Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2726630 A1 2009/12/10

(21) 2 726 630

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2009/06/04
(87) Date publication PCT/PCT Publication Date: 2009/12/10
(85) Entrée phase nationale/National Entry: 2010/12/01
(86) N° demande PCT/PCT Application No.: US 2009/046279
(87) N° publication PCT/PCT Publication No.: 2009/149271
(30) Priorité/Priority: 2008/06/06 (US61/059,535)

(51) Cl.Int./Int.Cl. *C12P 19/14* (2006.01), *C11D 3/386* (2006.01), *C12P 19/02* (2006.01), *D06M 16/00* (2006.01), *C12N 9/28* (2006.01), *C12P 7/06* (2006.01)

(71) Demandeur/Applicant:
DANISCO US INC., US

(72) Inventeurs/Inventors:
CASCAO-PEREIRA, LUIS G., US;
CHIN, REGINA, US;
CUEVAS, WILLIAM A., US;
ESTELL, DAVID A., US;
LEE, SANG-KYU, US;
PEPSIN, MICHAEL J., US; ...

(54) Titre : PRODUCTION DE GLUCOSE A PARTIR D'AMIDON A L'AIDE D'ALPHA-AMYLASES PROVENANT DE BACILLUS SUBTILIS
(54) Title: PRODUCTION OF GLUCOSE FROM STARCH USING ALPHA-AMYLASES FROM BACILLUS SUBTILIS

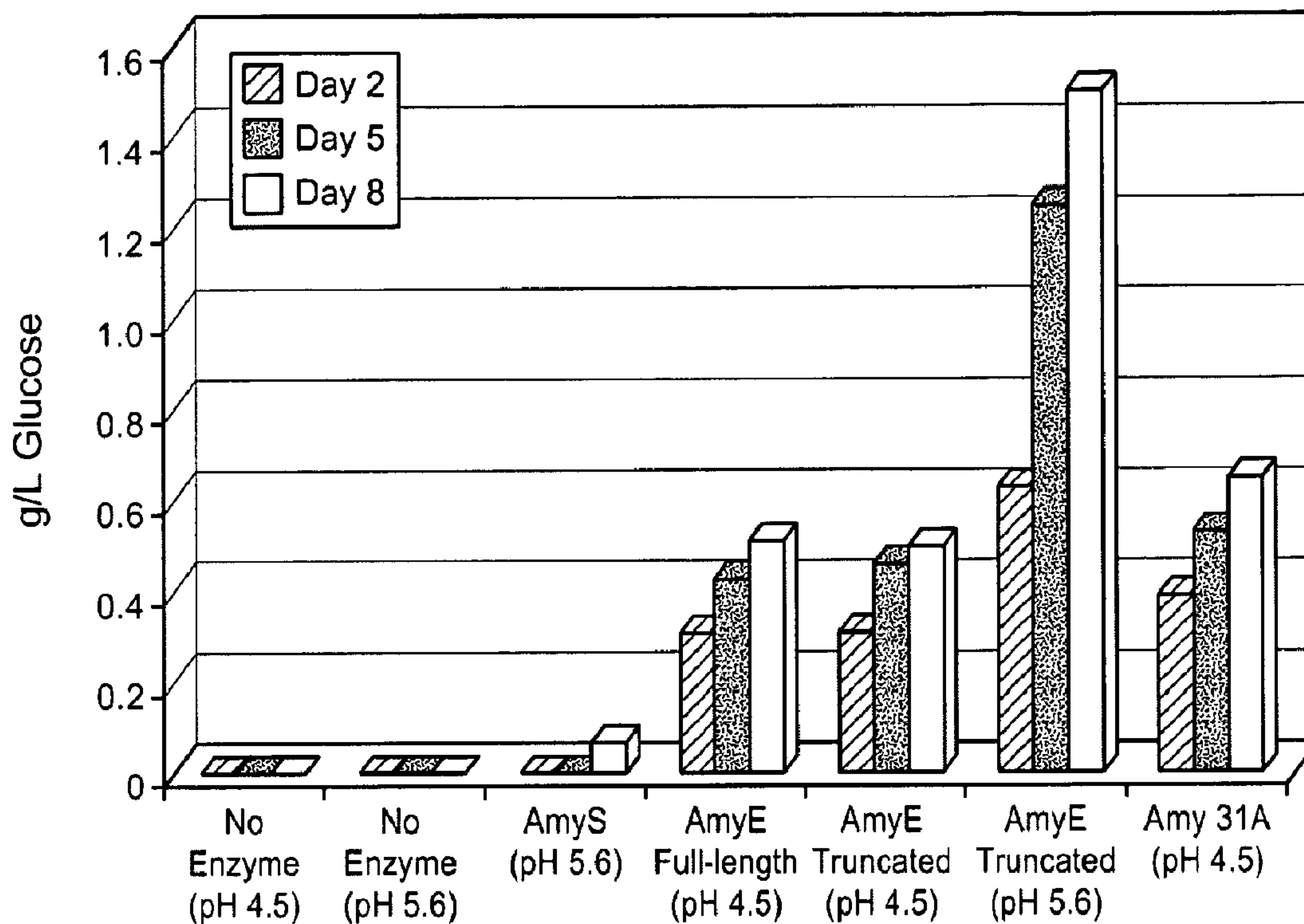


FIG. 5

(57) Abrégé/Abstract:
An alpha amylase from Bacillus subtilis (AmyE) produces significant amounts of glucose from various carbohydrate substrates, including vegetable starch, maltoheptaose, and maltotriose. Among other things, this advantageous property allows AmyE or

(21) 2 726 630

(13) A1

(72) **Inventeurs(suite)/Inventors(continued)**: POWER, SCOTT D., US; RAMER, SANDRA W., US; REQUADT, CAROL A., US; SHAW, ANDREW, US; TOPPOZADA, AMR R., US; WALLACE, LOUISE, US

(74) **Agent**: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(57) **Abrégé(suite)/Abstract(continued)**:
variants thereof to be used in a saccharification reaction having a reduced or eliminated requirement for glucoamylase. The reduction or elimination of the glucoamylase requirement significantly improves the efficiency of the production of ethanol or high fructose corn syrup, for example.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
10 December 2009 (10.12.2009)

(10) International Publication Number
WO 2009/149271 A3

(51) International Patent Classification:
C12P 19/02 (2006.01) *C11D 3/386* (2006.01)
C12N 9/28 (2006.01)

(21) International Application Number:
PCT/US2009/046279

(22) International Filing Date:
4 June 2009 (04.06.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/059,535 6 June 2008 (06.06.2008) US

(71) Applicant *(for all designated States except US)*: **DANISCO US INC.** [US/US]; 925 Page Mill Road, Palo Alto, CA 94304 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CASCAO-PEREIRA, Luis, G.** [BR/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **CHIN, Regina** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **CUEVAS, William, A.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **ESTELL, David, A.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **LEE, Sang-Kyu** [KR/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **PEPSIN, Michael, J.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **POWER, Scott, D.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **RAMER, Sandra, W.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **REQUADT, Carol, A.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **SHAW, Andrew** [US/US]; 25 Sierra Street, #E310, San Francisco, CA 94107 (US). **TOPPOZADA, Amr, R.** [US/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US). **WALLACE, Louise** [ZA/US]; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US).

