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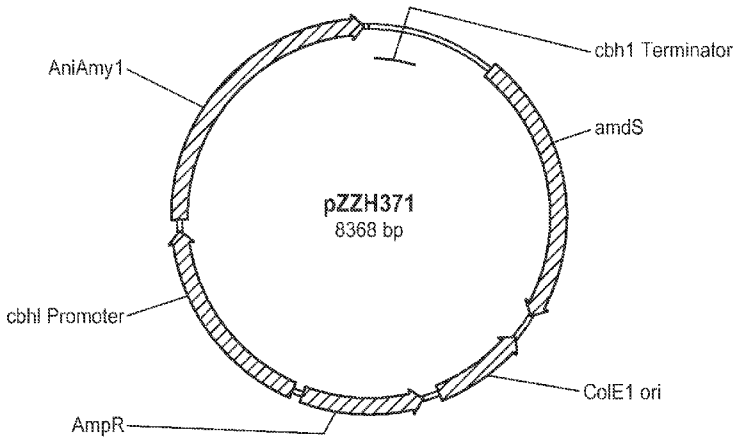



FIG. 1

(57) Abstract: Chimeric alpha-amylases are provided by fusing a catalytic domain derived from an *Aspergillus niger* alpha-amylase (AniAmy1) or a *Schwanniomyces occidentalis* alpha-amylase (SocAmy1) to a carbohydrate binding module derived from an *Aspergillus terreus* alpha-amylase. Embodiments of the present disclosure relate to the resulting chimeric alpha-amylases having improved properties (e.g., improved capability of hydrolyzing insoluble starch during a simultaneous saccharification and fermentation process). Also provided are compositions comprising the chimeric alpha-amylase and the use thereof. These compositions are useful in various starch process applications.

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# CHIMERIC FUNGAL ALPHA-AMYLASES COMPRISING CARBOHYDRATE BINDING MODULE AND THE USE THEREOF

## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from the International application Serial No. PCT/CN13/89935 filed 19 December 2013 and is incorporated by reference in its entirety.

## SEQUENCE LISTING

A Sequence Listing, comprising SEQ ID NOs: 1-20, is attached and incorporated herein by reference in its entirety.

## FIELD OF THE INVENTION

Compositions comprising a chimeric fungal alpha-amylase and methods of using the chimeric fungal alpha-amylase in starch processing, for example, simultaneous saccharification and fermentation (SSF), are provided.

## BACKGROUND

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.

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 alpha-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. The resulting syrup also may be fermented with microorganisms, such as yeast, to produce commercial products including ethanol, citric acid, lactic acid, succinic acid, itaconic acid, monosodium glutamate, gluconates, lysine, other organic acids, other amino acids, and other biochemicals, for example.

Fermentation and saccharification can be conducted simultaneously (i.e., an SSF process) to achieve greater economy and efficiency.

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

U.S. Patent No. 8,263,381 B2 describes the construction and characterization of a hybrid alpha-amylase obtained by attaching a carbohydrate binding module from an *Aspergillus kawacchii* alpha amylase to an *Aspergillus niger* acid stable alpha-amylase. The hybrid alpha-amylase, when used in a simultaneous saccharification and fermentation process with non-gelatinized starch, results in an approximate three-fold increase in the ethanol yield.

## SUMMARY

The carbohydrate binding module (CBM) from *Aspergillus terreus* alpha-amylase is fused to alpha-amylases naturally lacking a CBM (AniAmy1 and SocAmy1). The resulting chimeric fungal alpha-amylases (AniAmy1M and SocAmy1M) appear about 3 times or 9 times faster in hydrolyzing insoluble starch than the alpha-amylases naturally lacking a CBM. The insoluble starch could be raw uncooked starch for low temperature starch processing, cooked starch that has been exposed to steam or any other high temperature processes that gelatinize the starch granules, recalcitrant starch that remains after liquefaction and is present in the liquefact during saccharification, fermentation, or SSF, or a retrograded starch polymer. The chimeric alpha-amylase can be added to a fermentation or SSF process during which one or more amylases, glucoamylases, pullulanases, proteases, lipases, phytases, cellulases, cutinases, esterases, redox enzymes, transferases, or other enzymes are added or secreted by the production host. The chimeric alpha-amylases may also work in combination with endogenous non-secreted production host enzymes. In another example, the chimeric alpha-amylase can be secreted by a production host cell alone or with other enzymes during fermentation or SSF. The chimeric alpha-amylases may also be effective in direct hydrolysis of starch for syrup and/or biochemicals (e.g., alcohols, organic acids, amino acids, other biochemicals and biomaterials) where the reaction temperature is below the gelatinization temperature of substrate.

Accordingly, provided is a chimeric alpha-amylase having at least 90% sequence identity to residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13, comprising a catalytic domain (CD) derived from a first alpha-amylase having the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 10 fused to the N-terminus of a carbohydrate binding module (CBM) derived from a second alpha-amylase having the amino acid sequence of SEQ ID NO: 14. The chimeric alpha-amylase may have at least 92%, 95%, 98% or 99% sequence identity to residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13, or the chimeric alpha-amylase may comprise residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13. The chimeric alpha-amylase may be capable of hydrolyzing insoluble starch at least three times or at least nine times faster than the first alpha-amylase under the same reaction conditions. The reaction conditions may comprise mixing an insoluble starch substrate with the chimeric alpha-amylase or the first alpha-amylase, and incubating for 20-24 hours at 32°C to measure the glucose content in the supernatant, wherein the substrate is obtained from a simultaneous saccharification and fermentation (SSF) reaction having whole ground corn liquefact as a starting material, and wherein the substrate is a washed and sieved insoluble material containing no soluble starch.

The chimeric alpha-amylase may comprise the CD having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 17-492 of SEQ ID NO: 2, or residues 21-507 of SEQ ID NO: 10. Alternatively, the chimeric alpha-amylase may comprise a CD comprising residues 17-492 of SEQ ID NO: 2, or residues 21-507 of SEQ ID NO: 10. The chimeric alpha-amylase may comprise a CBM having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 498-607 of SEQ ID NO: 14. Alternatively, the chimeric alpha-amylase may comprise a CBM comprising residues 498-607 of SEQ ID NO: 14.

Also provided is (1) a nucleic acid encoding the chimeric alpha-amylase; (2) a vector comprising the nucleic acid encoding the chimeric alpha-amylase; and (3) a host cell comprising the vector comprising the nucleic acid encoding the chimeric alpha-amylase. The host cell may be a filamentous fungal cell. The typical host cell may be an *Aspergillus sp.* or *Trichoderma reesei* cell.

An enzyme composition comprising the chimeric alpha-amylase is also provided. The enzyme composition may further comprise glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, isoamylase, phytase, pullulanase, β-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, transglucosidase, or any combination thereof.

Also provided is a method of processing starch comprising contacting a composition comprising starch with the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase to produce a composition comprising glucose. The chimeric alpha-amylase may be purified. The composition comprising starch may comprise liquefied starch, gelatinized starch, granular starch, recalcitrant starch, or residual starch. The method of processing starch may further comprise adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, isoamylase, phytase, pullulanase, β-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, transglucosidase, or any combination thereof, to the composition comprising starch.

The method of processing starch may be conducted at a temperature range of 60°C – 85°C for the chimeric alpha-amylase having at least 90% sequence identity to residues 17-603 of SEQ ID NO: 5, or 42°C – 71°C for the chimeric alpha-amylase having at least 90% sequence identity to residues 21-616 of SEQ ID NO: 13. The method of processing starch may be conducted at a pH range of pH 3.0 – pH 7.3 for the chimeric alpha-amylase having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 17-603 of SEQ ID NO: 5, or pH 3.7 – pH 7.4 for the chimeric alpha-amylase having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 21-616 of SEQ ID NO: 13.

The method of processing starch may further comprise fermenting the glucose composition to produce an End of Fermentation (EOF) product. The method may comprise: contacting a mash and/or a wort with the chimeric alpha-amylase. The fermentation may be a simultaneous saccharification and fermentation (SSF) reaction. The fermentation may be conducted for 48 – 70 hours at pH 2 – 8 and in a temperature range of 25°C – 70°C. The EOF product typically may comprise ethanol. Alternatively, the EOF product may comprise citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta-lactone, sodium erythorbate, omega 3 fatty acid, butanol, an amino acid, lysine, itaconic acid, 1,3-propanediol, ethylene, or isoprene. For the fermentation, the chimeric alpha-amylase may be expressed and secreted by a host cell, the composition comprising starch may be contacted with the host cell, the host cell may further express and secrete a glucoamylase, or the host cell may be capable of fermenting the glucose composition.

Use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in the production of a fermented beverage is also provided. Also provided is a method of providing a fermented beverage, which may comprise the use of the chimeric alpha-

amylase or the enzymatic composition. A method of making a fermented beverage may comprise: (a) preparing a mash; (b) filtering the mash to obtain a wort; and (c) fermenting the wort to obtain a fermented beverage, where the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase is added to: (i) the mash of step (a) and/or (ii) the wort of step (b) and/or (iii) the wort of step (c).

The fermented beverage or end of fermentation product can be selected from the group consisting of a beer selected such as full malted beer, beer brewed under the "Reinheitsgebot", ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, and non-alcoholic malt liquor; or cereal or malt beverages such as fruit flavoured malt beverages, liquor flavoured malt beverages, and coffee flavoured malt beverages.

Also provided is a method of producing a food composition, comprising combining (i) one or more food ingredients, and (ii) the chimeric alpha-amylase the enzyme composition comprising the chimeric alpha-amylase, wherein the chimeric alpha-amylase catalyzes the hydrolysis of starch components present in the food ingredients to produce glucose. The food composition may comprise a dough, a dough product, or a processed dough product. The one or more food ingredients may comprise a baking ingredient or an additive. The one or more food ingredients may comprise flour and an enzyme selected from the group consisting an anti-staling amylase; a phospholipase; a phospholipid; a maltogenic alpha-amylase or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity; a bakery xylanase (EC 3.2.1.8); and a lipase. Alternatively, the one or more food ingredients may comprise an enzyme selected from the group consisting of (i) a maltogenic alpha-amylase from *Bacillus stearothermophilus*, (ii) a bakery xylanase is from *Bacillus*, *Aspergillus*, *Thermomyces* or *Trichoderma*, and (iii) a glycolipase from *Fusarium heterosporum.*

The food composition may be selected from the group consisting of a food product, a baking composition, a food additive, an animal food product, a feed product, a feed additive, an oil, a meat, and a lard. The method may further comprise baking the food composition to produce a baked good. The method may further comprise (i) providing a starch medium; (ii) adding to the starch medium the chimeric alpha-amylase; and (iii) applying heat to the starch medium during or after step (ii) to produce a bakery product.

Provided is a method of removing starchy stains from laundry, dishes, or textiles, comprising incubating a surface of said laundry, dishes, or textiles in the presence of an aqueous composition comprising an effective amount of the chimeric alpha-amylase, and allowing the chimeric alpha-amylase 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.

Also provided is a method of desizing a textile comprising contacting a desizing composition with a textile for a time sufficient to desize the textile, wherein the desizing composition comprises the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase and allowing the chimeric alpha-amylase to desize 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.

Accordingly, use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in the production of a composition comprising glucose is provided. A composition comprising glucose produced by the disclosed method is also provided. Use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in the production of a liquefied starch is provided. A liquefied starch produced by the disclosed method is also provided. Use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in preparing a food composition is provided. A food composition produced by the disclosed method is also provided. Use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in the production of a fermented beverage is provided. A fermented beverage produced by the disclosed methods is also provided. Moreover, use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in a dough product to retard or reduce staling or detrimental retrogradation of the dough product is provided. Use of the chimeric alpha-amylase or the enzyme composition comprising the chimeric alpha-amylase in (1) removing starchy stains from laundry, dishes, or textiles, or (2) desizing textiles is provided. Furthermore, use of a CBM having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 498-607 of SEQ ID NO: 14 in improving an alpha-amylase's capability of hydrolyzing insoluble starch is provided. The alpha-amylase may lack a CBM.

## BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are incorporated into the specification and provide non-limiting illustrations of various embodiments. In the drawings

FIG. 1 depicts a plasmid map of pZZH371 comprising a polynucleotide that encodes the AniAmy1 polypeptide.

FIG. 2 depicts a plasmid map of pZZH371.1 comprising a polynucleotide that encodes the AniAmy1M polypeptide.

FIG. 3 depicts a plasmid map of pZZH513 comprising a polynucleotide that encodes the SocAmy1 polypeptide.

FIG. 4 depicts a plasmid map of pZZH513.1 comprising a polynucleotide that encodes the SocAmy1M polypeptide.

FIGs. 5A, 5B, 5C, and 5D depict performance comparison between AniAmy1 and AniAmy1M in the cooked starch assay (FIG. 5A), the SSF assay (FIG. 5B), the residual starch assay (FIG. 5C), and the ceralpha assay (FIG. 5D). The assays were conducted as described in Examples 8-11.

FIGs. 6A, 6B, 6C, and 6D depict performance comparison between SocAmy1 and SocAmy1M in the cooked starch assay (FIG. 6A), the SSF assay (FIG. 6B), the residual starch assay (FIG. 6C), and the ceralpha assay (FIG. 6D). The assays were conducted as described in Examples 8-11.

FIGs. 7A, 7B, 7C, and 7D depict a ClustalW alignment of predicted mature alpha-amylases from *Aspergillus clavatus* (SEQ ID NO: 15), *Aspergillus kawachii* (SEQ ID NO: 16), *Aspergillus awamori* (SEQ ID NO: 17), *Aspergillus fumigatus* (SEQ ID NO: 18), *Aspergillus nidulans* (SEQ ID NO: 19), *Aspergillus terreus* (SEQ ID NO: 14), *Aspergillus niger* (AniAmy1) (SEQ ID NO: 2), *Schwanniomyces occidentalis* (SocAmy1) (SEQ ID NO: 9), and *Aspergillus niger* (TAKA-amylase) (SEQ ID NO: 20). Predicted signal peptide sequences are removed. The putative linkers and putative carbohydrate binding modules (CBMs) are shown in blocks. According to the alignment, the alpha-amylases from *A. niger* (SEQ ID NO: 2) and *S. occidentalis* (SEQ ID NO: 9) lack a linker or a carbohydrate binding module. Residues designated by an asterisk in FIGs. 7A, 7B, 7C, and 7D are conserved residues among SEQ ID NOs: 2, 9, and 14-20.

## DETAILED DESCRIPTION

By fusing the carbohydrate binding module (CBM) from *Aspergillus terreus* alpha-amylase to alpha-amylases naturally lacking a CBM (AniAmy1 and SocAmy1), chimeric fungal alpha-amylases (AniAmy1M and SocAmy1M) are provided. AniAmy1M appears about 9 times faster in hydrolyzing insoluble starch than AniAmy1, while SocAmy1M appears about 3 times faster in hydrolyzing insoluble starch than SocAmy1. Given such advantageous properties, exemplary applications for the chimeric alpha-amylases are in starch processing, e.g., SSF, the

preparation of cleaning compositions, such as detergent compositions for cleaning laundry, dishes, and other surfaces, for textile processing (e.g., desizing).

In some aspects, the embodiments of the present disclosure rely on routine techniques and methods used in the field of genetic engineering and molecular biology. The following resources include descriptions of general methodology useful in accordance with the embodiments Sambrook et al., MOLECULAR CLONING A LABORATORY MANUAL (2nd Ed., 1989); Kreigler, GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL (1990) and Ausubel et al., Eds. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994). Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the representative methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects or embodiments, which can be had by reference to the specification as a whole.

## 1. Definitions and Abbreviations

In accordance with this detailed description, the following abbreviations and definitions apply. It should be noted that as used herein, 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.

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

### 1.1. Abbreviations and Acronyms

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

| | |
|---|---|
| AniAmy1 | an alpha-amylase isolated from *Aspergillus niger* having the full-length amino acid sequence of SEQ ID NO: 2 |
| AniAmy1M | a chimera alpha-amylase comprising CBM20 of *Aspergillus terreus* fused to the C-terminus of AniAmy1 |
| SocAmy1 | an alpha-amylase isolated from *Schwanniomyces occidentalis* having the full length amino acid sequence of SEQ ID NO: 10 |
| SocAmy1M | a chimera alpha-amylase comprising CBM20 of *Aspergillus terreus* fused to the C-terminus of SocAmy1 |
| ABTS | 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid |
| AE | alcohol ethoxylate |
| AEO | alcohol ethoxylate |
| AEOS | alcohol ethoxysulfate |
| AES | alcohol ethoxysulfate |
| AnGA | *Aspergillus niger* glucoamylase |
| AOS | α-olefinsulfonate |
| AS | alkyl sulfate |
| cDNA | complementary DNA |
| CMC | carboxymethylcellulose |
| DE | dextrose equivalent |
| DNA | deoxyribonucleic acid |
| DPn | degree of saccharide polymerization having n subunits |
| ds or DS | dry solids |
| DTMPA | diethylenetriaminepentaacetic acid |
| EC | Enzyme Commission |
| EDTA | ethylenediaminetetraacetic acid |
| EO | ethylene oxide (polymer fragment) |
| EOF | End of Fermentation |
| FGSC | Fungal Genetics Stock Center |
| GA | glucoamylase |
| GAU/g ds | glucoamylase activity unit/gram dry solids |
| HFCS | high fructose corn syrup |
| HgGA | *Humicola grisea* glucoamylase |
| IPTG | isopropyl β-D-thiogalactoside |
| kDa | kiloDalton |

| | |
|---|---|
| LAS | linear alkylbenzenesulfonate |
| MW | molecular weight |
| NCBI | National Center for Biotechnology Information |
| NOBS | nonanoyloxybenzenesulfonate |
| NTA | nitriloacetic acid |
| PAHBAH | p-hydroxybenzoic acid hydrazide |
| PEG | polyethyleneglycol |
| pI | isoelectric point |
| ppm | parts per million, e.g., µg protein per gram dry solid |
| PVA | poly(vinyl alcohol) |
| PVP | poly(vinylpyrrolidone) |
| RNA | ribonucleic acid |
| SAS | alkanesulfonate |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SSF | simultaneous saccharification and fermentation |
| SSU/g solid | soluble starch unit/gram dry solids |
| sp. | species |
| TAED | tetraacetylethylenediamine |
| TrGA | *Trichoderma reesei* glucoamylase |
| w/v | weight/volume |
| w/w | weight/weight |
| v/v | volume/volume |
| wt% | weight percent |
| °C | degrees Centigrade |
| $H_2O$ | water |
| $dH_2O$ or DI | deionized water |
| $dIH_2O$ | deionized water, Milli-Q filtration |
| g or gm | grams |
| µg | micrograms |
| mg | milligrams |
| kg | kilograms |
| µL and µl | microliters |
| mL and ml | milliliters |
| 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 |
| ETOH | ethanol |
| eq. | equivalents |
| N | normal |

### 1.2. Definitions

The terms "amylase" or "amylolytic enzyme" refer to an enzyme that is, among other things, capable of catalyzing the degradation of starch. Alpha-amylases are hydrolases that cleave the α-D-(1→4) O-glycosidic linkages in starch. Generally, alpha-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 alpha-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 or enriched syrups of specific maltooligosaccharides.

As used herein, "glucoamylases" refer to the amyloglucosidase class of enzymes (EC 3.2.1.3, glucoamylase, α-1, 4-D-glucan glucohydrolase). These are exo-acting enzymes that release glucosyl residues from the non-reducing ends of amylose and/or amylopectin molecules. The enzymes are also capable of hydrolyzing α-1, 6 and α-1,3 linkages, however, at much slower rates than the hydrolysis of α-1, 4 linkages.

The term "pullulanase" (E.C. 3.2.1.41, pullulan 6-glucanohydrolase) refers to a class of enzymes that are capable of hydrolyzing α-1,6-D-glucosidic linkages present in amylopectin. Pullulanase hydrolyses the α-1,6-D-glucosidic linkages in pullulan to give the trisaccharide maltotriose.

The term "isoamylase," as used herein, refers to a debranching enzyme (E.C 3.2.1.68) capable of hydrolyzing the α-1,6-D-glucosidic linkages of starch, glycogen, amylopectin, glycogen, beta-limit dextrins, and oligosaccharides derived therefrom. It cannot hydrolyse pullulan.

"Enzyme units" herein refer to the amount of product formed per time under the specified conditions of the assay. For example, a "glucoamylase activity unit" (GAU) is defined as the amount of enzyme that produces 1 g of glucose per hour from soluble starch substrate (4% DS) at 60°C, pH 4.2. A "soluble starch unit" (SSU) is the amount of enzyme that produces 1 mg of glucose per minute from soluble starch substrate (4% DS) at pH 4.5, 50°C. DS refers to "dry solids."

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, cereal, grasses, tubers and roots, and more specifically materials obtained from wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, potato, sweet potato, and tapioca. The term "starch" includes granular starch. The term "granular starch" refers to raw, i.e., uncooked starch, e.g., starch that has not been subject to gelatinization.

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.

A "mature" polypeptide refers to a polypeptide in its final, functionally active form following translation and any post-translational modification, such as N-terminal process (e.g., cleavage of the signal peptide), C-terminal truncation, glycosylation, phosphorylation, etc. For

example, the mature AniAmy1 is predicted to be 497 amino acids in length covering positions 17-513 of SEQ ID NO: 2, where positions are counted from the N-terminus. The signal sequence of the wild-type AniAmy1 is expected to be 16 amino acids in length and has the sequence set forth in SEQ ID NO: 3. The mature SocAmy1 is predicted to be 487 amino acids in length covering positions 21-507 of SEQ ID NO: 10, where positions are counted from the N-terminus. The signal sequence of the wild-type SocAmy1 is expected to be 20 amino acids in length and has the sequence set forth in SEQ ID NO: 11.

As shown in FIG. 7, the "catalytic core" or "catalytic domain" of AniAmy1 is predicted to span residues 17-495 of SEQ ID NO: 2, while the "catalytic core" or "catalytic domain" of SocAmy1 is predicted to span residues 21-507 of SEQ ID NO: 10. Neither AniAmy1 nor SocAmy1 has an identifiable linker region or carbohydrate binding module. For the *A. terreus* alpha-amylase having the amino acid sequence of SEQ ID NO: 14, the putative catalytic domain, linker region, and carbohydrate binding module span residues 21-497, 498-499, and 500-607, respectively.

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

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

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

The terms "recovered" 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. As used herein, the term "isolated" refers to a protein, peptide, or nucleic acid that is free or substantially free from other macromolecular species found in a cellular environment. "Substantially free", as used herein, means the protein, peptide or nucleic acid of interest comprises more than 80% (on a molar basis) of the macromolecular species present, preferably more than 90% and more preferably more than 95%.

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.

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

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

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).

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).

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.

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

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.

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.

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., a chimeric alpha-amylase) has been introduced. Exemplary host strains are microorganism cells (e.g., bacteria, filamentous fungi, and yeast) capable of expressing the polypeptide of interest and/or fermenting saccharides. The term "host cell" includes protoplasts created from cells.

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.

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.

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.

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

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

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.

As used herein, "biologically active" refers to a sequence having a specified biological activity, such an enzymatic activity.

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.

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

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

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

Deletions are counted as non-identical residues, compared to a reference sequence. Deletions occurring at either termini are included. For example, a variant with five amino acid deletions of the C-terminus of the mature *A. terreus* alpha-amylase of SEQ ID NO: 14 would have a percent sequence identity of 99% (602 / 607 identical residues × 100, rounded to the nearest whole number) relative to the mature polypeptide. Such a variant would be encompassed by a variant having "at least 99% sequence identity" to the mature *A. terreus* alpha-amylase of SEQ ID NO: 14.

"Fused" polypeptide sequences are connected, i.e., operably linked, via a peptide bond between the two polypeptide sequences.

The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina.

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

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

The phrase "simultaneous saccharification and fermentation (SSF)" refers to a process in the production of biochemicals in which a microbial organism, such as an ethanologenic microorganism, and one or more enzymes, such as a chimeric alpha-amylase and a glucoamylase, are present during the same process step. SSF includes the contemporaneous hydrolysis of starch substrates (granular, liquefied, or solubilized) to saccharides, including glucose, and the fermentation of the saccharides into alcohol or other biochemical or biomaterial in the same reactor vessel.

As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol.

The term "fermented beverage" refers to any beverage produced by a method comprising a fermentation process, such as a microbial fermentation, e.g., a bacterial and/or yeast fermentation.

"Beer" is an example of such a fermented beverage, and the term "beer" is meant to comprise any fermented wort produced by fermentation/brewing of a starch-containing plant material. Often, beer is produced exclusively from malt or adjunct, or any combination of malt and adjunct. Examples of beers include: full malted beer, beer brewed under the "Reinheitsgebot," ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, non-alcoholic malt liquor and the like, but also alternative cereal and malt beverages such as fruit flavored malt beverages, e.g., citrus flavored, such as lemon-, orange-, lime-, or berry-flavored malt beverages, liquor flavored malt beverages, e.g., vodka-, rum-, or tequila-flavored malt liquor, or coffee flavored malt beverages, such as caffeine-flavored malt liquor, and the like.

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

The term "adjunct" refers to any starch and/or sugar containing plant material which is not malt, such as barley or wheat malt. Examples of adjuncts include common corn grits, refined corn grits, brewer's milled yeast, rice, sorghum, refined corn starch, barley, barley starch, dehusked barley, wheat, wheat starch, torrified cereal, cereal flakes, rye, oats, potato,

tapioca, cassava and syrups, such as corn syrup, sugar cane syrup, inverted sugar syrup, barley and/or wheat syrups, and the like.

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

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

As used herein, the term "insoluble starch" refers to (1) raw uncooked starch for low temperature starch processing; (2) cooked starch that has been exposed to steam or any other high temperature processes that gelatinizes the starch granules; (3) recalcitrant starch that remains after liquefaction and is present in the liquefact during SSF; or (4) a retrograded starch polymer. When saccharified starch or saccharide liquor is tested with iodine, the recalcitrant starch or the retrograded starch polymer binds iodine and produces a characteristic blue color. The saccharide liquor is thus termed "iodine-positive saccharide," "blue saccharide," or "blue sac."

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

**2. Chimeric Alpha-Amylases**

Chimeric alpha-amylases having at least 90%, at least 92%, at least 95%, at least 98%, or at least 99% sequence identity to residues of 17-603 of SEQ ID NO: 5 or resides 21-616 of SEQ ID NO: 13 are provided. The chimeric alpha-amylase can be the mature alpha-amylase having the amino acid SEQ ID NO: 5 or 13. Alternatively, the polypeptides may be fused to additional amino acid sequences at the N-terminus and/or C-terminus. Additional N-terminal sequences can be a signal peptide, which may have the sequence shown in SEQ ID NO: 3 or 11, for example. Other amino acid sequences fused at either termini include fusion partner polypeptides useful for labeling or purifying the protein.

The provided chimeric alpha-amylases comprise a catalytic domain (CD) derived from AniAmy1 (SEQ ID NO: 2) or SocAmy1 (SEQ ID NO: 10) fused to the N-terminus of a carbohydrate binding module (CBM) derived from an *A. terreus* alpha-amylase (SEQ ID NO: 14). The chimeric alpha-amylase may comprise a catalytic domain having at least 90%, at least 92%, at least 95%, at least 98%, or at least 99% sequence identity to residues 17-492 of SEQ ID NO: 2, or residues 21-507 of SEQ ID NO: 10. The chimeric alpha-amylase may comprise a

carbohydrate binding module (CBM) having at least 90%, at least 92%, at least 95%, at least 98%, or at least 99% sequence identity to residues 498-607 of SEQ ID NO: 14.

