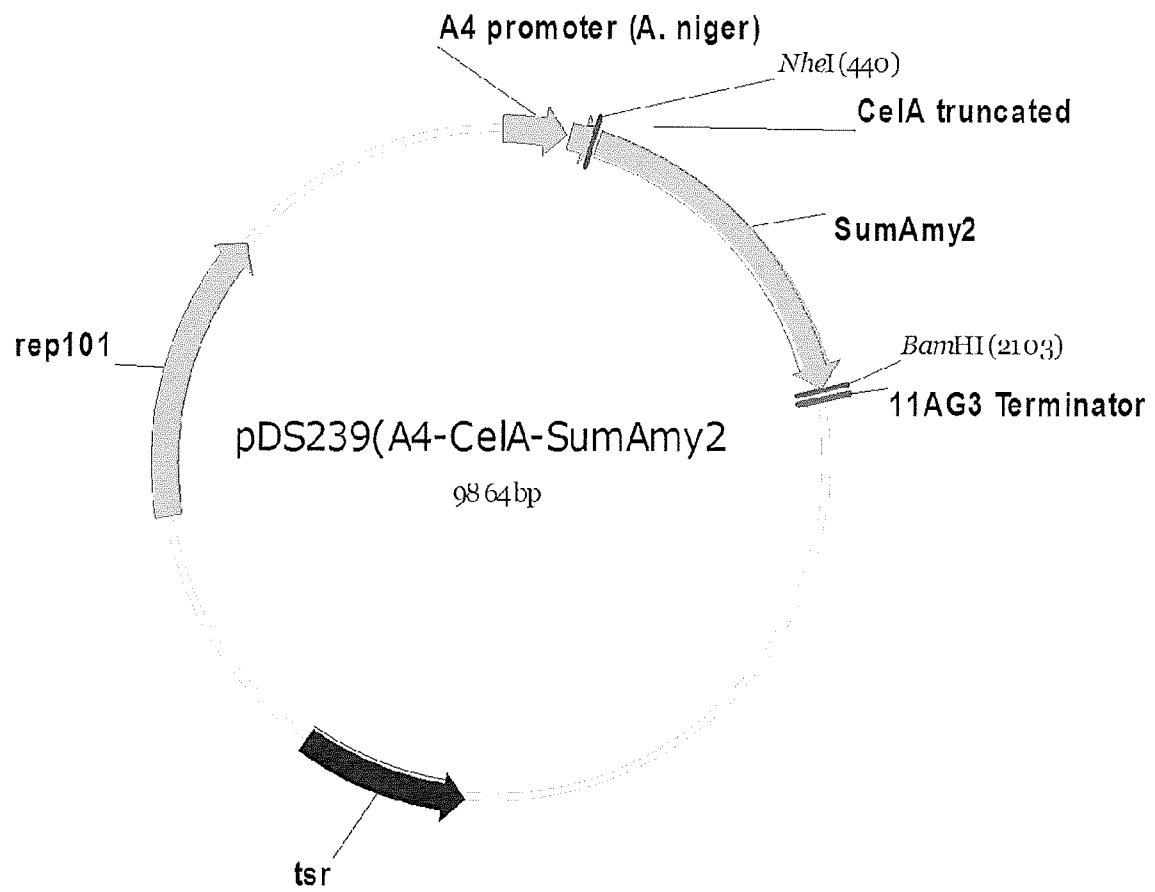
40. The polynucleotide of claim 38 or 39, wherein the polynucleotide hybridizes under stringent conditions to a nucleic acid that is complementary to a nucleic acid encoding SEQ ID NO: 4.