(74) Agent: **BACSI, Steven, G.**; Danisco US Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM,

[Continued on next page]

(54) Title: PRODUCTION OF GLUCOSE FROM STARCH USING ALPHA-AMYLASES FROM *BACILLUS SUBTILIS*

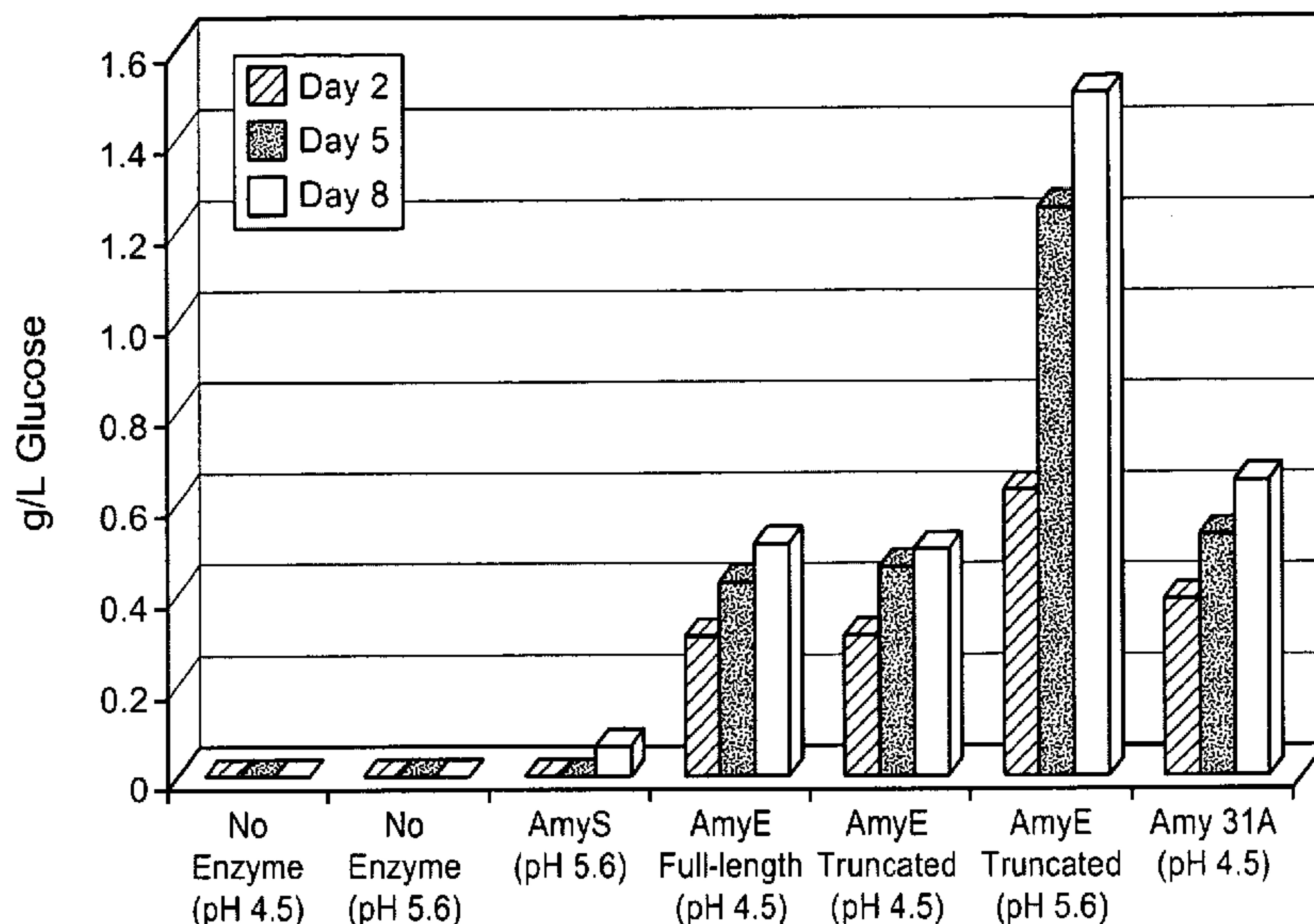


FIG. 5

(57) Abstract: An alpha amylase from *Bacillus subtilis* (AmyE) produces significant amounts of glucose from various carbohydrate substrates, including vegetable starch, maltoheptaose, and maltotriose. Among other things, this advantageous property allows AmyE or variants thereof to be used in a saccharification reaction having a reduced or eliminated requirement for glucoamylase. The reduction or elimination of the glucoamylase requirement significantly improves the efficiency of the production of ethanol or high fructose corn syrup, for example.

WO 2009/149271 A3

WO 2009/149271 A3

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report: 25 March 2010

PRODUCTION OF GLUCOSE FROM STARCH USING ALPHA-AMYLASES FROM *BACILLUS SUBTILIS*

CROSS-REFERENCE

[0001] This application claims priority to US Provisional Patent Application Serial No.
5 61/059,535 filed June 6, 2008, which is incorporated herein by reference.

SEQUENCE LISTING

[0002] A sequence listing comprising SEQ ID NOS: 1-24 is attached and incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 **[0003]** Alpha-amylases from *Bacillus subtilis* (AmyE), variants thereof, nucleic acids encoding the same, and host cells comprising the nucleic acids are provided. Methods of using AmyE or variants thereof are disclosed, including liquefaction and/or saccharification of starch, among others. Such methods may yield sugars useful for ethanol production or high fructose corn syrup production, for example.

15 ## BACKGROUND

[0004] Vegetable starches, e.g., cornstarch, are widely used in the industrial manufacture of products such as syrups and biofuels. For example, high fructose corn syrup (HFCS) is a processed form of corn syrup having high fructose content and a sweetness comparable to sugar, making HFCS useful as a sugar substitute in soft drinks and other processed
20 foods. HFCS production currently represents a billion dollar industry. The production of ethanol as a biofuel is also a growing industry.