An *Aspergillus niger* alpha-amylase precursor (AniAmy1), i.e., containing a signal peptide has the following amino acid sequence (SEQ ID NO: 2):

*MTIFLFLAIFVATALA*ATPAEWRSQSIYFLLTDRFARTDNSTTASCDLSARQYCGGSWQGIINQLDYIQG
MGFTAIWITPVTAQIPQDTGYGQAYHGYWQQDAYALNSHYGTADDLKALASALHSRGMYLMVDVVANHMG
HNGTGSSVDYSVYRPFNSQKYFHNLCWISDYNNQTNVEDCWLGDNTVALPDLDTTSTEVKNMWYDWVESL
VSNYSVDGLRVDTVKNVQKNFWPGYNNASGVYCIGEVFDGDASYTCPYQEDLDGVLNYPMYYPLLRAFES
TNGSISDLYNMINTVKSTCRDSTLLGTFVENHDNPRFANYTSDMSLAKNAATFTILADGIPIIYAGQEQH
YSGGNDPYNREATWLSGYKTTSELYTHIAASNKIRTHAIKQDTGYLTYKNYPIYQDTSTLAMRKGYNGTQ
TITVLSNLGASGSSYTLSLPGTGYTAGQKITEIYTCTNLTVNSNGSVPVPMKSGLPRILYPADKLVNGSS
FCSSAITVFKDAGGGVLFFSYTV

The italic amino acids above (SEQ ID NO: 3) constitute the predicted signal peptide. As suggested by the alignment of FIG. 7, AniAmy1 does not contain a linker region or a CBM, and AniAmy1's catalytic domain spans residues 17-495 of SEQ ID NO: 2. The polypeptide sequence of AniAmy1 is similar to other fungal alpha-amylases. For example, AniAmy1 has about 64% to 75% sequence identity to the alpha-amylases from *Aspergillus clavatus* (SEQ ID NO: 15), *Aspergillus kawachii* (SEQ ID NO: 16), *Aspergillus awamori* (SEQ ID NO: 17), *Aspergillus fumigatus* (SEQ ID NO: 18), *Aspergillus nidulans* (SEQ ID NO: 19), *Aspergillus terreus* (SEQ ID NO: 14), and *Aspergillus niger* (TAKA-amylase) (SEQ ID NO: 20). Sequence identity was determined by a BLAST alignment, using residues 17-495 of SEQ ID NO: 2 as the query sequence. *See* Altschul et al. (1990) *J. Mol. Biol.* 215: 403–410.

A *Schwanniomyces occidentalis* alpha-amylase precursor (SocAmy1), i.e., containing a signal peptide has the following amino acid sequence (SEQ ID NO: 10):

*MKFATILSTTALALSSLVAS*KPIFLSKRDAGSSAAAAWRSESIYQLVTDRFARTDGSTSATCNTGDRVYC
GGTFQGIIDKLDYIQGMGFTAIWISPVVEQIPDDTGYGYAYHGYWMKDIYAINSNFGTADDLKNLSNELH
KRNMKLMVDIVTNHYAWNGAGSSVAYSNYNPFNQQSYFHDYCLITNYDDQTNVEDCWEGDNTVSLPDLRT
EDSDVSSIFNLWVAELVSNYSIDGLRIDSAKHVDESFYPSFQSAAGVYLLGEVYDGDPAYTCPYQNYMSG
VTNYPLYYPMLRFFQGTSNSVDELNAMISSLESDCKDITLLGNFIENHDQPRLPSYTSDSALIKNAIAFN
LMSDGIPIIYYGQEQGYSGSSDPNNREALWLSGYSTSNGYYKLISSVNQIRNQAIYKDSKYTTYWSDVLY
ASGHVIALQRGADDQRIVSVFNNLGSSGSQTVTFSTKYSGGEKVVDVLTCQTSYANSDSTLTVSISGGAP
RIYAPASLIANSGICNF

The italic amino acids above (SEQ ID NO: 11) constitute the predicted signal peptide. As suggested by the alignment of FIG. 7, SocAmy1 does not contain a linker region or a CBM, and SocAmy1's catalytic domain spans residues 21-507 of SEQ ID NO: 10. The polypeptide

sequence of SocAmy1 is similar to other fungal alpha-amylases. For example, SocAmy1 has about 52% to 59% sequence identity to the alpha-amylases from *Aspergillus clavatus* (SEQ ID NO: 15), *Aspergillus kawachii* (SEQ ID NO: 16), *Aspergillus awamori* (SEQ ID NO: 17), *Aspergillus fumigatus* (SEQ ID NO: 18), *Aspergillus nidulans* (SEQ ID NO: 19), *Aspergillus terreus* (SEQ ID NO: 14), and *Aspergillus niger* (TAKA-amylase) (SEQ ID NO: 20). Sequence identity was determined by a BLAST alignment, using mature SocAmy1 (i.e., residues 21-507 of SEQ ID NO: 10) as the query sequence. *See* Altschul et al. (1990) *J. Mol. Biol.* 215: 403–410.

A ClustalW alignment of AniAmy1 (SEQ ID NO: 2), SocAmy1 (SEQ ID NO: 10), and alpha-amylases from *Aspergillus clavatus* (SEQ ID NO: 15), *Aspergillus kawachii* (SEQ ID NO: 16), *Aspergillus awamori* (SEQ ID NO: 17), *Aspergillus fumigatus* (SEQ ID NO: 18), *Aspergillus nidulans* (SEQ ID NO: 19), *Aspergillus terreus* (SEQ ID NO: 14), and *Aspergillus niger* (TAKA-amylase) (SEQ ID NO: 20) is shown in FIG. 7. *See* Thompson et al. (1994) *Nucleic Acids Res.* 22:4673-4680. As a general rule, the degree to which an amino acid is conserved in an alignment of related protein sequences is proportional to the relative importance of the amino acid position to the function of the protein. That is, amino acids that are common in all related sequences likely play an important functional role and cannot be easily substituted. Likewise, positions that vary between the sequences likely can be substituted with other amino acids or otherwise modified, while maintaining the activity of the protein.

The crystal structure of *A. niger* alpha-amylase (SEQ ID NO: 20) has been determined, including a complex of enzyme with maltose bound to its active site. *See, e.g.*, Vujicić-Zagar et al. (2006) "Monoclinic crystal form of *Aspergillus niger* α-amylase in complex with maltose at 1.8 Å resolution," *Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun.* 62(8): 716-21. The *A. niger* alpha-amylase disclosed in Vujicić-Zagar (2006) is also known as TAKA-amylase, an *A. oryzae* alpha-amylase homologue. The amino acid sequence of TAKA-amylase (SEQ ID NO: 20) has (1) a 75% sequence identity to AniAmy1 over residues 17-495 of SEQ ID NO: 2, and (2) a 59% sequence identity to SocAmy1 over residues 21-507 of SEQ ID NO: 10, when aligned using the BLAST algorithm. Given the relatively high amino acid sequence conservation between TAKA-amylase and AniAmy1 or SocAmy1, AniAmy1 or SocAmy1 can be predicted to adopt many of the secondary structures and possess similar structure/function relationships as TAKA-amylase. For example, AniAmy1 or SocAmy1 is expected to have a similar high affinity $Ca^{2+}$ binding site and maltose binding cleft as TAKA-amylase. Consistent with this expectation, the three acidic amino acids that participate in the hydrolysis reaction catalyzed by

TAKA-amylase, D206, E230, and D297 of SEQ ID NO: 20, all are conserved in AniAmy1 (D222, E246, and D313 of SEQ ID NO: 2) and SocAmy1 (D238, E262, and D329 of SEQ ID NO: 10). TAKA-amylase positions Y155, D233, and D235 of SEQ ID NO: 20, located near the binding cleft, also are conserved in AniAmy1 (Y171, D249, and D251) and SocAmy1 (Y187, D265, and D267). Additionally, TAKA-amylase positions N121, E162, and D175 of SEQ ID NO: 20, which constitute the high affinity $Ca^{2+}$ binding site, are also conserved in AniAmy1 (N137, E178, and D191) and SocAmy1 (N153, E194, and D207). See Vujičić-Zagar (2006).

The alignments shown in FIG. 7 and the structural relationships ascertained from the TAKA-amylase crystal structure, for example, can guide the construction of chimeric alpha-amylases comprising a catalytic domain derived from AniAmy1 or SocAmy1. The catalytic domains of the chimeric alpha-amylase may include, but are not limited to, those with an amino acid modification selected from a substitution, insertion, or deletion of a corresponding amino acid in SEQ ID NOs: 14-20. Correspondence between positions in AniAmy1 or SocAmy1 and the alpha-amylases of SEQ ID NOs: 14-20 can be determined with reference to the alignment shown in FIG. 7. For example, a chimeric alpha-amylase can have a catalytic domain derived from AniAmy1, wherein the catalytic domain contains the substitution of V216I or V216A, where isoleucine or alanine is the corresponding amino acid in SEQ ID NOs: 14-20, referring to the alignment in FIG. 7. Catalytic domains derived from AniAmy1 or SocAmy1 also include, but are not limited to, those with 1, 2, 3, or 4 randomly selected amino acid modifications. Amino acid modifications can be made using well-known methodologies, such as oligo-directed mutagenesis.

A known alpha-amylase from *Aspergillus terreus* is the alpha-amylase from *A. terreus* NIH2624. *A. terreus* NIH2624 alpha-amylase precursor, i.e., containing a signal peptide, has the following amino acid sequence (SEQ ID NO: 14):

*MKWTSSLLLLLSVIGQATHA*LTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTSDRVYCGGSWQGIINQLD
YIQGMGFTAIWITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLANALHERGMYLMVDVVA
NHMGYDGAGNTVDYSVFNPFSSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVRNIWYDW
VADLVANYSIDGLRVDTVKHVEKDFWPGYNSAAGVYCVGEVYSGDPAYTCPYQNYMDGVLNYPIYYQLLY
AFESSSGSISDLYNMISSVASSCKDPTLLGNFIENHDNPRFASYTSDYSQAKNVITFIFLSDGIPIVYAG
QEQHYSGGSDPANREATWLSGYSTSATLYTWIATTNQIRSLAISKDAGYVQAKNNPFYSDSNTIAMRKGT
TAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPMTSGLPRVFVPSSWV
NGSALCN**TECTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPEWYVSVTL
PVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