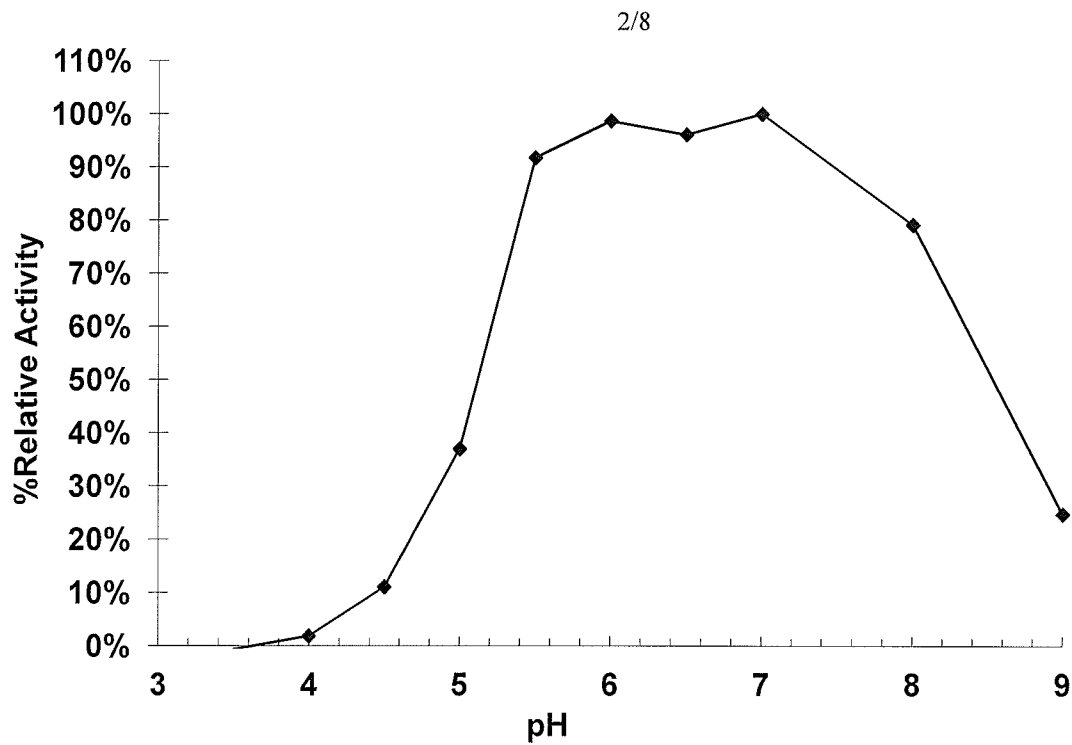
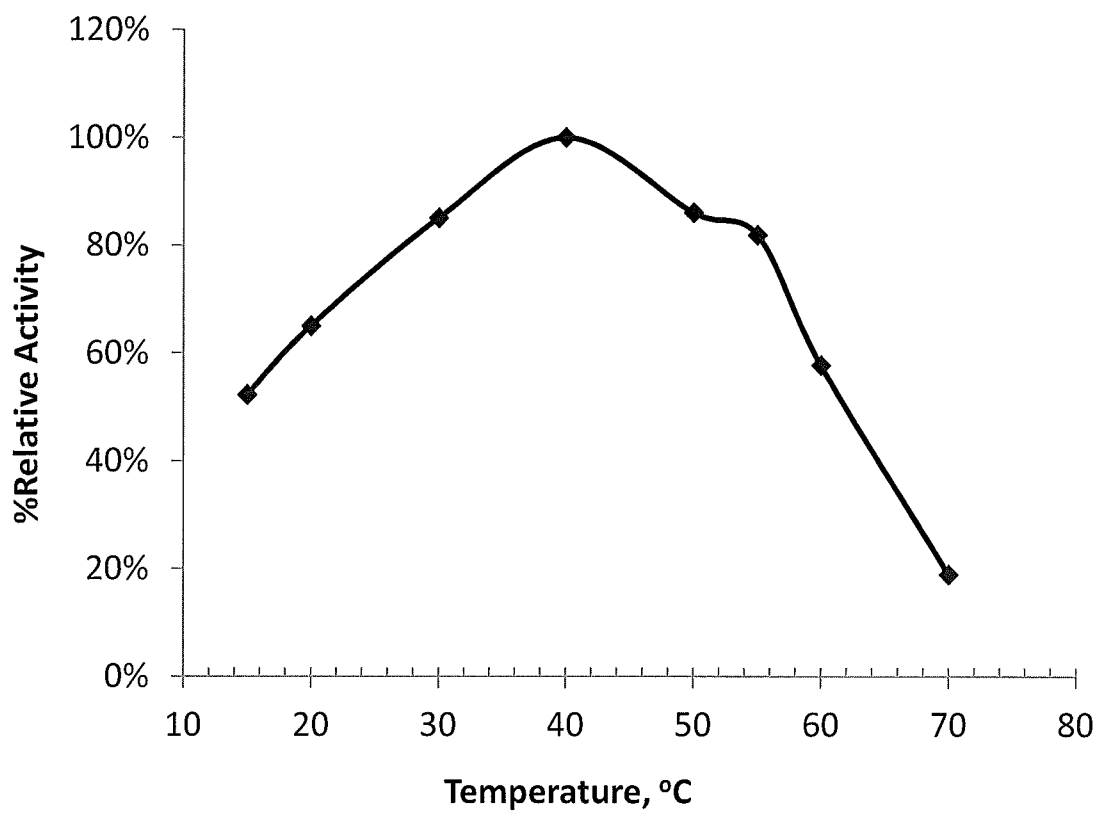
41. The polynucleotide of claim 38 or 39, wherein the polynucleotide hybridizes under stringent conditions to a nucleic acid that is complementary to the nucleic acid of SEQ ID NO: 1.