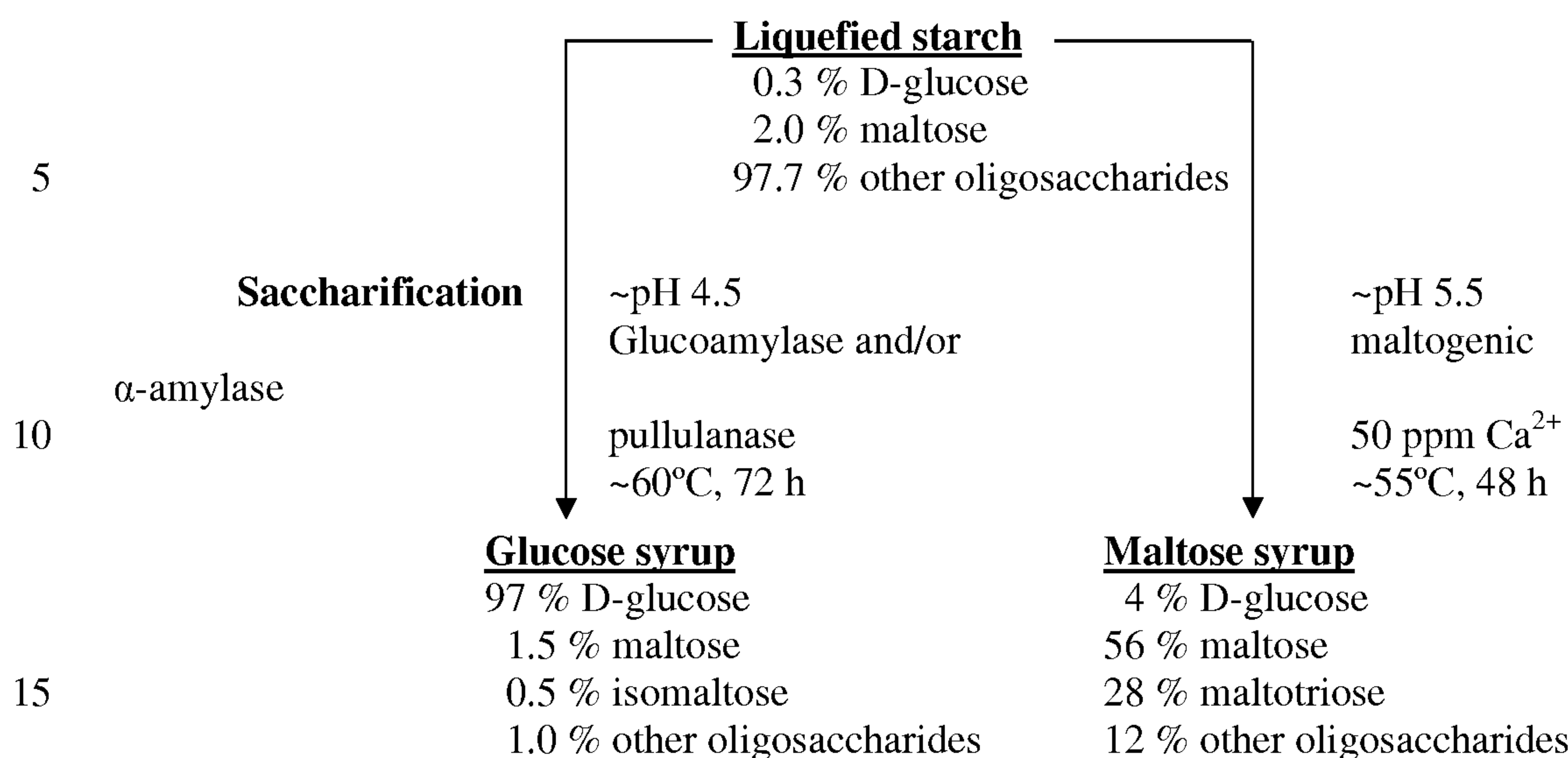
[0005] Syrups and biofuels can be produced from starch by an enzymatic process that catalyzes the breakdown of starch into glucose. This enzymatic process typically involves a sequence of enzyme-catalyzed reactions:

25 **[0006]** **(1) Liquefaction:** α-Amylases (EC 3.2.1.1) first catalyze the degradation of a starch suspension, which may contain 30-40% w/w dry solids (ds), to maltodextrans. α-Amylases are endohydrolases that catalyze the random cleavage of internal

α-1,4-D-glucosidic bonds. Because liquefaction typically is conducted at high temperatures, e.g., 90-100°C, thermostable α-amylases, such as an α-amylase from *Bacillus* sp., are preferred for this step. α-Amylases currently used for this step, e.g., α-amylases from *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus* (AmyS), do not produce significant amounts of glucose. Instead, the resulting liquefact has a low dextrose equivalent (DE) and contains maltose and sugars with high degrees of polymerization (DPn).

[0007] (2) **Saccharification:** Glucoamylases and/or maltogenic α-amylases catalyze the hydrolysis of non-reducing ends of the maltodextrans formed after liquefaction, releasing D-glucose, maltose and isomaltose. Saccharification produces either glucose-rich or high-maltose syrups. In the former case, glucoamylases typically catalyze saccharification under acidic conditions at elevated temperatures, e.g., 60°C, pH 4.3. Glucoamylases used in this process typically are obtained from fungi, e.g., *Aspergillus niger* glucoamylase used in Optidex® L400 or *Humincola grisea* glucoamylase. De-branching enzymes, such as pullulanases, can aid saccharification.

[0008] Maltogenic α-amylases alternatively may catalyze saccharification to form high-maltose syrups. Maltogenic α-amylases typically have a higher optimal pH and a lower optimal temperature than glucoamylase, and maltogenic amylases typically require Ca^{2+}. Maltogenic α-amylases currently used for this application include *B. subtilis* α-amylases, plant amylases, and the α-amylase from *Aspergillus oryzae*, the active ingredient of Clarase® L. Exemplary saccharification reactions used to produce various products are depicted below:



[0009] **(3) Further processing:** A branch point in the process occurs after the production of a glucose-rich syrup, shown on the left side of the reaction pathways above. If the final desired product is a biofuel, yeast can ferment the glucose-rich syrup to ethanol. On the other hand, if the final desired product is a fructose-rich syrup, glucose isomerase can catalyze the conversion of the glucose-rich syrup to fructose.

