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(54) Title: VARIANT ALPHA-AMYLASES AND METHODS OF USE, THEREOF

(57) Abstract: Compositions and methods relating to variant alpha-amylases are described.

VARIANT ALPHA-AMYLASES AND METHODS OF USE, THEREOF

PRIORITY

[001] This application claims priority to U.S. Provisional Application Serial No. 61/579,356, filed on December 22, 2011, which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[002] Disclosed are compositions and methods relating to variant α-amylase enzymes.

BACKGROUND

[003] Starch consists of a mixture of amylose (15-30% w/w) and amylopectin (70-85% w/w). Amylose consists of linear chains of 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] Sugars from starch, in the form of concentrated dextrose syrups, are currently produced by an enzyme catalyzed process involving: (1) liquefaction (or viscosity reduction) of solid starch with an α-amylase into dextrins having an average degree of polymerization of about 7-10, and (2) saccharification of the resulting liquefied starch (*i.e.* starch hydrolysate) with amyloglucosidase (also called glucoamylase or GA). The resulting syrup has a high glucose content. Much of the glucose syrup that is commercially produced is subsequently enzymatically isomerized to a dextrose/fructose mixture known as isosyrup.

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

[006] One characterized α-amylase is that of alkaliphilic *Bacillus* sp. strain TS-23, which produces at least five kinds of enzymes exhibiting starch hydrolyzing activity (Lin *et al.*, (1998) *Biotechnol. Appl. Biochem.* 28:61-68). The α-amylase of *Bacillus* sp. strain TS-23 has a pH optimum of 9 although it is stable over a broad pH range (*i.e.*, pH 4.7 to 10.8). Its temperature optimum is 45°C, although the enzyme has activity at lower temperatures, *e.g.*, 15-20°C. Variants of this enzyme have been described, *e.g.*, in international patent applications WO 2009/061380, WO 2009/100102, and WO 2010/115028.

SUMMARY

[007] The present compositions and methods relate to variant α-amylase polypeptides, and methods of use, thereof.

[008] In one aspect, a variant α-amylase polypeptide is provided, comprising at least one combinable mutation at a productive amino acid position; wherein: (i) the combinable mutation is a mutation that improves at least one desirable property of the variant α-amylase compared to the parental α-amylase, while not significantly decreasing either expression, activity, or stability of the variant α-amylase, compared to the parental α-amylase, (ii) the productive position is an amino acid position that can be substituted with a plurality of different amino acid residues, all of which substitutions result in a variant α-amylase that meets the requirements of (i), and (iii) the combinable mutation is listed in Table C or Table D, which uses SEQ ID NO: 2 for numbering.

[009] In some embodiments, the variant combinable mutation has a performance property listed in Table A. In some embodiments, the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.9, and in addition the PI for any one of these properties is greater than or equal to 1.0. In some embodiments, the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.8, and in in addition have a PI for any one of these tests that is greater than or equal to 1.2. in some embodiments, the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.5, and in in addition have a PI for any one of these tests that is greater than or equal to 1.5.

[0010] In some embodiments, the combinable mutation has a sustainability score of +++, ++++, or +++++. In some embodiments, the combinable mutation has a sustainability score of ++++, or +++++. In some embodiments, the combinable mutation has a sustainability score of +++++. In some embodiments, the combinable mutation has a productivity score of 1 or 2.

[0011] In some embodiments, the parental α-amylase has at least 60% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the parental α-amylase has at least 70% amino acid sequence identity to the amino acid sequence

of SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the parental α-amylase has at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the parental α-amylase has at least 90% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

[0012] In another aspect, a composition comprising any of the foregoing variant amylases is provided. In some embodiments, the composition is effective for removing starchy stains from laundry, dishes, or textiles. In some embodiments, the composition comprises a surfactant. In some embodiments, the composition is a detergent composition. In some embodiments, the composition is a laundry detergent or a laundry detergent additive. In some embodiments, the composition is manual or automatic dishwashing detergent.

[0013] In another aspect, a method for removing a starchy stain or soil from a surface is provided, comprising: incubating the surface in the presence of a aqueous composition comprising an effective amount of the variant amylase of any of the claims 1-13, allowing the polypeptide to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in the aqueous composition, and rinsing the surface, thereby removing the starchy stain from the surface.

[0014] In some embodiments, the aqueous composition further comprises a surfactant. In some embodiments, the surface is a textile surface. In some embodiments, the surface is on dishes. In some embodiments, the surface is a soiled hard surface.

[0015] In another aspect, an isolated polynucleotide encoding any of the forementioned polypeptides is provided, as is an expression vector comprising the polynucleotide and a host cell comprising the expression vector.

[0016] 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

[0017] Figure 1 is a map of plasmid pHPLT-Amy TS23t.

BRIEF DESCRIPTION OF THE SEQUENCES

[0018] SEQ ID NO: 1 sets forth the amino acid sequence of the mature form of *Bacillus* sp. strain TS-23amylase.

[0019] SEQ ID NO: 2 sets forth the amino acid sequence of the mature form of a C-terminal truncated form of *Bacillus* sp. strain TS-23amylase.

DETAILED DESCRIPTION

[0020] Described are compositions and methods relating to variant α-amylase enzymes. The variant amylases include mutations to impart a performance benefit, for example, increased hydrolysis of a starch substrate, increased cleaning performance, increased thermal stability, increased storage stability, increased solubility, an altered pH profile, decreased calcium dependence, and/or increased expression. In some cases, the performance benefit is realized at low temperatures.

[0021] The subject α-amylases are variants of *Bacillus* sp. strain TS-23 amylase (*i.e.*, AmyTS23), or variants of amylases that share at least 60%, at least 70%, at least 80%, or even at least 90%, sequence identity with AmyTS23.

[0022] Exemplary applications for the present amylases are in the preparation of cleaning compositions, such as detergent compositions for cleaning laundry, dishes [including manual and automatic dishwashing (ADW)], and other surfaces, for textile processing (*e.g.*, desizing), in animal feed for improved digestibility, and for starch liquefaction and saccharification. These and other aspects of the compositions and methods are described in detail, below.

1. Definitions and Acronyms

[0023] In accordance with this detailed description, the following abbreviations and definitions apply. Note that the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes a plurality of such enzymes, and reference to "the dosage" includes reference to one or more dosages and equivalents thereof known to those skilled in the art, and so forth.

[0024] 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.

[0025] 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 abbreviations and/or terms are defined for clarity:

1.1 Abbreviations/acronyms

[0026] The following abbreviations/acronyms have the following meanings unless otherwise specified:

AE	alcohol ethoxylate
AEO	alcohol ethoxylate
AEOS	alcohol ethoxysulfate
AES	alcohol ethoxysulfate
AOS	α-olefinsulfonate

AS	alkyl sulfate
cDNA	complementary DNA
CMC	carboxymethylcellulose
DNA	deoxyribonucleic acid
DTMPA	diethylenetriaminepentaacetic acid
EC	enzyme commission
EDTA	ethylenediaminetetraacetic acid
EO	ethylene oxide (polymer fragment)
GA	glucoamylase
IPTG	isopropyl β-D-thiogalactoside
kDa	kiloDalton
LAS	linear alkylbenzenesulfonate
LAT	*B. licheniformis* amylase
MW	molecular weight
MWU	modified Wohlgemuth unit; $1.6x10^{-5}$ mg/MWU = unit of activity
NOBS	nonanoyloxybenzenesulfonate
NTA	nitriloacetic acid
OxAm	Purastar HPAM 5000L (Danisco US Inc.)
PEG	polyethyleneglycol
pI	isoelectric point
PVA	poly(vinyl alcohol)
PVP	poly(vinylpyrrolidone)
RNA	ribonucleic acid
SAS	alkanesulfonate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp.	species
TAED	tetraacetylethylenediamine
w/v	weight/volume
w/w	weight/weight
v/v	volume/volume
wt%	weight percent
°C	degrees Centigrade
H_2O	water
dH_2O or DI	deionized water
dIH_2O	deionized water, Milli-Q filtration
g or gm	grams
µg	micrograms
mg	milligrams
kg	kilograms
µL and µl	microliters
mL and ml	milliliters
mm	millimeters
µm	micrometer
M	molar
mM	millimolar
µM	micromolar
U	units
sec	seconds
min(s)	minute/minutes
hr(s)	hour/hours
DO	dissolved oxygen

Ncm	Newton centimeter
ETOH	ethanol
eq.	equivalents
N	normal
ds or DS	dry solids content

1.2 Definitions

[0027] The terms "amylase" or "amylolytic enzyme" refer to an enzyme that is, among other things, capable of catalyzing the degradation of starch. α-amylases are hydrolases that cleave the α-D-(1→4) O-glycosidic linkages in starch. Generally, α-amylases (EC 3.2.1.1; α-D-(1→4)-glucan glucanohydrolase) are defined as endo-acting enzymes cleaving α-D-(1→4) O-glycosidic linkages within the starch molecule in a random fashion yielding polysaccharides containing three or more (1-4)-α-linked D-glucose units. In contrast, the exo-acting amylolytic enzymes, such as β-amylases (EC 3.2.1.2; α-D-(1→4)-glucan maltohydrolase) and some product-specific amylases like maltogenic α-amylase (EC 3.2.1.133) cleave the polysaccharide molecule from the non-reducing end of the substrate. β-amylases, α-glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; α-D-(1→4)-glucan glucohydrolase), and product-specific amylases like the maltotetraosidases (EC 3.2.1.60) and the maltohexaosidases (EC 3.2.1.98) can produce malto-oligosaccharides of a specific length. Some bacterial α-amylases predominantly produce maltotetraose (G4), maltopentaose (G5) or maltohexaose (G6) from starch and related α-1,4-glucans, while most α-amylases further convert them to glucose and or maltiose as final products.

[0028] As used herein 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, grasses, tubers and roots, and more specifically materials obtained from wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, potato, sweet potato, and tapioca.

[0029] 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.

[0030] 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 a man-made substitution, insertion, or deletion at one or more amino acid positions. 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.

[0031] The term "recombinant," when used in reference to a subject cell, nucleic acid, protein or vector, indicates that the subject has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. 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.

[0032] As used herein, "combinable mutations" are mutations at any amino acid position that can be used to make combinatorial variants. Combinable mutations improve at least one desired property of the molecule (in this case, an amylase), while not significantly decreasing either expression, activity, or stability. Combinable mutations can be grouped as follows:

Group A: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) activity, (iii) CS-28 microswatch activity at pH 8 (16°C, 32°C, or 50°C) or pH10 (16°C or 50°C), and (iv) detergent stability or thermostability are greater than or equal to 0.9, and in addition have a PI for any one of these tests that is greater than or equal to 1.0.

Group B: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) activity, (iii) CS-28 microswatch activity at pH 8 (16°C, 32°C, or 50°C) or pH10 (16°C or 50°C), and (iv) detergent stability or thermostability are greater than or equal to 0.8, and in in addition have a PI for any one of these tests that is greater than or equal to 1.2.

Group C: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) activity, (iii) CS-28 microswatch activity at pH 8 (16°C, 32°C, or 50°C) or pH10 (16°C or 50°C), and (iv) detergent stability or thermostability are greater than or equal to 0.5, and in addition have a PI for any one of these tests that is greater than or equal to 1.5.

[0033] The properties of combinable mutations are summarized in the following Table.

Table A. Performance properties for each group of combinable mutations

	Performance Index (PI)			
Group	Expression	Cleaning (pH 8 or 10)	Stability (detergent or thermal)	Minimum PI in one or more tests
A	≥ 0.9	≥ 0.9	≥ 0.9	X ≥ 1.0
B	≥ 0.8	≥ 0.8	≥ 0.8	X ≥ 1.2
C	≥ 0.5	≥ 0.5	≥ 0.5	X ≥ 1.5

[0034] Preferred combinable mutations are at "productive positions," as described, below. In the case of the present amylases, "activity" refers to amylase activity, which can be measured as described, herein.

[0035] As used herein, "productive positions" are amino acid positions that are tolerant to substitution with different amino acid residues, wherein the resulting variants meet a set of performance criteria for combinability, as set forth above. Productive positions can be assigned a Productivity Score as follows:

1. Positions where less than 15% of the substitutions at a given position fall within groups A, B, or C are given a Productivity Score of "1".
2. Positions where less than 40%, but greater than, or equal to 15% of the substitutions at a given position fall within groups A, B, or C are given a Productivity Score of "2".
3. Positions where less than 75%, but greater than, or equal to 40% of the substitutions at a given position fall within groups A, B, or C are given a Productivity Score of "3".
4. Positions where 75% or more of the substitutions at a given position fall within groups A, B, or C are given a Productivity Score of "4".

[0036] Preferred productive positions are combinable mutations.

[0037] As used herein, "suitability score" refers to the ability of one or more combinable mutations to be used to make combinatorial variants, based on the performance criteria for combinability, (*i.e.*, A, B, and C, as set forth, above) in which each of the mutations fall. A higher suitability score indicates a mutation or mutations that are more suitable for use in making combinatorial variants. Suitability scores are described in the following Table.

Table B. Definitions of suitability scores

Substitutions Occur in Group(s)	Suitability Score
A, B and C	+++++
A and B	++++
A or (B and C)	+++
B	++
C	+

[0038] 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.

[0039] As used herein, 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.

[0040] The term "enhanced stability" or "increased stability" in the context of an oxidating environment, the presence of chelators, the presence of detergents, exposure to elevated temperatures, and/or exposure to pH extremes, means that a subject amylase retains more amylolytic activity over time compared to another (*i.e.*, reference) amylase.