42. An expression vector comprising the polynucleotide of any of preceding claims 38-41.

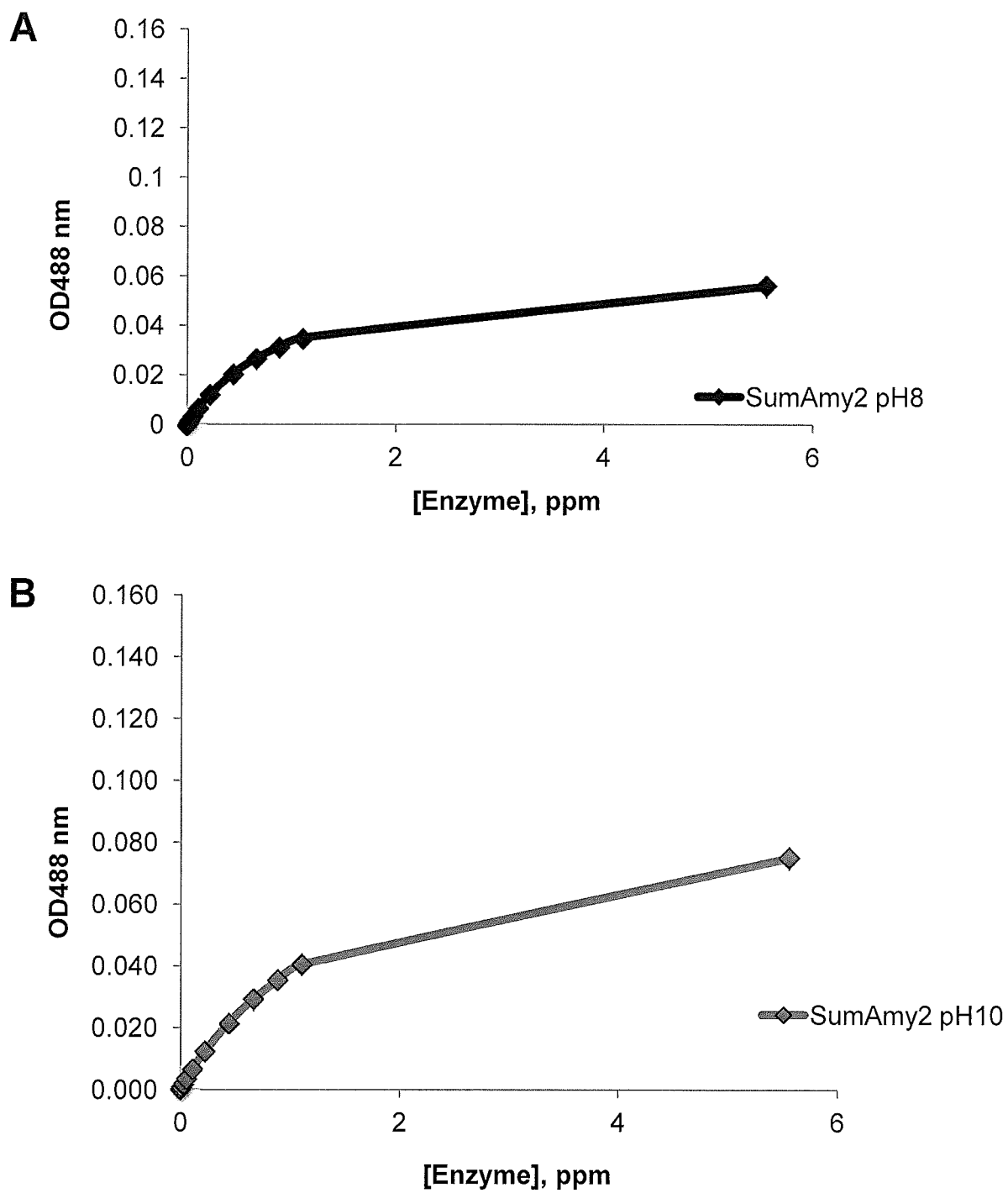
43. A host cell comprising the expression vector of claim 42.

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**Figure 1**

**Figure 2****Figure 3**

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**Figure 4**

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AmyS      -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANN-LSSLGITALWLPPAYKGTSRSDVGY-
AmyL      --ANLNGTLMQYFEWYMPNDGQHWKRLQND SAY-LAEHGITAVWIPPAYKGT SQADVGY-
SumAmy2   AAPGDKDVTAVMFEWK FASIAKACTDTLGPAGYGYVQVSP PQEHIQGGQWWT SYQPVS YR
          . :.. *** :.. . . : . . . : . ** *.
AmyS      GVDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYADV VFDHKGGADGTEWVD
AmyL      GAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDIN VYGDVVINHKG GADATEDVT
SumAmy2   IAGRLGDRAAFS AMVNTCHSAGVKV VADAVINHMAAGNGTGTGGSSYTKYDYPGIYSGAD
          . * * . * . . . *. * . *: : . . . : . . . .
AmyS      AVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNTYSSFKWRWYHFDGVDWDESRKLSRIYK
AmyL      AVEVDPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYK
SumAmy2   MDCRAQI SNYNDRG NVQNC ELVGLADLDTGEDYVRGRIAGYLNDLLSLG--VDGFRIDA
          : . * * . : . *. * : * * . . . **
AmyS      FRGIGKAWDWEVDTE NGNYDYL MYADLDM DHPEVVTELKNWGK WYVNTTNIDG-----FR
AmyL      FQ--GKAWDWEVSNENGN YDYL MYADIDYDHPDVAAEIKRWGTWYANELQLD G-----FR
SumAmy2   AKHMPAGDLANIKSRLTNPNVYWKQEA IYGAGEAVSPSEYLGTDGVQEF RYGRSLKQVFL
          : . . :... * : : . :... : *. . : . . *
AmyS      LDAVKHIKFSFFPDWLSYVRSQTGKPLFTVGEYWSYDINKLHNYITKT DGTMSLFDAP--
AmyL      LDAVKHIKFSFLRDWVNVH VREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVP--
SumAmy2   NENLAHLKN-FGEGWGFME SGKS AVFVDNH DTERGGDTLNYKNGSAYTLANVFMLAYPYG
          : : *. * * . * : : . . * . * * . : : *
AmyS      -----LHNKFYTASKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNH DTEPGQALQSWVD
AmyL      -----LHYQFHAASTQGGGYDMRKL LNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQ
SumAmy2   SPDVHSGYEFSDHDAGPPNGGQV NACYS DGWKCQHAWREISSMVGLRNAARGQSVTNWWDD
          . . : * . *. . . . : : *. : : * : : . :
AmyS      PWFKPLAY-----AFILTRQEGYPCVFYGDY YGIP---QYNIPSLKSKID
AmyL      TWFKPLAY-----AFILTRESGY PQVFYGD MYGTKGDSQREIPALKHKIE
SumAmy2   NGGDQIAFGRGDKAYVAINHEGSALNRTFQTSLPAGDYCDVQSGNGVT VNGSGQFTATVP
          . : *: : * : * : . * * * . : . :
AmyS      P-----LLIARRDYAYGTQH DYLDHSDIIGWTREGGTEKPGSGLAALIT-DGPGGSKWM
AmyL      P-----ILKARKQYAYGAQH DYFDHHDIVGWTREGDSSVANSGLAALIT-DGPGGAKRM
SumAmy2   AGTAVALHVNARTCSGGGGTNPDPGPGDGSSGASFGVNATTQPGQNIYVTGDQAALGNWN
          . : ** . * : . * . : * . . . * : * * . . :
AmyS      YVGKQHAGKV FYDLTGNRSDT V TINS DGWGEFKVNGGSVSVWVPR-----
AmyL      YVGRQNAGETWHDITGNRSEPVVINSEG WGEFHVNGGSVSIYVQR-----
SumAmy2   PANAPKLDPAAYPVWKL DVALPAGTT FAYKYVRKDGSGNVTWESGANRTATVPASGKVTL
          . . : . . : : . : : : : * . . :
AmyS      ----- (SEQ ID NO: 7)
AmyL      ----- (SEQ ID NO: 8)
SumAmy2   TADVWRG (SEQ ID NO: 4)