[0010] Saccharification is the rate-limiting step in the production of a glucose-rich syrup. Saccharification typically occurs over 48-72 hours, by which time many fungal glucoamylases lose significant activity. Further, although maltogenic α-amylases and glucoamylases both can catalyze saccharification, the enzymes typically operate at different optimal pH and temperatures, as shown above. If both enzymes are used sequentially, the difference in reaction conditions between the two enzymes necessitates adjusting the pH and temperature, which slows down the overall the process and may give rise to the formation of insoluble amylose aggregates.

[0011] Accordingly, there is a need in the art for an improved process of making industrial products from starch. In particular, there is a need for improved efficiencies in a saccharification step.

SUMMARY

[0012] An α-amylase from *Bacillus subtilis* (AmyE) produces significant amounts of glucose from various carbohydrate substrates, including vegetable starch, maltoheptaose, and

maltotriose. Among other things, this advantageous property allows AmyE or variants thereof to be used in a saccharification reaction having a reduced or eliminated requirement for glucoamylase. The reduction or elimination of the glucoamylase requirement significantly improves the efficiency of the production of high fructose corn
5 syrup (HFCS) or ethanol, for example.

[0013] To that end, a method of using a *Bacillus subtilis* α-amylase (AmyE) or a variant thereof to produce a solution comprising a significant amount of glucose can comprise: (i) contacting AmyE or variant thereof with a substrate solution comprising maltose, maltoheptaose, or maltotriose; and (ii) converting the substrate solution to a significant
10 amount of glucose, wherein the AmyE or variant thereof has the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least about 85% sequence identity to SEQ ID NO: 1 and with α-amylase activity. The final concentration of glucose in the solution may be 20% w/w or more. The substrate solution advantageously may be contacted with AmyE or a variant thereof in the absence of an added glucoamylase. In
15 one embodiment, the substrate solution is contacted with AmyE or a variant thereof in the presence of a glucoamylase, where the pH of the solution is at about pH 4.0 to pH 4.5, optionally in the absence of added Ca^{2+}.

[0014] In one embodiment the oligosaccharide solution comprises predominantly maltoheptaose (DP7) or higher oligosaccharides. In another embodiment the starch solution is
20 uncooked corn starch.

[0015] In yet another embodiment the pH of the substrate solution during the converting of the substrate solution is about pH 5.6 to about pH 5.8. In one embodiment the converting of the substrate solution does not comprise contacting the substrate solution with a glucoamylase.

25 **[0016]** In one embodiment step (i) further comprises contacting the starch substrate with a glucoamylase. In a particular embodiment the glucoamylase is added to a concentration of less than about 0.5 GAU/g ds. In an additional embodiment the glucoamylase is added to a concentration of less than about 0.02 GAU/g ds.

[0017] In one embodiment the solution comprising glucose contains at least about 0.2 g/L
30 glucose. In an alternative embodiment the solution comprising glucose contains at least

about 0.4 g/L glucose. In a further embodiment the solution comprising glucose contains at least about 1.4 g/L glucose.

[0018] Any naturally occurring AmyE is suitable for the present methods. For example, the AmyE may have the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence with at least about 85%, 90%, or 95% sequence identity to SEQ ID NO: 1, measured with the BLAST sequence alignment algorithm with default matching parameters, such as the AmyE having the amino acid sequence of SEQ ID NO: 3 (Amy31A). Amy31A is disclosed in Ohdan *et al.*, "Characteristics of two forms of alpha-amylases and structural implication," *Appl. Environ. Microbiol.* 65(10): 4652-58 (1999). Amy31A has about 86% sequence identity to the AmyE of SEQ ID NO: 1, using the BLAST algorithm. AmyE variants also are useful, which have amino acid sequences that differ from the sequence of a naturally occurring AmyE. Variants include an AmyE having a deletion of the C-terminal starch binding domain, such as the truncated AmyE having the amino acid sequence of SEQ ID NO: 2 (AmyE-tr), which is the AmyE truncated from residue D425 of SEQ ID NO: 1. Polynucleotides encoding the AmyE and AmyE variants also are provided. Vectors and host cells useful for expressing the polynucleotides are provided, as well.

[0019] In one embodiment the AmyE is selected from the group consisting of the AmyE comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, NCBI Accession No. ABW75769, NCBI Accession No. ABK54355, NCBI Accession No. AAF14358, NCBI Accession No. AAT01440, NCBI Accession No. AAZ30064, NCBI Accession No. NP_388186, NCBI Accession No. AAQ83841, and NCBI Accession No. BAA31528.

[0020] The method of saccharifying starch may further comprise fermenting the saccharified starch solution to produce a biofuel such as ethanol. In one embodiment, a batch fermentation process is used in a closed system, where the composition of the medium is set at the beginning of the fermentation and is not altered during the fermentation. In another embodiment, a "fed-batch fermentation" system is used, where the substrate is added in increments as the fermentation progresses. In yet another embodiment, a continuous fermentation system is used, where a defined fermentation medium is added continuously to a bioreactor, and an equal amount of conditioned medium is removed simultaneously for processing.

[0021] In one embodiment a further step includes (iii) fermenting the solution comprising glucose to produce ethanol. In a particular embodiment the ethanol concentration is at least about 6% v/v ethanol. In another embodiment the ethanol concentration is at least about 14 % v/v ethanol.

5

[0022] A method is also provided that further comprises contacting the saccharified starch solution with a glucose isomerase. Accordingly, in a particular embodiment a further step comprises (iii) contacting the solution comprising glucose with a glucose isomerase to produce high fructose corn syrup. In one embodiment, the saccharified starch solution

10 contains no exogenously added Ca^{2+}. The saccharified starch solution may be converted to fructose-starch based syrup (HFSS), such as HFCS. The conversion of saccharified starch to HFSS may be catalyzed at a pH of about 6.0 to about 8.0, e.g., pH 7.5. In one embodiment, the product contains about 40-45% fructose.

[0023] Also provided is a starch processing composition comprising an AmyE or variant thereof

15 and optionally a glucoamylase, a pullulanase, a β-amylase, a fungal α-amylase, a protease, a cellulase, a hemicellulase, a lipase, a cutinase, an isoamylase, or a combination thereof.

[0024] Also provided is a baking composition comprising an AmyE or variant thereof in a solution or in a gel. A method of baking comprises adding the baking composition to a

20 substance to be baked, and baking the substance.