[0041] 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 amylase activity following exposure to (*i.e.*, challenge by) an elevated temperature.

[0042] As used herein, the expression "substantially 100% stability in the presence of a protease-containing commercial laundry detergent composition," or similar language, means at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and up to 100% stability. Each and any of these values may be specified with reference to this stability property.

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

[0044] As used herein, the terms "pH stable" and "pH stability," with reference to an enzyme, relate to the ability of the enzyme to retain activity over a wide range of pH values for a predetermined period of time (*e.g.*, 15 min., 30 min., 1 hour).

[0045] As used herein, the term "amino acid sequence" is synonymous with the terms "polypeptide," "protein," and "peptide," and are used interchangeably. Where such amino acid sequences exhibit activity, they may be referred to as an "enzyme." The conventional one-letter or three-letter codes for amino acid residues are used, with amino acid sequences being presented in the standard amino-to-carboxy terminal orientation (*i.e.*, N→C).

[0046] The term "nucleic acid" encompasses DNA, RNA, heteroduplexes, and synthetic molecules capable of encoding a polypeptide. Nucleic acids may be single stranded or double stranded, and may be chemical modifications. The terms "nucleic acid" and "polynucleotide" are used interchangeably. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present compositions and methods encompass nucleotide sequences that encode a particular amino acid sequence. Unless otherwise indicated, nucleic acid sequences are presented in 5′-to-3′ orientation.

[0047] By "homologue" shall mean an entity having a specified degree of identity with the subject amino acid sequences and the subject nucleotide sequences. A homologous sequence is taken to include an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% identical to the subject sequence, using the well-known sequence alignment tool Clustal V with default parameters. Typically, homologues will include the same active site residues as the subject amino acid sequence, unless otherwise specified.

[0048] As used herein, "hybridization" refers to the process by which one strand of nucleic acid base pairs with a complementary strand, as occurs during blot hybridization techniques and PCR techniques. Stringent hybridization conditions are exemplified by the following: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0).

[0049] As used herein, a "synthetic" molecule is produced by *in vitro* chemical or enzymatic synthesis rather than by an organism.

[0050] As used herein, 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.

[0051] 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.

[0052] 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 bacterial cells. The term "host cell" includes protoplasts created from cells, such as those of a *Bacillus* sp.

[0053] 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.

[0054] The term "endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

[0055] As used herein, 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.

[0056] 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.

[0057] 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.

[0058] 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 that control termination of transcription and translation.

[0059] 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 if the expression of the coding sequence is under control of the regulatory sequences.

[0060] 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.

[0061] As used herein, "biologically active" refer to a sequence having a specified biological activity, such an enzymatic activity.

[0062] "Water hardness" is a measure of the minerals (*e.g.*, calcium and magnesium) present in water.

[0063] As used herein, 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, pigment, oil, or mixtures of these compounds.

[0064] As used herein, a "smaller swatch" is a section of the swatch that has been cut with a hole punch device, *e.g.*, a custom manufactured 96-hole punch device, where the pattern of the multi-hole punch is matched to standard 96-well microtiter plates, or the section has been otherwise removed from the swatch. The swatch can be of textile, paper, metal, or other suitable material. The smaller swatch can have the stain affixed either before or after it is placed into the well of a 24-, 48- or 96-well microtiter plate. The smaller swatch can also be made by applying a stain to a small piece of material. For example, the smaller swatch can be a stained piece of fabric 5/8" or 0.25" in diameter. The custom manufactured punch is designed in such a manner that it delivers 96 swatches simultaneously to all wells of a 96-well plate. The device allows delivery of more than one swatch per well by simply loading the same 96-well plate multiple times. Multi-hole punch devices can be conceived of to deliver simultaneously swatches to any format plate, including but not limited to 24-well, 48-well, and 96-well plates. In another conceivable method, the soiled test platform can be a bead made of metal, plastic, glass, ceramic, or another suitable material that is coated with the soil substrate. The one or more coated beads are then placed into wells of 96-, 48-, or 24- well plates or larger formats, containing suitable buffer and enzyme.

[0065] As used herein, "a cultured cell material comprising an α-amylase polypeptide," or similar language, refers to a cell lysate or supernatant (including media) that includes an α-amylase polypeptide as a component. The cell material is preferably from a heterologous host that is grown in culture for the purpose of producing the α-amylase polypeptide.

[0066] All references cited herein are expressly incorporated by reference.

2. <u>Amylase polypeptides and nucleic acids</u>

[0067] The mature form of the α-amylase from *Bacillus* sp. strain TS-23 (*i.e.*, AmyTS23) has the amino acid sequence of SEQ ID NO: 1:

```
NTAPINETMM QYFEWDLPND GTLWTKVKNE AANLSSLGIT ALWLPPAYKG   50
TSQSDVGYGV YDLYDLGEFN QKGTIRTKYG TKTQYIQAIQ AAKAAGMQVY  100
```

```
ADVVFNHKAG ADGTEFVDAV EVDPSNRNQE TSGTYQIQAW TKFDFPGRGN  150
TYSSFKWRWY HFDGTDWDES RKLNRIYKFR STGKAWDWEV DTENGNYDYL  200
MFADLDMDHP EVVTELKNWG TWYVNTTNID GFRLDAVKHI KYSFFPDWLT  250
YVRNQTGKNL FAVGEFWSYD VNKLHNYITK TNGSMSLFDA PLHNNFYTAS  300
KSSGYFDMRY LLNNTLMKDQ PSLAVTLVDN HDTQPGQSLQ SWVEPWFKPL  350
AYAFILTRQE GYPCVFYGDY YGIPKYNIPG LKSKIDPLLI ARRDYAYGTQ  400
RDYIDHQDII GWTREGIDTK PNSGLAALIT DGPGGSKWMY VGKKHAGKVF  450
YDLTGNRSDT VTINADGWGE FKVNGGSVSI WVAKTSNVTF TVNNATTTSG  500
QNVYVVANIP ELGNWNTANA IKMNPSSYPT WKATIALPQG KAIEFKFIKK  550
DQAGNVIWES TSNRTYTVPF SSTGSYTASW NVP                    583
```

[0068] An enzymatically active C-terminal-truncated form of the mature α-amylase from *Bacillus* sp. strain TS-23 (*i.e.*, AmyTS23t or BASE) has the amino acid sequence of SEQ ID NO: 2:

```
NTAPINETMMQYFEWDLPNDGTLWTKVKNEAANLSSLGITALWLPPAYKGTSQ
SDVGYGVYDLYDLGEFNQKGTIRTKYGTKTQYIQAIQAAKAAGMQVYADVVFN
HKAGADGTEFVDAVEVDPSNRNQETSGTYQIQAWTKFDFPGRGNTYSSFKWRW
YHFDGTDWDESRKLNRIYKFRSTGKAWDWEVDTENGNYDYLMFADLDMDHPEV
VTELKNWGTWYVNTTNIDGFRLDAVKHIKYSFFPDWLTYVRNQTGKNLFAVGE
FWSYDVNKLHNYITKTNGSMSLFDAPLHNNFYTASKSSGYFDMRYLLNNTLMK
DQPSLAVTLVDNHDTQPGQSLQSWVEPWFKPLAYAFILTRQEGYPCVFYGDYY
GIPKYNIPGLKSKIDPLLIARRDYAYGTQRDYIDHQDIIGWTREGIDTKPNSG
LAALITDGPGGSKWMYVGKKHAGKVFYDLTGNRSDTVTINADGWGEFKVNGGS
VSIWVAK
```

[0069] Variants of *Bacillus* sp. strain TS-23 have been described, *e.g.*, in international patent applications WO 2009/061380, WO 2009/100102, and WO 2010/115028. The present compositions and method relate to further variants of *Bacillus* sp. strain TS-23, which satisfy the various criteria described, herein. Preferred mutations are combinable substitutions at productive positions, in which the amino acid residue present in the parental amylase (*i.e.*, the "wild-type" residue) is replaced with a different amino acid residue. Specific substitutions are listed in Tables C and D.

Table C. Combinable substitutions at productive positions

Amino acid Postion	Substitutions
12	Y,D,H,Q,T,G,S
13	F,Y,S
20	D,N
23	L,A,E,F,G,M,N,S,T,W,Y,Q
27	V,E,G,N,S,T,Q
31	A,G,H,N,P,T,Y,Q,S
33	N,D,G,P
34	L,H,N,Q

Amino acid Postion	Substitutions
40	T,R
41	A,D,Q,R,S
43	W,Y
44	L,T
48	Y,Q,T
63	L,V,E,M,S
64	Y,T,S
66	L,I
68	E,D,N
74	T,L,W
79	Y,H,W
81	T,H
88	A,S
89	I,M,V,A
92	A,G
96	G,A,C,E,K
97	M,F,V
106	N,D
109	A,E,T
117	V,E,Q,P,W,A,S
120	V,A,N
122	V,C
133	G,N,D,P,E,H
139	A,P
146	P,A
173	L,Q,W
180	R,I,M,V,A,D,K,N,Q,T,C,G,W
181	S,A,G,C,D,H,M,P,Q,T
194	N,F,H
209	H,E,Y,A,C,W,M,N,Q,R
212	V,P
213	V,T,P
215	E,H
218	N,S
223	Y,F,L,S
224	V,K,R,Q
225	N,D
226	T,A,H,N,Q
229	I,V,S
232	F,W
242	Y,F
243	S,D,Q,A

Amino acid Postion	Substitutions
244	F,H,V,N,S,L,M,T
245	F,N,S,V,A,M,Q,T
249	L,Q
251	Y,H,M,Q,R,S,P
252	V,L
253	R,K,T,H
256	T,N,D,Q,R,Y
258	K,Q,C,N
260	L,M,R
262	A,G,M
271	V,Y,I,P,R,S,M,T,A,D,G,W,H,Q
282	N,D,L,M,E
285	M,Q,T,V,I,A
286	S,D,H,Q,T,N,R
287	L,I
294	N,R,E
295	N,R
302	S,E,K,Q,T,L,D
304	G,N,Q
312	L,I,K,M,Q,R,S,T
313	N,R,K,T
321	P,N,R,T,A
326	T,S
327	L,I,M
331	H,Q,C,G,T,V
335	P,D,G,H,I,L,M,N,Q,T,K
341	S,D,R,Q,E,H,N
342	W,Q,L,S
345	P,D
346	W,A,F
349	P,Q,T
350	L,I,M,S,Q
353	A,S
354	F,H,L,M,G,T
355	I,T
357	T,A
358	R,H
360	E,A,Q,S,F
362	Y,A,I,S,D,H,K,T,R,E
364	C,A,D,H,S,T,Q,M
366	F,D
367	Y,E,H,Q,F

Amino acid Postion	Substitutions
371	Y,A,H,N,T,R
378	I,A,H,M,N,S
380	G,D,E,N,P,Q,R,K
383	S,I,R,T,L
384	K,Y
385	I,L,M
386	D,Q
387	P,A,R,T
391	A,S
395	Y,F,H,N,Q,R
396	A,G,S
400	Q,E,H,N
404	I,E,S
405	D,S
406	H,D
410	I,A
413	T,S,A
415	E,D
417	I,D,E,G,K,L,M,Q,S,T,V,Y
418	D,H,Q,S,A,G
420	K,F,H,L,N,Q,R,S,Y,A
421	P,A,C,D,E,F,G,H,I,K,L,N,T,W,Y
422	N,A,C,D,H,I,L,Q,R,S,T,W,Y
423	S,A,D
424	G,S
425	L,A,F,I,T,V,Y
427	A,G,T
429	I,E,T
430	T,D
434	G,H,I,K,N,T,V,A,Q
435	G,C,M,T
440	Y,C,E,F,H,L,P,Q,S,T,W,D,G,N,R
442	G,D
443	K,A,C,D,E,F,G,H,Q,R,S,T,W,Y
449	V,A,C,D,E,G,H,K,M,P,T,Y,\Q
454	T,A,Q
455	G,A,D,K,N,S
456	N,T,H
457	R,A,L,Q,T,W
458	S,A,D,F,K,Q,T,V,W,L
460	T,A,E,I,L,P,Q
461	V,A,H,Q,T

Amino acid Postion	Substitutions
462	T,D,K,P,R,V
463	I,A,M,V,S
464	N,D,G,H,K,L,M,TA
466	D,E,Q,S,T
468	W,A,C,F,G,H,L,M,N,Q,R,T,Y
469	G,A,S
471	F,M,Y
472	K,A,I,R,T,D
474	N,A,D,E,F,I,K,M,S,L,T
476	G,A,N,P,Q,S,T,V,Y
477	S,H,K,R
478	V,C,L,M,W,N,Q,S,T,A,I
479	S,A,G,T,C
482	V,L,H

Table D. Combinable substitutions at productive positions

Amino Acid Position	Substitution(s)
15	H
25	G
50	D
59	C,K
107	C,S
140	K
162	M
165	F
166	C,G,H,P,Q
177	H
179	I
203	K,N,D,Q
236	E
337	P,V,Y
339	C,F,S,T
484	Q,S

[0070] In some embodiments, the present variant amylases include one or more of the substitutions listed in Table C and/or Table D. In some embodiments, the present variant amylases include, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more of the substitutions listed in Table C and/or Table D.

[0071] In some embodiments, the present amylase is a variant of *Bacillus* sp. strain TS-23 amylase having a defined degree of amino acid sequence homology/identity to SEQ ID NO: 1, for example, at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% amino acid sequence homology/identity.

[0072] In some embodiments, the present amylase is a variant of *Bacillus* sp. strain TS-23 amylase having a C-terminal truncations, as exemplified by the amino acid sequence of SEQ ID NO: 2, and having a defined degree of amino acid sequence homology/identity to SEQ ID NO: 2, for example, at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% amino acid sequence homology/identity.