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Figure 5

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ABF13430.1 -----VSPPPQEHVVLPAEDYPWWQDYQPVSYK
EHK88249.1 -----EHVVLGEQGYPPWWQDYQPVSYR
XP_003033038.1 -----YVQVSPQEHIQGPQWWTDYQPVSYN
SumAmy2 AAPGDKDVTAVMFEWKFASIAKACTDTLGPAGYGYVQVSPQEHIQGGQWWTSYQPVSYR
YP_001826792.1 -----QVSPPPQEHIQGGQWWTSYQPVSYR
ZP_06908805.1 -----EHIQGSQWWTSYQPVSYR
CAB06622.1 -----VQVSPPPQEHIQGGQWWTSYQPVSYR
CAA73775.1 -----SQWWTSYQPVSYK
AAA82875.1 -----PATEHIQGDQWWTSYQPVSYR
BAE94180.1 -----VAAECTSTIGPAGFGYVQVSPPPQETIQGTAWWTSYQPVSYK
1G94_A -----
AEV86508.1 -----APPQESVTLASTSDGAHPWWEVYQPVSYD

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```

ABF13430.1 LDQTRRGSRADFIDMVNTCREAGVKIYVDAVINHMTGTGSA-GAGPGSAGSSYSKYDYDPG
EHK88249.1 LDETRRGTRAEFAAMVDTCHDAGVQVYVDAVINHMTGQDEC-GTGSAGSAYCHYDYAEAG
XP_003033038.1 LV-SKRGDRTQFATMVATCHAAGVKVIVDTIWNHMSGQDS----GVTGGSFHETHYDYPG
SumAmy2 IA-GRLGDRAAFSAMVNTCHSAGVKVVDVINHMAA-----GNGTGTGSSSYTKYDYPG
YP_001826792.1 IA-GRLGDRAAFAMVNTCHAAGVKVIADSVINHMSA-----GSGTGTGSSSYTKYDYPG
ZP_06908805.1 IA-GRLGDRAAFKSMVDTCHAAGVKVIADSVINHMSA-----GSGTGTGSSAYTKYNYPG
CAB06622.1 IA-GRLGDRAQFKSMVDTCHAAGVKVVDVINHMSA-----GNGTGTGSSSYTKYDYPG
CAA73775.1 IA-GPVGDRTAFRNMVNTCHAAGVKVVDVINHMSA-----GSGTGTGSSSYTKYSYPG
AAA82875.1 IA-GRLGDRDGFAAMVDAACHSAGVKVVDVINHMAA-----GSGTGTGTTAYTKYDYPG
BAE94180.1 IE-GKLGTRAEFAAMVETCDAAGVEVIVDAVINHTTGADG--GSGTGTAGTPYGIIDFPG
1G94_A -----VNRCSAAGVDIYVDTLINHMAA-----GSGTGTAGNSFGNKSFPPI
AEV86508.1 VS-GRLGTQAQFTSMVTTCHNAGVRVYVDAVINHMAGSNNTGGSGYGGSTFNATGYSYPA
          * * *** : .*: : * * : . * . . .

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ABF13430.1 IYQSQDFNDCCR-----DITNWNKWEVQHCELVGLADLKTSSPYVQDRIAAYLNE
EHK88249.1 YGYDDFHHCGRNGD-----DNIIVDFHWRWEVQNCLELVNLADLDTGSEYVRDRIGAYLND
XP_003033038.1 IYQTQDFHHCGLEPY-----DDINNYGNRKEVQTCELVNLADLATDTVYVQARLAAYGND
SumAmy2 IYSGADMDDCRAQI-----SNYNDRGNVQNCLELVGLADLDTGEDYVRGRIAGYLND
YP_001826792.1 LYSGADMDDCRSQIG-----NNYNDRGNVQNCLELVGLADLDTGEEYVRGRIAGYLND
ZP_06908805.1 LYSSYDMDNCTSQI-----NNYQDRGNVQNCLELVGLADLDTGEEYVRGRIAGYLND
CAB06622.1 LYSSNDLDNCTSQI-----NNYGDRFNVQCELVGLADLDTGEAYVRGKIAGYLTD
CAA73775.1 LYSSYDFDDSTSQV-----TNYQDRWNVQHCELVGLADLDTGESYVRGGIARYMND
AAA82875.1 YFGDADFHTCRTAI-----KDYTDRGDVQNCLELVGLSDLDTGKDEVRSTIAAYLDG
BAE94180.1 IYGAADFNDCRTDI-----SSYQDRYQVQNCRLLSLQDLRTGSEYVQNTLAGYMND
1G94_A YSPQDFHESCTINN-----SDYGNDRYRVQNCLELVGLADLDTASNYVQNTIAAYIND
AEV86508.1 VPYSYNDFHHPNDGYCNDDDGQIDDYSNAAEVQNCLELVSLSDLKSQDTGVRTKIAGYLNK
          : ** *.*:.* ** : * : :. *