*See* NCBI Reference Number XP_001209405.1 (gi|115385717|ref|XP_001209405.1| alpha-amylase precursor [Aspergillus terreus NIH2624]). The bolded amino acids above constitute a putative C-terminal carbohydrate binding module (CBM) (residues 500-607 of SEQ ID NO: 14). A putative linker region (highlighted, bolded amino acids above; positions 498-499 of SEQ ID NO: 14) connects the N-terminal catalytic domain with the putative CBM. This putative CBM domain is conserved with a CBM20 domain found in a large number of starch degrading enzymes, including alpha-amylases, beta-amylases, glucoamylases, and cyclodextrin glucanotransferases. CBM20 folds as an antiparallel beta-barrel structure with two starch binding sites 1 and 2. These two sites are thought to differ functionally: site 1 may act as the initial starch recognition site, whereas site 2 may be involved in specific recognition of appropriate regions of starch. *See* Sorimachi et al. (1997) "Solution structure of the granular starch binding domain of *Aspergillus niger* glucoamylase bound to beta-cyclodextrin," *Structure* 5(5): 647-61. Residues in the *A. terreus* alpha amylase's putative CBM domain that are conserved with starch binding sites 1 and 2 are indicated in the sequence below by the numbers 1 and 2, respectively:

```
CTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPEWYVSVTLPVGTSFQYK
             222222        1    1 1111      2 2222  22
FIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR
            1
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residues 500-607 of SEQ ID NO: 14.

The alignment in FIG. 7 also includes the CBMs of the alpha-amylases from *Aspergillus clavatus* (SEQ ID NO: 15), *Aspergillus kawachii* (SEQ ID NO: 16), *Aspergillus awamori* (SEQ ID NO: 17), *Aspergillus fumigatus* (SEQ ID NO: 18), *Aspergillus nidulans* (SEQ ID NO: 19), and *Aspergillus terreus* (SEQ ID NO: 14). *See* Thompson et al. (1994) *Nucleic Acids Res.* 22:4673-4680. As a general rule, the degree to which an amino acid is conserved in an alignment of related protein sequences is proportional to the relative importance of the amino acid position to the function of the protein. That is, amino acids that are common in all related sequences likely play an important functional role and cannot be easily substituted. Likewise, positions that vary between the sequences likely can be substituted with other amino acids or otherwise modified, while maintaining the activity of the protein.

The CBMs of the chimeric alpha-amylase may include, but are not limited to, those with an amino acid modification selected from a substitution, insertion, or deletion of a corresponding amino acid in SEQ ID NOs: 15-19. Correspondence between CBMs positions in SEQ ID NO:

14 and the alpha-amylases of SEQ ID NOs: 15-19 can be determined with reference to the alignment shown in FIG. 7. For example, a chimeric alpha-amylase can have a CBM derived from SEQ ID NO: 14, wherein the CBM contains the substitution of L509V or L509T, where valine or threonine is the corresponding amino acid in SEQ ID NOs: 15-19, referring to the alignment in FIG. 7. CBMs derived from SEQ ID NO: 14 also include, but are not limited to, those with 1, 2, 3, or 4 randomly selected amino acid modifications. Amino acid modifications can be made using well-known methodologies, such as oligo-directed mutagenesis.

Nucleic acids encoding the chimeric alpha-amylases also are provided. Representative nucleic acids encoding the chimeric alpha-amylases include, for example, SEQ ID NOs: 4 and 12. As is well understood by one skilled in the art, the genetic code is degenerate, meaning that multiple codons in some cases may encode the same amino acid. Nucleic acids include all genomic DNA, mRNA and cDNA sequences that encode a described chimeric alpha-amylase. Chimeric alpha-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. Chimeric alpha-amylase may also be truncated at the N- or C-termini, so long as the resulting polypeptides retain alpha-amylase activity.

Given the addition of a CBM, the chimeric alpha-amylase may be capable of hydrolyzing insoluble starch more efficiently or effectively than AniAmy1 or SocAmy1. Chimeric alpha-amylases may have a specific activity higher or lower than AniAmy1 or SocAmy1. Additional altered characteristics of the chimeric alpha-amylase with reference to AniAmy1 or SocAmy1 may include stability, pH range, oxidation stability, and thermostability, for example.

## 3. Production of Chimeric Alpha-Amylases

The chimeric alpha-amylase can be isolated from a host cell, for example by secretion of the chimeric alpha-amylase from the host cell. A cultured cell material comprising a chimeric alpha-amylase can be obtained following secretion of the chimeric alpha-amylase from the host cell. The chimeric alpha-amylase optionally is purified prior to use. The nucleotide sequence encoding the chimeric alpha-amylase can be cloned and expressed according to methods well known in the art. Suitable host cells include bacterial, plant, yeast cells, algal cells or fungal cells, e.g., filamentous fungal cells. Particularly useful host cells include *Aspergillus terreus*, *Aspergillus niger*, yeast, *Trichoderma reesei*, *Rhizopus*, *Fusarium*, or other fungal hosts. Other host cells may include bacterial cells, e.g., *Bacillus subtilis* or *B. licheniformis*, plant, algal and animal host cells.

The host cell further may express a nucleic acid encoding a homologous or heterologous glucoamylase, i.e., a glucoamylase that is not the same species as the host cell, or one or more other enzymes. The glucoamylase may be a variant glucoamylase, such as one of the glucoamylase variants disclosed in U.S. Patent No. 8,058,033 (Danisco US Inc.), for example. Additionally, the host may express one or more accessory enzymes, proteins, and/or peptides. These may benefit pretreatment, liquefaction, saccharification, fermentation, SSF, stillage, etc processes. Furthermore, the host cell may produce biochemicals in addition to enzymes used to digest the various feedstock(s). Such host cells may be useful for fermentation or simultaneous saccharification and fermentation processes to reduce or eliminate the need to add enzymes.

### 3.1. Vectors

A DNA construct comprising a nucleic acid encoding a chimeric alpha-amylase can be constructed to be expressed in a host cell. Representative nucleic acids that encode the chimeric alpha-amylase include SEQ ID NO: 4 or 12. Because of the well-known degeneracy in the genetic code, variant polynucleotides that encode an identical amino acid sequence can be designed and made with routine skill. It is also well-known in the art to optimize codon use for a particular host cell. Nucleic acids encoding the chimeric alpha-amylase can be incorporated into a vector. Vectors can be transferred to a host cell using well-known transformation techniques, such as those disclosed below.

The vector may be any vector that can be transformed into and replicated within a host cell. For example, a vector comprising a nucleic acid encoding a chimeric alpha-amylase can be transformed and replicated in a bacterial host cell as a means of propagating and amplifying the

vector. The vector also may be transformed into an expression host, so that the encoding nucleic acids can be expressed as a functional chimeric alpha-amylase. Host cells that serve as expression hosts can include filamentous fungi, for example. The Fungal Genetics Stock Center (FGSC) Catalogue of Strains lists suitable vectors for expression in fungal host cells. *See* FGSC, Catalogue of Strains, University of Missouri, available on the Internet at hypertext transfer protocol:// www.fgsc.net (last modified June 4, 2013). Representative vectors include plasmids pZZH371.1 and pZZH513.1 (FIGs. 2 and 4, respectively), which comprises a pTrex3gM expression vector (U.S. Published Application No. 2011/0136197 A1). pZZH371.1 and pZZH513.1 also allow expression of a nucleic acid encoding a chimeric alpha-amylase under the control of the *cbh1* promoter in a fungal host. pZZH371.1 and pZZH513.1 can be modified with routine skill to comprise and express a nucleic acid encoding chimeric alpha-amylases having at least 90%, at least 92%, at least 95%, at least 98%, or at least 99% sequence identity to residues of 17-603 of SEQ ID NO: 5 or resides 21-616 of SEQ ID NO: 13.

A nucleic acid encoding a chimeric alpha-amylase can be operably linked to a suitable promoter, which allows transcription in the host cell. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Exemplary promoters for directing the transcription of the DNA sequence encoding the chimeric alpha-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* alpha-amylase gene (amyL), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (amyM), the promoters of the *Bacillus amyloliquefaciens* alpha-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 alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, or *A. nidulans* acetamidase. When a gene encoding a chimeric alpha-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 example, the pZZH371.1 and pZZH513.1 vectors depicted in FIGs. 2 and 4, respectively, contains a *cbh1*

promoter operably linked to the nucleic acid encoding a chimeric alpha-amylase. *cbh1* is an endogenous, inducible promoter from *T. reesei*. *See* Liu et al. (2008) "Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (cbh1) promoter optimization," *Acta Biochim. Biophys. Sin (Shanghai)* 40(2): 158-65.

The coding sequence can be operably linked to a signal sequence. The DNA encoding the signal sequence may be the DNA sequence naturally associated with the nucleic acid encoding the *AniAmy1* or *SocAmy1* gene. For example, the DNA may encode the AniAmy1 signal sequence of SEQ ID NO: 3 or the SocAmy1 signal sequence of SEQ ID NO: 11 operably linked to a nucleic acid encoding the chimeric alpha-amylase. The DNA may encode a signal sequence from a species other than *A. niger or S. occidentalis*. A signal sequence and a promoter sequence comprising a DNA construct or vector can be introduced into a fungal host cell and can be derived from the same source. For example, the signal sequence is the *cbh1* signal sequence that is operably linked to a *cbh1* promoter.

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

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.

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 *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, such as known in the art. See e.g., International PCT Application WO 91/17243.

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

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 chimeric alpha-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 serine-lysine-leucine (SKL), which is a known peroxisome target signal. For expression under the direction of control sequences, the nucleic acid sequence of the chimeric alpha-amylase is operably linked to the control sequences in proper manner with respect to expression.

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

### 3.2. Transformation and Culture of Host Cells

An isolated cell, either comprising a DNA construct or an expression vector, is advantageously used as a host cell in the recombinant production of a chimeric alpha-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.

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.

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia* sp., *Hansenula* sp., or *Kluyveromyces*, *Yarrowinia*, *Schizosaccharomyces* species or a species of *Saccharomyces*, including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces* such as, for example, *S. pombe* species. A strain of the methylotrophic yeast species, *Pichia pastoris*, can be used as the host organism. Alternatively, the host organism can be a *Hansenula* species. Suitable host organisms among filamentous fungi include species of *Aspergillus, e.g., Aspergillus niger, Aspergillus oryzae, Aspergillus tubigensis, Aspergillus awamori*, or *Aspergillus nidulans*. Alternatively, strains of a *Fusarium* species, *e.g., Fusarium oxysporum* or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species. In addition, *Trichoderma sp.* can be used as a host. A suitable procedure for transformation of *Aspergillus* host cells includes, for example, that described in EP 238023. The chimeric alpha-amylase expressed by a fungal host cell can be glycosylated, i.e., the chimeric alpha-amylase will comprise a glycosyl moiety. The glycosylation pattern can be the same as present in the wild-type AniAmy1 or SocAmy1. Alternatively, the host organism can be an algal, bacterial, yeast or plant expression host.

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

Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, e.g., lipofection mediated and DEAE-Dextrin mediated transfection; incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art. *See, e.g.*, Sambrook

et al. (2001), *supra*. The expression of heterologous protein in *Trichoderma* is described, for example, in U.S. Patent No. 6,022,725. Reference is also made to Cao et al. (2000) *Science* 9: 991-1001 for transformation of *Aspergillus* strains. Genetically stable transformants can be constructed with vector systems whereby the nucleic acid encoding a chimeric alpha-amylase is stably integrated into a host cell chromosome. Transformants are then selected and purified by known techniques.

The preparation of *Trichoderma* sp. for transformation, for example, may involve the preparation of protoplasts from fungal mycelia. *See* Campbell et al. (1989) *Curr. Genet.* 16: 53-56. The mycelia can be obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall, resulting in protoplasts. The protoplasts are protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M, e.g., a 1.2 M solution of sorbitol can be used in the suspension medium.

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

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

### 3.3. Expression

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

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of a chimeric alpha-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).

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

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, in some cases, concentrating the clarified broth. Further processes may include 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.

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

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

An expression host also can be cultured in the appropriate medium for the host, under aerobic conditions. Shaking or a combination of agitation and aeration can be provided, with production occurring at the appropriate temperature for that host, e.g., from about 25°C to about 75°C (e.g., 30°C to 45°C), depending on the needs of the host and production of the desired chimeric alpha-amylase. Culturing can occur from about 12 to about 100 hours or greater (and any hour value there between, *e.g.*, from 24 to 72 hours). Typically, the culture broth is at a pH of about 4.0 to about 8.0, again depending on the culture conditions needed for the host relative to production of a chimeric alpha-amylase or variant thereof.

### 3.4. Identification of the Expression and Enzymatic Activity of Chimeric Alpha-Amylases

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

### 3.5. Methods for Purifying Chimeric Alpha-Amylases

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

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.

It is desirable to concentrate a chimeric alpha-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.

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.

The enzyme solution is concentrated into a concentrated enzyme solution until the enzyme activity of the concentrated chimeric alpha-amylase polypeptide-containing solution is at a desired level.

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.

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

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 chimeric alpha-amylase polypeptide and on its concentration in the concentrated enzyme solution.

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

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.

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

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.

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.

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.

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.

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.

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.

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.

During fermentation, a chimeric alpha-amylase polypeptide accumulates in the culture broth. For the isolation and purification of the desired chimeric alpha-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.

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).

A more specific example of purification, is described in Sumitani et al. (2000) "New type of starch-binding domain: the direct repeat motif in the C-terminal region of *Bacillus* sp. 195 α-amylase contributes to starch binding and raw starch degrading," *Biochem. J.* 350: 477-484, and is briefly summarized here. The enzyme obtained from 4 liters of a *Streptomyces lividans* TK24 culture supernatant was treated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered by centrifugation at 10,000 × g (20 min. and 4°C) and re-dissolved in 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. The solubilized precipitate was then dialyzed against the same buffer. The dialyzed sample was then applied to a Sephacryl S-200 column, which had previously been equilibrated with 20 mM Tris/HCl buffer, (pH 7.0), 5 mM $CaCl_2$, and eluted at a linear flow rate of 7 mL/hr with the same buffer. Fractions from the column were collected and assessed for activity as judged by enzyme assay and SDS-PAGE. The protein was further purified as follows. A Toyopearl HW55 column (Tosoh Bioscience, Montgomeryville, PA; Cat. No. 19812) was equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 1.5 to 0 M $(NH_4)_2SO_4$ in 20 mM Tris/HCL buffer, pH 7.0 containing 5 mM $CaCl_2$. The active fractions were collected, and the enzyme precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was recovered, re-dissolved, and dialyzed as described above. The dialyzed sample was then applied to a Mono Q HR5/5 column (Amersham Pharmacia; Cat. No. 17-5167-01) previously equilibrated with 20 mM Tris/HCl buffer (pH 7.0) containing 5 mM $CaCl_2$, at a flow rate of 60 mL/hour. The active fractions are collected and added to a 1.5 M $(NH_4)_2SO_4$ solution. The active enzyme fractions were re-chromatographed on a Toyopearl HW55 column, as before,

to yield a homogeneous enzyme as determined by SDS-PAGE. *See* Sumitani et al. (2000) *Biochem. J.* 350: 477-484, for general discussion of the method and variations thereon.

For production scale recovery, a chimeric alpha-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. Compositions and Uses of Chimeric Alpha-Amylases

Chimeric alpha-amylases are useful for a variety of industrial applications. For example, chimeric alpha-amylases are useful in a starch conversion process, including but not limited to saccharification processes (at or below the gelatinization temperature of starch), simultaneous saccharification and fermentation (SSF), and direct conversion of starch to glucose. Chimeric alpha-amylases can be particularly useful in a saccharification process of a starch that has undergone liquefaction. The desired end-product may be any product that may be produced by the enzymatic conversion of the starch substrate. The end product can be alcohol, or optionally ethanol. The end product also can be organic acids, amino acids, biofuels, and other biochemical, including, but not limited to, ethanol, citric acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol, and biodiesel. For example, the desired product may be a syrup comprising glucose and maltose, which can be used in other processes, such as the preparation of HFCS, or which can be converted into a number of other useful products, such as ascorbic acid intermediates (e.g., gluconate; 2-keto-L-gulonic acid; 5-keto-gluconate; and 2,5-diketogluconate); 1,3-propanediol; aromatic amino acids (e.g., tyrosine, phenylalanine and tryptophan); organic acids (e.g., lactate, pyruvate, succinate, isocitrate, and oxaloacetate); amino acids (e.g., serine and glycine); antibiotics; antimicrobials; enzymes; vitamins; and hormones.

The starch conversion process may be a precursor to, or simultaneous with, a fermentation process designed to produce alcohol for fuel or for drinking (i.e., potable alcohol). One skilled in the art is aware of various fermentation conditions that may be used in the production of these end-products. Chimeric alpha-amylases also are useful in compositions and methods of food preparation. These various uses of chimeric alpha-amylases are described in more detail below.

### 4.1. Preparation of Starch Substrates

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

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

### 4.2. Gelatinization and Liquefaction of Starch

As used herein, the term "liquefaction" or "liquefy" means a process by which starch is converted to less viscous and shorter chain dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of an alpha-amylase, although additional liquefaction-inducing enzymes optionally may be added. In some embodiments, the starch substrate prepared as described above is slurried with water. The starch

slurry may contain starch as a weight percent of dry solids of about 10-55%, about 20-45%, about 30-45%, about 30-40%, or about 30-35%. Alpha-Amylase (EC 3.2.1.1) may be added to the slurry, with a metering pump, for example. The alpha-amylase typically used for this application is a thermally stable, bacterial alpha-amylase, such as a *Geobacillus stearothermophilus* alpha-amylase. The alpha-amylase is usually supplied, for example, at about 1500 units per kg dry matter of starch. To optimize alpha-amylase stability and activity, the pH of the slurry typically is adjusted to about pH 5.5-6.5 and about 1 mM of calcium (about 40 ppm free calcium ions) typically is added. *Geobacillus stearothermophilus* variants or other alpha-amylases may require different conditions. Bacterial alpha-amylase remaining in the slurry following liquefaction may be deactivated via a number of methods, including lowering the pH in a subsequent reaction step or by removing calcium from the slurry in cases where the enzyme is dependent upon calcium.

The slurry of starch plus the alpha-amylase may be pumped continuously through a jet cooker, which is steam heated to 105°C. Gelatinization occurs rapidly under these conditions, and the enzymatic activity, combined with the significant shear forces, begins the hydrolysis of the starch substrate. The residence time in the jet cooker is brief. The partly gelatinized starch may be passed into a series of holding tubes maintained at 105-110°C and held for 5-8 min. to complete the gelatinization process ("primary liquefaction"). Hydrolysis to the required DE is completed in holding tanks at 85-95°C or higher temperatures for about 1 to 2 hours ("secondary liquefaction"). These tanks may contain baffles to discourage back mixing. As used herein, the term "minutes of secondary liquefaction" refers to the time that has elapsed from the start of secondary liquefaction to the time that the Dextrose Equivalent (DE) is measured. The slurry is then allowed to cool to room temperature. This cooling step can be 30 minutes to 180 minutes, e.g. 90 minutes to 120 minutes.

The liquefied starch resulting from the process above typically contains about 98% oligosaccharides and about 2% maltose and 0.3% D-glucose. The liquefied starch typically is in the form of a slurry having a dry solids content (w/w) of about 10-50%; about 10-45%; about 15-40%; about 20-40%; about 25-40%; or about 25-35%.

Chimeric alpha-amylases can be used in the process of low temperature starch hydrolyisis instead of bacterial alpha-amylases. During low temperature starch hydrolysis, the starch granules are kept below its gelatinization temperature, so that the chimeric alpha-amylases can act on non-swollen starch granules. Low temperature liquefaction with chimeric alpha-amylases advantageously can be conducted at a lower pH, eliminating the requirement to

adjust the pH to about pH 5.5-6.5. Chimeric alpha-amylases thereof can be used for liquefaction at a pH range of 2 to 7, e.g., pH 3.0 – 7.5, pH 4.0 – 6.0, or pH 4.5 – 5.8.

### 4.3. Saccharification

The liquefied starch can be saccharified into a syrup rich in lower DP (e.g., DP1 + DP2) saccharides, using the chimeric alpha-amylase, optionally in the presence of another enzyme(s). This liquefied starch (liquidfact) may contain liquefied starch and residual starch. This residual or recalcitrant starch also may be a substrate for chimeric alpha-amylases. The exact composition of the products of saccharification depends on the combination of enzymes used, as well as the type of granular starch processed.

Whereas liquefaction is generally run as a continuous process, saccharification is often conducted as a batch process. Saccharification typically is most effective at temperatures of about 60-65°C and a pH of about 4.0-4.5, e.g., pH 4.3, necessitating cooling and adjusting the pH of the liquefied starch. Saccharification may be performed, for example, at a temperature between about 30°C, about 40°C, about 50°C, or about 55°C to about 60°C or about 65°C. Saccharification is normally conducted in stirred tanks, which may take several hours to fill or empty. Enzymes typically are added either at a fixed ratio to dried solids as the tanks are filled or added as a single dose at the commencement of the filling stage. A saccharification reaction to make a syrup typically is run over about 24-72 hours, for example, 24-48 hours. When a maximum or desired DE has been attained, the reaction is stopped by heating to 85°C for 5 min., for example. Further incubation will result in a lower DE, eventually to about 90 DE, as accumulated glucose re-polymerizes to isomaltose and/or other reversion products via an enzymatic reversion reaction and/or with the approach of thermodynamic equilibrium. When using a chimeric alpha-amylase, saccharification optimally is conducted at a temperature range of about 30°C to about 65°C, e.g., 47°C – 60°C. The saccharifying may be conducted over a pH range of about pH 2.0 to about pH 7.0, e.g., pH 3.5 – pH 7.0, pH 4.0 – pH 7.0, pH 3.5, pH 3.8, or pH 4.5.

A chimeric alpha-amylase also may be added to the slurry in the form of a composition. The chimeric alpha-amylase can be added to a slurry of a granular starch substrate in an amount of about 0.6 – 10 ppm ds, e.g., 2 ppm ds. The chimeric alpha-amylase can be added as a whole broth, clarified, partially purified, or purified enzyme. The specific activity of the purified chimeric alpha-amylase may be about 300 to 500 U/mg of enzyme, for example, measured with the PAHBAH assay. The chimeric alpha-amylase also can be added as a whole broth product.

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

### 4.4. Isomerization

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

### 4.5. Fermentation

The soluble starch hydrolysate, particularly a glucose rich syrup, can be fermented by contacting the starch hydrolysate with a fermenting organism typically at a temperature around 32°C, such as from 28°C to 65°C. EOF products include metabolites. The end product can be alcohol, or optionally ethanol. The end product also can be organic acids, amino acids, biofuels,

other biochemicals, and biochemical intermediates, including, but not limited to, ethanol, citric acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol, ethylene, and biodiesel.

Ethanologenic microorganisms include yeast, such as *Saccharomyces cerevisiae* and bacteria, e.g., *Zymomonas moblis*, expressing alcohol dehydrogenase and pyruvate decarboxylase. The ethanologenic microorganism can express xylose reductase and xylitol dehydrogenase, which convert xylose to xylulose. Improved strains of ethanologenic microorganisms, which can withstand higher temperatures, for example, are known in the art and can be used. *See* Liu et al. (2011) *Sheng Wu Gong Cheng Xue Bao* 27(7): 1049-56. Commercial sources of yeast include ETHANOL RED® (LeSaffre); Thermosacc® (Lallemand); RED STAR® (Red Star); FERMIOL® (DSM Specialties); and SUPERSTART® (Alltech). Useful microorganisms may be butanologenic. Butanologenic microorganisms may include, for example, butanologenic *Clostridia*, such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharobutylacetonicum*. *See, e.g.*, Ezeji et al. (2007) "Bioproduction of butanol from biomass: from genes to bioreactors," *Curr. Opin. Biotechnol.* 18(3): 220-27. Microorganisms that produce other metabolites, such as citric acid and lactic acid, by fermentation are also known in the art. *See, e.g.*, Papagianni (2007) "Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling," *Biotechnol. Adv.* 25(3): 244-63; John et al. (2009) "Direct lactic acid fermentation: focus on simultaneous saccharification and lactic acid production," *Biotechnol. Adv.* 27(2): 145-52.

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

As mentioned above, an SSF process can be conducted with fungal cells that express and secrete a chimeric alpha-amylase continuously throughout SSF. The fungal cells expressing the

chimeric alpha-amylase also can be the fermenting microorganism, e.g., an ethanologenic microorganism. Ethanol production thus can be carried out using a fungal cell that expresses sufficient chimeric alpha-amylase so that less or no enzyme has to be added exogenously. The fungal host cell can be from an appropriately engineered fungal strain. Fungal host cells that express and secrete other enzymes, in addition to the chimeric alpha-amylase also can be used. Such cells may express glucoamylase and/or a pullulanase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, protease, β-amylase, alpha-amylase, protease, cellulase, hemicellulase, lipase, cutinase, trehalase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, lyase, or other hydrolases, another enzyme, or a combination thereof. See e.g., WO 2009/099783.

A variation on this process is a "fed-batch fermentation" system, where the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression may inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. The actual substrate concentration in fed-batch systems is estimated by the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases, such as $CO_2$. Batch and fed-batch fermentations are common and well known in the art.

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

### 4.6. Compositions Comprising A Chimeric Alpha-Amylase

A chimeric alpha-amylase may be combined with a glucoamylase (EC 3.2.1.3), e.g., a *Trichoderma* glucoamylase or variant thereof. An exemplary glucoamylase is *Trichoderma reesei* glucoamylase (TrGA) and variants thereof that possess superior specific activity and thermal stability. *See* U.S. Published Applications Nos. 2006/0094080, 2007/0004018, and 2007/0015266 (Danisco US Inc.). Suitable variants of TrGA include those with glucoamylase

activity and at least 80%, at least 90%, or at least 95% sequence identity to wild-type TrGA. The chimeric alpha-amylase advantageously may increase the yield of glucose produced in a saccharification process catalyzed by TrGA.

Alternatively, the glucoamylase may be another glucoamylase derived from plants, fungi, algae, or bacteria. For example, the glucoamylases may be *Aspergillus niger* G1 or G2 glucoamylase or its variants (e.g., Boel et al. (1984) *EMBO J.* 3: 1097-1102; WO 92/00381; WO 00/04136 (Novo Nordisk A/S)); and *A. awamori* glucoamylase (e.g., WO 84/02921 (Cetus Corp.)). Other contemplated *Aspergillus* glucoamylase include variants with enhanced thermal stability, e.g., G137A and G139A (Chen et al. (1996) *Prot. Eng.* 9: 499-505); D257E and D293E/Q (Chen et al. (1995) *Prot. Eng.* 8: 575-582); N182 (Chen et al. (1994) *Biochem. J.* 301: 275-281); A246C (Fierobe et al. (1996) *Biochemistry*, 35: 8698-8704); and variants with Pro residues in positions A435 and S436 (Li et al. (1997) *Protein Eng.* 10: 1199-1204). Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *T. emersonii* (e.g., WO 99/28448 (Novo Nordisk A/S), *T. leycettanus* (e.g., U.S. Patent No. RE 32,153 (CPC International, Inc.)), *T. duponti*, or *T. thermophilus* (e.g., U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (e.g., EP 135,138 (CPC International, Inc.) and *C. thermohydrosulfuricum* (e.g., WO 86/01831 (Michigan Biotechnology Institute)). Suitable glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase shown in SEQ ID NO: 2 in WO 00/04136 (Novo Nordisk A/S). Also suitable are commercial glucoamylases, such as AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (Novozymes); OPTIDEX® 300 and OPTIDEX L-400 (Danisco US Inc.); AMIGASE™ and AMIGASE™ PLUS (DSM); G-ZYME® G900 (Enzyme Bio-Systems); and G-ZYME® G990 ZR (*A. niger* glucoamylase with a low protease content). Still other suitable glucoamylases include *Aspergillus fumigatus* glucoamylase, *Talaromyces* glucoamylase, *Thielavia* glucoamylase, *Trametes* glucoamylase, *Thermomyces* glucoamylase, *Athelia* glucoamylase, or *Humicola* glucoamylase (e.g., HgGA). Glucoamylases typically are added in an amount of about 0.1 – 2 glucoamylase units (GAU)/g ds, e.g., about 0.16 GAU/g ds, 0.23 GAU/g ds, or 0.33 GAU/g ds.

In particular, glucoamylases as contemplated herein may be used for starch conversion processes, and particularly in the production of dextrose for fructose syrups, specialty sugars and in alcohol and other end products (e.g., organic acids, amino acids, biofuels, and other biochemical) production from fermentation of starch containing substrates (e.g., G.M.A. van

Beynum et al., Eds. (1985) STARCH CONVERSION TECHNOLOGY, Marcel Dekker Inc. NY; see also U.S. Patent No. 8,178,326). The contemplated glucoamylase variant may also work synergistically with plant enzymes that are endogenously produced or genetically engineered. Additionally, the contemplated glucoamylase variant can work synergistically with endogenous, engineered, secreted, or non-secreted enzymes from a host producing the desired end product (*e.g.*, organic acids, amino acids, biofuels, other biochemicals, and biochemical intermediates, including, but not limited to, ethanol, citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, itaconic acid and other carboxylic acids, glucono delta-lactone, sodium erythorbate, lysine, omega 3 fatty acid, butanol, isoprene, 1,3-propanediol, ethylene, and biodiesel). Furthermore, the host cells expressing the contemplated glucoamylase variant may produce biochemicals in addition to enzymes used to digest the various feedstock(s). Such host cells may be useful for fermentation or simultaneous saccharification and fermentation processes to reduce or eliminate the need to add enzymes.

Other suitable enzymes that can be used with chimeric alpha-amylases include another glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, phytase, protease, pullulanase, β-amylase, α-amylase, protease, cellulase, hemicellulase, lipase, cutinase, trehalase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, lyase, or other hydrolases, or a combination thereof. See e.g., WO 2009/099783. For example, a debranching enzyme, such as an isoamylase (EC 3.2.1.68), may be added in effective amounts well known to the person skilled in the art. A pullulanase (EC 3.2.1.41), e.g., Promozyme®, is also suitable. Pullulanase typically is added at 100 U/kg ds. Further suitable enzymes include proteases, such as fungal, yeast, and bacterial proteases, plant proteases, and algal proteases. Fungal proteases include those obtained from *Aspergillus*, such as *A. niger*, *A. awamori*, *A. oryzae*; *Mucor* (e.g., *M. miehei*); *Rhizopus*; and *Trichoderma*.

β-Amylases (EC 3.2.1.2) are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-α-glucosidic linkages in amylopectin and related glucose polymers, thereby releasing maltose. β-Amylases have been isolated from various plants and microorganisms. *See* Fogarty et al. (1979) in PROGRESS IN INDUSTRIAL MICROBIOLOGY, Vol. 15, pp. 112-115. These β-Amylases have optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from about 4.5 to about 7.0. Contemplated β-amylases include, but are not limited to, β-amylases from barley Spezyme® BBA 1500, Spezyme® DBA, Optimalt™ ME, Optimalt™ BBA (Danisco US Inc.); and Novozym™ WBA (Novozymes A/S) .

## 5. Compositions and Methods for Baking and Food Preparation

The present embodiments also relate to a "food composition," including but not limited to a food product, animal feed and/or food/feed additives, comprising a chimeric alpha-amylase, and methods for preparing such a food composition comprising mixing the chimeric alpha-amylase with one or more food ingredients, or uses thereof.

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

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

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

A chimeric alpha-amylase further can be added alone or in a combination with other amylases to prevent or retard staling, i.e., crumb firming of baked products. The amount of anti-staling amylase will typically be in the range of 0.01-10 mg of enzyme protein per kg of flour, e.g., 0.5 mg/kg ds. Additional anti-staling amylases that can be used in combination with achimeric alpha-amylase include an endo-amylase, e.g., a bacterial endo-amylase from *Bacillus*. The additional amylase can be another maltogenic alpha-amylase (EC 3.2.1.133), e.g., from

*Bacillus*. Novamyl® is an exemplary maltogenic alpha-amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen et al. (1997) *Starch* 50: 39-45. Other examples of anti-staling endo-amylases include bacterial alpha-amylases derived from *Bacillus*, such as *B. licheniformis* or *B. amyloliquefaciens*. The anti-staling amylase may be an exo-amylase, such as β-amylase, e.g., from plant sources, such as soy bean, or from microbial sources, such as *Bacillus*.

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

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

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

The dough may also comprise other conventional dough ingredients, e.g., proteins, such as milk powder, gluten, and soy; eggs (e.g., whole eggs, egg yolks or egg whites); an oxidant, such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; or a salt, such as sodium

chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough further may comprise fat, e.g., triglyceride, such as granulated fat or shortening. The dough further may comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin. In particular, the dough can be made without addition of emulsifiers.

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

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

The xylanase is typically of microbial origin, e.g., derived from a bacterium or fungus, such as a strain of *Aspergillus*. Mammalian or plant derived xylanase is also envisioned. Xylanases include Pentopan® and Novozym 384®, for example, which are commercially available xylanase preparations produced from *Trichoderma reesei*. The amyloglucosidase may be an *A. niger* amyloglucosidase (such as AMG®). Other useful amylase products include Grindamyl® A 1000 or A 5000 (Grindsted Products, Denmark) and Amylase® H or Amylase® P (DSM). The glucose oxidase may be a fungal glucose oxidase, in particular an *Aspergillus niger* glucose oxidase (such as Gluzyme®). An exemplary protease is Neutrase®.

The process may be used for any kind of baked product prepared from dough, either of a soft or a crisp character, either of a white, light or dark type. Examples are bread, particularly white, whole-meal or rye bread, typically in the form of loaves or rolls, such as, but not limited

to, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

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

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

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

The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the alpha-amylase particles in such a fashion that the lipid material covers at least a portion of the surface of at least a majority, e.g., 100% of the alpha-amylase particles. Thus, each alpha-

amylase particle is individually enveloped in a lipid. For example, all or substantially all of the alpha-amylase particles are provided with a thin, continuous, enveloping film of lipid. This can be accomplished by first pouring a quantity of lipid into a container, and then slurrying the alpha-amylase particles so that the lipid thoroughly wets the surface of each alpha-amylase particle. After a short period of stirring, the enveloped alpha-amylase particles, carrying a substantial amount of the lipids on their surfaces, are recovered. The thickness of the coating so applied to the particles of alpha-amylase can be controlled by selection of the type of lipid used and by repeating the operation in order to build up a thicker film, when desired.

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

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

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

The loaded delivery vehicle, i.e., the lipid-enveloped enzyme, is added to the ingredients used to prepare a baked good in an effective amount to extend the shelf-life of the baked good. The baker computes the amount of enveloped alpha-amylase, prepared as discussed above, that will be required to achieve the desired anti-staling effect. The amount of the enveloped alpha-amylase required is calculated based on the concentration of enzyme enveloped and on the proportion of alpha-amylase to flour specified. A wide range of concentrations has been found

to be effective, although, as has been discussed, observable improvements in anti-staling do not correspond linearly with the alpha-amylase concentration, but above certain minimal levels, large increases in alpha-amylase concentration produce little additional improvement. The alpha-amylase concentration actually used in a particular bakery production could be much higher than the minimum necessary to provide the baker with some insurance against inadvertent under-measurement errors by the baker. The lower limit of enzyme concentration is determined by the minimum anti-staling effect the baker wishes to achieve.

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

The enveloped alpha-amylase can be added to the dough during the mix cycle, e.g., near the end of the mix cycle. The enveloped alpha-amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped alpha-amylase throughout the dough; however, the mixing stage is terminated before the protective coating becomes stripped from the alpha-amylase particle(s). Depending on the type and volume of dough, and mixer action and speed, anywhere from one to six minutes or more might be required to mix the enveloped alpha-amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped alpha-amylase should have a total volume sufficient to allow the enveloped alpha-amylase to be spread throughout the dough mix. If the preparation of enveloped alpha-amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped alpha-amylase is added to the dough. Recipes and production processes may require specific modifications; however, good results generally can be achieved when 25% of the oil specified in a bread dough formula is held out of the dough and is used as a carrier for a concentrated enveloped alpha-amylase when added near the end of the mix cycle. In bread or other baked goods, particularly those having a low fat content, e.g., French-style breads, an enveloped alpha-amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped alpha-amylase properly with the dough. The range of suitable percentages is wide and depends on the formula, finished product, and production methodology requirements of the individual baker. Second, the enveloped alpha-

amylase suspension should be added to the mix with sufficient time for complete mixture into the dough, but not for such a time that excessive mechanical action strips the protective lipid coating from the enveloped alpha-amylase particles.

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

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

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

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

The terms "animal feed composition," "feedstuff" and "fodder" are used interchangeably and may comprise one or more feed materials selected from the group comprising a) cereals, such as small grains (e.g., wheat, barley, rye, oats and combinations thereof) and/or large grains

such as maize or sorghum; b) by products from cereals, such as corn gluten meal, Distillers Dried Grain Solubles (DDGS) (particularly corn based Distillers Dried Grain Solubles (cDDGS), wheat bran, wheat middlings, wheat shorts, rice bran, rice hulls, oat hulls, palm kernel, and citrus pulp; c) protein obtained from sources such as soya, sunflower, peanut, lupin, peas, fava beans, cotton, canola, fish meal, dried plasma protein, meat and bone meal, potato protein, whey, copra, sesame; d) oils and fats obtained from vegetable and animal sources; e) minerals and vitamins.

### 6. Textile Desizing Compositions and Use

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

A chimeric alpha-amylase can be applied during or after the weaving of a textile, or during the desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives to increase their tensile strength and to prevent breaking. A chimeric alpha-amylase can be applied during or after the weaving to remove these sizing starch or starch derivatives. After weaving, a chimeric alpha-amylase can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

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

## 7. Cleaning Compositions

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

### 7.1. Overview

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

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.

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.

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).

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

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.

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.

The detergent may contain a bleaching system, which may comprise a $H_2O_2$ source such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids (*e.g.,* the amide, imide, or sulfone type peroxyacids). The bleaching system can also be an enzymatic bleaching system,

for example, perhydrolase, such as that described in International PCT Application WO 2005/056783.

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.

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.

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

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

### 7.2. Heavy Duty Liquid (HDL) Laundry Detergent Composition

Exemplary HDL laundry detergent compositions includes a detersive surfactant (10%-40% wt/wt), including an anionic detersive surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates, and/or mixtures thereof), and optionally non-ionic surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl alkoxylated alcohol, for example a $C_8$-$C_{18}$ alkyl ethoxylated alcohol and/or $C_6$-$C_{12}$ alkyl phenol alkoxylates), wherein the weight ratio of anionic detersive surfactant (with a hydrophilic index (HIc) of from 6.0 to 9) to non-ionic detersive surfactant is greater than 1: 1. Suitable detersive surfactants also include cationic detersive surfactants (selected from a group of alkyl pyridinium compounds, alkyl quarternary ammonium compounds, alkyl quarternary phosphonium compounds, alkyl ternary sulphonium compounds, and/or mixtures thereof); zwitterionic and/or amphoteric detersive surfactants (selected from a group of alkanolamine sulpho-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof.

The composition may optionally include, a surfactancy boosting polymer consisting of amphiphilic alkoxylated grease cleaning polymers (selected from a group of alkoxylated polymers having branched hydrophilic and hydrophobic properties, such as alkoxylated

polyalkylenimines in the range of 0.05wt%-10wt%) and/or random graft polymers (typically comprising of hydrophilic backbone comprising monomers selected from the group consisting of: unsaturated $C_1$-$C_6$ carboxylic acids, ethers, alcohols, aldehydes, ketones, esters, sugar units, alkoxy units, maleic anhydride, saturated polyalcohols such as glycerol, and mixtures thereof; and hydrophobic side chain(s) selected from the group consisting of: $C_4$-$C_{25}$ alkyl group, polypropylene, polybutylene, vinyl ester of a saturated $C_1$-$C_6$ mono-carboxylic acid, $C_1$-$C_6$ alkyl ester of acrylic or methacrylic acid, and mixtures thereof.

The composition may include additional polymers such as soil release polymers (include anionically end-capped polyesters, for example SRP1, polymers comprising at least one monomer unit selected from saccharide, dicarboxylic acid, polyol and combinations thereof, in random or block configuration, ethylene terephthalate-based polymers and co-polymers thereof in random or block configuration, for example Repel-o-tex SF, SF-2 and SRP6, Texcare SRA100, SRA300, SRN100, SRN170, SRN240, SRN300 and SRN325, Marloquest SL), anti-redeposition polymers (0.1 wt% to 10wt%, include carboxylate polymers, such as polymers comprising at least one monomer selected from acrylic acid, maleic acid (or maleic anhydride), fumaric acid, itaconic acid, aconitic acid, mesaconic acid, citraconic acid, methylenemalonic acid, and any mixture thereof, vinylpyrrolidone homopolymer, and/or polyethylene glycol, molecular weight in the range of from 500 to 100,000 Da); cellulosic polymer (including those selected from alkyl cellulose, alkyl alkoxyalkyl cellulose, carboxyalkyl cellulose, alkyl carboxyalkyl cellulose examples of which include carboxymethyl cellulose, methyl cellulose, methyl hydroxyethyl cellulose, methyl carboxymethyl cellulose, and mixures thereof) and polymeric carboxylate (such as maleate/acrylate random copolymer or polyacrylate homopolymer).

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

The composition may further include dye transfer inhibiting agents, examples of which include manganese phthalocyanine, peroxidases, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole,

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

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

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

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

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

Exemplary HDD laundry detergent compositions includes a detersive surfactant, including anionic detersive surfactants (e.g., linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates and/or mixtures thereof), non-ionic detersive surfactant

(e.g., linear or branched or random chain, substituted or unsubstituted $C_8$-$C_{18}$ alkyl ethoxylates, and/or $C_6$-$C_{12}$ alkyl phenol alkoxylates), cationic detersive surfactants (e.g., alkyl pyridinium compounds, alkyl quaternary ammonium compounds, alkyl quaternary phosphonium compounds, alkyl ternary sulphonium compounds, and mixtures thereof), zwitterionic and/or amphoteric detersive surfactants (e.g., alkanolamine sulpho-betaines), ampholytic surfactants, semi-polar non-ionic surfactants, and mixtures thereof; builders including phosphate free builders (for example zeolite builders examples which include zeolite A, zeolite X, zeolite P and zeolite MAP in the range of 0wt% to less than 10wt%), phosphate builders (for example sodium tri-polyphosphate in the range of 0wt% to less than 10wt%), citric acid, citrate salts and nitrilotriacetic acid, silicate salt (e.g., sodium or potassium silicate or sodium meta-silicate in the range of 0wt% to less than 10wt%, or layered silicate (SKS-6)); carbonate salt (e.g., sodium carbonate and/or sodium bicarbonate in the range of 0 wt% to less than 80 wt%); and bleaching agents including photobleaches (e.g., sulfonated zinc phthalocyanines, sulfonated aluminum phthalocyanines, xanthenes dyes, and mixtures thereof) hydrophobic or hydrophilic bleach activators (e.g., dodecanoyl oxybenzene sulfonate, decanoyl oxybenzene sulfonate, decanoyl oxybenzoic acid or salts thereof, 3,5,5-trimethy hexanoyl oxybenzene sulfonate, tetraacetyl ethylene diamine-TAED, nonanoyloxybenzene sulfonate-NOBS, nitrile quats, and mixtures thereof), sources of hydrogen peroxide (e.g., inorganic perhydrate salts examples of which include mono or tetra hydrate sodium salt of perborate, percarbonate, persulfate, perphosphate, or persilicate), preformed hydrophilic and/or hydrophobic peracids (e.g., percarboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts, and mixtures thereof), and/or bleach catalysts (e.g., imine bleach boosters (examples of which include iminium cations and polyions), iminium zwitterions, modified amines, modified amine oxides, N-sulphonyl imines, N-phosphonyl imines, N-acyl imines, thiadiazole dioxides, perfluoroimines, cyclic sugar ketones, and mixtures thereof, and metal-containing bleach catalysts (e.g., copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations along with an auxiliary metal cations such as zinc or aluminum and a sequestrate such as ethylenediaminetetraacetic acid, ethylenediaminetetra(methylenephosphonic acid), and water-soluble salts thereof).

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

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

### 7.4. Automatic Dishwashing (ADW) Detergent Composition

Exemplary ADW detergent composition includes non-ionic surfactants, including ethoxylated non-ionic surfactants, alcohol alkoxylated surfactants, epoxy-capped poly(oxyalkylated) alcohols, or amine oxide surfactants present in amounts from 0 to 10% by weight; builders in the range of 5-60% including phosphate builders (e.g., mono-phosphates, di-phosphates, tri-polyphosphates, other oligomeric-poylphosphates, sodium tripolyphosphate-STPP) and phosphate-free builders (e.g., amino acid-based compounds including methyl-glycine-diacetic acid (MGDA) and salts and derivatives thereof, glutamic-N,N-diacetic acid (GLDA) and salts and derivatives thereof, iminodisuccinic acid (IDS) and salts and derivatives thereof, carboxy methyl inulin and salts and derivatives thereof, nitrilotriacetic acid (NTA), diethylene triamine penta acetic acid (DTPA), B-alaninediacetic acid (B-ADA) and their salts, homopolymers and copolymers of poly-carboxylic acids and their partially or completely neutralized salts, monomeric polycarboxylic acids and hydroxycarboxylic acids and their salts in the range of 0.5% to 50% by weight; sulfonated/carboxylated polymers in the range of about 0.1 % to about 50% by weight to to provide dimensional stability; drying aids in the range of about 0.1 % to about 10% by weight (e.g., polyesters, especially anionic polyesters, optionally together with further monomers with 3 to 6 functionalities - typically acid, alcohol or ester functionalities which are conducive to polycondensation, polycarbonate-, polyurethane- and/or polyurea-polyorganosiloxane compounds or precursor compounds, thereof, particularly of the reactive cyclic carbonate and urea type); silicates in the range from about 1 % to about 20% by weight (including sodium or potassium silicates for example sodium disilicate, sodium meta-silicate and crystalline phyllosilicates); inorganic bleach (e.g., perhydrate salts such as perborate, percarbonate, perphosphate, persulfate and persilicate salts) and organic bleach (e.g., organic peroxyacids, including diacyl and tetraacylperoxides, especially diperoxydodecanedioc acid, diperoxytetradecanedioc acid, and diperoxyhexadecanedioc acid); bleach activators (i.e., organic peracid precursors in the range from about 0.1 % to about 10% by weight); bleach catalysts (e.g., manganese triazacyclononane and related complexes, Co, Cu, Mn, and Fe bispyridylamine and related complexes, and pentamine acetate cobalt(III) and related complexes); metal care agents

in the range from about 0.1% to 5% by weight (e.g., benzatriazoles, metal salts and complexes, and/or silicates); enzymes in the range from about 0.01 to 5.0 mg of active enzyme per gram of automatic dishwashing detergent composition (e.g., proteases, amylases, lipases, cellulases, choline oxidases, peroxidases/oxidases, pectate lyases, mannanases, cutinases, laccases, phospholipases, lysophospholipases, acyltransferase, perhydrolase, arylesterase, and mixtures thereof); and enzyme stabilizer components (e.g., oligosaccharides, polysaccharides, and inorganic divalent metal salts).

### 7.5. Additional Detergent Compositions

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

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

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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.

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%.

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%.

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.

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

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).

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.

As above, the present chimeric alpha-amylase 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.

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

The detergent composition may be formulated as a hand (manual) or machine (automatic) laundry detergent composition, including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated

as a detergent composition for use in general household hard surface cleaning operations, or be formulated for manual or automatic dishwashing operations.

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

*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.

*Lipases*: Suitable lipases include those of bacterial, fungal, plant, or animal 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).

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

*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.).

*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).

*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).

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

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.

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.

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.

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.

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").

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).

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.

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.

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

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.

### 7.6. Methods of Assessing Amylase Activity in Detergent Compositions

Numerous alpha-amylase cleaning assays are known in the art, including swatch and micro-swatch assays. In order to further illustrate the compositions and methods, and advantages thereof, the specific examples are given with the understanding that they are illustrative rather than limiting.

## 8. Brewing Compositions

A chimeric alpha-amylase may be a component of a brewing composition used in a process of providing a fermented beverage, such as brewing. It is believed that non-fermentable

carbohydrates form the majority of the dissolved solids in the final beer. This residue remains because of the inability of malt amylases to hydrolyze the alpha-1,6-linkages of the starch. The non-fermentable carbohydrates contribute about 50 calories per 12 ounces (about 340 grams) of beer. The chimeric alpha-amylase, usually in combination with a glucoamylase and optionally one or more other enzymes, assist in converting the starch into dextrins and fermentable sugars, lowering the residual non-fermentable carbohydrates in the final beer.

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

For a number of reasons, the malt, which is produced principally from selected varieties of barley, has an important effect on the overall character and quality of the beer. First, the malt is the primary flavoring agent in beer. Second, the malt provides the major portion of the fermentable sugar. Third, the malt provides the proteins, which will contribute to the body and foam character of the beer. Fourth, the malt provides the necessary enzymatic activity during mashing. Hops also contribute significantly to beer quality, including flavoring. In particular, hops (or hops constituents) add desirable bittering substances to the beer. In addition, the hops can act as protein precipitants, establish preservative agents and aid in foam formation and stabilization.

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

As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed

or coarsely ground forms. The methods described herein may be used to determine alpha-amylase activity levels in both flours and stock.

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

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

The brewing composition comprising the present chimeric alpha-amylase, often but not necessarily in combination with one or more exogenous enzymes, such as glucoamylase (s), optionally a pullulanase and/or isoamylase, and any combination thereof, may be added to the mash of step (a) above, such as during the preparation of the mash. Alternatively, or in addition, the brewing composition may be added to the mash of step (b) above, such as during the filtration of the mash. Alternatively, or in addition, the brewing composition may be added to the wort of step (c) above, such as during the fermenting of the wort.

One aspect of the embodiments relates to the use of the present chimeric alpha-amylase in the production of a fermented beverage, such as a beer.

Another aspect concerns a method of providing a fermented beverage comprising the step of contacting a mash and/or a wort with the present chimeric alpha-amylase.

A further aspect relates to a method or providing a fermented beverage comprising the steps of: (a) preparing a mash, (b) filtering the mash to obtain a wort, and (c) fermenting the wort to obtain a fermented beverage, such as a beer, wherein the chimeric alpha-amylase are added to: (i) the mash of step (a) and/or (ii) the wort of step (b) and/or (iii) the wort of step (c).

According to yet another aspect, a fermented beverage, such as a beer, is produced or provided by a method comprising the step(s) of (1) contact a mash and/or a wort with the chimeric alpha-amylase; and/or (2) (a) preparing a mash, (b) filtering the mash to obtain a wort, and (c) fermenting the wort to obtain a fermented beverage, such as a beer, wherein the chimeric alpha-amylase are added to: (i) the mash of step (a) and/or (ii) the wort of step (b) and/or (iii) the wort of step (c).

Particluar embodiments pertain to any of the above use, method, or fermented beverage, wherein said fermented beverage is a beer, such as full malted beer, beer brewed under the "Reinheitsgebot", ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, non-alcoholic malt liquor and the like, but also alternative cereal and malt beverages such as fruit flavored malt beverages, e.g., citrus flavoured, such as lemon-, orange-, lime-, or berry-flavoured malt beverages, liquor flavoured malt beverages, e.g., vodka-, rum-, or tequila-flavoured malt liquor, or coffee flavoured malt beverages, such as caffeine-flavoured malt liquor, and the like.

## 9. Reduction of Insoluble Starch

The present chimeric alpha-amylases may reduce the insoluble starch more efficiently and effectively, when used in a method of liquefaction and/or saccharification. One source of insoluble starch is from amylose that escapes hydrolysis and/or from retrograded starch polymer. Starch retrogradation occurs spontaneously in a starch paste, or gel on ageing, because of the tendency of starch molecules to bind to one another followed by an increase in crystallinity. Solutions of low concentration become increasingly cloudy due to the progressive association of starch molecules into larger particles. Spontaneous precipitation takes place and the precipitated starch appears to be reverting to its original condition of cold-water insolubility. Pastes of higher concentration on cooling set to a gel, which on ageing becomes steadily firmer due to the increasing association of the starch molecules. This arises because of the strong

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

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

## EXAMPLES

The following examples are provided in order to demonstrate and further illustrate certain representative embodiments and aspects of the present disclosure and are not to be construed as limiting the scope thereof.

### EXAMPLE 1 Amino acid and DNA sequences of the *Aspergillus niger* alpha-amylase *AniAmy1* and chimeric alpha-amylase *AniAmy1M*

*Aspergillus niger* was selected as a potential source for various glycosyl hydrolases and other enzymes, useful for industrial applications. The entire genomic sequence data of *A. niger* is available on the Internet at hypertext transfer protocol:// www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html. One of genes identified in *A. niger* encodes a glycosyl hydrolase with homology to various other fungal alpha-amylases as determined from a BLAST search. *See* Altschul et al. (1990) *J Mol Biol* 215: 403–410. The genomic sequence of this gene, the *AniAmy1* gene, is depicted as SEQ ID NO: 1, while the protein encoded by the *AniAmy1* gene is depicted as SEQ ID NO: 2. SEQ ID NO: 1, the nucleotide sequence with 8 introns of the *AniAmy1* gene, is shown below (introns are shown in lowercase)

ATGACAATCTTTCTGTTTCTGGCCATTTTCGTGGCTACAGCTCTGGCAGCCACGCCTGCAGAATGGCGCT
CCCAGTCGATATATTTCCTGCTCACCGATCGCTTTGCGCGAACGGATAATTCTACCACTGCTTCTTGTGA