[0073] In addition to the mutations described, herein, the present amylase may further include one or more previously described mutations. Previously described mutations are those known to confer beneficial properties in at least one amylase having a similar fold and/or having 60% or greater amino acid sequence identity to *Bacillus* amylases, or in any amylase that has heretofore been referred to as "Termamyl-like."

[0074] Furthermore, the present amylases may include any number of conservative amino acid substitutions, at positions not specifically mutated. Exemplary conservative amino acid substitutions are listed in the Table E

Table E. Conservative amino acid substitutions

For Amino Acid	*Code*	*Replace with any of*
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, b-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn

Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

[0075] The present amylases may be "precursor," "immature," or "full-length," in which case they include a signal sequence, or "mature," in which case they lack a signal sequence. Mature forms of the polypeptides are generally the most useful. Unless otherwise noted, the amino acid residue numbering used herein refers to the mature forms of the respective amylase polypeptides. The present amylase polypeptides may also be truncated to remove the N or C-termini (as exemplified by SEQ ID NO: 2), so long as the resulting polypeptides retain amylase activity.

[0076] The present amylases may be "chimeric" or "hybrid" polypeptides, in that they include at least a portion of a first amylase polypeptide, and at least a portion of a second amylase polypeptide (such chimeric amylases have recently been "rediscovered" as domain-swap amylases). The present amylases may further include heterologous signal sequence, an epitope to allow tracking or purification, or the like. Exemplary heterologous signal sequences are from *B. licheniformis* amylase (LAT), *B. subtilis* (AmyE or AprE), and *Streptomyces* CelA.

[0077] In another aspect, nucleic acids encoding any of the described amylase polypeptides are provided. The nucleic acid may encode a particular amylase polypeptide, or an amylase having a specified degree of amino acid sequence identity to the particular amylase. In one example, the nucleic acid encodes an amylase having at least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% homology/identity to a reference amylase. It will be appreciated that due to the degeneracy of the genetic code, a plurality of nucleic acids may encode the same polypeptide.

[0078] The nucleic acid may also have a specified degree of homology to an exemplary polynucleotide encoding an α-amylase polypeptide. For example, the nucleic acid may have at

least 60%, at least 65%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% nucleotide sequence identity to the exemplary sequence. In another example, the nucleic acid hybridizes under stringent or very stringent conditions to the exemplary sequence. Such conditions are described here but are also well known in the art. Indeed, in some embodiments, the parent enzyme is encoded by a nucleic acid sequence that hybridizes under stringent or very stringent conditions to a nucleic acid encoding *Bacillus* sp. strain TS-23 amylase, as exemplified by SEQ ID NO: 1 or 2.

[0079] 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.

[0080] A nucleic acid that encodes a 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. Method of Producing and Purifying Proteins

[0081] An aspect of the present compositions and method is that the amylases can be expressed as secreted polypeptides. Methods of producing and purifying proteins that are secreted in to the culture medium from *Bacillus* are known in the art, as are suitable host cells for producing amylases. Exemplary methods for producing the amylases are disclosed below.

3.1 Materials and Methods for Producing Amylases

[0082] A polypeptide can be expressed using an expression vector which will typically includes control sequences including a suitable promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. A large number of vectors are commercially available for use with recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, a bacteriophage or an extrachromosomal element, mini-chromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into an isolated host cell, is integrated into the

host cell genome and replicated together with the chromosome(s) into which it has been integrated. The integrated gene may also be amplified to create multiple copies of the gene in the chromosome by use of an amplifiable construct driven by antibiotic selection or other selective pressure, such as an essential regulatory gene or by complementation through dose effect of an essential metabolic pathway gene.

[0083] In the vector, the DNA sequence should be operably linked to a suitable promoter sequence. 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 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. For expression in *Trichoderma reesei*, the CBHII (cellobiohydrolase II) promoter may be used.

[0084] 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.

[0085] 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.

[0086] The vector may also comprise a selectable marker, *e.g.*, a gene the product of which complements a defect in the isolated host cell, such as the *dal* genes from *B. subtilis* or *B.*

licheniformis, or a gene that confers antibiotic resistance such as, *e.g.*, ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and xxs*C*, 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.

[0087] As noted above, while intracellular expression or solid-state fermentation may be advantageous in some respects, *e.g.*, when using certain bacteria or fungi as host cells, one aspect of the compositions and methods contemplates expression of an α-amylase into the culture medium.

[0088] In general, "full-length," "mature," or "precursor" amylases includes a signal sequence at the amino terminus that permits secretion into the culture medium. If desirable, this signal peptide may be replaced by a different sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective signal polypeptide.

[0089] The expression vector typically includes the components of a cloning vector, such as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences such as a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a sequence coding for an amino acid sequence capable of targeting the amylase to a host cell organelle such as a peroxisome, or to a particular host cell compartment. Such a targeting sequence includes but is not limited to the sequence, SKL. For expression under the direction of control sequences, the nucleic acid sequence of the amylase is operably linked to the control sequences in proper manner with respect to expression.

[0090] 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).

[0091] 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.

[0092] 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.

[0093] 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 reesei* can be used as a host. A suitable procedure for transformation of *Aspergillus* host cells includes, for example, that described in EP 238023.

[0094] In a yet further aspect, a method of producing an α-amylase is provided comprising 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.

[0095] 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).

[0096] In one aspect, an enzyme secreted from the host cells is used in a whole broth preparation. In the present methods, the preparation of a spent whole fermentation broth of a recombinant microorganism can be achieved using any cultivation method known in the art resulting in the expression of an alpha-amylase. Fermentation may, therefore, be understood as comprising shake flask cultivation, small- or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the amylase to be expressed or isolated. The term "spent whole fermentation broth" is defined herein as unfractionated contents of fermentation material that includes culture medium, extracellular proteins (*e.g.*, enzymes), and cellular biomass. It is understood that the term "spent whole fermentation broth" also encompasses cellular biomass that has been lysed or permeabilized using methods well known in the art.

[0097] 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.

[0098] An aspect contemplates the polynucleotide in a vector is 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.

[0099] 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 Sopharose. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TNT™ (Promega) rabbit reticulocyte system.

[00100] An amylase-expressing 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 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 5.5 to about 8.0, again depending on the culture conditions needed for the host relative to production of an amylase.

3.2 Materials and Methods for Protein Purification

[00101] Fermentation, separation, and concentration techniques are well-known in the art and conventional methods can be used in order to prepare a concentrated amylase polypeptide-containing solution.

[00102] 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 ultra-filtration, extraction, or chromatography, or the like, are generally used.

[00103] 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 purified enzyme precipitate.

[00104] 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 purification include but are not limited to rotary vacuum filtration and/or ultrafiltration.

[00105] The enzyme solution is concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated amylase polypeptide-containing solution is at a desired level.

[00106] 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.

[00107] The metal halide precipitation agent is used in an amount effective to precipitate the α-amylase polypeptide. 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.

[00108] 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 amylase polypeptide and on its concentration in the concentrated enzyme solution.

[00109] Another alternative to effect precipitation of 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 said 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.

[00110] 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.

[00111] Addition of the organic compound precipitation agent provides the advantage of high flexibility of the precipitation conditions with respect to pH, temperature, amylase polypeptide concentration, precipitation agent concentration, and time of incubation.

[00112] 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.

[00113] 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.

[00114] 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 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.

[00115] The incubation time necessary to obtain a 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.

[00116] 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.

[00117] The overall recovery of 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.

[00118] After the incubation period, the 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 purification of the purified enzyme precipitate can be obtained by washing the precipitate with water. For example, the 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.

[00119] During fermentation, an α-amylase polypeptide accumulates in the culture broth. For the isolation and purification of the desired amylase, the culture broth is centrifuged or filtered to eliminate cells, and the resulting cell-free liquid is used for enzyme 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 purification, a conventional procedure such as ion exchange chromatography may be used.

[00120] 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).

[00121] A more specific example of purification, is described in Sumitani, J. *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 $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered by centrifugation at 10,000 x g (20 min. and 4°C) and re-dissolved in 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. The solubilized precipitate was then dialyzed against the same buffer. The dialyzed sample was then applied to a Sephacryl S-200 column, which had previously been equilibrated with 20 mM Tris/HCl buffer, (pH 7.0), 5 mM $CaCl_2$, and eluted at a linear flow rate of 7 mL/hr with the same buffer. Fractions from the column were collected and assessed for activity as judged by enzyme assay and SDS-PAGE. The protein was further purified as follows. A Toyopearl HW55 column (Tosoh Bioscience, Montgomeryville, PA; Cat. No. 19812) was equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 1.5 to 0 M $(NH_4)_2SO_4$ in 20 mM Tris/HCL buffer, pH 7.0 containing 5 mM $CaCl_2$. The active fractions were collected, and the enzyme precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered, re-dissolved, and dialyzed as described above. The dialyzed sample was then applied to a Mono Q HR5/5 column (Amersham Pharmacia; Cat. No. 17-5167-01)

previously equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$, at a flow rate of 60 mL/hour. The active fractions are collected and added to a 1.5 M $(NH_4)_2SO_4$ solution. The active enzyme fractions were re-chromatographed on a Toyopearl HW55 column, as before, to yield a homogeneous enzyme as determined by SDS-PAGE. *See* Sumitani, J. *et al.* (2000) *Biochem. J.* 350: 477-484, for general discussion of the method and variations thereon.

[00122] For production scale recovery, an amylase polypeptide can be partially purified as generally described above by removing cells via flocculation with polymers. Alternatively, the enzyme can be purified by microfiltration followed by concentration by ultrafiltration using available membranes and equipment. However, for some applications, the enzyme does not need to be purified, and whole broth culture can be lysed and used without further treatment. The enzyme can then be processed, for example, into granules.

4. Cleaning Compositions

[00123] An aspect of the present compositions and methods is a cleaning composition that includes an amylase polypeptide 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. Preferably, an amylase polypeptide 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:

4.1 Laundry Detergent Composition

[00124] 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.

[00125] 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.

[00126] The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0% to about 50% of anionic surfactant, such as linear alkylbenzenesulfonate (LAS); α-olefinsulfonate (AOS); alkyl sulfate (fatty alcohol sulfate) (AS); alcohol ethoxysulfate (AEOS or AES); secondary alkanesulfonates (SAS); α-sulfo fatty acid methyl esters; alkyl- or alkenylsuccinic acid; or soap. The composition may also contain 0% to about 40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamineoxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (as described for example in WO 92/06154).

[00127] The detergent composition may additionally comprise one or more other enzymes, such as lipase, another amylolytic enzyme, cutinase, protease, cellulase, peroxidase, and/or laccase in any combination.

[00128] The detergent may contain about 1% to about 65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (*e.g.*, SKS-6 from Hoechst). The detergent may also be unbuilt, *i.e.* essentially free of detergent builder. The enzymes can be used in any composition compatible with the stability of the enzyme. Enzymes generally can be protected against deleterious components by known forms of encapsulation, for example, by granulation or sequestration in hydro gels. Enzymes, and specifically amylases, either with or without starch binding domains, can be used in a variety of compositions including laundry and dishwashing applications, surface cleaners, as well as in compositions for ethanol production from starch or biomass.

[00129] The detergent may comprise one or more polymers. Examples include carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG),

poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

[00130] The detergent may contain a bleaching system, which may comprise a H_2O_2 source such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids (*e.g.*, the amide, imide, or sulfone type peroxyacids). The bleaching system can also be an enzymatic bleaching system, for example, perhydrolase, such as those described in US patent documents US2008145353, US7754460, US7951566, US7723083, and US8062875.

[00131] 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.

[00132] The detergent may also contain other conventional detergent ingredients such as *e.g.*, fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, tarnish inhibiters, optical brighteners, or perfumes.

[00133] The pH (measured in aqueous solution at use concentration) is usually neutral or alkaline, *e.g.*, pH about 7.0 to about 11.0.

[00134] Particular forms of detergent compositions comprising an α-amylase can be formulated to include:

[00135] 1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 7% to about 12%; alcohol ethoxysulfate (*e.g.*, $C_{12\text{-}18}$ alcohol, 1-2 ethylene oxide (EO)) or alkyl sulfate (*e.g.*, $C_{16\text{-}18}$) about 1% to about 4%; alcohol ethoxylate (*e.g.*, $C_{14\text{-}15}$ alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na_2CO_3) about 14% to about 20%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 2 to about 6%; zeolite (*e.g.*, $NaA1SiO_4$) about 15% to about 22%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 6%; sodium citrate/citric acid (*e.g.*, $C_6H_5Na_3O_7/C_6H_8O_7$) about 0% to about 15%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 11% to about 18%; TAED about 2% to about 6%; carboxymethylcellulose (CMC) and 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid, copolymer, PVP, PEG) 0-3%; enzymes (calculated as pure enzyme) 0.0001-0.1% protein; and minor ingredients (*e.g.*, suds suppressors, perfumes, optical brightener, photobleach) 0-5%.