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ABF13430.1 LIDLGVAGFRIDAAKHIPEGDLQAILSRLKNVHPAWGGGKPYIFQEVIADSTISTGSYTH
EHK88249.1 LLSLGVGDGFRIDAAKHMPAEDVAALVSRLDR-----PAYVYQEVVLHGDGEFIPTEEYLD
XP_003033038.1 LLSLGVGDGLRLDAAKHIPTGEVATIIDMLAS-----HPYITQEVTTSSPDDVVKPEEYVQ
SumAmy2 LLSLGVGDGFRIDAAKHMPAGDLANIKSRLTNPN-----VYWKQEAIIYGAGEAVSPSEYLG
YP_001826792.1 LLSLGVGDGFRIDAAKHMPAGDLANIKSRLTNPN-----VYWKHEAIYGAGEAVSPSEYLG
ZP_06908805.1 LLTLGVGDGFRIDASKHMPADDLAAIKGKLTNPG-----IYWKHEAIIHGAGEAVSPSEYLG
CAB06622.1 LLSLGVGDGFRIDAAKHMAADLAAIKSRLSNPN-----VYWKHEAIYGAGEAVSPTEYVG
CAA73775.1 LLSLGVGDGFRIDAAKHIDAADLANIKSRLTNPS-----VYWKQEVIIYGAGEAVQPTEYTG
AAA82875.1 LRSMGVDGFRIDAAKHMAADDVA-IQQQDERPP-----GSGSPEVIQGGGEAVQPEEYTE
BAE94180.1 LLSLGVAGFRIDAAKHIPASDLAAIKAKLSDPN-----AYWVQEVIGAGEPQVQSEYTG
1G94_A LQAIGVKGFRFDASKHVAASDIQSLMAKVNGSP-----VVFQEVIDQGGGEAVGASEYLS
AEV86508.1 LVDWGVGDGFRVDAAKHMAADLSAIIKALLHNTTEGR-SPYFAQEVIPGSGGAIAPSAYTG
          * ** *.*:.**: : : : . : *

```

Figure 6A

ABF13430.1	LGSVTEFQYHRDISHAFANGNIAHLTGLGSGLT-PSDKAVFVFNHDTQR-----YEPIL
EHK88249.1	NGDVYDQRYARDLAKVFNSEKLAYLRDFGTPV--PSENVVVFVDNHSQR-----GDETL
XP_003033038.1	NGDVQEFYRYTRALQSAFLGGGIDQLKDLGKGWVPASAANVFVFNHDTQR----GDSL
SumAmy2	TGDVQEFYGRSLKQVFLNENLAHLKNFGEGWGFMESEKSAVFVDNHDTER----GGDTL
YP_001826792.1	SGDVQEFYGRSLKQVFLNENLAHLKNFGEGWGFMESEKSAVFVDNHDTER----GGDTL
ZP_06908805.1	SGDVQEFYGRGLKQVFTGGNLTSLKNFGEGWGFMESEKSAVFVANHDTER----GGDTL
CAB06622.1	SGDVQEFYARDLKRNVFNENLAYLKNFGGEAWGHLPSDEAAVFVDNHDTER----NGETL
CAA73775.1	NGDVQEFYAYDLKRNVFNENLAYLKNYGEAWGYLNSSVAGVFVDNHDTD-----NGSTL
AAA82875.1	IGDVDEFRYGGHLKSAFQGGGIAQLKAVADRK--LGSASARTFVDNWDTER----NGSTL
BAE94180.1	TGDVHEFDYARQLKSDFDGSIANLQRI SNGKL---PSAQAGVFVDNHDTER----NGETM
1G94_A	TGLVTEFKYSELGNTFRNGSLAWLSNFGEGWGFMPSSSAVFVDNHDNQRGHGGAGNVI
AEV86508.1	IGDVLGFSYAYGLKTQFANGTLSNLSGIPSWSLDATSDQTAAMVTNHDLER----NGSTL
	* * * : * :
ABF13430.1	THTDGARYDLAQ-----KFMLAHPYGTPKVMSSYTWSG-DDKAGPPMHS
EHK88249.1	TYRDGARYSLAN-----AFMLAWPVGTPKINSSYTFD--DYDAGPPSDA
XP_003033038.1	SKSPSNTYITAT-----VFSLAHPYGMPSVLSSYEFS---DHDAGAPGN
SumAmy2	NYKNGSAYTLAN-----VFMLAYPYGSPDVHSGYEFS--DHDAGPPNGG
YP_001826792.1	NYKNGSAYTLAA-----VFMLAYPYGSPDVHSGYEFS--DHDAGPPNGG
ZP_06908805.1	TYKDGANYTLAH-----VFMLAWPYGSPDVHSGYEFT--QHDAGPPNGG
CAB06622.1	TYKDGATYTLAH-----VFMLAWPYGSPDVHSGYEFT--DHDAGPPNGG
CAA73775.1	NYRDGANYTLAN-----VFMLAWPYGAPDINSGYEWS--DAAAGPPNA-
AAA82875.1	TYKDGAAAYTLAN-----VFMLASPYGSPNVYSGYEWS--DADAGPPSG-
BAE94180.1	NETWGAKYVLANGETMNETWGAKYVLANTFMLAYPYGSPSVYSGYTFT--DEDAGAPGAT
1G94_A	TFEDGRLYDLAN-----VFMLAYPYGYPKVMSSYDFHG-DTDAGGPNVP