CTTGAGCGCTCGGgttagtcacagcatgttctagaatctccaattgattcgctgacagatctagCAATAT
TGCGGTGGATCCTGGCAGGGCATCATCAATCAGgtcggtccgtccatcgttgcagcactatctacatcaa
cgtttgtttggcaaattaacatccattagCTGGACTATATTCAAGGAATGGGCTTTACAGCGATCTGGAT
CACACCCGTAACTGCACAGATCCCCCAAGATACTGGTTACGGACAGGCATATCACGGATACTGGCAGCAG
GACGCgtgagatgctacctctatcgcccggatgaatgtatatccttcttaccatgcagacagTTATGCCC
TGAACTCCCATTATGGTACGGCAGACGATCTCAAAGCTCTGGCTTCAGCTCTTCACTCACGGGGCATGTA
TCTCATGGTGGACGTTGTTGCCAATCACATGgtatgttcttagcctcccacgggaccttagctttatatc
tgacagcgatagGGCCACAATGGTACGGGGAGCTCTGTGGACTACAGTGTTTATAGGCCATTTAATTCGC
AAAAGTACTTTCACAACCTCTGTTGGATCTCTGATTACAATAACCAGACAAACGTTGAAGACTGCTGGCT
AGGCGATAACACCGTTGCCTTGCCGGATCTTGATACTACCAGTACGGAGGTGAAGAATATGTGGTATGAC
TGGGTCGAGTCTCTCGTCTCTAACTACTCCGgtaatcctacctttacttcgctattttctgcctcttatg
agacaaagactaacaaatatcaagTCGACGGCCTCCGCGTAGACACAGTCAAGAACGTACAGAAGAACTT
CTGGCCCGGCTACAACAATGCTTCAGGCGTGTACTGTATTGGAGAAGTCTTCGATGGGGACGCCTCATAC
ACCTGTCCTTATCAGGAAGACTTGGACGGAGTCCTTAATTACCCCATgtaagccctacatttaaccccat
tgaatgcttgccaacgacttgcaatagGTACTATCCACTCCTCCGGGCTTTCGAATCCACCAACGGCAGT
ATCAGCGACCTCTATAACATGATCAACACCGTGAAATCCACCTGCAGAGATTCTACGCTTCTAGGGACCT
TCGTCGAAAACCACGATAACCCACGCTTTGCCAAgtaagaatatcctctccgagttcaccattacaaaca
caagagctcacctcagaagCTACACAAGCGACATGTCCCTAGCCAAAAATGCCGCAACATTCACTATCCT
GGCTGACGGCATTCCCATCATATACGCCGGTCAGGAACAGCACTATAGCGGCGGTAATGACCCCTACAAC
CGCGAAGCGACCTGGCTTTCAGGCTACAAGACCACCAGCGAGCTCTACACCCATATCGCCGCATCGAACA
AGATTCGCACCCACGCTATAAAACAGGATACCGGATATCTCACCTACAAAgtaatcttcattcgagtcca
tgtgtggtacaatctatctgactagaacctattctagAACTACCCCATCTACCAAGACACCTCGACCCTT
GCCATGCGCAAAGGCTACAATGGCACCCAAACTATCACGGTCCTTTCTAACCTTGGCGCCTCGGGGTCCT
CATACACACTCTCCCTCCCAGGAACAGGCTACACAGCCGGCCAAAAGATTACTGAAATCTATACCTGCAC
GAATCTAACAGTCAACTCAAATGGCTCGGTGCCAGTACCCATGAAGAGCGGGTTACCGCGGATCCTCTAT
CCTGCAGATAAGTTGGTTAATGGAAGCTCATTTTGCAgttagttccctgcctatatgtttcaataagccg
tatttgcatgcgcgtccatcgcattgattctttatggattgatcaactgatacattcgcacagGCTCTGC
GATCACTGTTTTCAAGGATGCAGGAGGAGGCGTGTTGTTCTTTAGCTACACTGTGTAA

SEQ ID NO: 2, the amino acid sequence of the AniAmy1 protein, is shown below

*MTIFLFLAIFVATALA*ATPAEWRSQSIYFLLTDRFARTDNSTTASCDLSARQYCGGSWQGIINQLDYIQG
MGFTAIWITPVTAQIPQDTGYGQAYHGYWQQDAYALNSHYGTADDLKALASALHSRGMYLMVDVVANHMG
HNGTGSSVDYSVYRPFNSQKYFHNLCWISDYNNQTNVEDCWLGDNTVALPDLDTTSTEVKNMWYDWVESL
VSNYSVDGLRVDTVKNVQKNFWPGYNNASGVYCIGEVFDGDASYTCPYQEDLDGVLNYPMYYPLLRAFES
TNGSISDLYNMINTVKSTCRDSTLLGTFVENHDNPRFANYTSDMSLAKNAATFTILADGIPIIYAGQEQH
YSGGNDPYNREATWLSGYKTTSELYTHIAASNKIRTHAIKQDTGYLTYKNYPIYQDTSTLAMRKGYNGTQ
TITVLSNLGASGSSYTLSLPGTGYTAGQKITEIYTCTNLTVNSNGSVPVPMKSGLPRILYPADKLVNGSS
FCSSAITVFKDAGGGVLFFSYTV

At the N-terminus, AniAmy1 protein has a 16-amino-acid signal peptide (SEQ ID NO: 3, shown in italics) predicted by SignalP-NN. *See* Emanuelsson et al. (2007) *Nature Protocols* 2: 953-971. This suggests that AniAmy1 is a secreted glycosyl hydrolase.

The chimeric AniAmy1M contains the CBM20 domain of an alpha-amylase from *Aspergillus terreus* (SEQ IN NO: 14; NCBI Reference Number XP_001209405.1 (gi|115385717|ref|XP_001209405.1| alpha-amylase precursor [Aspergillus terreus NIH2624])) fused to the C-terminal of AniAmy1 protein. Residues 496-607 of SEQ ID NO: 14 are fused to the C-terminal of AniAmy1 protein (*i.e.*, residues 1 to 491 of SEQ ID NO: 2) to creat the chimeric AniAmy1M. The nucleotide sequence of AniAmy1M is depicted as SEQ ID NO: 4, while the amino acid sequence of AniAmy1M is depicted as SEQ ID NO: 5. SEQ ID NO: 4 is shown below (introns are shown in lowercase, and the nucleotide sequences encoding the CBM20 are shown in bold)

ATGACAATCTTTCTGTTTCTGGCCATTTTCGTGGCTACAGCTCTGGCAGCCACGCCTGCAGAATGGCGCT
CCCAGTCGATATATTTCCTGCTCACCGATCGCTTTGCGCGAACGGATAATTCTACCACTGCTTCTTGTGA
CTTGAGCGCTCGGgttagtcacagcatgttctagaatctccaattgattcgctgacagatctagCAATAT
TGCGGTGGATCCTGGCAGGGCATCATCAATCAGgtcggtccgtccatcgttgcagcactatctacatcaa
cgtttgtttggcaaattaacatccattagCTGGACTATATTCAAGGAATGGGCTTTACAGCGATCTGGAT
CACACCCGTAACTGCACAGATCCCCCAAGATACTGGTTACGGACAGGCATATCACGGATACTGGCAGCAG
GACGCgtgagatgctacctctatcgcccggatgaatgtatatccttcttaccatgcagacagTTATGCCC
TGAACTCCCATTATGGTACGGCAGACGATCTCAAAGCTCTGGCTTCAGCTCTTCACTCACGGGGCATGTA
TCTCATGGTGGACGTTGTTGCCAATCACATGgtatgttcttagcctcccacgggaccttagctttatatc
tgacagcgatagGGCCACAATGGTACGGGGAGCTCTGTGGACTACAGTGTTTATAGGCCATTTAATTCGC
AAAAGTACTTTCACAACCTCTGTTGGATCTCTGATTACAATAACCAGACAAACGTTGAAGACTGCTGGCT
AGGCGATAACACCGTTGCCTTGCCGGATCTTGATACTACCAGTACGGAGGTGAAGAATATGTGGTATGAC
TGGGTCGAGTCTCTCGTCTCTAACTACTCCGgtaatcctacctttacttcgctattttctgcctcttatg
agacaaagactaacaaatatcaagTCGACGGCCTCCGCGTAGACACAGTCAAGAACGTACAGAAGAACTT
CTGGCCCGGCTACAACAATGCTTCAGGCGTGTACTGTATTGGAGAAGTCTTCGATGGGGACGCCTCATAC
ACCTGTCCTTATCAGGAAGACTTGGACGGAGTCCTTAATTACCCCATgtaagccctacatttaaccccat
tgaatgcttgccaacgacttgcaatagGTACTATCCACTCCTCCGGGCTTTCGAATCCACCAACGGCAGT
ATCAGCGACCTCTATAACATGATCAACACCGTGAAATCCACCTGCAGAGATTCTACGCTTCTAGGGACCT
TCGTCGAAAACCACGATAACCCACGCTTTGCCAAgtaagaatatcctctccgagttcaccattacaaaca
caagagctcacctcagaagCTACACAAGCGACATGTCCCTAGCCAAAAATGCCGCAACATTCACTATCCT
GGCTGACGGCATTCCCATCATATACGCCGGTCAGGAACAGCACTATAGCGGCGGTAATGACCCCTACAAC
CGCGAAGCGACCTGGCTTTCAGGCTACAAGACCACCAGCGAGCTCTACACCCATATCGCCGCATCGAACA
AGATTCGCACCCACGCTATAAAACAGGATACCGGATATCTCACCTACAAAgtaatcttcattcgagtcca
tgtgtggtacaatctatctgactagaacctattctagAACTACCCCATCTACCAAGACACCTCGACCCTT

GCCATGCGCAAAGGCTACAATGGCACCCAAACTATCACGGTCCTTTCTAACCTTGGCGCCTCGGGGTCCT
CATACACACTCTCCCTCCCAGGAACAGGCTACACAGCCGGCCAAAAGATTACTGAAATCTATACCTGCAC
GAATCTAACAGTCAACTCAAATGGCTCGGTGCCAGTACCCATGAAGAGCGGGTTACCGCGGATCCTCTAT
CCTGCAGATAAGTTGGTTAATGGAAGCTCATTT**TGCAACACAGAATGCACGGCCGCCACGTCCATCTCTG**
**TTCTCTTCGAGGAACTGGTTACGACGACCTACGGGGAGAACATTTATCTGAGCGGCTCGATCAGCCAGCT**
**GGGTAGTTGGAATACGGCCTCGGCTGTTGCCCTGTCGGCGAGTCAATATACCTCGTCCAACCCGGAGTGG**
**TATGTGAGTGTGACCTTGCCTGTGGGCACGTCGTTCCAGTACAAGTTCATCAAGAAGGGGTCGGACGGAA**
**GTGTTGTGTGGGAGAGTGATCCGAACCGGTCGTATACCGTTCCGGCTGGGTGCGAGGGCGCGACGGTGAC**
**AGTTGCTGATACTTGGAGGTGA**

SEQ ID NO: 5, the amino acid sequence of the AniAmy1M protein, is shown below (the putative signal peptide is shown in italics, and the CBM20 is shown in bold)

*MTIFLFLAIFVATALA*ATPAEWRSQSIYFLLTDRFARTDNSTTASCDLSARQYCGGSWQGIINQLDYIQG
MGFTAIWITPVTAQIPQDTGYGQAYHGYWQQDAYALNSHYGTADDLKALASALHSRGMYLMVDVVANHMG
HNGTGSSVDYSVYRPFNSQKYFHNLCWISDYNNQTNVEDCWLGDNTVALPDLDTTSTEVKNMWYDWVESL
VSNYSVDGLRVDTVKNVQKNFWPGYNNASGVYCIGEVFDGDASYTCPYQEDLDGVLNYPMYYPLLRAFES
TNGSISDLYNMINTVKSTCRDSTLLGTFVENHDNPRFANYTSDMSLAKNAATFTILADGIPIIYAGQEQH
YSGGNDPYNREATWLSGYKTTSELYTHIAASNKIRTHAIKQDTGYLTYKNYPIYQDTSTLAMRKGYNGTQ
TITVLSNLGASGSSYTLSLPGTGYTAGQKITEIYTCTNLTVNSNGSVPVPMKSGLPRILYPADKLVNGSS
F**CNTECTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPEWYVSVTLPVGT**
**SFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

## Example 2 Expression and purification of AniAmy1 and AniAmy1M proteins

For expression, genomic DNA of *Aspergillus niger* ATCC#9142 was used for amplifying the *AniAmy1* gene for expression. The *AniAmy1* gene (SEQ ID NO: 1) was amplified using the following primers

Primer 1(Not I)

5'- ccgcggccgcaccATGACAATCTTTCTGTTTCTGG -3' (SEQ ID NO: 6), and

Primer 2 (Asc I)

5'- ccggcgcgcccttaTCACGTCATTATAGCAATAAGC-3' (SEQ ID NO: 7).

After digestion with Not I and Asc I, the PCR product was cloned into pTrex3gM expression vector (described in U.S. Published Patent Application No. 2011/0136197 A1) digested with the same restriction enzymes, resulting in plasmid pZZH371 (FIG. 1). The sequence of the *AniAmy1* gene within pZZH371was confirmed by DNA sequencing.

The CBM20 domain was amplified from an alpha-amylase from *Aspergillus terreus* (SEQ IN NO 14; NCBI Reference Number XP_001209405.1 (gi|115385717|ref|XP_001209405.1| alpha-amylase precursor [Aspergillus terreus NIH2624])) ) and fused with *AniAmy1* gene at the 3' end by overlap PCR to generate *AniAmy1M* gene (SEQ ID NO: 4). The *AniAmy1M* gene was amplified using the following primers

Primer 1(Not I)

5'- ccgcggccgcaccATGACAATCTTTCTGTTTCTGG -3' (SEQ ID NO: 6), and

Primer 3 (Asc I)

5'- ccggcgcgcccttaTCACCTCCAAGTATCAGCAAC -3'(SEQ ID NO: 8).

After digested with Not I and Asc I, the PCR product of the *AniAmy1M* gene was cloned into pTrex3gM expression vector digested with the same restriction enzymes, resulting in plasmid pZZH371.1 (FIG. 2). The sequence of the *AniAmy1M* gene within pZZH371.1was confirmed by DNA sequencing.

The plasmids pZZH371 and pZZH371.1 were transformed into a quad deleted *Trichoderma reesei* strain (described in WO 05/001036) using biolistic method. *See* Te'o et al. (2002) *J Microbiol Methods* 51: 393-99. The protein was secreted into the extracellular medium and filtered culture medium was used to perform the SDS-PAGE assay and alpha-amylase activity assay to confirm the enzyme expression.

AniAmy1 was purified via a one-step purification procedure. For this, ammonium sulfate was added to 0.8 M to 160 mL concentrated fermentor broth. The sample was loaded onto a 50 mL Hydrophobic Interaction Chromatography column Phenyl FF pre-equilibrated with 20 mM Tris-HCl, pH 7.0 with 0.8 M ammonium sulfate (buffer A). The column was washed by linear gradient of ammonium sulfate from 0.8-0 M. The target protein was in the flowthrough. The flowthrough was concentrated using a 10K Amicon Ultra-15 device. The final product was above 95% pure and stored in 40% glycerol at -80°C for further studies.

AniAmy1M was purified via the beta-cyclodextrin coupled Sepharose 6 affinity chromatography, taking advantage of its carbohydrate binding domain. The 700 mL crude broth from the shake flask was concentrated and adjusted to pH 4.3. The solution was then loaded onto a 30 mL beta-cyclodextrin coupled Sepharose 6 column pre-equilibrated with 20 mM sodium acetate pH 4.3. The column was applied with a gradient of 0-10 mM alpha-cyclodextrin in 20 mM sodium acetate pH 4.3 in 2 column volumes, followed by 1 column volume of 10 mM alpha-cyclodextrin in 20 mM sodium acetate pH 4.3. The fractions from the column were assayed for amylase activity and SDS-PAGE. The target protein was found in the gradient

elution step. The fractions containing the target protein were pooled and buffer-exchanged to 20 mM sodium acetate pH 4.3 to remove the cyclodextrin during the elution step. The sample was concentrated using an Amicon Ultra-15 device with 10 K MWCO. The purified sample is above 95% pure and stored in 40% glycerol at -80°C until usage.

**EXAMPLE 3 Amino acid and DNA sequences of the *Schwanniomyces occidentalis* alpha-amylase SocAmy1 and chimeric alpha-amylase SocAmy1M**

*Schwanniomyces occidentalis* was further selected as a potential source for various glycosyl hydrolases and other enzymes, useful for industrial applications. One of genes identified in *S.occidentalis* encodes a glycosyl hydrolase with homology to various other fungal alpha-amylases as determined from a BLAST search. *See* Altschul et al. (1990) *J Mol Biol* 215: 403–410. The nucleotide sequence of this gene, the *SocAmy1* gene, is depicted as SEQ ID NO: 9, while the protein encoded by the *SocAmy1* gene is depicted as SEQ ID NO: 10. SEQ ID NO: 9 is shown below (no intron)

ATGAAATTTGCCACTATTCTCTCCACCACCGCCCTCGCCCTGTCCTCCCTCGTTGCCTCCAAGCCCATCT
TCCTCAGCAAACGAGACGCCGGTTCCTCCGCCGCTGCTGCCTGGCGCTCCGAGAGCATTTATCAGCTGGT
CACTGACCGCTTCGCTCGCACTGACGGCTCCACTAGCGCCACTTGCAACACTGGCGACCGCGTCTATTGT
GGCGGCACTTTCCAAGGCATTATCGACAAGCTGGACTACATCCAGGGCATGGGTTTCACCGCCATCTGGA
TCTCCCCCGTCGTCGAGCAAATCCCTGACGACACCGGCTATGGCTATGCCTACCACGGTTATTGGATGAA
GGACATCTACGCCATCAATTCCAATTTCGGCACCGCTGATGATCTGAAGAACCTGTCCAACGAACTCCAC
AAGCGCAATATGAAGCTGATGGTCGACATCGTCACCAATCACTACGCCTGGAACGGCGCTGGCTCCTCCG
TCGCCTATAGCAACTACAACCCCTTCAACCAGCAGTCCTATTTCCACGACTATTGCCTCATCACTAATTA
CGACGACCAGACCAACGTCGAGGATTGCTGGGAGGGTGACAACACCGTCAGCCTGCCCGACCTGCGCACC
GAGGACTCCGACGTCTCCAGCATTTTCAATCTCTGGGTCGCTGAGCTCGTTTCCAACTATTCCATTGACG
GCCTCCGCATCGACTCCGCCAAACACGTTGACGAGAGCTTTTACCCTAGCTTCCAGTCCGCTGCCGGCGT
TTACCTCCTCGGCGAAGTCTACGACGGTGACCCCGCTTACACTTGTCCCTACCAGAACTACATGAGCGGC
GTCACTAACTACCCCCTCTATTATCCCATGCTGCGCTTCTTTCAGGGCACCAGCAACTCCGTTGATGAGC
TGAACGCCATGATCAGCTCCCTGGAGAGCGACTGCAAAGACATTACTCTCCTGGGCAACTTTATCGAGAA
CCACGACCAACCTCGACTGCCTTCCTACACCAGCGATAGCGCCCTGATCAAGAATGCCATCGCCTTTAAT
CTCATGTCCGACGGCATCCCCATCATCTATTACGGCCAAGAGCAGGGTTACAGCGGCAGCTCCGATCCCA
ATAATCGAGAGGCCCTGTGGCTGTCCGGCTACAGCACCTCCAACGGCTACTATAAACTGATTTCCAGCGT
TAATCAAATTCGCAACCAGGCCATCTATAAGGACAGCAAGTACACCACCTACTGGAGCGATGTTCTGTAC
GCCAGCGGCCACGTTATCGCTCTGCAGCGAGGCGCTGACGATCAGCGCATTGTCTCCGTCTTTAACAACC
TGGGTAGCTCCGGCTCCCAGACCGTCACTTTCAGCACTAAGTACTCCGGTGGCGAGAAGGTTGTCGACGT

CCTGACCTGCCAAACCAGCTACGCTAATTCCGACAGCACTCTGACTGTCAGCATCTCCGGCGGTGCTCCC
CGAATCTATGCCCCTGCCAGCCTGATCGCCAATAGCGGCATCTGTAATTTCTAA

SEQ ID NO: 10, the amino acid sequence of the SocAmy1 protein, is shown below

*MKFATILSTTALALSSLVAS*KPIFLSKRDAGSSAAAAWRSESIYQLVTDRFARTDGSTSATCNTGDRVYC
GGTFQGIIDKLDYIQGMGFTAIWISPVVEQIPDDTGYGYAYHGYWMKDIYAINSNFGTADDLKNLSNELH
KRNMKLMVDIVTNHYAWNGAGSSVAYSNYNPFNQQSYFHDYCLITNYDDQTNVEDCWEGDNTVSLPDLRT
EDSDVSSIFNLWVAELVSNYSIDGLRIDSAKHVDESFYPSFQSAAGVYLLGEVYDGDPAYTCPYQNYMSG
VTNYPLYYPMLRFFQGTSNSVDELNAMISSLESDCKDITLLGNFIENHDQPRLPSYTSDSALIKNAIAFN
LMSDGIPIIYYGQEQGYSGSSDPNNREALWLSGYSTSNGYYKLISSVNQIRNQAIYKDSKYTTYWSDVLY
ASGHVIALQRGADDQRIVSVFNNLGSSGSQTVTFSTKYSGGEKVVDVLTCQTSYANSDSTLTVSISGGAP
RIYAPASLIANSGICNF

At the N-terminus, SocAmy1 protein has a 20-amino-acid signal peptide (SEQ ID NO: 11, shown in italics) predicted by SignalP-NN. *See* Emanuelsson et al. (2007) *Nature Protocols* 2: 953-971. This suggests that AniAmy1 is a secreted glycosyl hydrolase.

The chimeric SocAmy1M contains the CBM20 domain of an alpha-amylase from *Aspergillus terreus* (SEQ IN NO: 14; NCBI Reference Number XP_001209405.1 (gi|115385717|ref|XP_001209405.1| alpha-amylase precursor [Aspergillus terreus NIH2624])) fused to the C-terminal of SocAmy1 protein. Residues 498-607 of SEQ ID NO: 14 are fused to the C-terminal of SocAmy1 protein (*i.e.*, residues 1 to 506 of SEQ ID NO: 2) to creat the chimeric SocAmy1M. The nucleotide sequence of SocAmy1M is depicted as SEQ ID NO: 12, while the amino acid sequence of SocAmy1M is depicted as SEQ ID NO: 13. SEQ ID NO: 12 is shown below (the nucleotide sequences encoding the CBM20 are shown in bold)

ATGAAATTTGCCACTATTCTCTCCACCACCGCCCTCGCCCTGTCCTCCCTCGTTGCCTCCAAGCCCATCT
TCCTCAGCAAACGAGACGCCGGTTCCTCCGCCGCTGCTGCCTGGCGCTCCGAGAGCATTTATCAGCTGGT
CACTGACCGCTTCGCTCGCACTGACGGCTCCACTAGCGCCACTTGCAACACTGGCGACCGCGTCTATTGT
GGCGGCACTTTCCAAGGCATTATCGACAAGCTGGACTACATCCAGGGCATGGGTTTCACCGCCATCTGGA
TCTCCCCCGTCGTCGAGCAAATCCCTGACGACACCGGCTATGGCTATGCCTACCACGGTTATTGGATGAA
GGACATCTACGCCATCAATTCCAATTTCGGCACCGCTGATGATCTGAAGAACCTGTCCAACGAACTCCAC
AAGCGCAATATGAAGCTGATGGTCGACATCGTCACCAATCACTACGCCTGGAACGGCGCTGGCTCCTCCG
TCGCCTATAGCAACTACAACCCCTTCAACCAGCAGTCCTATTTCCACGACTATTGCCTCATCACTAATTA
CGACGACCAGACCAACGTCGAGGATTGCTGGGAGGGTGACAACACCGTCAGCCTGCCCGACCTGCGCACC
GAGGACTCCGACGTCTCCAGCATTTTCAATCTCTGGGTCGCTGAGCTCGTTTCCAACTATTCCATTGACG
GCCTCCGCATCGACTCCGCCAAACACGTTGACGAGAGCTTTTACCCTAGCTTCCAGTCCGCTGCCGGCGT
TTACCTCCTCGGCGAAGTCTACGACGGTGACCCCGCTTACACTTGTCCCTACCAGAACTACATGAGCGGC
GTCACTAACTACCCCCTCTATTATCCCATGCTGCGCTTCTTTCAGGGCACCAGCAACTCCGTTGATGAGC

TGAACGCCATGATCAGCTCCCTGGAGAGCGACTGCAAAGACATTACTCTCCTGGGCAACTTTATCGAGAA
CCACGACCAACCTCGACTGCCTTCCTACACCAGCGATAGCGCCCTGATCAAGAATGCCATCGCCTTTAAT
CTCATGTCCGACGGCATCCCCATCATCTATTACGGCCAAGAGCAGGGTTACAGCGGCAGCTCCGATCCCA
ATAATCGAGAGGCCCTGTGGCTGTCCGGCTACAGCACCTCCAACGGCTACTATAAACTGATTTCCAGCGT
TAATCAAATTCGCAACCAGGCCATCTATAAGGACAGCAAGTACACCACCTACTGGAGCGATGTTCTGTAC
GCCAGCGGCCACGTTATCGCTCTGCAGCGAGGCGCTGACGATCAGCGCATTGTCTCCGTCTTTAACAACC
TGGGTAGCTCCGGCTCCCAGACCGTCACTTTCAGCACTAAGTACTCCGGTGGCGAGAAGGTTGTCGACGT
CCTGACCTGCCAAACCAGCTACGCTAATTCCGACAGCACTCTGACTGTCAGCATCTCCGGCGGTGCTCCC
CGAATCTATGCCCCTGCCAGCCTGATCGCCAATAGCGGCATCTGTAAC**ACTGAATGCACCGCTGCCACTT**
**CCATCAGCGTTCTGTTCGAGGAACTGGTCACCACTACCTACGGCGAGAATATCTACCTGAGCGGTAGCAT**
**CAGCCAACTGGGTAGCTGGAACACCGCTTCCGCTGTCGCCCTCAGCGCCTCCCAATATACTAGCAGCAAC**
**CCCGAGTGGTATGTCAGCGTCACTCTCCCTGTCGGCACCTCCTTCCAGTACAAATTCATCAAGAAGGGCA**
**GCGACGGCTCCGTCGTCTGGGAGAGCGATCCTAACCGCAGCTACACCGTTCCTGCCGGCTGCGAGGGCGC**
**CACTGTTACCGTCGCCGATACTTGGCGATAA**

SEQ ID NO: 13, the amino acid sequence of the SocAmy1M protein, is shown below (the putative signal peptide is shown in italics, and the CBM20 is shown in bold)

*MKFATILSTTALALSSLVAS*KPIFLSKRDAGSSAAAAWRSESIYQLVTDRFARTDGSTSATCNTGDRVYC
GGTFQGIIDKLDYIQGMGFTAIWISPVVEQIPDDTGYGYAYHGYWMKDIYAINSNFGTADDLKNLSNELH
KRNMKLMVDIVTNHYAWNGAGSSVAYSNYNPFNQQSYFHDYCLITNYDDQTNVEDCWEGDNTVSLPDLRT
EDSDVSSIFNLWVAELVSNYSIDGLRIDSAKHVDESFYPSFQSAAGVYLLGEVYDGDPAYTCPYQNYMSG
VTNYPLYYPMLRFFQGTSNSVDELNAMISSLESDCKDITLLGNFIENHDQPRLPSYTSDSALIKNAIAFN
LMSDGIPIIYYGQEQGYSGSSDPNNREALWLSGYSTSNGYYKLISSVNQIRNQAIYKDSKYTTYWSDVLY
ASGHVIALQRGADDQRIVSVFNNLGSSGSQTVTFSTKYSGGEKVVDVLTCQTSYANSDSTLTVSISGGAP
RIYAPASLIANSGICN**TECTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSN**
**PEWYVSVTLPVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

**Example 4 Expression and purification of SocAmy1 and SocAmy1M proteins**

The synthetic *SocAmy1* and *SocAmy1M* genes were cloned into pTrex3gM expression vector (described in US 2011/0136197 A1) and the resulting plasmids were labeled pZZH513 (FIG. 3) and pZZH513.1 (FIG. 4). The sequence of the *SocAmy1* and *SocAmy1M* gene was confirmed by DNA sequencing. The plasmids pZZH513 and pZZH513.1 were transformed into a quad-deleted *Trichoderma reesei* strain (described in WO 05/001036) using biolistic method. *See* Te'o et al. (2002) *J Microbiol Methods* 51: 393-99. The protein was secreted into the extracellular medium and filtered culture medium was used to perform the SDS-PAGE assay and alpha-amylase activity assay to confirm the enzyme expression.

SocAmy1 was purified via a two-step purification procedure. For this, ammonium sulfate was added to 0.5 M to 650 mL fermentation broth from shake flask. The sample was loaded onto a 20 mL Hydrophobic Interaction Chromatography column Phenyl HP pre-equilibrated with 20 mM Tris-HCl, pH 8.0 with 0.5 M ammonium sulfate (buffer A). The column was washed by linear gradient of ammonium sulfate from 0.5-0 M. The target protein was in the flowthrough. The flowthrough was desalted and loaded onto a 20 mL Anion Exchange Chromatography column Q XL pre-equilibrated with 20 mM HEPES, pH 7.0 (buffer B). The column was washed by a linear gradient of sodium chloride from 0-0.5 M. The target protein was eluted in the end of the salt gradient. The active fractions were concentrated using a 10K Amicon Ultra-15 device. The final product was above 98% pure and stored in 40% glycerol at -80°C for further studies.

SocAmy1M was purified via the beta-cyclodextrin coupled Sepharose 6 affinity chromatography, taking advantage of its carbohydrate binding domain. The 700 mL crude broth from the shake flask was concentrated and adjusted to pH 4.3. The solution was then loaded onto a 30 mL beta-cyclodextrin coupled Sepharose 6 column pre-equilibrated with 20 mM sodium acetate pH 4.3. The column was applied with a gradient of 0-10 mM alpha-cyclodextrin in 20 mM sodium acetate pH 4.3 in 2 column volumes, followed by 1 column volume of 10 mM alpha-cyclodextrin in 20 mM sodium acetate pH 4.3. The fractions from the column were assayed for amylase activity and SDS-PAGE. The target protein was found in the gradient elution step. The fractions containing the target protein were pooled and concentrated using an Amicon Ultra-15 device with 10 K MWCO. The concentrated sample was then loaded onto a Superdex 75 XK26X60 column in 20 mM HEPES pH 8 buffer with 2 mM $CaCl_2$. The fractions containing the protein of interest were again pooled and concentrated. The purified sample is above 95% pure and stored in 40% glycerol at -80°C until usage.

**Example 5 Alpha-amylase activity characterization of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M**

Alpha-amylase activities were determined using a colorimetric assay to monitor the release of reducing sugars from potato amylopectin. The activity is reported as equivalents of glucose released per minute. Substrate solutions were prepared by mixing 9 mL of 1% (w/w, in water) potato amylopectin (Sigma, Cat. No. 10118), 1 mL of 0.5 M buffer (pH 5.0 sodium acetate or pH 8.0 HEPES), and 40 µL of 0.5 M $CaCl_2$ into a 15-mL conical tube. Stock solutions of purified alpha-amylase samples were made by diluting original samples to 0.4 mg/mL (400 ppm) in water. Serial dilutions of enzyme samples and glucose standard were prepared in water in non-binding microtiter plates (MTP, Corning 3641). Then 90 µL of substrate solution (preincubated at 50°C for 5 min at 600 rpm) and 10 µL of the enzyme serial dilution were added and mixed into non-binding microtiter plates (MTP, Corning 3641). All the incubations were done at 50°C for 10 min at 600 rpm in a thermomixer (Eppendorf). After incubation, 50 µL of 0.5 N NaOH were added to each well to stop the reaction. Total reducing sugars present in each well were measured using a PAHBAH method 80 µL of 0.5 N NaOH was aliquoted into a microtiter plate, followed by the addition of 20 µL of PAHBAH reagent (5% w/v 4-hydroxybenzoic acid hydrazide in 0.5 N HCl) and 10 µL of each reaction mixture. Plates were incubated at 95°C for 5 min and cooled down at 4°C for 5 sec. Samples (80 µL) were then transferred to polystyrene microtiter plates (Costar 9017) and absorbance was read at 410 nm. Resulting absorbance values were plotted against enzyme concentration and linear regression was used to determine the slope of the linear region of the plot. Alpha-amylase activity can be quantified using the following equation

$$\text{Specific Activity (Unit/mg)} = \text{Slope (enzyme)} / \text{slope (standard)} \times 100,$$

where 1 Unit = 1 µmol glucose eq. /min.

The specific activity of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M was 227, 425, 327, and 389 units/mg, respectively, at pH 5.0, and 121, 257, 71, and 50 units/mg, respectively, at pH 8.0 using the above method.

**Example 6 pH profile of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M**

The effect of pH (from 3.0 to 10.0) on alpha-amylase activities was monitored. Buffer working solutions consisted of the combination of glycine/sodium acetate/HEPES (250 mM),

with pH varying from 3.0 to 10.0. Substrate solutions were prepared by mixing 896 µL of 1% (w/w, in water) potato amylopectin (Sigma, Cat. No. 10118), 100 µL of 250 mM buffer working solution (pH from 3.0 to 10.0), and 4 µL of 0.5 M $CaCl_2$. Enzyme working solutions were prepared in water at a certain dose (showing signal within linear range as per dose response curve). All the incubations were done using the same protocol as described for alpha-amylase activity assay in Example 5. The absorbance from a control (water-only) was subtracted, and the resulting values were converted to percentages of relative activity, by defining the activity at the optimal pH as 100%. AniAmy1 and, AniAmy1M showed an optimum pH below 3, while SocAmy1 and SocAmy1M showed an optimum pH of 4-6 and 4-7, respectively. AniAmy1 and AniAmy1M retained greater than 70% of maximum activity within pH 3.0-6.7 and pH 3.0-7.3, respectively. Both Socamy1 and Socamy1M retained greater than 70% of maximum activity between pH 3.7-7.4.

**Example 7 Temperature profile of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M**

The effect of temperature (from 30 to 95°C) on alpha-amylase activities was monitored. Substrate solutions were prepared by mixing 3.6 mL of 1% (w/w, in water) potato amylopectin (Sigma, Cat. No. 10118), 0.4 mL of 0.5 M buffer (pH 5.0 sodium acetate), and 16 µL of 0.5 M $CaCl_2$ into a 15-mL conical tube. Enzyme working solutions were prepared in water at a certain dose (showing signal within linear range as per dose response curve). Incubations were done at temperatures from 30 to 95°C, respectively, for 10 min at 600 rpm in a thermomixer (Eppendorf). After incubation, samples were quenched and measured using the same protocol as described for alpha-amylase activity assay in Example 5. The absorbance from a control (water-only) was subtracted, and the resulting values were converted to percentages of relative activity by defining the activity at the optimal temperature as 100%. AniAmy1 showed an optimum temperature of 60°C and retained greater than 70% of maximum activity between 49°C and 69°C, while AniAmy1M showed an optimum temperature of 75°C and retained greater than 70% of maximum activity between 60°C and 85°C. SocAmy1 showed an optimum temperature of 60°C and retained greater than 70% of maximum activity between 41°C and 69°C, while SocAmy1M showed an optimum temperature of 55°C and retained greater than 70% of maximum activity between 42°C and 71°C.

**Example 8 Characterization of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M in the residual starch assays**

The goal of residual starch assay is to evaluate an enzyme's capability of removing insoluble residual starch after a SSF reaction by measuring the reducing ends at the end of the assay. This insoluable, residual starch comprises both recalcitrant and non-recalcitrant starch.

The substrate was isolated from an SSF reaction in a 14 L fermentor. The starting material for the SSF was whole ground corn liquefact at 32.8% dry solids (ds), which was obtained from a typical dry grind ethanol facility. The glucoamylase used was CS4, a glucoamylase variant disclosed in U.S. Patent No. 8,058,033 (Danisco US Inc.). No alpha-amylase was added. The SSF was stopped at 30 hrs. The whole mixture was then autoclaved to kill the yeast and the CS4. The mixture was centrifuged to separate the insoluble material from the soluble material. The insoluble material was resuspended in water and blended in an industrial blender to remove large chunks that would clog pipette tips. The material was then sieved to remove any remaining large particles that would clog the pipette tips. The sieved material was then washed twice by diluting it with water and then centrifuging. This step was to remove any soluble starch material that could inflate the observed activity of the alpha-amylases, so that the most difficult substrate can be obtained for the assays. After the second centrifugation, the material was resuspended in minimal water and stored at -80°C. For this assay, the washed insoluble material is then diluted 10× with 100 mM NaOAc, pH 3.7.

The substrate (150 µL) was mixed well (stirring) while it was added to a 96-well plate. Wide bore tips were required for transferring substrate. The enzyme solution (10 µL) was added to each well with final concentrations from 0 to 25 ppm. The plate was then incubated for 20-24 hrs while shaking at 32°C. After 20-24 hrs, the plate was briefly mixed to make sure that the particles were suspended. 10 µl of the supernatant was removed (being careful to avoid the pellet) and added to 90 µl of buffer. 10 µl of the diluted supernatant was added to PCR plates containing the BCA reagent (Thermo Scientific). Plates were incubated at 95°C for 2 minutes. 80 µl of this reaction was transferred to microtiter plate (Costar 9017) and read at 560 nm using a SpectraMAX MTP Reader (Molecular Devices). The data were compiled and presented in FIGs. 5C and 6C.

**Example 9 Characterization of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M in cooked starch assay**

The goal of the cooked starch assay is to evaluate an enzyme's capability of removing insoluble residual starch, i.e., by measuring the remaining reducing end generated using BCA substrate.

The substrate for the cooked starch assay was made from the SSF substrate (50/50 amylogel cornstarch) by diluting it 25-fold into 100 mM sodium acetate (pH 3.7). The diluted SSF substrate was then autoclaved for 60 minutes with a stir bar. As the mixture cooled, it was stirred overnight on a stir plate to prevent gelling. After that, the substrate was stored at 4°C and ready for use. The SSF substrate was prepared by washing sieved (250 μm) corn starch (Cargill Foods, Lot # OX 0293A B) and amylogel (70% amylase content, Hylon VII) in water via successive centrifugation / decanting steps. Material was suspended at 30% (w/w) such that corn starch and amylogel were present in equal proportions in 50 mM potassium formate (pH 3.5), 10 mM sodium chloride, 0.25 mM potassium chloride, 0.005% TWEEN® 80, and 0.005% sodium azide.

The autoclaved SSF substrate (150 μL) was mixed well (stirring) while it was added to a 96-well plate. Wide bore tips were required for transferring substrate. The enzyme solution (10 μL) was added to each well with final concentration from 0 to 25 ppm. The plate was then incubated for 20-24 h while shaking at 32°C. After 20-24 hrs, the plate was briefly mixed to make sure that the particles were suspended. 10 μl of the supernatant was removed (being careful to avoid the pellet) and added to 90 μl of buffer. 10 μl of the diluted supernatant was added to PCR plates containing the BCA reagent (Thermo Scientific). Plates were incubated at 95°C for 2 minutes. 80 μl of this reaction was transferred to microtiter plate (Costar 9017) and read at 560 nm using a SpectraMAX MTP Reader (Molecular Devices). The data were compiled and presented in FIGs. 5A and 6A.

**Example 10 Characterization of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M in the SSF assays**

The goal of the SSF assay to is assess an enzyme's performance under conditions that approximate simultaneous SSF (for example, ethanol production), using the SSF substrate described in Example 8.

The SSF substrate (150 μL) was mixed well (stirring) while it was added to a 96-well plate. Wide bore tips were required for transferring substrate. The enzyme solution (10 μL) was

added to each well with final concentration from 0 to 25 ppm. The plate was then incubated for 20-24 h while shaking at 32 °C. After 20-24 h, the plate was briefly mixed to make sure that the particles were suspended. 10 µl of the supernatant was removed (being careful to avoid the pellet) and added to 90 µl of buffer. 10 µl of the diluted supernatant was added to PCR plates containing the BCA reagent (Thermo Scientific). Plates were incubated at 95 °C for 2 minutes. 80 µl of this reaction was transferred to microtiter plate (Costar 9017) and read at 560 nm using a SpectraMAX MTP Reader (Molecular Devices). The data were compiled and presented in FIGs. 5B and 6B.

**Example 11 Characterization of purified AniAmy1, AniAmy1M, SocAmy1, and SocAmy1M in the ceralpha assays**

The principle of the ceralpha alpha-amylase assay is based on the hydrolysis of a defined oligosaccharide (BPNPG7), in the presence of excess levels of a thermostable alpha-glucosidase, to glucose and free *p*-nitrophenol. The absorbance at 400 nm is measured and $OD_{400nm}$ corresponds directly to the level of active amylase in the sample analyzed.

The equipment used was a SpectraMAX MTP Reader (Molecular Devices) and iEMS incubator/shaker (Thermo/Labsystems). In this assay system, the reagent and solutions used included 1) *p*-nitrophenyl maltoheptaoside (BPNPG7) substrate (Megazyme HR kit); 2) 50 mM malate, 50 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 buffer, pH 5; and 3) 200 mM Boric acid / NaOH buffer, pH 10.2 (STOP buffer). A vial containing 54.5 mg BPNPG7 substrate was dissolved in 10 mL of milliQ water. The amylase samples (fermentation supernatant) were diluted in malate buffer. The assay was performed by adding 5 µl of diluted amylase solution into the wells of a MTP followed by the addition of 54.5 mg/mL BPNPG7 substrate solution. The solutions were mixed and the microtiter plate was sealed with a plate seal and placed in an incubator/shaker (iEMSThermo/Labsystems) for 30 minutes at 25°C and 900 rpm. The reaction was terminated by adding 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. The data were compiled and presented in FIGs. 5D and 6D.

**Example 12 Analysis of the effect of CBM20 fusion to AniAmy and SocAmy1 on breakdown of insoluble starch**

When an alpha-amylase breaks down a large insoluble starch granule, it releases smaller polysaccharides and oligosaccharides. These polysaccharides and oligosaccharides are not only

products of the alpha-amylase reaction but they also can be substrates of the alpha-amylase. Ideally, the target substrate for an alpha-amylase in industrial starch processing should be large insoluble starch granules and not the smaller oligosaccharides, because the smaller oligosaccharides can be more effciently hydrolyzed by the glucoamylase present. One strategy for engineering an alpha-amylase to better target the large starch granules is to improve the enzyme's substrate specificity (kcat/KM) for large starch granules over that of smaller oligosaccharides. This can be achieved by adding a starch binding domain (SBD) to an alpha-amylase that does not naturally have one to create a chimeric alpha-amylase. Ideally, the addition of this SBD should not significantly affect the hydrolysis of oligosaccharides, nor should it have an effect when the substrate is 100% large starch granules (at a saturating level). The SBD of the resulting chimeric alpha-amyase should have an effect when there is competition between large starch granules and smaller oligosaccharides. This scenario is expected to be true for most cases, since even if the substrate comprises 100% large starch granules at the beginning of the reaction, as the alpha-amylase acts on the starch granules it will create more and more oligosaccharides. As the reaction proceeds and moves toweard completion, the concentration of the oligosaccharides gradually should increase, so that the effect of SBD addition should be more dramatic. As for the breakdown of the insoluble starch present in liquefact during SSF, the above discussed scenario should be true from the very beginning.

The importance of a SBD for breaking down insoluble starch is highlighted in the following data. In the above Examples 8-10, both alpha-amylase lacking SBD (AniAmy1 and SocAmy1) and chimeric alpha-amylase having SBD were analyzed with the solubilization assay, SSF assay, and ceralpha assay. By comparing the performance of an alpha-amylase without a SBD to that of a chimera version with an SBD, the true effect of an SBD can be evaluated.

The BPNPG7 substrate for the ceralpha assay is a modified DP7; thus it is too small a substract for the benefit of the addition of an SBD to be realized. The substrate for the solubilization and SSF assays is insoluble starch; thus the addition of a SBD is expected to have an effect in these assays. The ceralpha assay serves as a control to ensure that any increase in activity of the SBD-containg chimeric alpha-amylase is not due to any slight modifications, if any, of the active site that improves its activity (kcat). For the ceralpha assay of Example 11, the addition of a SBD either showed no effect or resulted in marginal benefit on activity (FIGs. 5D and 6D). The benefit in activity of adding a SBD to an alpha-amylase naturally lacking a SBD

can be seen in the cooked starch assay, SSF assay, and residual starch assay (FIGs. 5A-C and 6A-C). Linear portions of the SSF curves shown in FIGs. 5B and 6B were analyzed and summarized in Table 1 below:

**Table 1 Fold improvement in the SSF assay for AniAmy1 and SocAmy1 due to the addition of a CBM**

| Enzyme | Rate calculated based on FIGs. 5B and 6B (OD560nm $\mu m^{-1}$) | Fold improvement due to the addition of a CBM |
|---|---|---|
| AniAmy1 | 0.16 | 9.0 |
| AniAmy1M | 1.43 | |
| SocAmy1 | 0.20 | 3.1 |
| SocAmy1M | 0.64 | |

Here the CBM-chimera AniAmy1M showed a 9.0-fold improvement in activity over AniAmy1, while the CBM-chimera SocAmy1M showed a 3.1-fold improvement in activity over SocAmy1.

Various modifications and variations of the described methods and system of the disclosure will be apparent to those skilled in the art without departing from the scope and spirit of the disclosure. Although the disclosure has been described in connection with specific representative embodiments, it should be understood that the subject matters as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the disclosure that are obvious to those skilled in the art are intended to be within the scope of the following claims.

## SEQUENCE LISTING

**SEQ ID NO: 1** (nucleotide sequence encoding AniAmy1, an alpha-amylase isolated from *Aspergillus niger*, containing 8 introns)

ATGACAATCTTTCTGTTTCTGGCCATTTTCGTGGCTACAGCTCTGGCAGCCACGCCTGCAGA
ATGGCGCTCCCAGTCGATATATTTCCTGCTCACCGATCGCTTTGCGCGAACGGATAATTCTA
CCACTGCTTCTTGTGACTTGAGCGCTCGGgttagtcacagcatgttctagaatctccaattg
attcgctgacagatctagCAATATTGCGGTGGATCCTGGCAGGGCATCATCAATCAGgtcgg
tccgtccatcgttgcagcactatctacatcaacgtttgtttggcaaattaacatccattagC
TGGACTATATTCAAGGAATGGGCTTTACAGCGATCTGGATCACACCCGTAACTGCACAGATC
CCCCAAGATACTGGTTACGGACAGGCATATCACGGATACTGGCAGCAGGACGCgtgagatgc
tacctctatcgcccggatgaatgtatatccttcttaccatgcagacagTTATGCCCTGAACT
CCCATTATGGTACGGCAGACGATCTCAAAGCTCTGGCTTCAGCTCTTCACTCACGGGGCATG
TATCTCATGGTGGACGTTGTTGCCAATCACATGgtatgttcttagcctcccacgggacctta
gctttatatctgacagcgatagGGCCACAATGGTACGGGGAGCTCTGTGGACTACAGTGTTT
ATAGGCCATTTAATTCGCAAAAGTACTTTCACAACCTCTGTTGGATCTCTGATTACAATAAC
CAGACAAACGTTGAAGACTGCTGGCTAGGCGATAACACCGTTGCCTTGCCGGATCTTGATAC
TACCAGTACGGAGGTGAAGAATATGTGGTATGACTGGGTCGAGTCTCTCGTCTCTAACTACT
CCGgtaatcctacctttacttcgctattttctgcctcttatgagacaaagactaacaaatat
caagTCGACGGCCTCCGCGTAGACACAGTCAAGAACGTACAGAAGAACTTCTGGCCCGGCTA
CAACAATGCTTCAGGCGTGTACTGTATTGGAGAAGTCTTCGATGGGGACGCCTCATACACCT
GTCCTTATCAGGAAGACTTGGACGGAGTCCTTAATTACCCCATgtaagccctacatttaacc
ccattgaatgcttgccaacgacttgcaatagGTACTATCCACTCCTCCGGGCTTTCGAATCC
ACCAACGGCAGTATCAGCGACCTCTATAACATGATCAACACCGTGAAATCCACCTGCAGAGA
TTCTACGCTTCTAGGGACCTTCGTCGAAAACCACGATAACCCACGCTTTGCCAAgtaagaat
atcctctccgagttcaccattacaaacacaagagctcacctcagaagCTACACAAGCGACAT
GTCCCTAGCCAAAAATGCCGCAACATTCACTATCCTGGCTGACGGCATTCCCATCATATACG
CCGGTCAGGAACAGCACTATAGCGGCGGTAATGACCCCTACAACCGCGAAGCGACCTGGCTT
TCAGGCTACAAGACCACCAGCGAGCTCTACACCCATATCGCCGCATCGAACAAGATTCGCAC
CCACGCTATAAAACAGGATACCGGATATCTCACCTACAAAgtaatcttcattcgagtccatg
tgtggtacaatctatctgactagaacctattctagAACTACCCCATCTACCAAGACACCTCG
ACCCTTGCCATGCGCAAAGGCTACAATGGCACCCAAACTATCACGGTCCTTTCTAACCTTGG
CGCCTCGGGGTCCTCATACACACTCTCCCTCCCAGGAACAGGCTACACAGCCGGCCAAAAGA
TTACTGAAATCTATACCTGCACGAATCTAACAGTCAACTCAAATGGCTCGGTGCCAGTACCC
ATGAAGAGCGGGTTACCGCGGATCCTCTATCCTGCAGATAAGTTGGTTAATGGAAGCTCATT

TTGCAgttagttccctgcctatatgtttcaataagccgtatttgcatgcgcgtccatcgcat
tgattctttatggattgatcaactgatacattcgcacagGCTCTGCGATCACTGTTTTCAAG
GATGCAGGAGGAGGCGTGTTGTTCTTTAGCTACACTGTGTAA

**SEQ ID NO: 2** (full-length amino acid sequence of AniAmy1)

MTIFLFLAIFVATALAATPAEWRSQSIYFLLTDRFARTDNSTTASCDLSARQYCGGSWQGII
NQLDYIQGMGFTAIWITPVTAQIPQDTGYGQAYHGYWQQDAYALNSHYGTADDLKALASALH
SRGMYLMVDVVANHMGHNGTGSSVDYSVYRPFNSQKYFHNLCWISDYNNQTNVEDCWLGDNT
VALPDLDTTSTEVKNMWYDWVESLVSNYSVDGLRVDTVKNVQKNFWPGYNNASGVYCIGEVF
DGDASYTCPYQEDLDGVLNYPMYYPLLRAFESTNGSISDLYNMINTVKSTCRDSTLLGTFVE
NHDNPRFANYTSDMSLAKNAATFTILADGIPIIYAGQEQHYSGGNDPYNREATWLSGYKTTS
ELYTHIAASNKIRTHAIKQDTGYLTYKNYPIYQDTSTLAMRKGYNGTQTITVLSNLGASGSS
YTLSLPGTGYTAGQKITEIYTCTNLTVNSNGSVPVPMKSGLPRILYPADKLVNGSSFCSSAI
TVFKDAGGGVLFFSYTV

**SEQ ID NO: 3** (predicted signal peptide of AniAmy1 or AniAmy1M)

MTIFLFLAIFVATALA

**SEQ ID NO: 4** (nucleotide sequence encoding AniAmy1M, a chimera alpha-amylase comprising CBM20 of *Aspergillus terreus* fused to the C-terminus of AniAmy1, containing 8 introns)

ATGACAATCTTTCTGTTTCTGGCCATTTTCGTGGCTACAGCTCTGGCAGCCACGCCTGCAGA
ATGGCGCTCCCAGTCGATATATTTCCTGCTCACCGATCGCTTTGCGCGAACGGATAATTCTA
CCACTGCTTCTTGTGACTTGAGCGCTCGGgttagtcacagcatgttctagaatctccaattg
attcgctgacagatctagCAATATTGCGGTGGATCCTGGCAGGGCATCATCAATCAGgtcgg
tccgtccatcgttgcagcactatctacatcaacgtttgtttggcaaattaacatccattagC
TGGACTATATTCAAGGAATGGGCTTTACAGCGATCTGGATCACACCCGTAACTGCACAGATC
CCCCAAGATACTGGTTACGGACAGGCATATCACGGATACTGGCAGCAGGACGCgtgagatgc
tacctctatcgcccggatgaatgtatatccttcttaccatgcagacagTTATGCCCTGAACT
CCCATTATGGTACGGCAGACGATCTCAAAGCTCTGGCTTCAGCTCTTCACTCACGGGGCATG
TATCTCATGGTGGACGTTGTTGCCAATCACATGgtatgttcttagcctcccacgggaccttа
gctttatatctgacagcgatagGGCCACAATGGTACGGGGAGCTCTGTGGACTACAGTGTTT
ATAGGCCATTTAATTCGCAAAAGTACTTTCACAACCTCTGTTGGATCTCTGATTACAATAAC
CAGACAAACGTTGAAGACTGCTGGCTAGGCGATAACACCGTTGCCTTGCCGGATCTTGATAC

TACCAGTACGGAGGTGAAGAATATGTGGTATGACTGGGTCGAGTCTCTCGTCTCTAACTACT
CCGgtaatcctacctttacttcgctattttctgcctcttatgagacaaagactaacaaatat
caagTCGACGGCCTCCGCGTAGACACAGTCAAGAACGTACAGAAGAACTTCTGGCCCGGCTA
CAACAATGCTTCAGGCGTGTACTGTATTGGAGAAGTCTTCGATGGGGACGCCTCATACACCT
GTCCTTATCAGGAAGACTTGGACGGAGTCCTTAATTACCCCATgtaagccctacatttaacc
ccattgaatgcttgccaacgacttgcaatagGTACTATCCACTCCTCCGGGCTTTCGAATCC
ACCAACGGCAGTATCAGCGACCTCTATAACATGATCAACACCGTGAAATCCACCTGCAGAGA
TTCTACGCTTCTAGGGACCTTCGTCGAAAACCACGATAACCCACGCTTTGCCAAgtaagaat
atcctctccgagttcaccattacaaacacaagagctcacctcagaagCTACACAAGCGACAT
GTCCCTAGCCAAAAATGCCGCAACATTCACTATCCTGGCTGACGGCATTCCCATCATATACG
CCGGTCAGGAACAGCACTATAGCGGCGGTAATGACCCCTACAACCGCGAAGCGACCTGGCTT
TCAGGCTACAAGACCACCAGCGAGCTCTACACCCATATCGCCGCATCGAACAAGATTCGCAC
CCACGCTATAAAACAGGATACCGGATATCTCACCTACAAAgtaatcttcattcgagtccatg
tgtggtacaatctatctgactagaacctattctagAACTACCCCATCTACCAAGACACCTCG
ACCCTTGCCATGCGCAAAGGCTACAATGGCACCCAAACTATCACGGTCCTTTCTAACCTTGG
CGCCTCGGGGTCCTCATACACACTCTCCCTCCCAGGAACAGGCTACACAGCCGGCCAAAAGA
TTACTGAAATCTATACCTGCACGAATCTAACAGTCAACTCAAATGGCTCGGTGCCAGTACCC
ATGAAGAGCGGGTTACCGCGGATCCTCTATCCTGCAGATAAGTTGGTTAATGGAAGCTCATT
T**TGCAACACAGAATGCACGGCCGCCACGTCCATCTCTGTTCTCTTCGAGGAACTGGTTACGA**
**CGACCTACGGGGAGAACATTTATCTGAGCGGCTCGATCAGCCAGCTGGGTAGTTGGAATACG**
**GCCTCGGCTGTTGCCCTGTCGGCGAGTCAATATACCTCGTCCAACCCGGAGTGGTATGTGAG**
**TGTGACCTTGCCTGTGGGCACGTCGTTCCAGTACAAGTTCATCAAGAAGGGGTCGGACGGAA**
**GTGTTGTGTGGGAGAGTGATCCGAACCGGTCGTATACCGTTCCGGCTGGGTGCGAGGGCGCG**
**ACGGTGACAGTTGCTGATACTTGGAGGTGA**

**SEQ ID NO: 5** (full-length amino acid of AniAmy1M)

*MTIFLFLAIFVATALA*ATPAEWRSQSIYFLLTDRFARTDNSTTASCDLSARQYCGGSWQGII
NQLDYIQGMGFTAIWITPVTAQIPQDTGYGQAYHGYWQQDAYALNSHYGTADDLKALASALH
SRGMYLMVDVVANHMGHNGTGSSVDYSVYRPFNSQKYFHNLCWISDYNNQTNVEDCWLGDNT
VALPDLDTTSTEVKNMWYDWVESLVSNYSVDGLRVDTVKNVQKNFWPGYNNASGVYCIGEVF
DGDASYTCPYQEDLDGVLNYPMYYPLLRAFESTNGSISDLYNMINTVKSTCRDSTLLGTFVE
NHDNPRFANYTSDMSLAKNAATFTILADGIPIIYAGQEQHYSGGNDPYNREATWLSGYKTTS
ELYTHIAASNKIRTHAIKQDTGYLTYKNYPIYQDTSTLAMRKGYNGTQTITVLSNLGASGSS
YTLSLPGTGYTAGQKITEIYTCTNLTVNSNGSVPVPMKSGLPRILYPADKLVNGSSF**CNTEC**

**TAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTSSNPEWYVSVTLPV GTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

**SEQ ID NO: 6** (primer 1 (NotI))

5′- ccgcggccgcaccATGACAATCTTTCTGTTTCTGG -3′

**SEQ ID NO: 7** (primer 2 (AscI))

5′- ccggcgcgcccttaTCACGTCATTATAGCAATAAGC -3′

**SEQ ID NO: 8** (primer 3 (AscI))

5′- ccggcgcgcccttaTCACCTCCAAGTATCAGCAAC -3′

**SEQ ID NO: 9** (nucleotide sequence encoding SocAmy1, an alpha-amylase isolated from *Schwanniomyces occidentalis*, no intron)

ATGAAATTTGCCACTATTCTCTCCACCACCGCCCTCGCCCTGTCCTCCCTCGTTGCCTCCAA
GCCCATCTTCCTCAGCAAACGAGACGCCGGTTCCTCCGCCGCTGCTGCCTGGCGCTCCGAGA
GCATTTATCAGCTGGTCACTGACCGCTTCGCTCGCACTGACGGCTCCACTAGCGCCACTTGC
AACACTGGCGACCGCGTCTATTGTGGCGGCACTTTCCAAGGCATTATCGACAAGCTGGACTA
CATCCAGGGCATGGGTTTCACCGCCATCTGGATCTCCCCCGTCGTCGAGCAAATCCCTGACG
ACACCGGCTATGGCTATGCCTACCACGGTTATTGGATGAAGGACATCTACGCCATCAATTCC
AATTTCGGCACCGCTGATGATCTGAAGAACCTGTCCAACGAACTCCACAAGCGCAATATGAA
GCTGATGGTCGACATCGTCACCAATCACTACGCCTGGAACGGCGCTGGCTCCTCCGTCGCCT
ATAGCAACTACAACCCCTTCAACCAGCAGTCCTATTTCCACGACTATTGCCTCATCACTAAT
TACGACGACCAGACCAACGTCGAGGATTGCTGGGAGGGTGACAACACCGTCAGCCTGCCCGA
CCTGCGCACCGAGGACTCCGACGTCTCCAGCATTTTCAATCTCTGGGTCGCTGAGCTCGTTT
CCAACTATTCCATTGACGGCCTCCGCATCGACTCCGCCAAACACGTTGACGAGAGCTTTTAC
CCTAGCTTCCAGTCCGCTGCCGGCGTTTACCTCCTCGGCGAAGTCTACGACGGTGACCCCGC
TTACACTTGTCCCTACCAGAACTACATGAGCGGCGTCACTAACTACCCCCTCTATTATCCCA
TGCTGCGCTTCTTTCAGGGCACCAGCAACTCCGTTGATGAGCTGAACGCCATGATCAGCTCC
CTGGAGAGCGACTGCAAAGACATTACTCTCCTGGGCAACTTTATCGAGAACCACGACCAACC
TCGACTGCCTTCCTACACCAGCGATAGCGCCCTGATCAAGAATGCCATCGCCTTTAATCTCA
TGTCCGACGGCATCCCCATCATCTATTACGGCCAAGAGCAGGGTTACAGCGGCAGCTCCGAT
CCCAATAATCGAGAGGCCCTGTGGCTGTCCGGCTACAGCACCTCCAACGGCTACTATAAACT
GATTTCCAGCGTTAATCAAATTCGCAACCAGGCCATCTATAAGGACAGCAAGTACACCACCT
ACTGGAGCGATGTTCTGTACGCCAGCGGCCACGTTATCGCTCTGCAGCGAGGCGCTGACGAT

CAGCGCATTGTCTCCGTCTTTAACAACCTGGGTAGCTCCGGCTCCCAGACCGTCACTTTCAG
CACTAAGTACTCCGGTGGCGAGAAGGTTGTCGACGTCCTGACCTGCCAAACCAGCTACGCTA
ATTCCGACAGCACTCTGACTGTCAGCATCTCCGGCGGTGCTCCCCGAATCTATGCCCCTGCC
AGCCTGATCGCCAATAGCGGCATCTGTAATTTCTAA

**SEQ ID NO: 10** (full-length amino acid sequence of SocAmy1)

*MKFATILSTTALALSSLVAS*KPIFLSKRDAGSSAAAAWRSESIYQLVTDRFARTDGST
SATCNTGDRVYCGGTFQGIIDKLDYIQGMGFTAIWISPVVEQIPDDTGYGYAYHGYWM
KDIYAINSNFGTADDLKNLSNELHKRNMKLMVDIVTNHYAWNGAGSSVAYSNYNPFNQ
QSYFHDYCLITNYDDQTNVEDCWEGDNTVSLPDLRTEDSDVSSIFNLWVAELVSNYSI
DGLRIDSAKHVDESFYPSFQSAAGVYLLGEVYDGDPAYTCPYQNYMSGVTNYPLYYPM
LRFFQGTSNSVDELNAMISSLESDCKDITLLGNFIENHDQPRLPSYTSDSALIKNAIA
FNLMSDGIPIIYYGQEQGYSGSSDPNNREALWLSGYSTSNGYYKLISSVNQIRNQAIY
KDSKYTTYWSDVLYASGHVIALQRGADDQRIVSVFNNLGSSGSQTVTFSTKYSGGEKV
VDVLTCQTSYANSDSTLTVSISGGAPRIYAPASLIANSGICNF

**SEQ ID NO: 11** (predicted signal peptide of SocAmy1 or SocAmy1M)

*MKFATILSTTALALSSLVAS*

**SEQ ID NO: 12** (nucleotide sequence encoding SocAmy1M, a chimera alpha-amylase comprising CBM20 of *Aspergillus terreus* fused to the C-terminus of SocAmy1)

ATGAAATTTGCCACTATTCTCTCCACCACCGCCCTCGCCCTGTCCTCCCTCGTTGCCTCCAA
GCCCATCTTCCTCAGCAAACGAGACGCCGGTTCCTCCGCCGCTGCTGCCTGGCGCTCCGAGA
GCATTTATCAGCTGGTCACTGACCGCTTCGCTCGCACTGACGGCTCCACTAGCGCCACTTGC
AACACTGGCGACCGCGTCTATTGTGGCGGCACTTTCCAAGGCATTATCGACAAGCTGGACTA
CATCCAGGGCATGGGTTTCACCGCCATCTGGATCTCCCCCGTCGTCGAGCAAATCCCTGACG
ACACCGGCTATGGCTATGCCTACCACGGTTATTGGATGAAGGACATCTACGCCATCAATTCC
AATTTCGGCACCGCTGATGATCTGAAGAACCTGTCCAACGAACTCCACAAGCGCAATATGAA
GCTGATGGTCGACATCGTCACCAATCACTACGCCTGGAACGGCGCTGGCTCCTCCGTCGCCT
ATAGCAACTACAACCCCTTCAACCAGCAGTCCTATTTCCACGACTATTGCCTCATCACTAAT
TACGACGACCAGACCAACGTCGAGGATTGCTGGGAGGGTGACAACACCGTCAGCCTGCCCGA
CCTGCGCACCGAGGACTCCGACGTCTCCAGCATTTTCAATCTCTGGGTCGCTGAGCTCGTTT
CCAACTATTCCATTGACGGCCTCCGCATCGACTCCGCCAAACACGTTGACGAGAGCTTTTAC
CCTAGCTTCCAGTCCGCTGCCGGCGTTTACCTCCTCGGCGAAGTCTACGACGGTGACCCCGC
TTACACTTGTCCCTACCAGAACTACATGAGCGGCGTCACTAACTACCCCCTCTATTATCCCA

TGCTGCGCTTCTTTCAGGGCACCAGCAACTCCGTTGATGAGCTGAACGCCATGATCAGCTCC
CTGGAGAGCGACTGCAAAGACATTACTCTCCTGGGCAACTTTATCGAGAACCACGACCAACC
TCGACTGCCTTCCTACACCAGCGATAGCGCCCTGATCAAGAATGCCATCGCCTTTAATCTCA
TGTCCGACGGCATCCCCATCATCTATTACGGCCAAGAGCAGGGTTACAGCGGCAGCTCCGAT
CCCAATAATCGAGAGGCCCTGTGGCTGTCCGGCTACAGCACCTCCAACGGCTACTATAAACT
GATTTCCAGCGTTAATCAAATTCGCAACCAGGCCATCTATAAGGACAGCAAGTACACCACCT
ACTGGAGCGATGTTCTGTACGCCAGCGGCCACGTTATCGCTCTGCAGCGAGGCGCTGACGAT
CAGCGCATTGTCTCCGTCTTTAACAACCTGGGTAGCTCCGGCTCCCAGACCGTCACTTTCAG
CACTAAGTACTCCGGTGGCGAGAAGGTTGTCGACGTCCTGACCTGCCAAACCAGCTACGCTA
ATTCCGACAGCACTCTGACTGTCAGCATCTCCGGCGGTGCTCCCCGAATCTATGCCCCTGCC
AGCCTGATCGCCAATAGCGGCATCTGTAAC**ACTGAATGCACCGCTGCCACTTCCATCAGCGT**
**TCTGTTCGAGGAACTGGTCACCACTACCTACGGCGAGAATATCTACCTGAGCGGTAGCATCA**
**GCCAACTGGGTAGCTGGAACACCGCTTCCGCTGTCGCCCTCAGCGCCTCCCAATATACTAGC**
**AGCAACCCCGAGTGGTATGTCAGCGTCACTCTCCCTGTCGGCACCTCCTTCCAGTACAAATT**
**CATCAAGAAGGGCAGCGACGGCTCCGTCGTCTGGGAGAGCGATCCTAACCGCAGCTACACCG**
**TTCCTGCCGGCTGCGAGGGCGCCACTGTTACCGTCGCCGATACTTGGCGATAA**

**SEQ ID NO: 13** (full-length amino acid of SocAmy1M)

*MKFATILSTTALALSSLVAS*KPIFLSKRDAGSSAAAAWRSESIYQLVTDRFARTDGSTSATC
NTGDRVYCGGTFQGIIDKLDYIQGMGFTAIWISPVVEQIPDDTGYGYAYHGYWMKDIYAINS
NFGTADDLKNLSNELHKRNMKLMVDIVTNHYAWNGAGSSVAYSNYNPFNQQSYFHDYCLITN
YDDQTNVEDCWEGDNTVSLPDLRTEDSDVSSIFNLWVAELVSNYSIDGLRIDSAKHVDESFY
PSFQSAAGVYLLGEVYDGDPAYTCPYQNYMSGVTNYPLYYPMLRFFQGTSNSVDELNAMISS
LESDCKDITLLGNFIENHDQPRLPSYTSDSALIKNAIAFNLMSDGIPIIYYGQEQGYSGSSD
PNNREALWLSGYSTSNGYYKLISSVNQIRNQAIYKDSKYTTYWSDVLYASGHVIALQRGADD
QRIVSVFNNLGSSGSQTVTFSTKYSGGEKVVDVLTCQTSYANSDSTLTVSISGGAPRIYAPA
SLIANSGICN**TECTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSASQYTS**
**SNPEWYVSVTLPVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

**SEQ ID NO: 14** (Alpha-amylase from *Aspergillus terreus*; gi|115385717|ref|XP_001209405.1| alpha-amylase precursor [Aspergillus terreus NIH2624]; the region used for CBM20 fusion 496-607)

MKWTSSLLLLLSVIGQATHALTPAEWRSQSIYFLLTDRFGRTDNSTTAACDTSDRVYCGGSWQGIINQ
LDYIQGMGFTAIWITPVTGQFYENTGDGTSYHGYWQQDIYDLNYNYGTAQDLKNLANALHERGMYLMV

DVVANHMGYDGAGNTVDYSVFNPFSSSSYFHPYCLISNYDNQTNVEDCWLGDTTVSLPDLDTTSTAVR
NIWYDWVADLVANYSIDGLRVDTVKHVEKDFWPGYNSAAGVYCVGEVYSGDPAYTCPYQNYMDGVLNY
PIYYQLLYAFESSSGSISDLYNMISSVASSCKDPTLLGNFIENHDNPRFASYTSDYSQAKNVITFIFL
SDGIPIVYAGQEQHYSGGSDPANREATWLSGYSTSATLYTWIATTNQIRSLAISKDAGYVQAKNNPFY
SDSNTIAMRKGTTAGAQVITVLSNKGASGSSYTLSLSGTGYSAGATLVETYTCTTVTVDSSGNLPVPM
TSGLPRVFVPSSWVNGSAL**CNTECTAATSISVLFEELVTTTYGENIYLSGSISQLGSWNTASAVALSA**
**SQYTSSNPEWYVSVTLPVGTSFQYKFIKKGSDGSVVWESDPNRSYTVPAGCEGATVTVADTWR**

**SEQ ID NO: 15** (Alpha-amylase from *Aspergillus clavatus*; gi|121708778|ref|XP_001272245.1| alpha-amylase, putative [Aspergillus clavatus NRRL 1])

MKLLALTTAFALLGKGVFGLTPAEWRGQSIYFLITDRFARTDGSTTAPCDLSQRAYCGGSWQGII
KQLDY
IQGMGFTAIWITPITEQIPQDTAEGSAFHGYWQKDIYNVNSHFGTADDIRALSKALHDRGMYLMI
DVVAN
HMGYNGPGASTDFSTFTPFNSASYFHSYCPINNYNDQSQVENCWLGDNTVALADLYTQHSDVRN
IWYSWI
KEIVGNYSADGLRIDTVKHVEKDFWTGYTQAAGVYTVGEVLDGDPAYTCPYQGYVDGVLNYPIYY
PLLRA
FESSSGSMGDLYNMINSVASDCKDPTVLGSFIENHDNPRFASYTKDMSQAKAVISYVILSDGIPI
IYSGQ
EQHYSGGNDPYNREAIWLSGYSTTSELYKFIATTNKIRQLAISKDSSYLTSRNNPFYTDSNTIA
MRKGSG
GSQVITVLSNSGSNGGSYTLNLGNSGYSSGANLVEVYTCSSVTVGSDGKIPVPMASGLPRVLVPA
SWMSG
SGLCGSSSTTTLVTATTTPTGSSSSTTLATAVTTPTGSCKTATTVPVVLEESVRTSYGENIFISG
SIPQL
GSWNPDKAVALSSSQYTSSNPLWAVTLDLPVGTSFEYKFLKKEQNGGVAWENDPNRSYTVPEACA
GTSQK
VDSSWR

**SEQ ID NO: 16** (Acid-stable alpha-amylase from *Aspergillus kawachii*; gi|2570150|dbj|BAA22993.1| acid-stable alpha-amylase [Aspergillus kawachii])

MRVSTSSIALAVSLFGKLALGLSAAEWRTQSIYFLLTDRFGRTDNSTTATCNTGDQIYCGGS
WQGIINHLDYIQGMGFTAIWISPITEQLPQDTSDGEAYHGYWQQKIYYVNSNFGTADDLKSL
SDALHARGMYLMVDVVPNHMGYAGNGNDVDYSVFDPFDSSSYFHPYCLITDWDNLTMVQDCW
EGDTIVSLPDLNTTETAVRTIWYDWVADLVSNYSVDGLRIDSVEEVEPDFFPGYQEAAGVYC

VGEVDNGNPALDCPYQKYLDGVLNYPIYWQLLYAFESSSGSISNLYNMIKSVASDCSDPTLL
GNFIENHDNPRFASYTSDYSQAKNVLSYIFLSDGIPIVYAGEEQHYSGGDVPYNREATWLSG
YDTSAELYTWIATTNAIRKLAISADSDYITYKNDPIYTDSNTIAMRKGTSGSQIITVLSNKG
SSGSSYTLTLSGSGYTSGTKLIEAYTCTSVTVDSNGDIPVPMASGLPRVLLPASVVDSSSLC
GGSGNTTTTTTAATSTSKATTSSSSSSAAATTSSSCTATSTTLPITFEELVTTTYGEEVYLS
GSISQLGEWHTSDAVKLSADDYTSSNPEWSVTVSLPVGTTFEYKFIKVDEGGSVTWESDPNR
EYTVPECGSGSGETVVDTWR

**SEQ ID NO: 17** (Alpha-amylase from *Aspergillus awamori*; gi|40313278|dbj|BAD06003.1| alpha-amylase [Aspergillus awamori])

MRVSTSSLALSVSLFGKLALGLSAAEWRSQSIYFLLTDRFGRTDNSTTATCDTGDQIYCGGS
WQGIINHLDYIQGMGFTAIWISPITEQLPQDTSDGEAYHGYWQQKIYDVNSNFGTADDLKSL
SDALHARGMYLMVDVVPNHMGYAGNGNDVDYSVFDPFDSSSYFHPYCLITDWDNLTMVQDCW
EGDTIVSLPDLNTTETAVRTIWYDWVADLVSNYSVDGLRIDSVLEVEPDFFPGYQEAAGVYC
VGEVDNGNPALDCPYQDYLDGVLNYPIYWQLLYAFESSSGSISDLYNMIKSVASDCSDPTLL
GNFIENHDNPRFASYTSDYSQAKNVLSYIFLSDGIPIVYAGEEQHYSGGDVPYNREATWLSG
YDTSAELYTWIATTNAIRKLAISADSDYITYANDPIYTDSNTIAMRKGTSGSQVITVLSNKG
SSGSSYTLTLSGSGYTSGTELIEAYTCTSVTVDSNGDIPVPMASGLPRVLLPAWVVDSSSSL
WGGSTTTTTSSSTSTSTSKATSSSSTTTSSSCTATSTTLPITLEELVTTTYGEEIYLSGSIS
QLGEWDTSDAVKLSADDYTSSNPEWYVTVSLPVGTTFEYKFIKVEEDGSVTWESDPNREYTV
PECGSGETVVDTWR

**SEQ ID NO: 18** (Alpha-amylase from *Aspergillus fumigatus*; gi|70988703|ref|XP_749208.1| alpha-amylase [Aspergillus fumigatus Af293])

MKWIAQLFPLSLCSSLLGQAAHALTPAEWRSQSIYFLLTDRFGREDNSTTAACDVTQRLYCG
GSWQGIINHLDYIQGMGFTAIWITPVTEQFYENTGDGTSYHGYWQQNIHEVNANYGTAQDLR
DLANALHARGMYLMVDVVANHMGYNGAGNSVNYGVFTPFDSATYFHPYCLITDYNNQTAVED
CWLGDTTVSLPDLDTTSTAVRSIWYDWVKGLVANYSIDGLRIDTVKHVEKDFWPGYNDAAGV
YCVGEVFSGDPQYTCPYQNYLDGVLNYPIYYQLLYAFQSTSGSISNLYNMISSVASDCADPT
LLGNFIENHDNPRFASYTSDYSQAKNVISFMFFSDGIPIVYAGQEQHYSGGADPANREAVWL
SGYSTSATLYSWIASTNKIRKLAISKDSAYITSKNNPFYYDSNTLAMRKGSVAGSQVITVLS
NKGSSGSSYTLSLSGTGYSAGATLVEMYTCTTLTVDSSGNLAVPMVSGLPRVFVPSSWVSGS
GLCGDSISTTATAPSATTSATATRTACAAATAIPILFEELVTTTYGESIYLTGSISQLGNWD

TSSAIALSASKYTSSNPEWYVTVTLPVGTSFEYKFVKKGSDGSIAWESDPNRSYTVPTGCAG
TTVTVSDTWR

**SEQ ID NO: 19** (Alpha-amylase from *Aspergillus nidulans*; gi|67525889|ref|XP_661006.1| hypothetical protein AN3402.2 [Aspergillus nidulans FGSC A4])

MRLLALTSALALLGKAVHGLDADGWRSQSIYFLLTDRFARTDGSTTAACDLAQRRYCGGSWQ
GIINQLDYIQDMGFTAIWITPITEQIPDVTAVGTGFHGYWQKNIYGVDTNLGTADDIRALSE
ALHDRGMYLMLDVVANHMSYGGPGGSTDFSIFTPFDSASYFHSYCAINNYDNQWQVENCFLG
DDTVSLTDLNTQSSEVRDIWYDWIEDIVANYSVDGLRIDTVKHVEKDFWPGYIDAAGVYSVG
EIFHGDPAYTCPYQDYMDGVMNYPIYYPLLNAFKSSSGSMSDLYNMINTVASNCRDPTLLGN
FIENHDNPRFPNYTPDMSRAKNVLAFLFLTDGIPIVYAGQEQHYSGSNDPYNREPVWWSSYS
TSSELYKFIATTNKIRKLAISKDSSYLTSRNTPFYSDSNYIAMRKGSGGSQVLTLLNNIGTS
IGSYTFDLYDHGYNSGANLVELYTCSSVQVGSNGAISIPMTSGLPRVLVPAAWVSGSGLCGL
TNPTSKTTTATTTSTTTCASATATAITVVFQERVQTAYGENVFLAGSISQLGNWDTTEAVAL
SAAQYTATDPLWTVAIELPVGTSFEFKFLKKRQDGSIVWESNPNRSAKVNEGCARTTQTIST
SWR

SEQ ID NO: 20 (alpha-amylase from *Aspergillus niger* (Protein Data Base entry 2GUY|A))

ATPADWRSQSIYFLLTDRFARTDGSTTATCNTADQKYCGGTWQGIIDKLDYIQGMGFTAIWI
TPVTAQLPQTTAYGDAYHGYWQQDIYSLNENYGTADDLKALSSALHERGMYLMVDVVANHMG
YDGAGSSVDYSVFKPFSSQDYFHPFCFIQNYEDQTQVEDCWLGDNTVSLPDLDTTKDVVKNE
WYDWVGSLVSNYSIDGLRIDTVKHVQKDFWPGYNKAAGVYCIGEVLDGDPAYTCPYQNVMDG
VLNYPIYYPLLNAFKSTSGSMDDLYNMINTVKSDCPDSTLLGTFVENHDNPRFASYTNDIAL
AKNVAAFIILNDGIPIIYAGQEQHYAGGNDPANREATWLSGYPTDSELYKLIASANAIRNYA
ISKDTGFVTYKNWPIYKDDTTIAMRKGTDGSQIVTILSNKGASGDSYTLSLSGAGYTAGQQL
TEVIGCTTVTVGSDGNVPVPMAGGLPRVLYPTEKLAGSKICSSS

## WHAT IS CLAIMED IS

Claim 1. A chimeric alpha-amylase having at least 90% sequence identity to residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13, comprising a catalytic domain (CD) derived from a first alpha-amylase having the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 10 fused to the N-terminus of a carbohydrate binding module (CBM) derived from a second alpha-amylase having the amino acid sequence of SEQ ID NO: 14.

Claim 2. The chimeric alpha-amylase of claim 1, wherein the chimeric alpha-amylase has at least 92%, 95%, 98% or 99% sequence identity to residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13.

Claim 3. The chimeric alpha-amylase of claim 1 or 2, wherein the chimeric alpha-amylase comprises residues 17-603 of SEQ ID NO: 5 or residues 21-616 of SEQ ID NO: 13.

Claim 4. The chimeric alpha-amylase of any one of claims 1-3, wherein the CD derived from the first alpha-amylase has at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 17-492 of SEQ ID NO: 2, or residues 21-507 of SEQ ID NO: 10.

Claim 5. The chimeric alpha-amylase of any one of claims 1-4, wherein the CD derived from the first alpha-amylase comprises residues 17-492 of SEQ ID NO: 2, or residues 21-507 of SEQ ID NO: 10.

Claim 6. The chimeric alpha-amylase of any one of claims 1-5, wherein the CBM derived from the second alpha-amylase has at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 498-607 of SEQ ID NO: 14.

Claim 7. The chimeric alpha-amylase of any one of claims 1-6, wherein the CBM derived from the second alpha-amylase comprises residues 498-607 of SEQ ID NO: 14.

Claim 8. The chimeric alpha-amylase of any one of claims 1-7, wherein the chimeric alpha-amylase is capable of hydrolyzing insoluble starch at least three times or at least nine times faster than the first alpha-amylase under the same reaction conditions.

Claim 9. The chimeric alpha-amylase of claim 8, wherein the reaction conditions comprise mixing an insoluble starch substrate with the chimeric alpha-amylase or the first alpha-amylase, and incubating for 20-24 hours at 32 °C to measure the glucose content in the supernatant, wherein the substrate is obtained from a simultaneous saccharification and fermentation (SSF) reaction having whole ground corn liquefact as a starting material, and wherein the substrate is a washed and sieved insoluble material containing no soluble starch.

Claim 10. An enzyme composition comprising the chimeric alpha-amylase of any one of claims 1-9.

Claim 11. The enzyme composition of claim 10, further comprising glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, isoamylase, phytase, pullulanase, β-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, transglucosidase, or any combination thereof.

Claim 12. A method of processing starch comprising contacting a composition comprising starch with the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 to produce a composition comprising glucose.

Claim 13. The method of claim 12, wherein the composition comprising starch comprises liquefied starch, gelatinized starch, granular starch, recalcitrant starch, or residual starch.

Claim 14. The method of claim 12 or 13, further comprising adding glucoamylase, hexokinase, xylanase, glucose isomerase, xylose isomerase, phosphatase, isoamylase, phytase, pullulanase, β-amylase, protease, cellulase, hemicellulase, lipase, cutinase, isoamylase, redox enzyme, esterase, transferase, pectinase, alpha-glucosidase, beta-glucosidase, transglucosidase, or any combination thereof, to the composition comprising starch.

Claim 15. The method of any one of claims 12-14, wherein starch processing is conducted at a temperature range of 60°C – 85°C for the chimeric alpha-amylase having at least 90% sequence identity to residues 17-603 of SEQ ID NO: 5, or 42°C – 71°C for the

chimeric alpha-amylase having at least 90% sequence identity to residues 21-616 of SEQ ID NO: 13.

Claim 16. The method of any one of claims 12-15, wherein starch processing is conducted at a pH range of pH 3.0 – pH 7.3 for the chimeric alpha-amylase having at least 90% sequence identity to residues 17-603 of SEQ ID NO: 5, or pH 3.7 – pH 7.4 for the chimeric alpha-amylase having at least 90% sequence identity to residues 21-616 of SEQ ID NO: 13.

Claim 17. The method of any one of claims 12-16, further comprising fermenting the glucose composition to produce an End of Fermentation (EOF) product.

Claim 18. The method of any one of claims 12-17, wherein said fermentation is a simultaneous saccharification and fermentation (SSF) reaction.

Claim 19. The method of any one of claims 12-18, wherein said fermentation is conducted for 48 – 70 hours at pH 2 – 8 and in a temperature range of 25°C – 70°C.

Claim 20. The method of any one of claims 17-19, wherein the EOF product comprises ethanol.

Claim 21. The method of any one of claims 17-19, wherein the EOF product comprises citric acid, lactic acid, succinic acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta-lactone, sodium erythorbate, omega 3 fatty acid, butanol, an amino acid, lysine, itaconic acid, 1,3-propanediol, ethylene, or isoprene.

Claim 22. The method of any one of claims 17-21, further comprising contacting a mash and/or a wort with the chimeric alpha-amylase or the enzyme composition.

Claim 23. A method of providing a fermented beverage comprising the use of the chimeric alpha-amylase of any one of claims 1-9 or the enzymatic composition of claim 10 or 11.

Claim 24. The method of claim 23, comprising:

(a) preparing a mash;

(b) filtering the mash to obtain a wort; and

(c) fermenting the wort to obtain a fermented beverage,

wherein the chimeric alpha-amylase or the enzyme composition is added to:

(i) the mash of step (a) and/or

(ii) the wort of step (b) and/or

(iii) the wort of step (c).

Claim 25. The method of claim 23 or 24, wherein the fermented beverage is

i) a beer selected from the group consisting of full malted beer, beer brewed under the "Reinheitsgebot," ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, and non-alcoholic malt liquor; or

ii) a cereal or malt beverage selected from the group consisting of fruit flavoured malt beverages, liquor flavoured malt beverages, and coffee flavoured malt beverages.

Claim 26. The method of any one of claims 17-25, wherein the chimeric alpha-amylase is purified.

Claim 27. The method of any one of claims 17-26, wherein the chimeric alpha-amylase is expressed and secreted by a host cell.

Claim 28. The method of claim 27, wherein the composition comprising starch is contacted with the host cell.

Claim 29. The method of claim 27 or 28, wherein the host cell further expresses and secretes a glucoamylase.

Claim 30. The method of any one of claims 27-29, wherein the host cell is capable of fermenting the glucose composition.

Claim 31. The method of any one of claims 17-30, wherein fermenting the glucose composition results in a product selected from the group consisting of

i) a beer selected from the group consisting of full malted beer, beer brewed under the "Reinheitsgebot," ale, IPA, lager, bitter, Happoshu (second beer), third beer, dry beer, near beer, light beer, low alcohol beer, low calorie beer, porter, bock beer, stout, malt liquor, non-alcoholic beer, and non-alcoholic malt liquor; and

ii) cereal or malt beverages selected from the group consisting of fruit flavoured malt beverages, liquor flavoured malt beverages, and coffee flavoured malt beverages.

Claim 32. A method of producing a food composition, comprising combining

(i) one or more food ingredients, and

(ii) the chimeric alpha-amylase of any of claims 1-9 or or the enzyme composition of claim 10 or 11,

wherein the chimeric alpha-amylase catalyzes the hydrolysis of starch components present in the food ingredients to produce glucose.

Claim 33. The method of claim 32, wherein the food composition is selected from the group consisting of a food product, a baking composition, a food additive, an animal food product, a feed product, a feed additive, an oil, a meat, and a lard.

Claim 34. The method of claim 32 or 33, wherein the one or more food ingredients comprise a baking ingredient or an additive.

Claim 35. The method of any one of claims 32-34, wherein one or more food ingredients comprises flour and an enzyme selected from the group consisting an anti-staling amylase; a phospholipase; a phospholipid; a maltogenic alpha-amylase or a variant, homologue, or mutants thereof which has maltogenic alpha-amylase activity; a bakery xylanase (EC 3.2.1.8); and a lipase.

Claim 36. The method of any one of claims 32-35, wherein one or more food ingredients comprises an enzyme selected from the group consisting of

(i) a maltogenic alpha-amylase from *Bacillus stearothermophilus*,

(ii) a bakery xylanase is from *Bacillus*, *Aspergillus*, *Thermomyces* or *Trichoderma*, and

(iii) a glycolipase from *Fusarium heterosporum.*

Claim 37. The method of any one of claims 32-36, wherein the food composition comprises a dough, a dough product, or a processed dough product.

Claim 38. The method of any one of claims 32-37, further comprising baking the food composition to produce a baked good.

Claim 39. The method of any one of claims 32-38, further comprising

(i) providing a starch medium;

(ii) adding to the starch medium the chimeric alpha-amylase; and

(iii) applying heat to the starch medium during or after step (ii) to produce a bakery product.

Claim 40. A method of removing starchy stains from laundry, dishes, or textiles, comprising incubating a surface of said laundry, dishes, or textiles in the presence of an aqueous composition comprising an effective amount of the chimeric alpha-amylase of any one of claims 1-9, and allowing the chimeric alpha-amylase 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.

Claim 41. A method of desizing a textile comprising contacting a desizing composition with a textile for a time sufficient to desize the textile, wherein the desizing composition comprises the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 and allowing the chimeric alpha-amylase to desize 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.

Claim 42. A composition comprising glucose produced by the method of any one of claims 12-16.

Claim 43. A liquefied starch produced by the method of claim 12.

Claim 44. A fermented beverage produced by the method of any one of claims 17-31.

Claim 45. A food composition produced by the method of any one of claims 32-39.

Claim 46. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in the production of a composition comprising glucose.

Claim 47. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in the production of a liquefied starch.

Claim 48. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in the production of a fermented beverage.

Claim 49. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in preparing a food composition.

Claim 50. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in a dough product to retard or reduce staling or detrimental retrogradation of the dough product.

Claim 51. Use of the chimeric alpha-amylase of any one of claims 1-9 or the enzyme composition of claim 10 or 11 in desizing textiles.

Claim 52. Use of a CBM having at least 90%, 92%, 95%, 98% or 99% sequence identity to residues 498-607 of SEQ ID NO: 14 in improving an alpha-amylase's capability of hydrolyzing insoluble starch,

Claim 53. The use of claim 52, wherein the alpha-amylase lacks a CBM.

Claim 54. A nucleic acid encoding the chimeric alpha-amylase of any one of claims 1-9.

Claim 55. A vector comprising the nucleic acid of claim 54.

Claim 56. A host cell comprising the vector of claim 55.

Claim 57. The host cell of claim 56, wherein the host cell is a filamentous fungal cell.

Claim 58. The host cell of claim 57, wherein the host cell is an *Aspergillus sp.* or *Trichoderma reesei* cell.

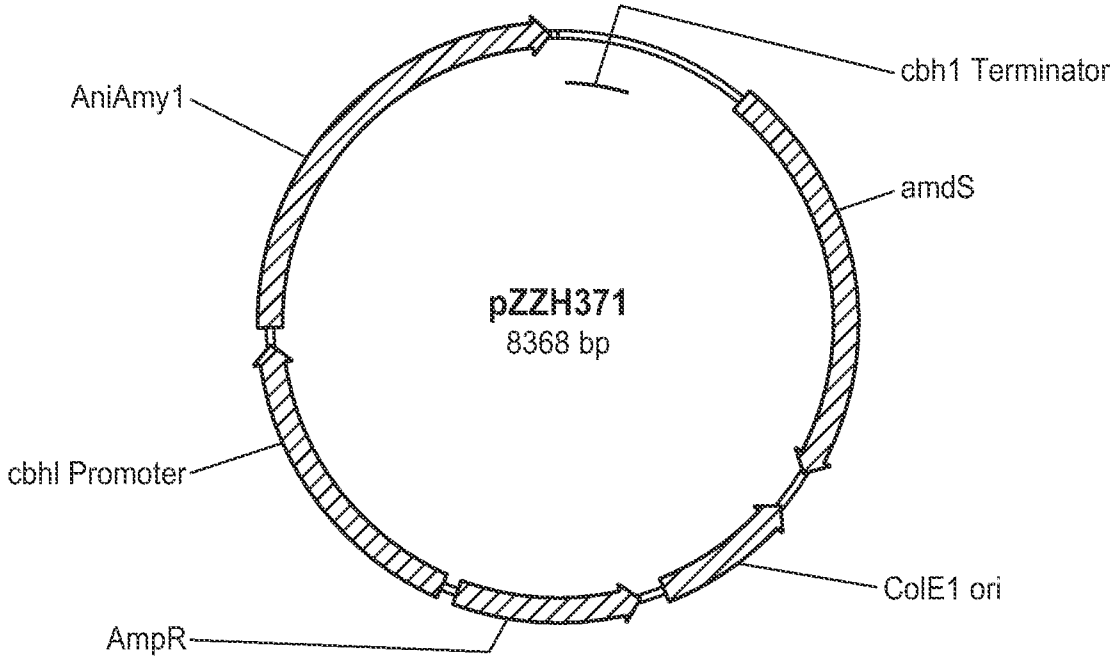

cbh1 Terminator
AniAmy1
amdS
pZZH371
8368 bp
cbhI Promoter
ColE1 ori
AmpR

FIG. 1

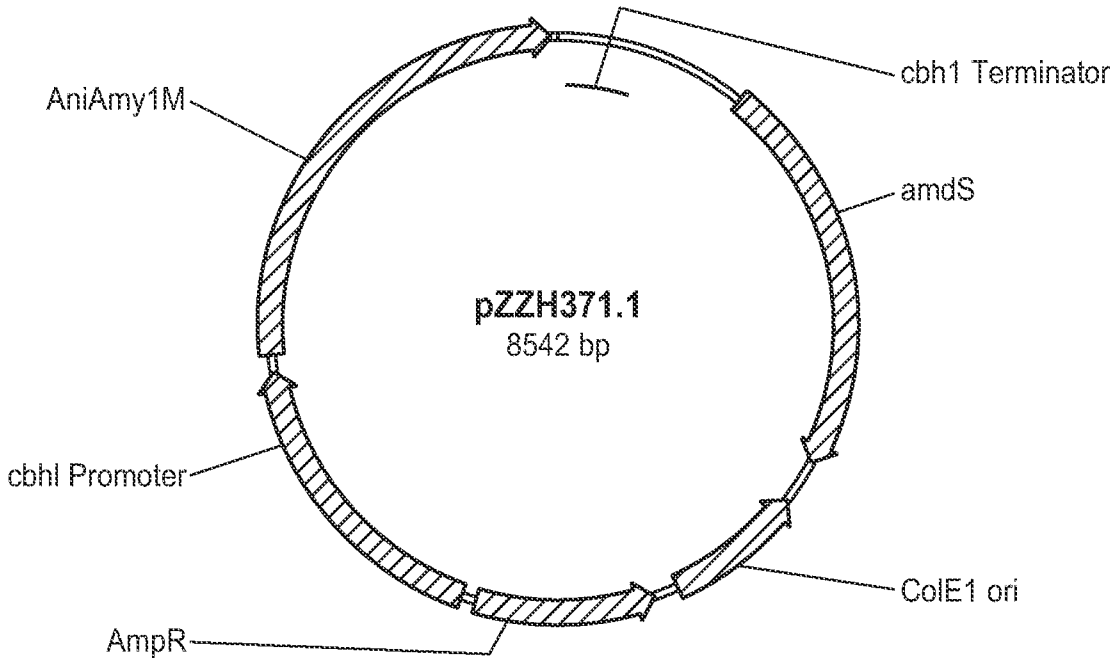

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amdS
pZZH371.1
8542 bp
cbhI Promoter
ColE1 ori
AmpR

FIG. 2

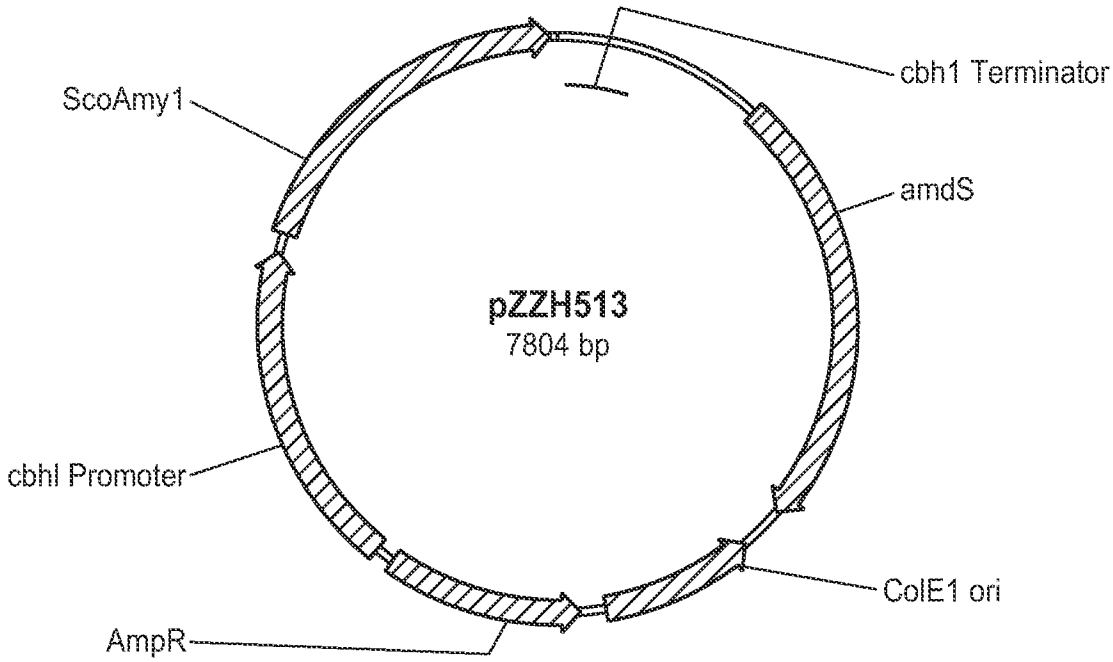

cbh1 Terminator
ScoAmy1
amdS
pZZH513
7804 bp
cbhl Promoter
ColE1 ori
AmpR

FIG. 3

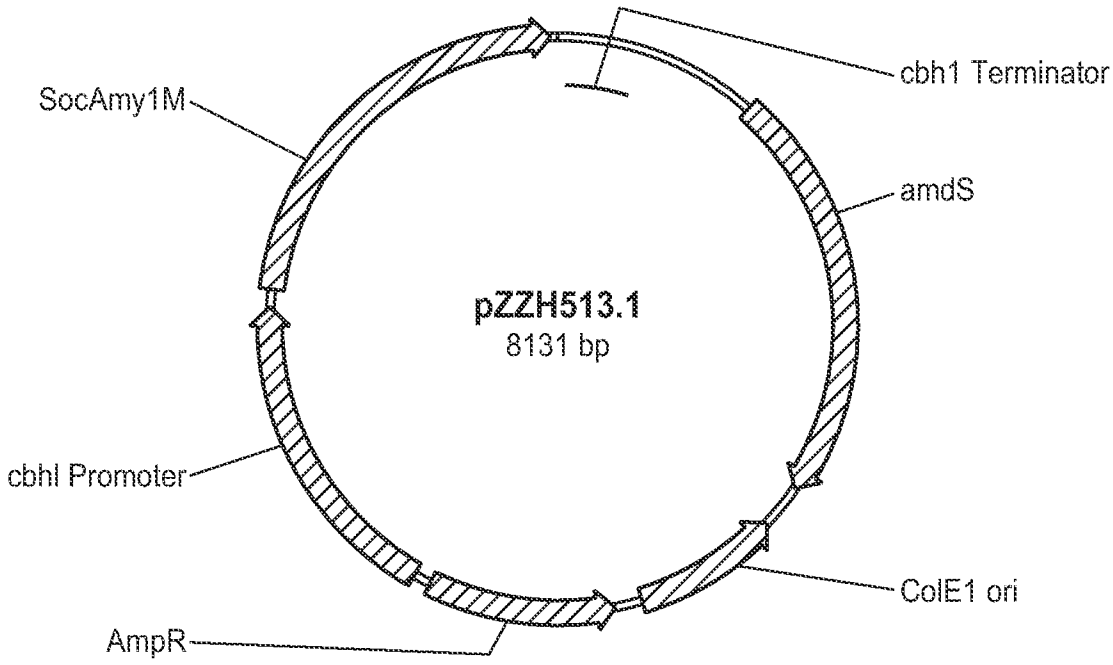

cbh1 Terminator
SocAmy1M
amdS
pZZH513.1
8131 bp
cbhl Promoter
ColE1 ori
AmpR

FIG. 4

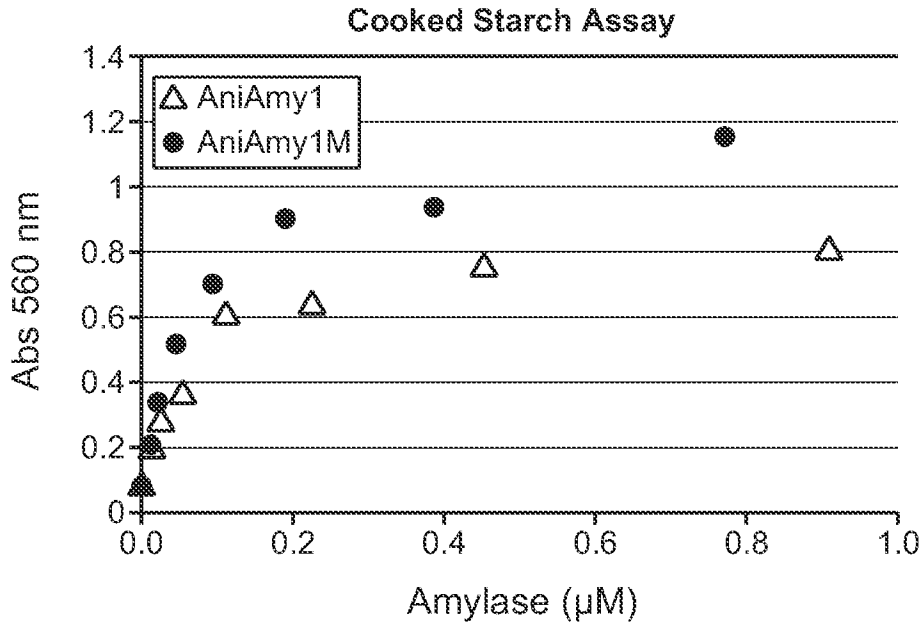

Cooked Starch Assay
AniAmy1
AniAmy1M
Abs 560 nm
Amylase (μM)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 5A

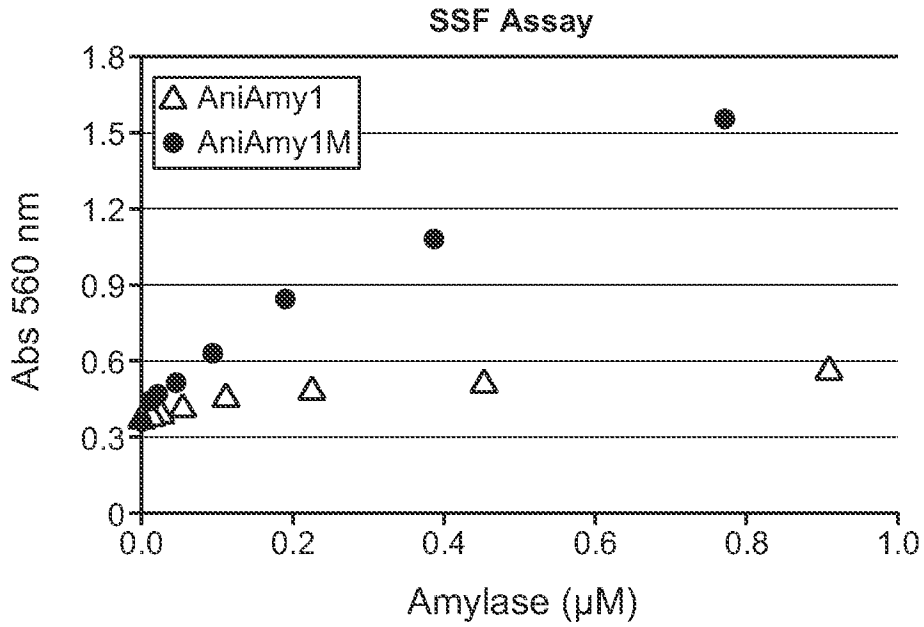

SSF Assay
AniAmy1
AniAmy1M
Abs 560 nm
Amylase (μM)
0
0.3
0.6
0.9
1.2
1.5
1.8
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 5B

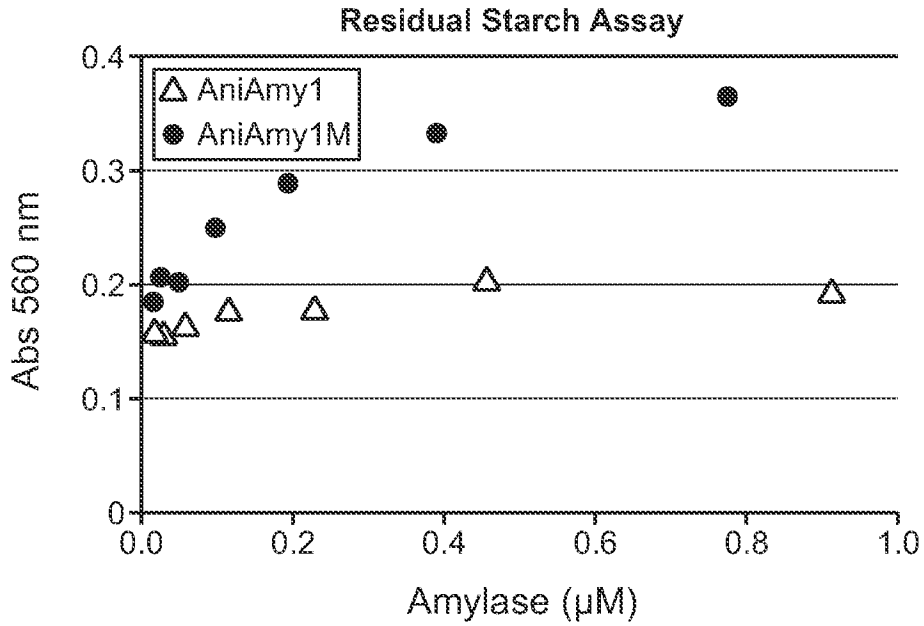

Residual Starch Assay
AniAmy1
AniAmy1M
Abs 560 nm
0.4
0.3
0.2
0.1
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 5C

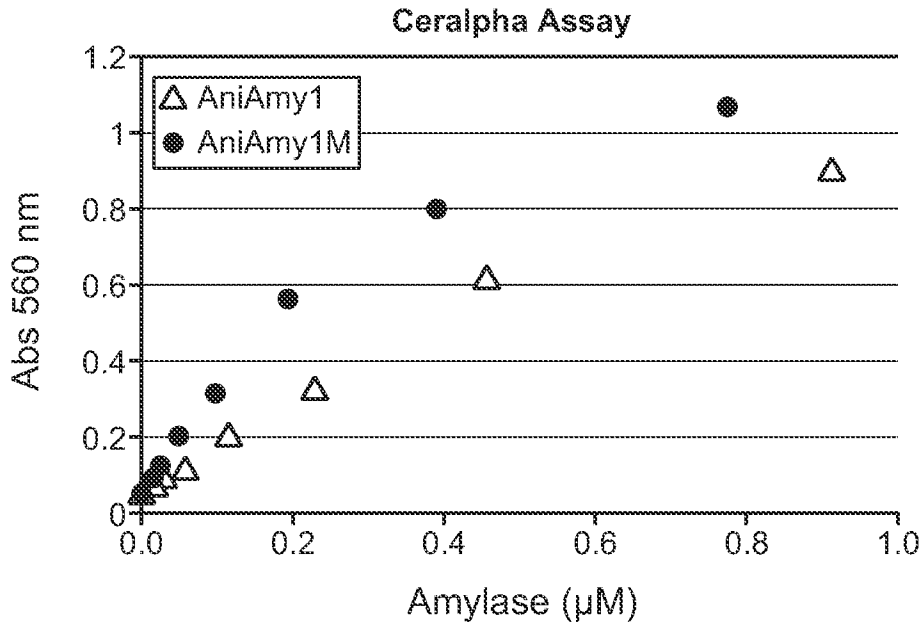

Ceralpha Assay
AniAmy1
AniAmy1M
Abs 560 nm
1.2
1
0.8
0.6
0.4
0.2
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 5D

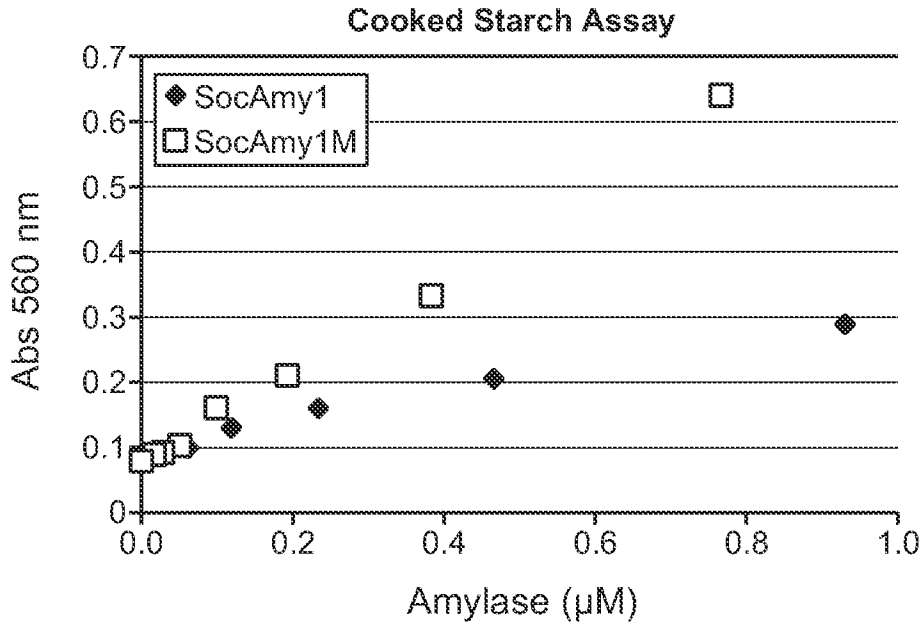

Cooked Starch Assay
SocAmy1
SocAmy1M
Abs 560 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 6A

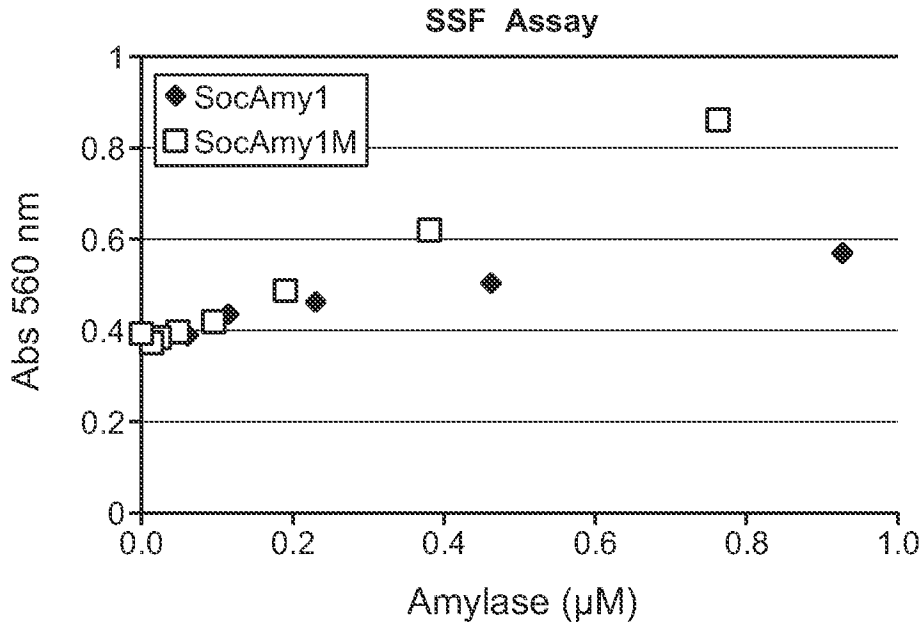

SSF Assay
SocAmy1
SocAmy1M
Abs 560 nm
1
0.8
0.6
0.4
0.2
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 6B

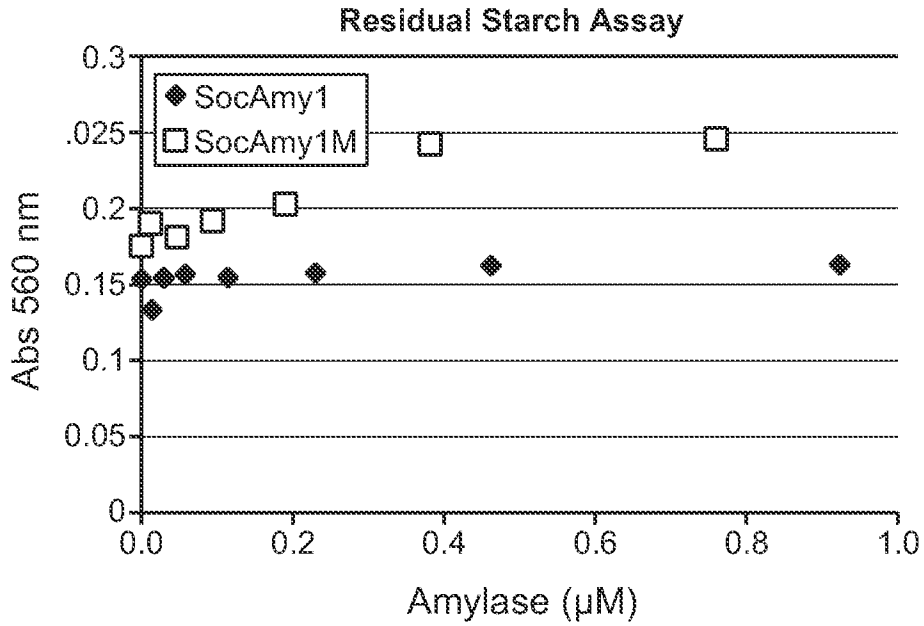

Residual Starch Assay
SocAmy1
SocAmy1M
Abs 560 nm
0.3
.025
0.2
0.15
0.1
0.05
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 6C

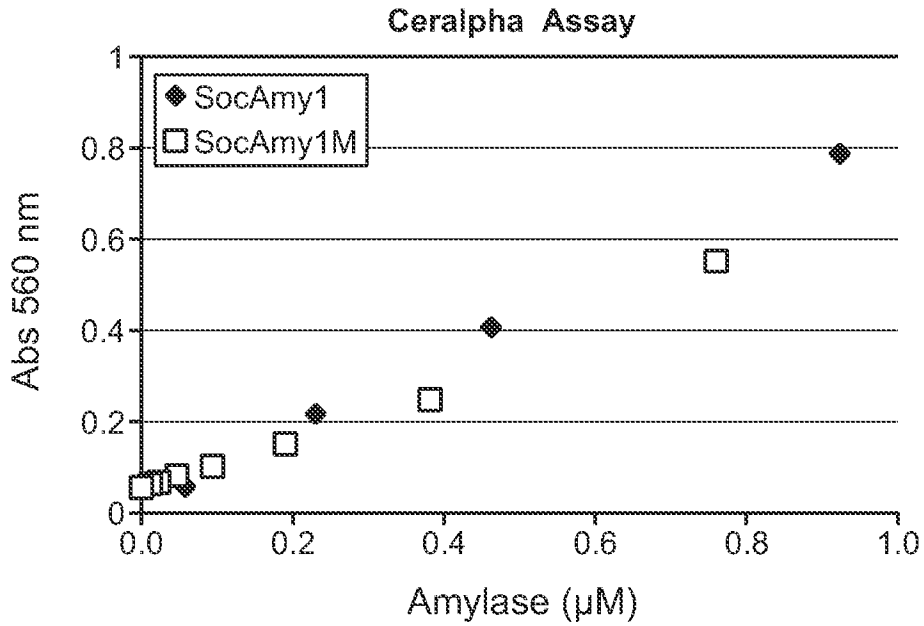

Ceralpha Assay
SocAmy1
SocAmy1M
Abs 560 nm
1
0.8
0.6
0.4
0.2
0
0.0
0.2
0.4
0.6
0.8
1.0
Amylase (μM)

FIG. 6D