[00136] 2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 6% to about

11%; alcohol ethoxysulfate (*e.g.*, C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (*e.g.*, C_{16-18}) about 1% to about 3%; alcohol ethoxylate (*e.g.*, C_{14-15} alcohol, 7 EO) about 5% to about 9%; sodium carbonate (*e.g.*, Na_2CO_3) about 15% to about 21%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 24% to about 34%; sodium sulfate (*e.g.*, Na_2SO_4) about 4% to about 10%; sodium citrate/citric acid (*e.g.*, $C_6H_5Na_3O_7$/ $C_6H_8O_7$) 0% to about 15%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 1-6%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00137] 3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 5% to about 9%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO) about 7% to about 14%; Soap as fatty acid (*e.g.*, C_{16-22} fatty acid) about 1 to about 3%; sodium carbonate (as Na_2CO_3) about 10% to about 17%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 3% to about 9%; zeolite (as $NaA1SiO_4$) about 23% to about 33%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 4%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 8% to about 16%; TAED about 2% to about 8%; phosphonate (*e.g.*, EDTMPA) 0% to about 1%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 0-3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, suds suppressors, perfume, optical brightener) 0-5%.

[00138] 4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising linear alkylbenzenesulfonate (calculated as acid) about 8% to about 12%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO) about 10% to about 25%; sodium carbonate (as Na_2CO_3) about 14% to about 22%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 5%; zeolite (*e.g.*, $NaA1SiO_4$) about 25% to about 35%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 10%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, maleic/acrylic acid copolymer, PVP, PEG) 1-3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00139] 5) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO) about 12% to about 18%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 13%; alkenylsuccinic acid (C_{12-14}) 0% to about 13%; aminoethanol about 8% to about 18%; citric acid about 2% to about 8%; phosphonate 0% to about 3%; polymers (*e.g.*, PVP, PEG) 0% to about 3%; borate (*e.g.*, B_4O_7) 0% to about 2%; ethanol 0% to about 3%; propylene glycol about 8% to about 14%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, dispersants, suds suppressors, perfume, optical brightener) 0-5%.

[00140] 6) An aqueous structured liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 21%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) 3-9%; soap as fatty acid (*e.g.*, oleic acid) about 3% to about 10%; zeolite (as $NaA1SiO_4$) about 14% to about 22%; potassium citrate about 9% to about 18%; borate (*e.g.*, B_4O_7) 0% to about 2%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*, PEG, PVP) 0% to about 3%; anchoring polymers such as, *e.g.*, lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1, MW 3800) 0% to about 3%;glycerol 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, dispersants, suds suppressors, perfume, optical brighteners) 0-5%.

[00141] 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising fatty alcohol sulfate about 5% to about 10%; ethoxylated fatty acid monoethanolamide about 3% to about 9%; soap as fatty acid 0-3%; sodium carbonate (*e.g.*, Na_2CO_3) about 5% to about 10%; Soluble silicate (*e.g.*, Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 20% to about 40%; Sodium sulfate (*e.g.*, Na_2SO_4) about 2% to about 8%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 12% to about 18%; TAED about 2% to about 7%; polymers (*e.g.*, maleic/acrylic acid copolymer, PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, suds suppressors, perfume) 0-5%.

[00142] 8) A detergent composition formulated as a granulate comprising linear alkylbenzenesulfonate (calculated as acid) about 8% to about 14%; ethoxylated fatty acid monoethanolamide about 5% to about 11%; soap as fatty acid 0% to about 3%; sodium carbonate (*e.g.*, Na_2CO_3) about 4% to about 10%; soluble silicate (Na_2O, $2SiO_2$) about 1% to about 4%; zeolite (*e.g.*, $NaA1SiO_4$) about 30% to about 50%; sodium sulfate (*e.g.*, Na_2SO_4) about 3% to about 11%; sodium citrate (*e.g.*, $C_6H_5Na_3O_7$) about 5% to about 12%; polymers (*e.g.*, PVP, maleic/acrylic acid copolymer, PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, suds suppressors, perfume) 0-5%.

[00143] 9) A detergent composition formulated as a granulate comprising linear alkylbenzenesulfonate (calculated as acid) about 6% to about 12%; nonionic surfactant about 1% to about 4%; soap as fatty acid about 2% to about 6%; sodium carbonate (*e.g.*, Na_2CO_3) about 14% to about 22%; zeolite (*e.g.*, $NaA1SiO_4$) about 18% to about 32%; sodium sulfate (*e.g.*, Na_2SO_4) about 5% to about 20%; sodium citrate (*e.g.*, $C_6H_5Na_3O_7$) about 3% to about 8%; sodium perborate (*e.g.*, $NaBO_3H_2O$) about 4% to about 9%; bleach activator (*e.g.*, NOBS or TAED) about 1% to about 5%; carboxymethylcellulose (CMC) 0% to about 2%; polymers (*e.g.*,

polycarboxylate or PEG) about 1% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, perfume) 0-5%.

[00144] 10) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 15% to about 23%; alcohol ethoxysulfate (*e.g.*, C_{12-15} alcohol, 2-3 EO) about 8% to about 15%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) about 3% to about 9%; soap as fatty acid (*e.g.*, lauric acid) 0% to about 3%; aminoethanol about 1% to about 5%; sodium citrate about 5% to about 10%; hydrotrope (*e.g.*, sodium toluensulfonate) about 2% to about 6%; borate (*e.g.*, B_4O_7) 0% to about 2%; carboxymethylcellulose 0% to about 1%; ethanol about 1% to about 3%; propylene glycol about 2% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, polymers, dispersants, perfume, optical brighteners) 0-5%.

[00145] 11) An aqueous liquid detergent composition comprising linear alkylbenzenesulfonate (calculated as acid) about 20% to about 32%; alcohol ethoxylate (*e.g.*, C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO) 6-12%; aminoethanol about 2% to about 6%; citric acid about 8% to about 14%; borate (*e.g.*, B_4O_7) about 1% to about 3%; polymer (*e.g.*, maleic/acrylic acid copolymer, anchoring polymer such as, *e.g.*, lauryl methacrylate/acrylic acid copolymer) 0% to about 3%; glycerol about 3% to about 8%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, hydrotropes, dispersants, perfume, optical brighteners) 0-5%.

[00146] 12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, α-olefinsulfonate, α-sulfo fatty acid methyl esters, alkanesulfonates, soap) about 25% to about 40%; nonionic surfactant (*e.g.*, alcohol ethoxylate) about 1% to about 10%; sodium carbonate (*e.g.*, Na_2CO_3) about 8% to about 25%; soluble silicates (*e.g.*, Na_2O, $2SiO_2$) about 5% to about 15%; sodium sulfate (*e.g.*, Na_2SO_4) 0% to about 5%; zeolite ($NaA1SiO_4$) about 15% to about 28%; sodium perborate (*e.g.*, $NaBO_3{\cdot}4H_2O$) 0% to about 20%; bleach activator (TAED or NOBS) about 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; minor ingredients (*e.g.*, perfume, optical brighteners) 0-3%.

[00147] 13) Detergent compositions as described in compositions 1)-12) *supra,* wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

[00148] 14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C_{12}-C_{18}) alkyl sulfate about 9% to about 15%; alcohol ethoxylate about 3% to about 6%; polyhydroxy alkyl fatty acid amide about 1% to about 5%; zeolite (*e.g.*, $NaA1SiO_4$) about 10% to about 20%; layered disilicate (*e.g.*, SK56 from Hoechst) about 10% to

about 20%; sodium carbonate (*e.g.*, Na_2CO_3) about 3% to about 12%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) 0% to about 6%; sodium citrate about 4% to about 8%; sodium percarbonate about 13% to about 22%; TAED about 3% to about 8%; polymers (*e.g.*, polycarboxylates and PVP) 0% to about 5%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, photobleach, perfume, suds suppressors) 0-5%.

[00149] 15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C_{12}-C_{18}) alkyl sulfate about 4% to about 8%; alcohol ethoxylate about 11% to about 15%; soap about 1% to about 4%; zeolite MAP or zeolite A about 35% to about 45%; sodium carbonate (as Na_2CO_3) about 2% to about 8%; soluble silicate (*e.g.*, Na_2O, $2SiO_2$) 0% to about 4%; sodium percarbonate about 13% to about 22%; TAED 1-8%; carboxymethylcellulose (CMC) 0% to about 3%; polymers (*e.g.*, polycarboxylates and PVP) 0% to about 3%; enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (*e.g.*, optical brightener, phosphonate, perfume) 0-3%.

[00150] 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.

[00151] 17) Detergent compositions as described supra in 1), 3), 7), 9), and 12), wherein perborate is replaced by percarbonate.

[00152] 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).

[00153] 19) Detergent composition formulated as a non-aqueous detergent liquid comprising a liquid nonionic surfactant such as, *e.g.*, linear alkoxylated primary alcohol, a builder system (*e.g.*, phosphate), an enzyme(s), and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

[00154] An amylase polypeptide may be incorporated at a concentration conventionally employed in detergents. It is at present contemplated that, in the detergent composition, the enzyme may be added in an amount corresponding to 0.00001-1.0 mg (calculated as pure enzyme protein) of amylase polypeptide per liter of wash liquor.

[00155] In another embodiment, other enzymes, such as 2,6-β-D-fructan hydrolase, can be incorporated in detergent compositions comprising an α-amylase polypeptide and used for removal/cleaning of biofilm present on household and/or industrial textile/laundry.

[00156] The detergent composition may for example 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.

[00157] In a specific aspect, the detergent composition can comprise 2,6-β-D-fructan hydrolase in addition to an α-amylase polypeptide, and one or more other cleaning enzymes, such as a protease, a lipase, a cutinase, a carbohydrase, a cellulase, a pectinase, a pectate lyase, a mannanase, an arabinase, a galactanase, another amylolytic enzyme, a xylanase, an oxidase, a laccase, an aryl esterase, a perhydrolase, and/or a peroxidase, and/or combinations thereof.

[00158] In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (*e.g.*, pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, *etc.*), and the enzyme(s) should be present in effective amounts.

[00159] *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.

[00160] *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).

[00161] *Polyesterases*: Suitable polyesterases can be included in the composition, such as those described in, for example, WO 01/34899 and WO 01/14629.

[00162] 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.).

[00163] *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).

[00164] *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).

[00165] The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive, *i.e.* a separate additive or a combined additive, can be formulated *e.g.*, as a granulate, a liquid, a slurry, and the like. Exemplary detergent additive formulations include but are not limited to granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids or slurries.

[00166] Non-dusting granulates may be produced, *e.g.*, as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (*e.g.*, polyethyleneglycol, PEG) with mean molar weights of 1,000 to 20,000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in, for example, GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

[00167] The detergent composition may be in any convenient form, *e.g.*, a bar, a tablet, a powder, a granule, a paste, or a liquid. A liquid detergent may be aqueous, typically containing up to about 70% water, and 0% to about 30% organic solvent. Compact detergent gels containing about 30% or less water are also contemplated. The detergent composition can optionally comprise one or more surfactants, which may be non-ionic, including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants can be present in a wide range, from about 0.1% to about 60% by weight.

[00168] When included therein the detergent will typically contain from about 1% to about 40% of an anionic surfactant, such as linear alkylbenzenesulfonate, α-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, α-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid, or soap.

[00169] 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").

[00170] 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).

[00171] 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.

[00172] 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.

[00173] It is at present 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).

4.2 Cleaning Compositions

[00174] In the detergent applications, an α-amylase polypeptide is usually used in a liquid composition containing propylene glycol. The enzyme is solubilized in, for example, propylene glycol by mixing in a 25% volume/volume propylene glycol solution containing 10% calcium chloride.

[00175] An α-amylase polypeptide thereof discussed herein can be formulated in detergent compositions for use in cleaning dishes or other cleaning compositions. These can be powders, gels, or liquids. The compositions can comprise the enzyme alone, or with other amylolytic enzymes and/or with other cleaning enzymes or bleach activating enzymes, and other components common to cleaning compositions.

[00176] Thus, a dishwashing detergent composition can comprise a surfactant. The surfactant may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent can contain 0% to about 90% by weight of a non-ionic surfactant, such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

[00177] The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains about 1% to about 90% of detergent builders. Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, and polyphosphates. An example of phosphorus-containing organic alkaline detergent builder, when present, includes the water-soluble salts of phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates, and silicates, as well as the various types of water-insoluble crystalline or amorphous alumino silicates, of which zeolites are the best-known representatives.

[00178] Examples of suitable organic builders include the alkali metal; ammonium and substituted ammonium; citrates; succinates; malonates; fatty acid sulphonates; carboxymethoxy succinates; ammonium polyacetates; carboxylates; polycarboxylates; aminopolycarboxylates; polyacetyl carboxylates; and polyhydroxysulponates.

[00179] Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers, and their salts.

[00180] The cleaning composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite, and hypobromite, as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo-and N-chloro-imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric, and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

[00181] The cleaning composition may contain oxygen bleaches, for example in the form of an inorganic persalt, optionally with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates, and perphosphates. Exemplary activator materials are TAED, and glycerol triacetate. Enzymatic bleach activation systems may also be present in the formulation, *e.g.*, such as perborate or percarbonate, glycerol triacetate and perhydrolase (*see, e.g.,* WO 2005/056783).

[00182] The cleaning composition may be stabilized using conventional stabilizing agents for the enzyme(s), *e.g.*, a polyol such as, *e.g.*, propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative (*e.g.*, an aromatic borate ester).

[00183] The cleaning composition may also contain other conventional detergent ingredients, *e.g.*, deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners, and perfumes.

[00184] Although the present compositions and methods have been described with reference to the details below, it would be understood that various modifications can be made.

4.3 Methods of Assessing Amylase Activity in Detergent Compositions

[00185] Numerous amylase cleaning assays are known in the art, including swatch and micro-swatch assays. The appended Examples describe only a few such assays.