AEV86508.1	RYQDGSTYLLAN-----YFLLAYPYGQPFVYDGFATSTSATGASPPADA
	. * * * * * : :
ABF13430.1	DGTRPTDCSADRWLCEH--RAVAGMVG FHNNAVAGQ-GIGSAVTDGNRLAFARGSAGYA
EHK88249.1	AGNTTDAACDSAAWQCEHDWQPIRNMVAFHNEVGDE-PVVQWWDNGNDTIAFGRGDKGYV
XP_003033038.1	GGGTCSQKGGASGWLCQHRYPAISGMVGFERNMVGDV-PMSDWVSPSSQRIAFGRGALGFV
SumAmy2	QVNA----CYSDGWKCQHAWREISSMVGLRNAARGQ-SVTNWWDNGGDQIAFGRGDKAYV
YP_001826792.1	QVNA----CYSDGWKCQHAWREISSMVGFERNARGQ-SVGNWWDNGGDQIAFGRGNKAYV
ZP_06908805.1	AVTA----CYSDGWKCQHSWREISSMVGLRNARGQ-GVTNWWDNGADQIAFGRGTKAYV
CAB06622.1	QVNA----CYSDGWKCQHAWREISSMVAFRNARGQ-GVTDWWDNGGDQIAFGRGSKAYV
CAA73775.1	TVNA----CWQDGWKCQHKWPEIIRMVGFERNARGQ-AVTDWWDNGGDAIAFGRGSKGFV
AAA82875.1	-----ADGWTDTTHAQQTITGLVGFERNVAGSA-ELTDWWDNGGSALAFGRGDKGFV
BAE94180.1	ATSVPDANCDSDAWTCTQRWTEIQGMVGFYNTVAGT-ALTNWWDDGGNQIAFGRGDKGFV
1G94_A	VHNNGNLECFASNWKCEHRSYIAGGVDFRNNTADNNAVNTNWDNTNNQISFGRGSSGHM
AEV86508.1	NGYVTSTSTCNGAWQCTTQSAGVKGMVAWHNATRSATTVSNTNTASNVIGFSRGLGWFE
	* : * * . . : . : . * . *
ABF13430.1	AFNATNTAWT-RTFTTSLPDGVYCDVANGTFVDGV--CDGPSYQVSGGK-FTATVPANGA
EHK88249.1	VINDESSAVTGRSFQALPAGTYCDVLHGDVVEGA--CTGPTYTVDGGGWFRADVGAGHDG
XP_003033038.1	AINNVQDEWT-TTFATSLPDGKYCDVDAQVVAGK--CSGTTYTVSGGK-FEGKVPARSA
SumAmy2	AINHEGSALN-RTFQTSPLAGDYCDVQ-----SGNGVTVNGSGQFTATVPAGTA
YP_001826792.1	AINHEGSALT-RTFQTSPLAGDYCDVQ-----SGKGVTVNGSGQFTATVPAGTA
ZP_06908805.1	AINQEGAALT-RTFQTSPLSGEITYCDVQ-----SGRSVAVDGSGRFTATLGAGTA
CAB06622.1	AINHEGTSALT-RTFQTSPLAGDYCDVQ-----TGKGVTVDGAGRFTATLGAGTA
CAA73775.1	AINHESATIS-RTYQTSPLPPGPYCNVQ-----NNTTCSVNGSGQLTATLAANTA
AAA82875.1	ALNNADDALT-ETFTTSLPAGTYCNVA-----AASPDGCGNTVTVGDDGAVQA
BAE94180.1	AINNTDSATT-RTYSSSLPAGTYCDVV-----AASDCSETITVAADGTFSATLP
1G94_A	AINKEDSTLT-ATVQTDMSAGQYCNVLKGE LSADAKSCSGEVITVNSDGTINLNIGAWDA
AEV86508.1	GVNRSGSAST-ATYTTGLADGAYRDRI-----AGTTITVSGGRASVTIPANGA
	. * . . : : . * * :

Figure 6B

7/8

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ABF13430.1      VALHVEAPGSCG---PDGCGTPPGGGDDCTTVTARFHATVTTWYGQEVAVVGSIPELGSW
EHK88249.1      VALHAGARVG-----
XP_003033038.1  LALHVGAMSL-----
SumAmy2         VALHVNARTCS-----GGGGTNPDPGPGDGSSGASFGVNATTQPGQNIYVTGDQAAALGNW
YP_001826792.1  VALHTGARTC-----GGGGSNPDPGPGNGTSGASFGVNATTQLGQNIHVTGDQAAALGNW
ZP_06908805.1   LALHAGARDC-----GGGSTDPGPVSAGASFVNATTVVGQNIYVTGNQPALGNW
CAB06622.1      VALHVGARTC-----DGGDPGDPDPVSSGVSFAVDATTSWGQDIYVTGNRPGLGHW
CAA73775.1      LAVYAGKSSC-----
AAA82875.1      TVPARGALALH----TGAQAG-----
BAE94180.1      AYGALALHVEKT---SGGTTPDPDPGTDVPVAGKVSASVHASTVLGQEVRIVGSVPPELGGW
1G94_A          MAIHKNAKLN-----
AEV86508.1      VAIDVNARSGSSASPSTSPSVSTSPSTSTGTVQATFNVYAATTAGTSVYVVGSTAALGNW