```
SEQ ID NO: 15     20 ----------LTPAEWRG QSIYFLITDRFARTDGSTTA PCDLSQRAYCGGSWQGIIKQ  67
SEQ ID NO: 16     22 ----------LSAAEWRT QSIYFLLTDRFGRTDNSTTA TCNTGDQIYCGGSWQGIINH  69
SEQ ID NO: 17     22 ----------LSAAEWRS QSIYFLLTDRFGRTDNSTTA TCDTGDQIYCGGSWQGIINH  69
SEQ ID NO: 18     24 ----------LTPAEWRS QSIYFLLTDRFGREDNSTTA ACDVTQRLYCGGSWQGIINH  71
SEQ ID NO: 19     20 ----------LDADGWRS QSIYFLLTDRFARTDGSTTA ACDLAQRRYCGGSWQGIINQ  67
SEQ ID NO: 14     21 ----------LTPAEWRS QSIYFLLTDRFGRTDNSTTA ACDTSDRVYCGGSWQGIINQ  68
SEQ ID NO: 2      17 ----------ATPAEWRS QSIYFLLTDRFARTDNSTTA SCDLSARQYCGGSWQGIINQ  64
SEQ ID NO: 10     21 KPIFLSKRDAGSSAAAAWRS ESIYQLVTDRFARTDGSTSA TCNTGDRVYCGGTFQGIIDK  80
SEQ ID NO: 20      1 ----------ATPADWRS QSIYFLLTDRFARTDGSTTA TCNTADQKYCGGTWQGIIDK  48
                                    **   *** * **** * * ** *  *     ****  ****