[00186] The following numbered paragraphs further describe aspects and embodiments of the present compositions and methods:

1. In one aspect, a variant α-amylase polypeptide is provided, comprising at least one combinable mutation at a productive amino acid position; wherein: (i) the combinable mutation is a mutation that improves at least one desirable property of the variant α-amylase compared to the parental α-amylase, while not significantly decreasing either expression, activity, or stability of the variant α-amylase, compared to the parental α-amylase, (ii) the productive position is an amino acid position that can be substituted with a plurality of different amino acid residues, all of which substitutions result in a variant α-amylase that meets the requirements of (i), and (iii) the combinable mutation is listed in Table C or Table D, which uses SEQ ID NO: 2 for numbering.

2. In some embodiments of the variant amylase of paragraph 1 the combinable mutation has a performance property listed in Table A.

3. In some embodiments of the variant amylase of paragraph 1 the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.9, and in addition the PI for any one of these properties is greater than or equal to 1.0.

4. In some embodiments of the variant amylase of paragraph 1 the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.8, and in in addition have a PI for any one of these tests that is greater than or equal to 1.2.

5. In some embodiments of the variant amylase of paragraph 1 the combinable mutation

produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.5, and in in addition have a PI for any one of these tests that is greater than or equal to 1.5.

6. In some embodiments of the variant amylase of any of the preceding paragraphs the combinable mutation has a sustainability score of +++, ++++, or +++++.

7. In some embodiments of the variant amylase of any of the preceding paragraphs the combinable mutation has a sustainability score of ++++, or +++++.

8. In some embodiments of the variant amylase of any of the preceding paragraphs the combinable mutation has a sustainability score of +++++.

9. In some embodiments of the variant amylase of any of the preceding paragraphs the combinable mutation has a productivity score of 1 or 2.

10. In some embodiments of the variant amylase of any of the preceding paragraphs the parental α-amylase has at least 60% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

11. In some embodiments of the variant amylase of any of the preceding paragraphs the parental α-amylase has at least 70% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

12. In some embodiments of the variant amylase of any of the preceding paragraphs the parental α-amylase has at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

13. In some embodiments of the variant amylase of any of the preceding paragraphs the parental α-amylase has at least 90% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

14. In another aspect, a composition comprising the variant amylase of any of the preceding paragraphs is provided.

15. In some embodiments of the composition of paragraph 14, the composition is effective for removing starchy stains from laundry, dishes, or textiles.

16. In some embodiments, the composition of either of paragraphs 14 or 15 further comprises a surfactant.

17. In some embodiments, the composition of any of paragraphs 14-16 is a detergent composition.

18. In some embodiments, the composition of any of paragraphs 14-17 is a laundry detergent or a laundry detergent additive.

19. In some embodiments, the composition of any of paragraphs 14-18 is manual or automatic dishwashing detergent.

20. In another aspect, a method for removing a starchy stain or soil from a surface is provided, comprising: incubating the surface in the presence of a aqueous composition comprising an effective amount of the variant amylase of any of the claims 1-13, allowing the polypeptide to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in the aqueous composition, and rinsing the surface, thereby removing the starchy stain from the surface.

21. In some embodiments of the method of paragraph 20, the aqueous composition further comprises a surfactant.

22. In some embodiments of the method of either of paragraphs 20 or 21, the surface is a textile surface.

23. In some embodiments of the method of any of paragraphs 20-22, the surface is on dishes.

24. In some embodiments of the method of any of paragraphs 20-23, the surface is a soiled hard surface.

25. In another aspect, an isolated polynucleotide encoding a polypeptide of any of paragraphs 1-13 is provided.

26. In another aspect, an expression vector comprising the polynucleotide of paragraph 25 is provided.

27. In another aspect, a host cell comprising the expression vector of paragraph 26 is provided.

[00187] 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: Assays

[00188] In the following examples, various assays were used as set forth below for ease in reading. Any deviations from the protocols provided below are indicated in the relevant

sections. In these experiments, a spectrophotometer was used to measure the absorbance of the products formed after the completion of the reactions.

A. Performance index

[00189] The performance index (PI) compares the performance or stability of the variant (measured value) and the standard enzyme (theoretical value) at the same protein concentration. In addition, the theoretical values can be calculated, using the parameters of the Langmuir equation of the standard enzyme. A performance index (PI) that is greater than 1 (PI>1) indicates improved performance by a variant as compared to the standard (*e.g.*, wild-type Amy TS23t, SEQ ID NO: 2), while a PI of 1 (PI=1) identifies a variant that performs the same as the standard, and a PI that is less than 1 (PI<1) identifies a variant that performs worse than the standard.

B. Protein Determination Assay

[00190] This assay was performed using filtered culture supernatant from cultures grown in 96-well micro-titer plates (MTPs) over 3 days at 37°C with shaking at 300 rpm and 80% humidity. A fresh 96-well round-bottom MTP containing 25 µL supernatant per well was used for the High Performance Liquid Chromatography (HPLC) protein determination method. Supernatants were diluted three fold into 25 mM sodium acetate, pH 5.5 and 20 µL of each diluted sample was analyzed. An Agilent 1100 (Hewlet Packard) HPLC equipped with a Poroshell 300SB-C8 (Agilent Technologies) column was used. Sample was bound to the column using 25 mM sodium acetate, pH 5.5 and eluted over a gradient up to 70% acetonitrile. Absorbance was read at 222 nm, integrated using ChemStation software (Agilent Technologies) and the protein concentration of samples was determined based on a standard curve of purified Amy TS23t protein.

C. Ceralpha α-Amylase Activity Assay

[00191] The Ceralpha α-amylase assay was performed using the Ceralpha HR Kit (Megazyme, Wicklow, Ireland). The assay involves incubating culture supernatant with a substrate mixture under defined conditions. The reaction is terminated (and color developed) by the addition of borate buffer (200 mM Boric acid/NaOH buffer, pH 10). The substrate used was a mixture of the defined oligosaccharide "nonreducing-end blocked *p*-nitrophenyl maltoheptaoside" (BPNPG7) and excess levels of α-glucosidase (which has no action on the native substrate due to the presence of the "blocking group"). On hydrolysis of the

oligosaccharide by *endo*acting α-amylase (or G6 amylase), the excess quantities of α-glucosidase present in the mixture give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The absorbance at 405 nm was measured, which relates directly to the level of amylase in the sample analysed.

[00192] The equipment used for this assay included a Biomek FX Robot (Beckman Coulter); a SpectraMAX MTP Reader (type 340-Molecular Devices) and iEMS incubator/shaker (Thermo Scientific). The reagent and solutions used were:

1) *p*-nitrophenyl maltoheptaoside (BPNPG7) substrate (Megazyme Ceralpha HR kit);
2) 50 mM MOPS, 0.005% TWEEN® 80 buffer, pH 7 (dilution buffer); and
3) 200 mM Boric acid / NaOH buffer, pH 10 (STOP buffer).

[00193] A vial containing 54.5 mg BPNPG7 substrate was dissolved in 10 mL of MilliQ water and then diluted into 30 mL of dilution buffer to make up 40 mL of the working substrate (1.36 mg/mL). The amylase samples (fermentation supernatant) were diluted 40X with dilution buffer. The assay was performed by adding 5μL of diluted amylase solution into the wells of a MTP followed by the addition of 55 μL of diluted BPNPG7 working substrate solution. The solutions were mixed and the MTP was sealed with a plate seal and placed in an incubator/shaker (iEMS- Thermo Scientific) for 4 minutes at 25°C. The reaction was terminated by adding 70 μL STOP buffer and the absorbance was read at wavelength 400 nm in an MTP-Reader. A non-enzyme control was used to correct for background absorbance values.

D. CS-28 Rice Starch Microswatch Assay

[00194] The principle of this amylase assay is the liberation of an orange-dye due to the hydrolysis of rice starch incorporated in a cotton microswatch. The absorbance at 488 nm of the wash liquid was measured and related to the level of amylase activity in the sample analyzed at the desired conditions (pH, temperature, and buffer).

[00195] The equipment used for this assay included a Biomek FX Robot (Beckman Coulter), a SpectraMAX MTP Reader (type 340-Molecular Devices) and iEMS incubator/shaker (Thermo Scientific). The reagent and solutions used were:

1) CS-28 Microswatches (rice starch, colored);
2) 10 mM HEPES, 2 mM $CaCl_2$, 0.005% TWEEN 80 buffer, pH 8.0, conductivity 1mS/cm;
3) 10 mM HEPES, 2 mM $CaCl_2$, 0.005% TWEEN 80 buffer, pH 8.0, conductivity 5mS/cm (adjusted with 5M NaCl);

3) 25 mM CAPS, 2 mM $CaCl_2$, 0.005% TWEEN 80 buffer, pH 10.0; conductivity 5mS/cm (adjusted with 5M NaCl); and

4) 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN 80.

[00196] CS-28 Microswatches of 5.5 mm circular diameter were provided by the Center for Testmaterials (CFT, Vlaardingen, The Netherlands). Two microswatches were placed in each well of a 96-well Corning 9017 flat bottomed polystyrene MTP. The culture supernatants were diluted in 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN®80 solution to approximately 1ppm.

[00197] The incubator/shaker was set at the desired temperature, 25°C (room temperature) 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 solution 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 pH8, both low (1mS/cm) and high (5mS/cm) conductivity and for 30 minutes at 1150 rpm at 32°C for cleaning at pH 10, high conductivity (5mS/cm). Following incubation under the appropriate conditions, 100 µL of solution from each well was transferred to a new MTP, and the absorbance at 488 nm was measured using a MTP-spectrophotometer. Controls containing two microswatches and buffer but no enzyme were included for subtraction of background cleaning performance.

[00198] The obtained absorbance value was corrected for the blank value (obtained after incubation of microswatches in the absence of enzyme), and the resulting absorbance provided a measure of the hydrolytic activity. A performance index (PI) was calculated for each sample. For the PI calculation for the wash performance indices, a curve-fit was made based on the wild-type Amy TS23t enzyme (SEQ ID NO: 2), using the Langmuir equation. Using the protein concentration of the variants, the expected performance based on the curve-fit was calculated. The observed performance was divided by the calculated performance. This value was then divided by the performance of the wild-type AmyTS23t enzyme.

E. Thermostability Assay – Determination of Initial and Residual Activities

[00199] The thermostability of the amylase variant in relation to a reference amylase (wild-type Amy TS23t, SEQ ID NO: 2) was determined by incubating the amylase samples under defined conditions in MOPS buffer, pH 7. The temperature of the incubation was selected such that approximately 40% of the initial reference amylase activity was lost. The initial and

residual amylase activities were determined using the Ceralpha α-amylase method described in section C above.

[00200] The equipment used for this assay included a Biomek FX Robot (Beckman Coulter); a SpectraMAX MTP Reader (type 340-Molecular Devices), a Tetrad2 DNA Engine PCR machine (BioRad), and iEMS incubator/shaker (Thermo Scientific). The reagent solutions used were:

1) p-nitrophenyl maltoheptaoside (BPNPG7) substrate (Megazyme Ceralpha HR kit);
2) 50 mM MOPS, 1.0 mM $CaCl_2$, 0.005% TWEEN®80 buffer, pH 7 (dilution buffer); and
3) 200 mM Boric acid / NaOH buffer, pH 10 (STOP buffer)

[00201] The culture supernatants were diluted 40X in dilution buffer to yield samples in a concentration range of 0-4 ppm. 5 µL of diluted sample was used to determine the initial amylase activity and 70 µL was used for heat incubation. 70 µL samples were put in each well of a 96 well PCR plate (VWR 211-0297) that was sealed with an aluminum seal and incubated at 75°C for 4 minutes in the Tetrad2 DNA Engine PCR machine. Initial ($t_{initial}$) and residual ($t_{residual}$) amylase activity was determined by the Ceralpha α-amylase assay as described above in Section C using a 5 µL sample.

[00202] For each variant, the ratio of the residual and initial amylase activities was used to calculate thermostability as follows: Thermostability = [$t_{residual}$ value] / [$t_{initial}$ value], so the thermostability activity ratio was calculated based on enzyme activity after the heat incubation, divided by enzyme activity before the heat incubation. The performance index for thermostability was determined by dividing the activity ratio of the variant enzyme, with that of the similarly treated wild-type AmyTS23t enzyme (SEQ ID NO: 2).

F. Detergent Stability Assay

[00203] The stability of the reference amylase and variants thereof was measured after incubation under defined conditions in the presence of 10% commercially purchased Persil Color Gel detergent, Henkel (purchased in 2011). The detergent was heat inactivated before use, and the initial and residual amylase activities were determined using the Ceralpha α-amylase assay as described in section C above.

[00204] The equipment used for this assay included a Biomek FX Robot (Beckman Coulter); a SpectraMAX MTP Reader (type 340-Molecular Devices), a Tetrad2DNA Engine PCR machine (Biorad), and iEMS incubator/shaker (Thermo Scientific). The reagent solutions used were:

1) p-nitrophenyl maltoheptaoside (BPNPG7) substrate (Megazyme Ceralpha HR kit):
2) Liquid detergent (Persil color gel, enzyme– inactivated, 2 hrs at 80°C);
3) 50 mM MOPS, 0.1 mM $CaCl_2$, 0.005% TWEEN®80 buffer, pH 7 (dilution buffer);
4) 10% detergent solution diluted in dilution buffer;
5) 200 mM Boric acid / NaOH buffer, pH 10 (STOP buffer)
6) Amylase culture supernatants containing 0-260 µg/mL protein

[00205] 90 µL of a 10% detergent solution was added to a 96 well PCR plate and mixed with 10 µL of original culture supernatant. A sample from the PCR plate was diluted 3X in dilution buffer and a 5 µL aliquot of this dilution was used to determine initial amylase activity. The PCR plate was incubated in a Tetrad PCR block at 64°C for 5 minutes. After incubation, detergent-enzyme mix was diluted 3X in dilution buffer and residual activity was measured. Initial ($t_{initial}$) and residual ($t_{residual}$) amylase activity was determined by the Ceralpha α-amylase assay as described above in Section C using a 5 µL sample.