[0025] Also provided is a textile desizing composition comprising an AmyE or variant thereof in an aqueous solution, and optionally with another enzyme. A method of desizing a textile comprises contacting the desizing composition with a textile for a time sufficient to desize the textile. In one embodiment a method of desizing textiles comprises (i)

25 contacting a textile with AmyE and (ii) desizing the textile, wherein the AmyE comprises an amino acid sequence with at least about 85% sequence identity to the AmyE of SEQ ID NO: 1.

[0026] Also provided is a cleaning composition comprising an AmyE or variant thereof in an aqueous solution, and optionally another enzyme, detergent and/or bleach. The cleaning

30 solution is used for laundering or washing dishes, for example. A method is provided

that comprises contacting the cleaning composition with an article to be cleaned, e.g., dishes or laundry, for a sufficient time for the article to be cleaned.

[0027] In one embodiment a method of washing an item is provided, comprising (i) contacting an item to be washed with a detergent composition comprising AmyE and (ii) washing 5 the item, wherein the AmyE comprises an amino acid sequence with at least about 85% sequence identity to the AmyE of SEQ ID NO: 1.

[0028] In a particular embodiment the item to be washed is dishware or clothing. In another embodiment the detergent composition is a non-dusting granulate or a stabilized liquid. In a furhter embodiment the detergent composition further comprises a cellulase, a 10 protease, an amylase, or a combination thereof. In one embodiment the amylase is an α-amylase, a β-amylase, or a glucoamylase. In a further embodiment the detergent composition further comprises a lipase, a peroxidase, a mannanase, a pectate lyase, or a combination thereof. In one embodiment the detergent composition is a manual or automatic dishwashing detergent composition. In another embodiment the detergent 15 composition further comprises a protease, a lipase, a peroxidase, an amylase, a cellulase, a mannanase, a pectate lyase, or a combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The accompanying drawings are incorporated into the specification and provide non-limiting illustrations of various embodiments. In the drawings:

20 **[0030]** FIG. 1 depicts a sequence alignment between the AmyE having the amino acid sequence of SEQ ID NO: 1 ("AmyE full length") and the AmyE having the amino acid sequence of SEQ ID NO: 25 (mature "Amy31A"). Differences in the amino acid sequences are shown in bold font. Residues are numbered from the first amino acid in the mature form of the enzymes.

25 **[0031]** FIG. 2 depicts plasmid pME630-7, which comprises a polynucleotide (labeled "SAMY 425aa") that encodes AmyE-tr (SEQ ID NO: 2). The plasmid comprises a polynucleotide in-frame with the SAMY gene that encodes a signal sequence from *B. licheniformis* α-amylase (labeled "pre LAT").

[0032] FIG. 3 depicts ethanol formation by AmyE-tr ("AmyE truncated") and Spezyme® Xtra 30 amylase ("Xtra") in conventional fermentation at pH 4.3 and pH 5.8.

[0033] FIG. 4 depicts hydrolysis of insoluble granular (uncooked) starch into ethanol by full length AmyE ("AmyE FL") and AmyE-tr compared to Aspergillus kawachii α-amylase (AkAA) alone or a mixture of A. kawachii α-amylase and Trichoderma reesei glucoamylase (TrGA), at pH 4.3 and pH 5.8.

5 **[0034]** FIG. 5 depicts glucose formation by AmyE ("AmyE full-length"), AmyE-tr ("AmyE truncated"), and Amy 31A compared to *Geobacillus stearothermophilus* α-amylase (AmyS; SEQ ID NO: 4) at pH 4.5 and 5.6.

[0035] FIG. 6 depicts breakdown products detected by HPLC following a 0h (top panel) and 72h incubation (bottom panel) of AmyE-tr with maltoheptaose (DP7).

10 **[0036]** FIG. 7 depicts breakdown products detected by HPLC following a 0h, 2h, 4h, and 24h (panels from top to bottom) incubation of AmyS with a DP7 substrate.

[0037] FIG. 8 depicts breakdown products detected by HPLC following a 0h, 1h, 2h, and 3h (panels from top to bottom) incubation of Spezyme® FRED ("Fred") with a DP7 substrate.

15 **[0038]** FIG. 9 depicts breakdown products detected by HPLC following a 0 min, 30 min, and 90 (panels from top to bottom) min incubation of AmyE (SEQ ID NO: 1) with raw corn flour starch.

DETAILED DESCRIPTION

[0039] An α-amylase from *Bacillus subtilis* (AmyE) produces significant amounts of glucose
20 from various carbohydrate substrates, including vegetable starch, maltoheptaose, and maltotriose. Among other things, this advantageous property allows AmyE or variants thereof to be used in a saccharification reaction having a reduced or eliminated requirement for glucoamylase. The reduction or elimination of the glucoamylase requirement significantly improves the efficiency of the production of ethanol or high
25 fructose corn syrup, for example.

1. Definitions and Abbreviations

[0040] 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 formulation" includes reference to one or more formulations and equivalents thereof known to those skilled in the art, and so forth.

[0041] 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. Definitions

[0042] As used herein, "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein." In some instances, the term "amino acid sequence" is synonymous with the term "peptide"; in some instances, the term "amino acid sequence" is synonymous with the term "enzyme."

[0043] As used herein, "hybridization" includes the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies. Hybridized nucleic acid may exist as single- or double-stranded DNA or RNA, an RNA/DNA heteroduplex, or an RNA/DNA copolymer. As used herein, "copolymer" refers to a single nucleic acid strand that comprises both ribonucleotides and deoxyribonucleotides. Nucleic acids include those that hybridize under "highly stringent conditions" to a nucleic acid disclosed herein. Highly stringent conditions are defined as hybridization at 50°C in 0.2X SSC or at 65°C in 0.1X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0).

[0044] As used herein, "nucleotide sequence" or "nucleic acid sequence" refer to a sequence of genomic, synthetic, or recombinant origin and may be double-stranded or single-stranded, whether representing the sense or anti-sense strand. As used herein, the term "nucleic acid" may refer to genomic DNA, cDNA, synthetic DNA, or RNA. The residues of a nucleic acid may contain any of the chemically modifications commonly known and used in the art.

[0045] "Isolated" means that the material is at least substantially free from at least one other component that the material is naturally associated and found in nature.

[0046] "Purified" means that the material is in a relatively pure state, e.g., at least about 90% pure, at least about 95% pure, or at least about 98% pure.

[0047] "Thermostable" means the enzyme retains activity after exposure to elevated temperatures. The thermostability of an AmyE is measured by its half-life ($t_{1/2}$), where 5 half of the enzyme activity is lost by the half-life. The half-life is measured by determining the specific α-amylase activity of the enzyme remaining over time at a given temperature, particularly at a temperature used for a specific application.