LDYIQGMGFTAIWITPITEQ IPQDTAEGSAFHGYWQKDIY NVNSHFGTADDIRALSKALH DRGMYLMIDVVANHMGYNGP 147
LDYIQGMGFTAIWISPITEQ LPQDTSDGEAYHGYWQQKIY YVNSNFGTADDLKSLSDALH ARGMYLMVDVVPNHMGYAGN 149
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LDYIQGMGFTAIWITPVTAQ IPQDTGYGQAYHGYWQQDAY ALNSHYGTADDLKALASALH SRGMYLMVDVVANHMGHNGT 144
LDYIQGMGFTAIWISPVVEQ IPDDTGYGYAYHGYWMKDIY AINSNFGTADDLKNLSNELH KRNMKLMVDIVTNHYAWNGA 160
LDYIQGMGFTAIWITPVTAQ LPQTTAYGDAYHGYWQQDIY SLNENYGTADDLKALSSALH ERGMYLMVDVVANHMGYDGA 128
***** ******** *   *     *  *   ****          *** *   *   **  * * ** * * **    *
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FIG. 7A

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GASTDFSTFTPFNSASYFHS YCPINNYNDQSQVENCWLGD NTVALADLYTQHSDVRNIWY SWIKEIVGNYSADGLRIDTV 227
GNDVDYSVFDPFDSSSYFHP YCLITDWDNLTMVQDCWEGD TIVSLPDLNTTETAVRTIWY DWVADLVSNYSVDGLRIDSV 229
GNDVDYSVFDPFDSSSYFHP YCLITDWDNLTMVQDCWEGD TIVSLPDLNTTETAVRTIWY DWVADLVSNYSVDGLRIDSV 229
GNSVNYGVFTPFDSATYFHP YCLITDYNNQTAVEDCWLGD TTVSLPDLDTTSTAVRSIWY DWVKGLVANYSIDGLRIDTV 231
GGSTDFSIFTPFDSASYFHS YCAINNYDNQWQVENCFLGD DTVSLTDLNTQSSEVRDIWY DWIEDIVANYSVDGLRIDTV 227
GNTVDYSVFNPFSSSSYFHP YCLISNYDNQTNVEDCWLGD TTVSLPDLDTTSTAVRNIWY DWVADLVANYSIDGLRVDTV 228
GSSVDYSVYRPFNSQKYFHN LCWISDYNNQTNVEDCWLGD NTVALPDLDTTSTEVKNMWY DWVESLVSNYSVDGLRVDTV 224
GSSVAYSNYNPFNQQSYFHD YCLITNYDDQTNVEDCWEGD NTVSLPDLRTEDSDVSSIFN LWVAELVSNYSIDGLRIDSA 240
GSSVDYSVFKPFSSQDYFHP FCFIQNYEDQTQVEDCWLGD NTVSLPDLDTTKDVVKNEWY DWVGSLVSNYSIDGLRIDTV 208
*        **    ***   * *       *  *  **   * * ** *    *      *    * *** **** *