[00206] For each variant, the ratio of the residual and initial amylase activities was used to calculate the detergent stability as follows: Detergent stability = [$t_{residual}$ value] / [$t_{initial}$ value], so the detergent stability activity ratio was calculated based on enzyme activity after the heat incubation, divided by enzyme activity before the heat incubation.

[00207] For each sample (variants) the performance index (PI) was calculated. The performance index for detergent stability was determined by comparing the detergent stability of the variant enzyme with that of the similarly treated wild-type AmyTS23t enzyme (SEQ ID NO: 2).

Example 2: Generation of *B.subtilis* strains expressing and secreting Amy TS23t α-amylase and variants thereof

[00208] This Example describes the construction of *Bacillus subtilis* strains expressing Amy TS23t and variants of Amy TS23t. The mature α-amylase from *Bacillus* sp. strain TS-23 (*i.e.*, AmyTS23) has the amino acid sequence of SEQ ID NO: 1:

```
NTAPINETMM QYFEWDLPND GTLWTKVKNE AANLSSLGIT ALWLPPAYKG   50
TSQSDVGYGV YDLYDLGEFN QKGTIRTKYG TKTQYIQAIQ AAKAAGMQVY  100
ADVVFNHKAG ADGTEFVDAV EVDPSNRNQE TSGTYQIQAW TKFDFPGRGN  150
TYSSFKWRWY HFDGTDWDES RKLNRIYKFR STGKAWDWEV DTENGNYDYL  200
MFADLDMDHP EVVTELKNWG TWYVNTTNID GFRLDAVKHI KYSFFPDWLT  250
YVRNQTGKNL FAVGEFWSYD VNKLHNYITK TNGSMSLFDA PLHNNFYTAS  300
KSSGYFDMRY LLNNTLMKDQ PSLAVTLVDN HDTQPGQSLQ SWVEPWFKPL  350
AYAFILTRQE GYPCVFYGDY YGIPKYNIPG LKSKIDPLLI ARRDYAYGTQ  400
RDYIDHQDII GWTREGIDTK PNSGLAALIT DGPGGSKWMY VGKKHAGKVF  450
```

```
YDLTGNRSDT VTINADGWGE FKVNGGSVSI WVAKTSNVTF TVNNATTTSG  500
QNVYVVANIP ELGNWNTANA IKMNPSSYPT WKATIALPQG KAIEFKFIKK  550
DQAGNVIWES TSNRTYTVPF SSTGSYTASW NVP                    583
```

[00209] A C-terminal truncated form of the mature α-amylase from *Bacillus* sp. strain TS-23 (*i.e.*, AmyTS23t or BASE) has the amino acid sequence of SEQ ID NO: 2:

```
NTAPINETMMQYFEWDLPNDGTLWTKVKNEAANLSSLGITALWLPPAYKGTSQ
SDVGYGVYDLYDLGEFNQKGTIRTKYGTKTQYIQAIQAAKAAGMQVYADVVFN
HKAGADGTEFVDAVEVDPSNRNQETSGTYQIQAWTKFDFPGRGNTYSSFKWRW
YHFDGTDWDESRKLNRIYKFRSTGKAWDWEVDTENGNYDYLMFADLDMDHPEV
VTELKNWGTWYVNTTNIDGFRLDAVKHIKYSFFPDWLTYVRNQTGKNLFAVGE
FWSYDVNKLHNYITKTNGSMSLFDAPLHNNFYTASKSSGYFDMRYLLNNTLMK
DQPSLAVTLVDNHDTQPGQSLQSWVEPWFKPLAYAFILTRQEGYPCVFYGDYY
GIPKYNIPGLKSKIDPLLIARRDYAYGTQRDYIDHQDIIGWTREGIDTKPNSG
LAALITDGPGGSKWMYVGKKHAGKVFYDLTGNRSDTVTINADGWGEFKVNGGS
VSIWVAK
```

[00210] A synthetic DNA fragment (produced by GeneArt AG / Life Technologies, Im Gewerbepark B35, 93059 Regensburg, Germany), served as template DNA for the construction of *Bacillus subtilis* strains expressing Amy TS23t and variants of Amy TS23t. To express Amy TS23t, the Amy TS23t DNA fragment was cloned in the pHPLT vector (Solingen et al. (2001) *Extremophiles* 5:333-341) by GeneArt, using the unique *Pst*I and *Hpa*I restriction sites. The pHPLT expression vector contains the *B. licheniformis* LAT promoter (Plat), Lat leader sequence, and additional elements from pUB110 plasmid (McKenzie *et al.* (1986) *Plasmid* 15: 93-103) including a replicase gene (reppUB), a neomycin/kanamycin resistance gene (neo), and a bleomycin resistance marker (bleo). pHPLT-Amy TS23t is shown in Figure 1. A *B. subtilis* strain (Δ*aprE*, Δ*nprE*) was transformed using the pHPLT-Amy TS23t vector DNA as described in WO2002/14490, incorporated herein by reference.

[00211] The *B. subtilis* transformants were selected on agar plates containing Heart infusion agar (Difco, Cat.no 244400) and 10 mg/L neomycin sulfate (Sigma, Catalog No. N-1876; contains 732 µg neomycin per mg). Selective growth of *B. subtilis* transformants harboring the pHPLT-Amy TS23t vector was performed in shake flasks containing MBD medium (a MOPS based defined medium), 5 mM $CaCl_2$ and 10 mg/L neomycin. MBD medium was made essentially as known in the art (see, *e.g.*, Neidhardt et al. (1974) *J. Bacteriol.* 119: 736-747), except that NH_4Cl_2, $FeSO_4$, and $CaCl_2$ were omitted from the base medium, 3 mM K_2HPO_4 was used, and the base medium was supplemented with 60 mM urea, 75 g/L glucose, and 1% soytone. The micronutrients were made up as a 100X stock solution containing in one liter, 400 mg $FeSO_4$ $7H_2O$, 100 mg $MnSO_4$ $.H_2O$, 100 mg $ZnSO_4$ $7H_2O$, 50 mg $CuCl_2$ $2H_2O$, 100 mg $CoCl_2$ $6H_2O$, 100 mg $NaMoO_4$ $2H_2O$, 100 mg $Na_2B_4O_7$ $10H_2O$, 10 ml of 1M $CaCl_2$, and 10 mL

of 0.5 M sodium citrate. Growth resulted in the production of secreted AmyTS23t amylase with starch hydrolyzing activity.

Example 3: Generation of Amy TS23t Site Evaluation Libraries (SELs)

[00212] Site Evaluation Library (SEL) production was performed by GeneArt AG using a proprietary process (WO2004/$_{059556A3}$). Methods and devices for optimizing a nucleotide sequence for the purpose of expression of a protein by PCR, and the manufacture of DNA molecules utilized technology owned by or licensed to GeneArt (European Patent Nos. 0 200 362 and 0 201 184; and US Patent Nos. 4,683,195, 4,683,202 and 6,472,184). The construction of Amy TS23t SELs described in this example was performed by GeneArt using their technology platform for gene optimization, gene synthesis and library generation under proprietary GeneArt know how and/or intellectual property. The sequential permutation approach of GeneArt, to produce SELs, is described in general on the company's web site (http://www.geneart.com/english/products-services/directed-evolution/sequential-permutation-libraries/index.html).

[00213] The pHPLT-Amy TS23t plasmid DNA (Figure 1) served as template to produce the SELs. Amy TS23t SELs were produced by GeneArt at all positions pre-selected by the inventors. The accompanying DNA codons were each substituted into randomly mutated codons, coding for at least 15 different amino acids. The codon mutagenized pHPLT-Amy TS23t mixtures were used to transform competent *B. subtilis* cells as described (WO2002/014490), in order to generate the Amy TS23t Site Evaluation Libraries.

[00214] Transformation mixtures were plated on Heart Infusion agar plates containing 10 mg/L neomycin sulfate. For each library, single colonies were picked and grown in TSB (tryptone and soy based broth) liquid media under 10 mg/L neomycin selection for subsequent DNA isolation and gene sequence analysis. Sequence analysis data revealed a maximum of 19 Amy TS 23t mature single variants per library. To generate Amy TS 23t and Amy TS23t variant enzyme samples for biochemical characterization experiments, selective growth of the Amy TS23t SEL variant clones was performed in 96 well MTP (Costar 3599) at 37°C for 68 hours in 200µL MBD medium per well containing 10 mg/L neomycin and 5 mM $CaCl_2$.

Example 4: Identification of Combinable and Productive Mutations

[00215] Performance index (PI) values were determined for all the Amy TS23t amylase variants tested using the assays described in Example 1: CS-28 microswatch assay (at both pH8 and pH10), detergent stability, thermostability, and protein determination.

[00216] Productive positions are described as those positions within a molecule that are most useful for making combinatorial variants exhibiting an improved characteristic, where the position itself allows for at least one combinable mutation. Combinable mutations are mutations at any amino acid position that can be used to make combinatorial variants. Combinable mutations improve at least one desired property of the molecule, while not significantly decreasing expression, activity, or stability. Combinable mutations can be grouped as follows:

Group A: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) CS-28 microswatch activity at pH 8 (25°C) or pH10 (32°C), and (iii) detergent stability or thermostability are greater than or equal to 0.9, and in addition have a PI for any one of these tests that is greater than or equal to 1.0.

Group B: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) CS-28 microswatch activity at pH 8 (25°C) or pH10 (32°C), and (iii) detergent stability or thermostability are greater than or equal to 0.8, and in addition have a PI for any one of these tests that is greater than or equal to 1.2.

Group C: A mutation that produces a variant wherein the minimum performance indices (PI) relative to a defined parental protein for: (i) protein expression, (ii) CS-28 microswatch activity at pH 8 (25°C) or pH10 (32°C), and (iii) detergent stability or thermostability are greater than or equal to 0.5, and in addition have a PI for any one of these tests that is greater than or equal to 1.5.

[00217] The properties of combinable mutations are summarized in Table 4.1.

Table 4.1. Properties for each group of combinable mutations

	Performance Index (PI)			
Group	Expression	Cleaning (pH 8 or 10)	Stability (detergent or thermal)	Minimum PI in one or more tests
A	≥ 0.9	≥ 0.9	≥ 0.9	X ≥ 1.0
B	≥ 0.8	≥ 0.8	≥ 0.8	X ≥ 1.2
C	≥ 0.5	≥ 0.5	≥ 0.5	X ≥ 1.5

[00218] Preferred combinable mutations are at "productive positions," as described, below. In the case of the present amylases, "activity" refers to α-amylase activity, which can be measured as described, herein.

[00219] Productive positions are amino acid positions that are tolerant to substitution with different amino acid residues, wherein the resulting variants meet a set of performance criteria for combinability, as set forth above. Productive positions can be assigned a Productivity Score as follows:

- Positions where less than 15% of the substitutions at a given position fall within groups A, B, or C are given a **Productivity Score of "1".**
- Positions where less than 40%, but greater than, or equal to 15% of the substitutions at a given position fall within groups A, B, or C are given a **Productivity Score of "2".**
- Positions where less than 75%, but greater than, or equal to 40% of the substitutions at a given position fall within groups A, B, or C are given a **Productivity Score of "3".**
- Positions where 75% or more of the substitutions at a given position fall within groups A, B, or C are given a **Productivity Score of "4".**

[00220] Preferred productive positions are combinable mutations.

[00221] Suitability score refers to the ability of one or more combinable mutations to be used to make combinatorial variants, based on the performance criteria for combinability, (*i.e.*, A, B, and C, as set forth, above) in which each of the mutations fall. A higher suitability score indicates a mutation or mutations that are more suitable for use in making combinatorial variants. Suitability scores are described in Table 4.2.

Table 4.2. Definitions of suitability scores

Substitutions Occur in Group(s)	Suitability Score
A, B and C	+++++
A and B	++++
A or (B and C)	+++
B	++
C	+

[00222] Table 4.3 shows the Productivity Score (4, 3, or 2,) calculated for each position in the Amy TS23t protein. Subsets of these positions are listed in Tables 4.4 and 4.5. No positions

were calculated to have a productivity score of 1. For each Amy TS23t position, variants are listed according to the suitability score they received (+, ++, +++, ++++, or +++++). Position numbering is based on the mature Amy TS23t polypeptide (SEQ ID NO: 2).

Table 4.3. Productivity score for positions in the Amy TS23t protein.