[0048] As used herein, "food" includes both prepared food, as well as an ingredient for a food, such as flour, that is capable of providing any beneficial effect to the consumer. "Food 10 ingredient" includes a formulation that is or can be added to a food or foodstuff and includes formulations used at low levels in a wide variety of products that require, for example, acidifying or emulsifying. The food ingredient may be in the form of a solution or as a solid, depending on the use and/or the mode of application and/or the mode of administration.

15 **[0049]** "Oligosaccharide" means a carbohydrate molecule composed of 3–20 monosaccharides.

[0050] "Homologue" means an entity having a certain degree of identity or "homology" with the subject amino acid sequences and the subject nucleotide sequences. A "homologous sequence" includes an amino acid sequence having at least 85% sequence identity to the subject sequence, e.g., at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the 20 subject sequence. Typically, homologues will comprise the same active site residues as the subject amino acid sequence.

[0051] As used herein, "transformed cell" includes cells that have been transformed by use of recombinant DNA techniques. Transformation typically occurs by insertion of one or more nucleotide sequences into a cell. The inserted nucleotide sequence may be a 25 heterologous nucleotide sequence, i.e., is a sequence that is not natural to the cell that is to be transformed, such as a fusion protein.

[0052] As used herein, "operably linked" means that the described components are in a relationship permitting them to function in their intended manner. For example, a regulatory sequence operably linked to a coding sequence is ligated in such a way that

expression of the coding sequence is achieved under condition compatible with the control sequences.

[0053] As used herein, "biologically active" refers to a sequence having a similar structural, regulatory, or biochemical function as the naturally occurring sequence, although not
5 necessarily to the same degree.

1.2. Abbreviations

The following abbreviations apply unless indicated otherwise:

AE	alcohol ethoxylate
AEO	alcohol ethoxylate
AEOS	alcohol ethoxysulfate
AES	alcohol ethoxysulfate
AGU	glucoamylase activity unit
AkAA	*Aspergillus kawachii* α-amylase
AmyE	*Bacillus subtilis* α-amylase
AmyR	Spezyme® Xtra amylase
AmyS	*Geobacillus stearothermophilus* α-amylase
AS	alcohol sulfate
BAA	bacterial α-amylase
cDNA	complementary DNA
CMC	carboxymethylcellulose
DE	Dextrose Equivalent
DI	distilled, deionized
DNA	deoxyribonucleic acid
DP3	degree of polymerization with three subunits
DPn	degree of polymerization with n subunits
DS or ds	dry solid
DTMPA	diethyltriaminepentaacetic acid
EC	enzyme commission for enzyme classification
EDTA	ethylenediaminetetraacetic acid
EDTMPA	ethylenediaminetetramethylene phosphonic acid
EO	ethylene oxide
F&HC	fabric and household care

GAU	glucoamylase units
HFCS	high fructose corn syrup
HFSS	high fructose starch based syrup
IPTG	isopropyl β-D-thiogalactoside
LA	Lauria agar
LB	Lauria broth
LU	Lipase Units
L1T	leucine (L) residue at position 1 is replaced with a threonine (T) residue, where amino acids are designated by single letter abbreviations commonly known in the art
MW	molecular weight
NCBI	National Center for Biotechnology Information
nm	nanometer
NOBS	nonanoyloxybenzenesulfonate
NTA	nitrilotriacetic acid
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
ppm	parts per million
PVA	poly(vinyl alcohol)
PVP	poly(vinylpyrrolidone)
RAU	Reference Amylase Units
RNA	ribonucleic acid
SAS	secondary alkane sulfonates
1X SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0
SSF	simultaneous saccharification and fermentation
SSU	soluble starch unit, equivalent to the reducing power of 1 mg of glucose released per minute
TAED	tetraacetylethylenediamine
TNBS	trinitrobenzenesulfonic acid
TrGA	*Trichoderma reesei* glucoamylase
w/v	weight/volume
w/w	weight/weight

wt	wild-type
μL	microliter
μNm	microNewton × meter

2. AmyE and Variants Thereof

5 [0054] Amy E enzymes and variants thereof are provided, which are useful for carrying out the methods disclosed herein. Nucleic acids encoding AmyE and variants thereof also are provided, as are vectors and host cells comprising the nucleic acids.

[0055] “AmyE” for the purpose of this disclosure means a naturally occurring α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) from *B. subtilis*. A representative AmyE
10 sequence is set forth in SEQ ID NO: 1. The amino acid sequence of AmyE shown in SEQ ID NO: 1 is that of the mature form, without the native signal sequence. The amino acid sequence of the native signal sequence of this AmyE is shown in SEQ ID NO: 17. The mature form of this AmyE is referred to elsewhere in the present disclosure as “AmyE full-length.” Other AmyE sequences have at least about 85% sequence identity
15 to the AmyE of SEQ ID NO: 1, using the BLAST sequence alignment algorithm with default alignment parameters. For example, an AmyE known as Amy31A, disclosed in UniProtKB/TrEMBL Accession No. O82953 (SEQ ID NO: 3), has an 86% sequence identity to the AmyE of SEQ ID NO: 1. The N-terminal 45 amino acid residues of SEQ ID NO: 3 are the signal sequence of Amy31A. A sequence alignment between AmyE
20 (SEQ ID NO: 1) and Amy31A (without the signal sequence) is depicted in FIG. 1. AmyE enzymes include, but are not limited to, the AmyE having the amino acid sequence disclosed in NCBI Accession No. ABW75769. Further AmyE protein sequences include those disclosed in NCBI Accession Nos. ABK54355, AAF14358, AAT01440, AAZ30064, NP_388186, AAQ83841, and BAA31528, the contents of
25 which are incorporated here by reference.

[0056] An AmyE “variant” comprises an amino acid sequence modification of a naturally occurring AmyE sequence. As used herein, a naturally occurring AmyE is also a “parent enzyme,” “parent sequence,” “parent polypeptide,” or “wild-type AmyE.” The amino acid modification may comprise an amino acid substitution, addition, or deletion. The
30 amino acid modification in the AmyE variant may be the result of a naturally occurring mutation or the result of deliberate modification of the amino sequence using one of the well known methods in the art for this purpose, described further below. Representative

AmyE variants are disclosed in co-pending application Attorney Docket No. 48452-0019-P1-US, which is incorporated herein in its entirety.