KHVEKDFWTGYTQAAGVYTV GEVLDGDPAYTCPYQGYVDG VLNYPIYYPLLRAFESSSGS MGDLYNMINSVASDCKDPTV 307
EEVEPDFFPGYQEAAGVYCV GEVDNGNPALDCPYQKYLDG VLNYPIYWQLLYAFESSSGS ISNLYNMIKSVASDCSDPTL 309
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KHVEKDFWPGYIDAAGVYSV GEIFHGDPAYTCPYQDYMDG VMNYPIYYPLLNAFKSSSGS MSDLYNMINTVASNCRDPTL 307
KHVEKDFWPGYNSAAGVYCV GEVYSGDPAYTCPYQNYMDG VLNYPIYYQLLYAFESSSGS ISDLYNMISSVASSCKDPTL 308
KNVQKNFWPGYNNASGVYCI GEVFDGDASYTCPYQEDLDG VLNYPMYYPLLRAFESTNGS ISDLYNMINTVKSTCRDSTL 304
KHVDESFYPSFQSAAGVYLL GEVYDGDPAYTCPYQNYMSG VTNYPLYYPMLRFFQGTSNS VDELNAMISSLESDCKDITL 320
KHVQKDFWPGYNKAAGVYCI GEVLDGDPAYTCPYQNVMDG VLNYPIYYPLLNAFKSTSGS MDDLYNMINTVKSDCPDSTL 288
  *   *     * ***   **   *    ****   * * *** *   *  *    *   *  **   * * * *
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FIG. 7B