```

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ABF13430.1      QPAQGVRLRTDSGTYPVWSGAVDLPAGVGFEYKYVKLKP DGTVEWEQGGNRIATVDDSGG
EHK88249.1      -----
XP_003033038.1  -----
SumAmy2         NPANAPKLDPAAYPVWKLDVALPAGTTFAYKYVRKDGSGNVTWESGANRTATVPASGKVT
YP_001826792.1  NPATAPKLDPAAYPVWKLDVNLPA GTTFAYKYVRKDAAGNVTWESGANRTATVPASGKVT
ZP_06908805.1   NPAGAVKLDPAAYPVWKAEVNLAAGTSFEYKYIRKDGAGNVTWESGANRTATVPSSGKVA
CAB06622.1      DPGGGLQLDPAAYPVWKRDELPE GTTFEYKSLRKADAGNVTWESGANRTATVNTTKTTL
CAA73775.1      -----
AAA82875.1      -----
BAE94180.1      QPASGVALDASGYPSWTGGVDLPAGTSFEYKYVKVDDSGAVVWESGANRTATVGADGTLA
1G94_A          -----
AEV86508.1      DTAKAVALSAGGYPIWSSTVAVPSGSSFEYKYLLKKDAAGNVTWESNANRAVTTGSSAVTF

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ABF13430.1      GCSQNFYDSWR-----
EHK88249.1      -----
XP_003033038.1  -----
SumAmy2         LTADVWRG-----
YP_001826792.1  LTADVWRG-----
ZP_06908805.1   LNDTWRN-----
CAB06622.1      NDTWRN-----
CAA73775.1      -----
AAA82875.1      -----
BAE94180.1      LNDTWR-----
1G94_A          -----
AEV86508.1      TNSFGVANAGATAVTFTESATGTGLYVVGSIASLGSWNTADAIPLTSTGASGWSRLVALP

```