[0057] An AmyE variant, unless otherwise specified, has at least one amino acid modification, but the variant retains at least 85% sequence identity with the AmyE of SEQ ID NO: 1, measured by a BLAST alignment of the protein sequences with default alignment parameters. The AmyE variant may have at least 85%, 90%, 95%, 97%, 98%, or 99% amino acid sequence identity to the AmyE of SEQ ID NO: 1. For example, the variant may have one, two, three, up to five, up to ten, or up to 20 amino acid substitutions compared to the amino acid sequence of SEQ ID NO: 1. Typically, modifications are made to amino acid residues that are not required for biological function. The selection of amino acid residues to be modified may be guided by sequence homology among AmyE sequences. Generally, amino acids that are well conserved in AmyE sequences are more likely to be required for biological activity. Conversely, amino acid positions that vary among AmyE sequences are less likely to be required for biological activity. For example, amino acid residues that differ in the alignment between AmyE and Amy31A, shown in bold font in FIG. 1, likely can be modified in an AmyE variant without loss of biological activity.

[0058] AmyE or variants thereof may be expressed as a fusion protein that comprises sequences at the N- and/or C-terminus of the mature form of AmyE that facilitate expression, detection, and/or purification, e.g., a signal sequence or a His-tag. Such a sequence includes a signal sequence, which facilitates secretion and expression of the AmyE in a host organism. Additional amino acid residues may be cleaved from the N-terminus of an AmyE, following cleavage of the signal sequence, as discussed in Yang *et al.*, "Nucleotide sequence of the amylase gene from *Bacillus subtilis*," *Nucleic Acids Res.* 11: 237-49 (1983). A "mature form" of an AmyE is defined as the product of all such post-translational modifications of the expressed AmyE sequence. Sequences found at the N-terminus of the primary translation product that are cleaved to form the mature AmyE may be designated alternatively as a "signal sequence," "leader sequence," or "pro-sequence."

[0059] The signal sequence may encoded by the same gene as the AmyE. For example, the AmyE set forth in SEQ ID NO: 1 is expressed naturally with a signal sequence and additional N-terminal amino acids having the sequence

MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASAETANKSNE (SEQ ID NO: 17). The signal sequence alternatively may be a *B. subtilis* sp. signal sequence from a different AmyE or even a different protein. Further, the signal sequence may be from a different species, e.g., *B. licheniformis*. The signal sequence may be chosen to provide optimal expression of the AmyE or variant thereof in a particular host cell, for example. The mature AmyE may be produced as a result of proteolytic cleavage of additional sequences from the N-terminus that are not signal sequences. For example, a 31-amino acid residue signal sequence from *B. licheniformis* ("LAT leader sequence") may be fused in frame with an AmyE sequence.

[0060] An AmyE variant for the purpose of this disclosure has at least partial 1,4-α-D-glucan glucanohydrolase activity, compared to a naturally occurring AmyE. Variants may have the same activity and properties as a wild-type AmyE, or variants may have an altered property, compared to a wild-type AmyE. The altered property may be an altered, e.g., two- or three-fold higher, specific activity toward maltoheptaose and/or maltotriose substrates. The thermostability of the protein alternatively or additionally may be altered. For example, the variant may be more thermostable than AmyE. The altered property alternatively or additionally may be the optimal pH for enzymatic activity. For example, the variant may have a more acidic or alkaline optimum pH.

[0061] A "truncated" AmyE ("AmyE-tr") means an AmyE with a sequence deletion of all or part of the C-terminal starch binding domain. In the AmyE-tr of SEQ ID NO: 2, for example, the AmyE of SEQ ID NO: 1 is truncated at residue D425. A 2.5 Å resolution crystal structure of this AmyE-tr is available at Protein Databank Accession No. 1BAG, which is disclosed in Fujimoto *et al.*, "Crystal structure of a catalytic-site mutant alpha-amylase from *B. subtilis* complexed with maltopentaose," *J. Mol. Biol.* 277: 393-407 (1998). AmyE-tr may be truncated at other positions, e.g., Y423, P424, D426 or I427 of the AmyE of SEQ ID NO: 1, provided all or part of the C-terminal starch binding domain is removed.

[0062] Nucleic acids encoding AmyE or a variant thereof include, but are not limited to, the polynucleotide disclosed in SEQ ID NO: 9 and NO: 10, which encode the AmyE of SEQ ID NO: 1 and AmyE-tr (SEQ ID NO: 2), respectively. Further representative polynucleotides include that disclosed in SEQ ID NO: 11, which encodes Amy31A (SEQ ID NO: 3). The AmyE disclosed in NCBI Accession Nos. ABK54355, AAF14358,

AAT01440, AAZ30064, NP_388186, AAQ83841, and BAA31528 likewise are encoded by polynucleotides disclosed in publicly accessible databases, which sequences are incorporated herein by reference. Nucleic acids may be DNA, mRNA, or cDNA sequences. Nucleic acids further include "degenerate sequences" of any of the aforementioned nucleic acids. A degenerate sequence contains at least one codon that encodes the same amino acid residue but has a different nucleotide sequence from the aforementioned nucleic acid sequences. For example, nucleic acids include any nucleic acid sequence that encodes an AmyE or variant thereof. Degenerate sequences may be designed for optimal expression by using codons preferred by a particular host organism.

[0063] Vectors comprising the nucleic acids encoding AmyE or variants thereof also are provided. Host cells comprising the vectors are provided. The host cell may express the polynucleotide encoding the AmyE variant. The host may be a *Bacillus* sp., e.g., *B. subtilis*.

2.1. Characterization of AmyE Variants

[0064] AmyE variants can be characterized by their nucleic acid and primary polypeptide sequences, by 3D structural modeling, and/or by their specific activity. Additional characteristics of the AmyE variant include stability, Ca^{2+} dependence, pH range, oxidation stability, and thermostability. In one aspect, the AmyE variants are expressed at higher levels than the wild-type AmyE, while retaining the performance characteristics of the wild-type AmyE. Levels of expression and enzyme activity can be assessed using standard assays known to the artisan skilled in this field. In another aspect, variants demonstrate improved performance characteristics relative to the wild-type enzyme, such as improved stability at high temperatures or improved activity at various pH values, e.g., pH 4.0 to 6.0 or pH 8.0 to 11.0.

[0065] The AmyE variant may be expressed at an altered level in a host cell compared to AmyE. Expression generally relates to the amount of active variant that is recoverable from a fermentation broth using standard techniques known in this art over a given amount of time. Expression also can relate to the amount or rate of variant produced within the host cell or secreted by the host cell. Expression also can relate to the rate of translation of the mRNA encoding the variant enzyme.