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LGSFIENHDNPRFASYTKDM SQAKAVISYVILSDGIPIIY SGQEQHYSGGNDPYNREAIW LSGYSTTSELYKFIATTNKI 387
LGNFIENHDNPRFASYTSDY SQAKNVLSYIFLSDGIPIVY AGEEQHYSGGDVPYNREATW LSGYDTSAELYTWIATTNAI 389
LGNFIENHDNPRFASYTSDY SQAKNVLSYIFLSDGIPIVY AGEEQHYSGGDVPYNREATW LSGYDTSAELYTWIATTNAI 389
LGNFIENHDNPRFASYTSDY SQAKNVISFMFFSDGIPIVY AGQEQHYSGGADPANREAVW LSGYSTSATLYSWIASTNKI 391
LGNFIENHDNPRFPNYTPDM SRAKNVLAFLFLTDGIPIVY AGQEQHYSGSNDPYNREPVW WSSYSTSSELYKFIATTNKI 387
LGNFIENHDNPRFASYTSDY SQAKNVITFIFLSDGIPIVY AGQEQHYSGGSDPANREATW LSGYSTSATLYTWIATTNQI 388
LGTFVENHDNPRFANYTSDM SLAKNAATFTILADGIPIIY AGQEQHYSGGNDPYNREATW LSGYKTTSELYTHIAASNKI 384
LGNFIENHDQPRLPSYTSDS ALIKNAIAFNLMSDGIPIIY YGQEQGYSGSSDPNNREALW LSGYSTSNGYYKLISSVNQI 400
LGTFVENHDNPRFASYTNDI ALAKNVAAFIILNDGIPIIY AGQEQHYAGGNDPANREATW LSGYPTDSELYKLIASANAI 368
** * **** **   ** *    *       ***** *  * ** * *   * ***  *  * * *   *  *   * *
```

```
RQLAISKDSSYLTSRNNPFY TDSNTIAMRKG-SGGSQVIT VLSNSGSNGGSYTLNLGNSG YSSGANLVEVYTCSSVTVGS 466
RKLAISADSDYITYKNDPIY TDSNTIAMRKG-TSGSQIIT VLSNKGSSGSSYTLTLSGSG YTSGTKLIEAYTCTSVTVDS 468
RKLAISADSDYITYANDPIY TDSNTIAMRKG-TSGSQVIT VLSNKGSSGSSYTLTLSGSG YTSGTELIEAYTCTSVTVDS 468
RKLAISKDSAYITSKNNPFY YDSNTLAMRKGSVAGSQVIT VLSNKGSSGSSYTLSLSGTG YSAGATLVEMYTCTTLTVDS 471
RKLAISKDSSYLTSRNTPFY SDSNYIAMRKG-SGGSQVLT LLNNIGTSIGSYTFDLYDHG YNSGANLVELYTCSSVQVGS 466
RSLAISKDAGYVQAKNNPFY SDSNTIAMRKGTTAGAQVIT VLSNKGASGSSYTLSLSGTG YSAGATLVETYTCTTVTVDS 468
RTHAIKQDTGYLTYKNYPIY QDTSTLAMRKG-YNGTQTIT VLSNLGASGSSYTLSLPGTG YTAGQKITEIYTCTNLTVNS 463
RNQAIYKDSKYTTYWSDVLY ASGHVIALQRG-ADDQRIVS VFNNLGSSG-SQTVTFS-TK YSGGEKVVDVLTCQTSYANS 477
RNYAISKDTGFVTYKNWPIY KDDTTIAMRKG-TDGSQIVT ILSNKGASGDSYTLSLSGAG YTAGQQLTEVIGCTTVTVGS 447
*  **  *          *      *   *           * *    * *       *  *       *     *
```

FIG. 7C

Putative Linker

```
DGKIPVPMASGLPRVLVPAS WMSGSG-LCGSS 497 | 498 STTTLVTATTTPTGSSSSTTLATAVT----------TPTG 527
NGDIPVPMASGLPRVLLPAS VVDSSS-LCGGS 499 | 500 --------GNTTTTTTAATSTSKATTSSSSSSAAATTSSS 531
NGDIPVPMASGLPRVLLPAW VVDSSSSLWGGS 500 | 501 --------TTTTTSSSTSTSTSKATSSSS-----TTTSSS 527
SGNLAVPMVSGLPRVFVPSS WVSGSG-LCGDS 502 | 503 --------ISTTATAPSATTSATA------------TRTA 522
NGAISIPMTSGLPRVLVPAA WVSGSG-LCG-- 495 | 496 -----------LTNPTSKTTTATTTS----------TTTC 514
SGNLPVPMTSGLPRVFVPSS WVNGSA-LCN-- 497 | 498 ------------------------------------TE 499

NGSVPVPMKSGLPRILYPAD KLVNGSSFCSSAITVFKDAGGGVLFFSYTV 513
DSTLTVSISGGAPRIYAPAS LIANSG-ICNF------------------- 507
DGNVPVPMAGGLPRVLYPTE KLA-GSKICSSS----------------- 478
         * **   *

SCKTATTVPVVLEESVRTSY GENIFISGSIPQLGSWNPDK AVALSSSQYTSSNPLWAVTL DLPVGTSFEYKFLKKEQNGG 607
CTATSTTLPITFEELVTTTY GEEVYLSGSISQLGEWHTSD AVKLSADDYTSSNPEWSVTV SLPVGTTFEYKFIKVDEGGS 611
CTATSTTLPITLEELVTTTY GEEIYLSGSISQLGEWDTSD AVKLSADDYTSSNPEWYVTV SLPVGTTFEYKFIKVEEDGS 607
CAAA-TAIPILFEELVTTTY GESIYLTGSISQLGNWDTSS AIALSASKYTSSNPEWYVTV TLPVGTSFEYKFVKKGSDGS 601
ASATATAITVVFQERVQTAY GENVFLAGSISQLGNWDTTE AVALSAAQYTATDPLWTVAI ELPVGTSFEFKFLKKRQDGS 594
CTAA-TSISVLFEELVTTTY GENIYLSGSISQLGSWNTAS AVALSASQYTSSNPEWYVSV TLPVGTSFQYKFIKKGSDGS 578
    *      * * * * **    *** *** *    *  **   **   * * *   ***** *  ** *   *


VAWENDPNRSYTVPEACAGT SQKVDSSWR 636
VTWESDPNREYTVPECGSGS GETVVDTWR 640
VTWESDPNREYTVPECGSG- -ETVVDTWR 634
IAWESDPNRSYTVPTGCAGT TVTVSDTWR 630
IVWESNPNRSAKVNEGCART TQTISTSWR 623
VVWESDPNRSYTVPAGCEGA TVTVADTWR 607
  **  ***   *            **
```

Putative Carbohydrate Binding Module (CBM)

FIG. 7D

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/069277

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/26 C12N9/30
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 March 2015 | 18/03/2015 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Brero, Alessandro |

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
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