VARIANTS SUITABILITY SCORE						
POS	Productivity Score	(+)	(++)	(+++) WT AA 1ST	(++++)	(+++++)
1	2			NHK	QR	
2	2		A	TEQ		
3	2	W	G	ADEHPT		
5	4			IDEHKNRTVY	S	
7	2	R	N	EHKP	Q	
12	2		S	YDHQT	G	
13	3		S	FY		
15	2		AH	WY		
16	1			DE		
17	4			LCDEFGHIMNQRSTV	AY	
18	2	E		PQ	H	
19	3	G		NDEHPS	AKT	R
20	3			DN		
22	3	C	DL	TAHQ	KS	
23	4			LAEFGMNSTWY	Q	
25	3			TDGLNPSW	H	
26	2			KAGHRS	N	
27	3			VEGNST	Q	
28	3		D	KGHNQRST		
29	3			NDGKPRST	AH	
30	3			ED		
31	3			AGHNPTY	QS	
32	1			AGP		
33	2			NDGP		
34	2			LHNQ		
36	2		D	SPQT		
37	3			LHIMQRSTVY		
40	1			TR		
41	2			ADQRS		
43	1			WY		
44	1			LT		
48	1			YQT		
50	2	D		GS	A	
51	1		R	T	Q	
52	1		KR	S		
53	2		K	QAS		
54	3		W	SDEHKNRT	AQ	
56	4			VAEHINPQRST		
59	2	CK		GS		
60	2		IL	VQ	GT	AS
63	2		S	LV	EM	
64	1			YT	S	
66	1			L	I	
68	1	N		ED		
70	3			NAHKPQRS	D	
71	1			QAS		
73	2		AH	GQR	D	
74	1	LW		T		
75	4		LT	IAEGHKMRSW	FQ	
79	1			YHW		
81	1			TH		
82	2		I	KAEFLNY		
83	3			TADFHIKLQWY		

87	2			QLNSTW	P	
88	1			AS		
89	2		A	IMV		
90	2			QAGH		
91	3		E	AFGHIRW	K	
92	1			AG		
93	1		R	K		
94	3			AFHILSTW	R	
95	3			AGLQRST	IK	
96	2		K	GACE		
97	2			MFV		
103	1		I	VA		
105	1	I		F		
106	3	D		N		
107	1	CS		H		
108	2		AL	KR		
109	1	ET		A		
110	1			GAS		
113	3		LT	GER	AHQ	D
114	1	R		TS		
116	2	W	N	FAHIQR		
117	2	W	AS	VEQ	P	
118	2	AKS		D		
120	2	AN		V		
122	1			V		C
123	1			DAS		
125	2		K	SQ	FHR	
126	1			N	D	
128	2	C	Y	N	W	
129	3		LMT	QAV	KR	I
131	1			T		I
133	2		H	GN	DP	E
134	2		DS	TA	P	
135	2	HIKLM		Y		
136	2	CHT	AGPR	Q		
138	2	CLMT	DN	Q		
139	1			AP		
140	1			WHK		
142	3		GHTV	KAILQSW		
144	1	H		D		
146	1			PA		
147	2		AL	GH		W
149	3			GAHILQRWY	D	
150	2		GK	N	D	
152	1	S		YH		
154	2	FIV	K	SNQ	G	
156	1		F	KR		
158	2		A	RKW	LS	
160	4		M	YS	AEKLPQRT VW	DIN
162	1	M		F		
165	2	DF	C	T		IV
166	3	CGHPQ		D		
167	2	ANS	L	W		
168	2	N		D		
169	2			EFHIQT	A	
170	4	FL	CDM	SAQTWY	H	
171	1		L	R		
172	3			KADLNQRY	F	
173	1			LQW		
174	4			NACDS	HQT	R
175	2	CL	K	R		
177	2	H		YFW		
178	2	AI		KG		LQ
179	1	I		F		

180	4	CG	W	RIMV		ADKNQT
181	3	CDHMPQT		S		AG
182	4	H	VY	TW	FR	ACDEGLMNPQ
183	1	EN		G		
185	3	C	G	ANS	Q	DE
192	1	I	D	T		
194	2			NF	H	
195	2	M	V	GA	H	C
197	4	AN	F	Y		D
202	2		GH	FLMNY	S	
203	2	CKN	E	AD	Q	
207	1			M	Y	
209	4	MNQR		HEY	A	CW
210	4		LM	P	CEGHKNQT	DR
212	1	P		V		
213	1			VT		P
214	3			TAKQRS	DE	
215	1		H	E		
217	2	AS	Q	K		
218	1			NS		
221	4		EW	THNP	ADKQRSY	
223	2	L	S	YF		
224	2			VKR	Q	
225	1			ND		
226	3			TAHNQ		
228	3			NDEFGHL	PQS	
229	2		S	IV		
232	1			FW		
234	2		GS	LIM		A
236	1	E		A		
237	1		I	V		
242	1	F		Y		
243	2	A		S		DQ
244	3			FHV	NS	LMT
245	4		T	FNSV		AMQ
246	3		F	PK	DQRS	AEY
249	1			LQ		
250	4		I	TADEGHKQRY		
251	2	P		YHMQRS		
252	1			VL		
253	2		H	RKT		
254	3		AEH	NDKMQRT		
255	1			QR		
256	2		DQRY	T	N	
257	2			GDKNRST		
258	2	C	N	KQ		
260	1			LM	R	
262	2	M		AG		
264	1			GS		
268	2			SN	T	
269	2	EHLM	AS	Y		
271	4	ADGW	HQ	VY	IPRS	MT
272	3		HQ	NEMR	ADKST	
273	1	R		K		
275	2	D	R	HQ		
279	3			TDHNQRS		
282	2	LM	E	N		D
283	3	DFT	Q	GE	AHKNRS	
284	2			SDEHKRT	Q	
285	2		A	M	QTV	I
286	2			SDHQT	NR	
287	1			LI		

294	2	E		NR		
295	1			NR		
298	2			TEHKNR	Q	
301	1	S		K		
302	3		D	SEKQT	L	
303	3	DT		SAGQY		
304	2	NQ		G		
305	4	E		YDHKMNV		AGQRST
306	1	A		F		
310	4		IL	YCEGHKNQRST	D	
312	3			LIKMQRST		
313	2		T	NR	K	
314	1		E	ND		
318	2			KHQR		
319	2	IMR	ST	D		
320	3	G	DES	QKNR	H	
321	2	NRT	A	P		
322	2		R	SDQ		
323	2	DE	AHQ	L		
326	1			TS		
327	1	M		LI		
331	2	CGTV		HQ		
335	4			PDGHILMNQT	K	
336	2			GAHK		
337	2	PVY		Q		
338	1			S	A	
339	2	CFS	M	L		T
340	3			QADGHKS	T	
341	3		EHN	SDR	Q	
342	2	L	S	WQ		
345	1	D		P		
346	3	AF		W		
349	1	QT		P		
350	2		Q	LIMS		
353	2			A	S	
354	2	G	T	FHLM		
355	2			IT		
357	1			TA		
358	1			RH		
359	2			QAD		S
360	2	F		EAQ	S	
362	4		E	YAIS	DHKT	R
364	3		M	CADH	ST	Q
366	2	D		F		
367	2	F		YEHQ		
371	3			YAHNT	R	
374	2			PAKQR		
376	2	ADS		Y		
377	2			NKR	H	
378	2	AHMNS		I		
379	3			PHKRV		
380	3			GDENPQR	K	
381	2		S	LAIMQ		
382	1		R	K		
383	3			SIRT	L	
384	1	Y		K		
385	2			ILM		
386	2			DQ		
387	2	T		PAR		
391	2			A	S	
393	2			RHK	T	
394	2			DAELQ	ST	
395	2			YFHNQ	R	
396	2			A	GS	
399	3			TEIKPQRS		

400	2			QEHN		
401	4		I	RDEHKLNQST	A	
404	1			IES		
405	1	S		D		
406	1			HD		
407	2		K	QAFR		
410	2			IA		
413	2			TS	A	
415	1			E	D	
417	4			IDEGKLMQSTVY		
418	4			DHQS	AG	
419	3			TACGHKNQRS		D
420	3			KFHLNQRSY	A	
421	4			PACDEFGHIKLNTWY		
422	4			NACDHILQRSTWY		
423	1			SAD		
424	1			GS		
425	3			LAFITVY		
427	2			AGT		
429	1			IET		
430	1	D		T		
433	4	DE	KSV	PT	AQ	
434	3	HIKNTV	AQ	G		
435	2	CMT		G		
436	2			SDKLNQT		
438	4			WCFGHIKLMNPQRSTY		
440	4		R	YCEFHLPQSTW	DGN	
442	1			GD		
443	4			KACDEFGHQRSTWY		
444	4			KCDFGILMNPRSTVWY		
447	2			GDHNQT		
448	4			KACDGNQRST		
449	4			VACDEGHKMPTY	Q	
451	4			YDEGKLNSTV	ACQR	
453	4			LACDEFGHINSTVWY	KQR	
454	1			TAQ		
455	2			GADKNS		
456	1		H	NT		
457	2		QTW	RAL		
458	3			SADFKQTVW	L	
459	2	C		DEGHNQ		
460	2			TAEILPQ		
461	2			VAHQT		
462	2			TDKPRV		
463	3		S	IAMV		
464	4			NDGHKLMT	A	
465	3		I	AGNPQSTWY		
466	2			DEQST		
468	4			WACFGHLMNQRTY		
469	1			GAS		
470	3		C	EADHIQST		
471	2			FMY		
472	2		D	KAIRT		
474	3			NADEFIKMS	LT	
475	4	EKR		GDN	S	AQ
476	4	ANPQSTVY		G		
477	2	HKR		S		
478	4	A	I	VCLMW	NQST	
479	2	C		SAGT		
482	3			VL	H	
483	3		KR	ACFGQSW	D	

484	2			KADGHNQS		

Table 4.4. Productivity score for selected positions in the Amy TS23t protein.

POS	Productivity score	VARIANTS SUITABILITY SCORE				
		(+)	(++)	(+++) WT AA 1ST	(++++)	(+++++)
12	2		S	YDHQT	G	
13	3		S	FY		
20	3			DN		
23	4			LAEFGMNSTWY	Q	
27	3			VEGNST	Q	
31	3			AGHNPTY	QS	
33	2			NDGP		
34	2			LHNQ		
40	1			TR		
41	2			ADQRS		
43	1			WY		
44	1			LT		
48	1			YQT		
63	2		S	LV	EM	
64	1			YT	S	
66	1			L	I	
68	1	N		ED		
74	1	LW		T		
79	1			YHW		
81	1			TH		
88	1			AS		
89	2		A	IMV		
92	1			AG		
96	2		K	GACE		
97	2			MFV		
106	3	D		N		
109	1	ET		A		
117	2	W	AS	VEQ	P	
120	2	AN		V		
122	1			V		C
133	2		H	GN	DP	E
139	1			AP		
146	1			PA		
173	1			LQW		
180	4	CG	W	RIMV		ADKNQT
181	3	CDHMPQT		S		AG

POS	Productivity score	VARIANTS SUITABILITY SCORE				
		(+)	(++)	(+++) WT AA 1ST	(++++)	(+++++)
194	2			NF	H	
209	4	MNQR		HEY	A	CW
212	1	P		V		
213	1			VT		P
215	1		H	E		
218	1			NS		
223	2	L	S	YF		
224	2			VKR	Q	
225	1			ND		
226	3			TAHNQ		
229	2		S	IV		
232	1			FW		
242	1	F		Y		
243	2	A		S		DQ
244	3			FHV	NS	LMT
245	4		T	FNSV		AMQ
249	1			LQ		
251	2	P		YHMQRS		
252	1			VL		
253	2		H	RKT		
256	2		DQRY	T	N	
258	2	C	N	KQ		
260	1			LM	R	
262	2	M		AG		
271	4	ADGW	HQ	VY	IPRS	MT
282	2	LM	E	N		D
285	2		A	M	QTV	I
286	2			SDHQT	NR	
287	1			LI		
294	2	E		NR		
295	1			NR		
302	3		D	SEKQT	L	
304	2	NQ		G		
312	3			LIKMQRST		
313	2		T	NR	K	
321	2	NRT	A	P		
326	1			TS		
327	1	M		LI		
331	2	CGTV		HQ		

POS	Productivity score	VARIANTS SUITABILITY SCORE				
		(+)	(++)	(+++) WT AA 1ST	(++++)	(+++++)
335	4			PDGHILMNQT	K	
341	3		EHN	SDR	Q	
342	2	L	S	WQ		
345	1	D		P		
346	3	AF		W		
349	1	QT		P		
350	2		Q	LIMS		
353	2			A	S	
354	2	G	T	FHLM		
355	2			IT		
357	1			TA		
358	1			RH		
360	2	F		EAQ	S	
362	4		E	YAIS	DHKT	R
364	3		M	CADH	ST	Q
366	2	D		F		
367	2	F		YEHQ		
371	3			YAHNT	R	
378	2	AHMNS		I		
380	3			GDENPQR	K	
383	3			SIRT	L	
384	1	Y		K		
385	2			ILM		
386	2			DQ		
387	2	T		PAR		
391	2			A	S	
395	2			YFHNQ	R	
396	2			A	GS	
400	2			QEHN		
404	1			IES		
405	1	S		D		
406	1			HD		
410	2			IA		
413	2			TS	A	
415	1			E	D	
417	4			IDEGKLMQSTVY		
418	4			DHQS	AG	
420	3			KFHLNQRSY	A	
421	4			PACDEFGHIKLNTWY		

POS	Productivity score	VARIANTS SUITABILITY SCORE				
		(+)	(++)	(+++) WT AA 1ST	(++++)	(+++++)
422	4			NACDHILQRSTWY		
423	1			SAD		
424	1			GS		
425	3			LAFITVY		
427	2			AGT		
429	1			IET		
430	1	D		T		
434	3	HIKNTV	AQ	G		
435	2	CMT		G		
440	4		R	YCEFHLPQSTW	DGN	
442	1			GD		
443	4			KACDEFGHQRSTWY		
449	4			VACDEGHKMPTY	Q	
454	1			TAQ		
455	2			GADKNS		
456	1		H	NT		
457	2		QTW	RAL		
458	3			SADFKQTVW	L	
460	2			TAEILPQ		
461	2			VAHQT		
462	2			TDKPRV		
463	3		S	IAMV		
464	4			NDGHKLMT	A	
466	2			DEQST		
468	4			WACFGHLMNQRTY		
469	1			GAS		
471	2			FMY		
472	2		D	KAIRT		
474	3			NADEFIKMS	LT	
476	4	ANPQSTVY		G		
477	2	HKR		S		
478	4	A	I	VCLMW	NQST	
479	2	C		SAGT		
482	3			VL	H	

Table 4.5. Productivity score for selected positions in the Amy TS23t protein.