```

ABF13430.1      ----- (SEQ ID NO: 15)
EHK88249.1      ----- (SEQ ID NO: 16)
XP_003033038.1  ----- (SEQ ID NO: 17)
SumAmy2         ----- (SEQ ID NO: 4)
YP_001826792.1  ----- (SEQ ID NO: 9)
ZP_06908805.1   ----- (SEQ ID NO: 10)
CAB06622.1      ----- (SEQ ID NO: 11)
CAA73775.1      ----- (SEQ ID NO: 12)
AAA82875.1      ----- (SEQ ID NO: 14)
BAE94180.1      ----- (SEQ ID NO: 13)
1G94_A          ----- (SEQ ID NO: 19)
AEV86508.1      QRTTFQYKYLRKDSSGTVTWESGSNRSYTTGTASSYTI SDTWK (SEQ ID NO: 18)

```

Figure 6C

Phylogenetic tree

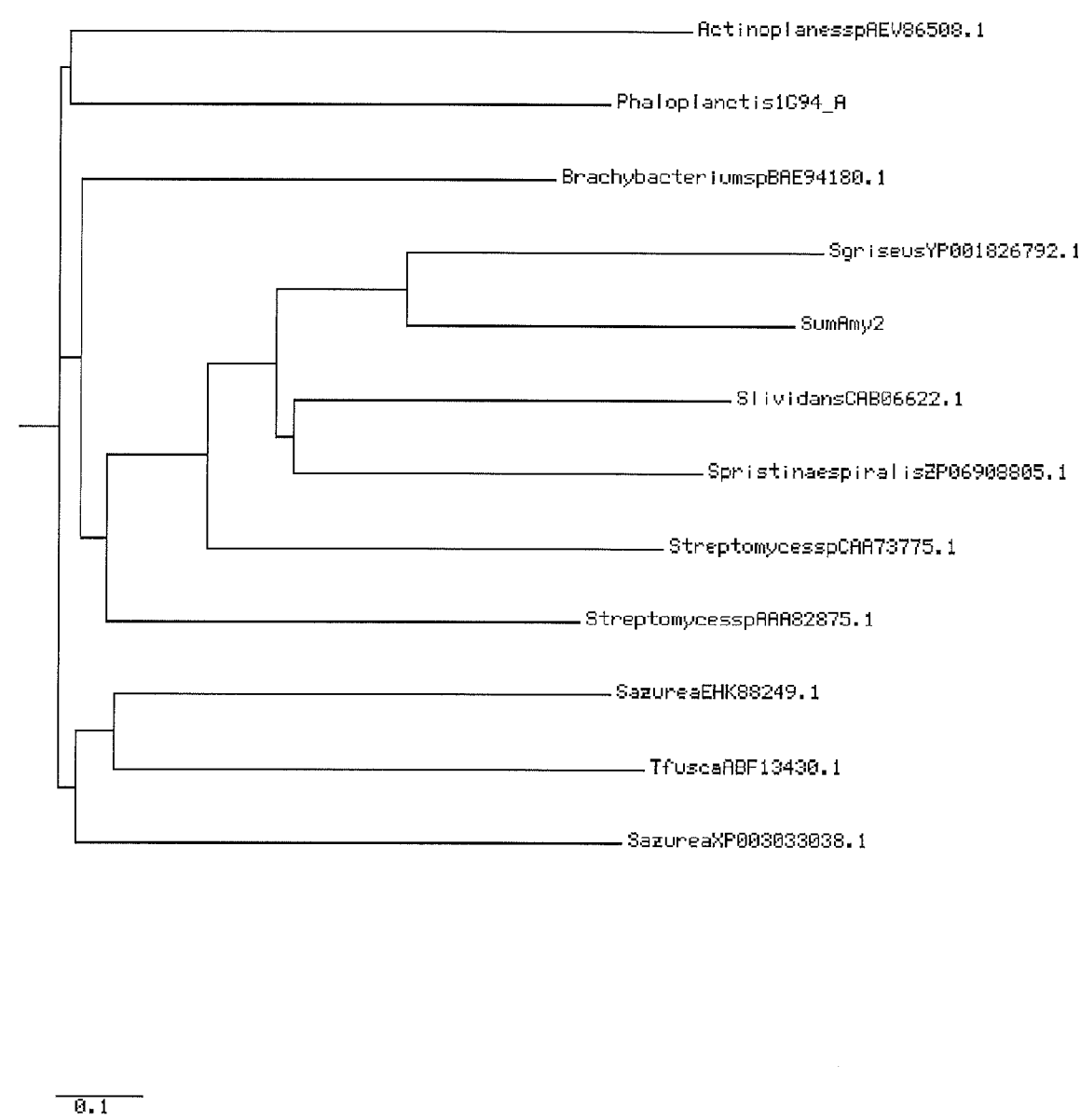


Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/038122

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/26 A23L1/09 C11D3/386
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 A23L C11D

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, Sequence Search, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	DATABASE UniProt [Online] 14 May 2014 (2014-05-14), "RecName: Full=Alpha-amylase; EC=3.2.1.1;"; XP002728803, retrieved from EBI accession no. UNIPROT:W9FRJ9 Database accession no. W9FRJ9 The query sequence SEQ ID NO:4 has 94.90 % identity (99.60 % similarity) over 544 positions in a common overlap (range (q:s): 1-544:34-577) with subject UNIPROT:W9FRJ9 (length: 577).; sequence ----- -/-	1-43



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 August 2014

Date of mailing of the international search report

15/09/2014

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Authorized officer

Rojo Romeo, Elena

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2014/038122

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE UniProt [Online] 22 February 2012 (2012-02-22), "RecName: Full=Alpha-amylase; EC=3.2.1.1;"; XP002728804, retrieved from EBI accession no. UNIPROT:H0BF45 Database accession no. H0BF45 The query sequence SEQ ID NO:4 has 94.30 % identity (99.30 % similarity) over 543 positions in a common overlap (range (q:s): 1-543:22-563) with subject UNIPROT:H0BF45 (length: 564).; sequence & QIN SONG ET AL: "Draft Genome Sequence of Marine Streptomyces sp Strain W007, Which Produces Angucyclinone Antibiotics with a Benz[a] anthracene Skeleton", JOURNAL OF BACTERIOLOGY, vol. 194, no. 6, March 2012 (2012-03), pages 1628-1629, DOI: 10.1128/JB.06701-11.	1-43
Y	----- DATABASE UniProt [Online] 19 October 2011 (2011-10-19), "RecName: Full=Alpha-amylase; EC=3.2.1.1; Flags: Precursor;"; XP002728805, retrieved from EBI accession no. UNIPROT:G0PWV5 Database accession no. G0PWV5 The query sequence SEQ ID NO:4 has 94.10 % identity (98.90 % similarity) over 545 positions in a common overlap (range (q:s): 1-544:35-578) with subject UNIPROT:G0PWV5 (length: 578).; sequence & GRUBBS KIRK J ET AL: "Genome Sequence of Streptomyces griseus Strain XylebKG-1, an Ambrosia Beetle-Associated Actinomycete", JOURNAL OF BACTERIOLOGY, vol. 193, no. 11, June 2011 (2011-06), pages 2890-2891, DOI: 10.1128/JB.00330-11 ----- -/--	1-43

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/038122

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 2014/127753 A1 (FUKUYAMA SHIRO [JP] ET AL) 8 May 2014 (2014-05-08) The query sequence SEQ ID NO:4 has 80.90 % identity (93.20 % similarity) over 544 positions in a common overlap (range (q:s): 1-544:29-566) with subject GSP:BBF92647 (length: 566) from US2014127753-A1 published on 2014-05-08. -----	1-43
Y	US 2003/138786 A1 (CALLEN WALTER [US] ET AL) 24 July 2003 (2003-07-24) the whole document in particular, paragraphs 4-12 -----	1-43
Y	US 2011/195481 A1 (SVENDSEN ALLAN [DK] ET AL) 11 August 2011 (2011-08-11) in particular paragraphs 445-453 -----	1-43
Y	OHNISHI YASUO ET AL: "Genome sequence of the streptomycin-producing microorganism Streptomyces griseus IFO 13350", JOURNAL OF BACTERIOLOGY, vol. 190, no. 11, June 2008 (2008-06), pages 4050-4060, XP002728806, ISSN: 0021-9193 The query sequence SEQ ID NO:1 has 94.10 % identity (94.10 % similarity) over 1638 positions in a common overlap (range (q:s): 1-1635:6213595-6215229) with subject EM_STD:AP009493 (length: 8545929). -----	1-43

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2014/038122

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014127753	A1	08-05-2014	NONE

US 2003138786	A1	24-07-2003	AU 2003225947 A1 13-10-2003
			US 2003138786 A1 24-07-2003
			WO 03083054 A2 09-10-2003

US 2011195481	A1	11-08-2011	CN 102869759 A 09-01-2013
			EP 2534236 A1 19-12-2012
			JP 2013519365 A 30-05-2013
			US 2011195481 A1 11-08-2011
			WO 2011098531 A1 18-08-2011