		VARIANTS SUITABILITY SCORE					
POS	Product-ivity score	(+)	(++)	(+++) WT AA 1ST		(++++)	(+++++)
1	2			NHK		QR	
2	2		A	TEQ			
3	2	W	G	ADEHPT			
5	4			IDEHKNRTVY		S	
7	2	R	N	EHKP		Q	
15	2		AH	WY			
16	1			DE			
17	4			LCDEFGHIMNQRSTV		AY	
18	2	E		PQ		H	
19	3	G		NDEHPS		AKT	R
22	3	C	DL	TAHQ		KS	
25	3			TDGLNPSW		H	
26	2			KAGHRS		N	
28	3		D	KGHNQRST			
29	3			NDGKPRST		AH	
30	3			ED			
32	1			AGP			
36	2		D	SPQT			
37	3			LHIMQRSTVY			
50	2	D		GS		A	
51	1		R	T		Q	
52	1		KR	S			
53	2		K	QAS			
54	3		W	SDEHKNRT		AQ	
56	4			VAEHINPQRST			
59	2	CK		GS			
60	2		IL	VQ		GT	AS
70	3			NAHKPQRS		D	
71	1			QAS			
73	2		AH	GQR		D	
75	4		LT	IAEGHKMRSW		FQ	
82	2		I	KAEFLNY			
83	3			TADFHIKLQWY			
87	2			QLNSTW		P	

90	2			QAGH		
91	3		E	AFGHIRW	K	
93	1		R	K		
94	3			AFHILSTW	R	
95	3			AGLQRST	IK	
103	1		I	VA		
105	1	I		F		
107	1	CS		H		
108	2		AL	KR		
110	1			GAS		
113	3		LT	GER	AHQ	D
114	1	R		TS		
116	2	W	N	FAHIQR		
118	2	AKS		D		
123	1			DAS		
125	2		K	SQ	FHR	
126	1			N	D	
128	2	C	Y	N	W	
129	3		LMT	QAV	KR	I
131	1			T		I
134	2		DS	TA	P	
135	2	HIKLM		Y		
136	2	CHT	AGPR	Q		
138	2	CLMT	DN	Q		
140	1			WHK		
142	3		GHTV	KAILQSW		
144	1	H		D		
147	2		AL	GH		W
149	3			GAHILQRWY	D	
150	2		GK	N	D	
152	1	S		YH		
154	2	FIV	K	SNQ	G	
156	1		F	KR		
158	2		A	RKW	LS	
160	4		M	YS	AEKLPQRT VW	DIN
162	1	M		F		
165	2	DF	C	T		IV
166	3	CGHPQ		D		
167	2	ANS	L	W		
168	2	N		D		
169	2			EFHIQT	A	
170	4	FL	CDM	SAQTWY	H	
171	1		L	R		

172	3			KADLNQRY	F	
174	4			NACDS	HQT	R
175	2	CL	K	R		
177	2	H		YFW		
178	2	AI		KG		LQ
179	1	I		F		
182	4	H	VY	TW	FR	ACDEGLMNPQ
183	1	EN		G		
185	3	C	G	ANS	Q	DE
192	1	I	D	T		
195	2	M	V	GA	H	C
197	4	AN	F	Y		D
202	2		GH	FLMNY	S	
203	2	CKN	E	AD	Q	
207	1			M	Y	
210	4		LM	P	CEGHKNQT	DR
214	3			TAKQRS	DE	
217	2	AS	Q	K		
221	4		EW	THNP	ADKQRSY	
228	3			NDEFGHL	PQS	
234	2		GS	LIM		A
236	1	E		A		
237	1		I	V		
246	3		F	PK	DQRS	AEY
250	4		I	TADEGHKQRY		
254	3		AEH	NDKMQRT		
255	1			QR		
257	2			GDKNRST		
264	1			GS		
268	2			SN	T	
269	2	EHLM	AS	Y		
272	3		HQ	NEMR	ADKST	
273	1	R		K		
275	2	D	R	HQ		
279	3			TDHNQRS		
283	3	DFT	Q	GE	AHKNRS	
284	2			SDEHKRT	Q	
298	2			TEHKNR	Q	
301	1	S		K		
303	3	DT		SAGQY		
305	4	E		YDHKMNV		AGQRST
306	1	A		F		

310	4		IL	YCEGHKNQRST	D	
314	1		E	ND		
318	2			KHQR		
319	2	IMR	ST	D		
320	3	G	DES	QKNR	H	
322	2		R	SDQ		
323	2	DE	AHQ	L		
336	2			GAHK		
337	2	PVY		Q		
338	1			S	A	
339	2	CFS	M	L		T
340	3			QADGHKS	T	
359	2			QAD		S
374	2			PAKQR		
376	2	ADS		Y		
377	2			NKR	H	
379	3			PHKRV		
381	2		S	LAIMQ		
382	1		R	K		
393	2			RHK	T	
394	2			DAELQ	ST	
399	3			TEIKPQRS		
401	4		I	RDEHKLNQST	A	
407	2		K	QAFR		
419	3			TACGHKNQRS		D
433	4	DE	KSV	PT	AQ	
436	2			SDKLNQT		
438	4			WCFGHIKLMNPQRSTY		
444	4			KCDFGILMNPRSTVWY		
447	2			GDHNQT		
448	4			KACDGNQRST		
451	4			YDEGKLNSTV	ACQR	
453	4			LACDEFGHINSTVWY	KQR	
459	2	C		DEGHNQ		
465	3		I	AGNPQSTWY		
470	3		C	EADHIQST		
475	4	EKR		GDN	S	AQ
483	3		KR	ACFGQSW	D	
484	2			KADGHNQS		

[00223] All references cited herein are herein incorporated by reference in their entirety for all purposes.

CLAIMS

What is claimed is:

1. A variant α-amylase polypeptide, comprising at least one combinable mutation at a productive amino acid position; wherein:

(i) the combinable mutation is a mutation that improves at least one desirable property of the variant α-amylase compared to the parental α-amylase, while not significantly decreasing either expression, activity, or stability of the variant α-amylase, compared to the parental α-amylase,

(ii) the productive position is an amino acid position that can be substituted with a plurality of different amino acid residues, all of which substitutions result in a variant α-amylase that meets the requirements of (i), and

(iii) the combinable mutation is listed in Table C or Table D, which uses SEQ ID NO: 2 for numbering.

2. The variant amylase of claim 1, wherein the combinable mutation has a performance property listed in Table A.

3. The variant amylase of claim 1, wherein the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.9, and in addition the PI for any one of these properties is greater than or equal to 1.0.

4. The variant amylase of claim 1, wherein the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.8, and in in addition have a PI for any one of these tests that is greater than or equal to 1.2.

5. The variant amylase of claim 1, wherein the combinable mutation produces a variant wherein the minimum performance indices (PI) relative to the parental amylase for (i) protein expression, (ii) activity, (iii) microswatch activity, and (iv) detergent stability or thermostability are greater than or equal to 0.5, and in in addition have a PI for any one of these tests that is greater than or equal to 1.5.

6. The variant amylase of any of the preceding claims, wherein the combinable mutation has a sustainability score of +++, ++++, or +++++.

7. The variant amylase of any of the preceding claims, wherein the combinable mutation has a sustainability score of ++++, or +++++.

8. The variant amylase of any of the preceding claims, wherein the combinable mutation has a sustainability score of +++++.

9. The variant amylase of any of the preceding claims, wherein the combinable mutation has a productivity score of 1 or 2.

10. The variant amylase of any of the preceding claims, wherein the parental α-amylase has at least 60% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

11. The variant amylase of any of the preceding claims, wherein the parental α-amylase has at least 70% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

12. The variant amylase of any of the preceding claims, wherein the parental α-amylase has at least 80% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

13. The variant amylase of any of the preceding claims, wherein the parental α-amylase has at least 90% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

14. A composition comprising the variant amylase of any of the preceding claims.

15. The composition of claim 14, wherein the composition is effective for removing starchy stains from laundry, dishes, or textiles.

16. The composition of claims 14 or 15, further comprising a surfactant.

17. The composition of any of claims 14-16, wherein the composition is a detergent composition.

18. The composition of any of claims 14-17, wherein the composition is a laundry detergent or a laundry detergent additive.

19. The composition of any of claims 14-18, wherein the composition is manual or automatic dishwashing detergent.

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

incubating the surface in the presence of a aqueous composition comprising an effective amount of the variant amylase of any of the claims 1-13,

allowing the polypeptide to hydrolyze starch components present in the starchy stain to produce smaller starch-derived molecules that dissolve in the aqueous composition, and

rinsing the surface,

thereby removing the starchy stain from the surface.

21. The method of claim 20, wherein the aqueous composition further comprises a surfactant.

22. The method of any of claims 20 or 21, wherein the surface is a textile surface.

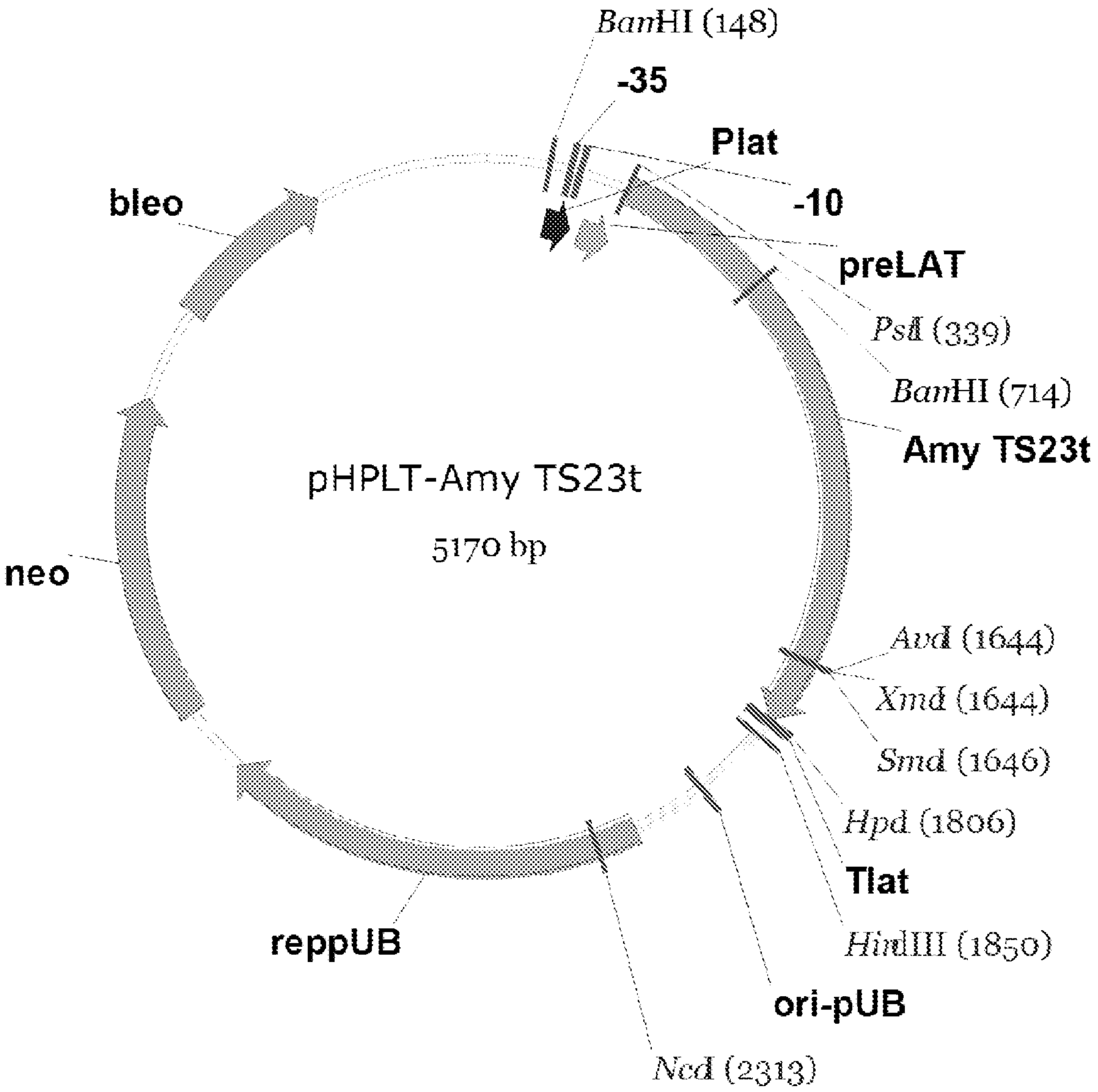
23. The method of any of claims 20-22, wherein the surface is on dishes.

24. The method of any of claims 20-23, wherein the surface is a soiled hard surface.

25. An isolated polynucleotide encoding a polypeptide of any of claims 1-13.

26. An expression vector comprising the polynucleotide of claim 25.

27. A host cell comprising the expression vector of claim 26.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Plat
bleo
-10
preLAT
PstI (339)
BamHI (714)
Amy TS23t
pHPLT-Amy TS23t
5170 bp
neo
AvaI (1644)
XmaI (1644)
SmaI (1646)
HpaI (1806)
TIat
HindIII (1850)
reppUB
ori-pUB
NcoI (2313)

FIGURE 1