[0066] In a further aspect, important mutations exhibit altered stability or specific activity, especially at temperatures around 60°C, e.g., 50-70°C, for use in saccharification, for example. Variants may have altered stability or specific activity at other temperatures, depending on whether the variant is to used in other applications or compositions. For example, in baking products, variant may exhibit altered specific activity at higher temperature ranges.

[0067] AmyE variants also may have altered oxidation stability, in particular higher oxidation stability, in comparison to the parent AmyE. For example, increased oxidation stability is advantageous in detergent compositions, and decreased oxidation stability may be advantageous in composition for starch liquefaction.

[0068] The AmyE variants described herein can also have mutations that extend half-life relative to the parent enzyme by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or more, particularly at elevated temperatures of about 55°C to about 95°C or more, particularly at about 80°C. In one embodiment, the AmyE variant can be heated for about 1-10 minutes at 80°C or higher.

[0069] The AmyE variants may have exo-specificity, measured by exo-specificity indices described herein, for example. AmyE variants include those having higher or increased exo-specificity compared to the parent enzymes or polypeptides from which they were derived, optionally when measured under identical conditions. Thus, for example, the AmyE variant polypeptides may have an exo-specificity index 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 500%, 1000%, 5000%, 10,000% or higher compared to their parent polypeptides.

[0070] In one aspect, the AmyE variant has the same pH stability as the parental sequence. In another aspect, the variant comprises a mutation that confers a greater pH stability range or shifts the pH range to a desired area for the end commercial purpose of the enzyme. For example, in one embodiment, the variant can degrade starch at about pH 5.0 to about pH 10.5. The AmyE variant polypeptide may have a longer half-life or higher activity (depending on the assay) compared to the parent polypeptide under identical conditions, or the AmyE variant may have the same activity as the parent polypeptide. The AmyE variant polypeptide also may have about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200% or longer half-life compared to their parent polypeptide under

identical pH conditions. Alternatively, or in addition, the AmyE variant may have higher specific activity compared to the parent polypeptide under identical pH conditions.

[0071] In another aspect, a nucleic acid complementary to a nucleic acid encoding any of the AmyE variants set forth herein is provided. Additionally, a nucleic acid capable of hybridizing to the complement is provided. In another embodiment, the sequence for use in the methods and compositions described here is a synthetic sequence. It includes, but is not limited to, sequences made with optimal codon usage for expression in a particular host organism.

3. Production of AmyE and Variants Thereof

[0072] A DNA sequence encoding the enzyme variant produced by methods described herein, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a suitable promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

3.1 Vectors

[0073] The recombinant expression vector carrying the DNA sequence encoding an AmyE or variant thereof may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, mini-chromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The integrated gene may also be amplified to create multiple copies of the gene in the chromosome by use of an amplifiable construct driven by antibiotic selection or other selective pressure, such as an essential regulatory gene or by complementation of an essential metabolic pathway gene.

[0074] An expression vector typically includes the components of a cloning vector, e.g., 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 encoding a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. In one aspect, all the signal sequences used target the material to the cell culture media for easier enzyme collection and optionally
5 purification. The procedures used to ligate the DNA construct encoding an AmyE or variant thereof, 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).

10 **[0075]** In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Suitable promoters for directing the transcription of the DNA sequence encoding an AmyE or variant thereof, especially in a
15 bacterial host, include various *Bacillus*-derived promoters, such as an α-amylase promoter derived from *B. subtilis*, *B. licheniformis*, *B. stearothermophilus*, or *B. amyloliquefaciens*, the promoter of the lac operon of *E. coli*, the *Streptomyces coelicolor* agarase gene dagA or celA promoters, and the promoters of the *Bacillus subtilis xylA* and *xylB* genes, etc. For transcription in a fungal host, examples of useful promoters are
20 those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, or *A. nidulans* acetamidase. When the gene encoding the AmyE or variant thereof is expressed in a bacterial species such as *E. coli*, a
25 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.

30 **[0076]** The expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant. 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 in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1, pICatH, and pIJ702.

5 **[0077]** The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or a gene which confers antibiotic resistance, e.g., ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD*, and *xxsC*, a marker giving rise
10 to hygromycin resistance, or the selection may be accomplished by co-transformation as known in the art. *See, e.g.*, WO 91/17243.

3.2 Variant expression and host organisms

[0078] It is generally advantageous if the AmyE or variant thereof is secreted into the culture medium, when expressed in a host cell. To this end, the AmyE or variants thereof may
15 comprise a signal sequence that permits secretion of the expressed enzyme into the culture medium. If desirable, this original signal sequence may be replaced by a different signal sequence, which is conveniently accomplished by substitution of the DNA sequences encoding the respective signal sequence. For example, a nucleic acid encoding AmyE is operably linked to a *B. licheniformis* signal sequence in the
20 expression vector shown in FIG. 2. Signal sequences are discussed in more detail above.

[0079] An isolated cell, either comprising a DNA construct or an expression vector, is advantageously used as a host cell in the recombinant production of an AmyE or variant thereof. The cell may be transformed with the DNA construct encoding the AmyE or variant thereof, optionally by integrating the DNA construct (in one or more copies) in
25 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
30 different types of host cells.

[0080] Examples of suitable bacterial host organisms are Gram positive bacterial species such as Bacillaceae, including B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. lautus, B. megaterium, and B. thuringiensis; Streptomyces sp., such as S. murinus; lactic acid bacterial species including Lactococcus sp., such as L. lactis; Lactobacillus sp., including L. reuteri; Leuconostoc sp.; Pediococcus sp.; and Streptococcus sp. Still other useful hosts include Bacillus sp. A 7-7, for example. Alternatively, strains of a Gram negative bacterial species belonging to Enterobacteriaceae, including E. coli, or to Pseudomonadaceae can be selected as the host organism.

[0081] A suitable yeast host organism can be selected from biotechnologically relevant yeasts species, such as, but not limited to, *Pichia* sp., *Hansenula* sp., *Kluyveromyces* sp., *Yarrowinia* sp., *Saccharomyces* sp., including *S. cerevisiae*, or a species belonging to *Schizosaccharomyces*, such as *S. pombe*. 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., *A. niger*, *A. oryzae*, *A. tubigensis*, *A. awamori*, or *A. nidulans*. Alternatively, a strain of *Fusarium* sp., e.g., *Fusarium oxysporum* or *Rhizomucor* sp., such as *R. miehei*, can be used as the host organism. Other suitable yeasts include *Thermomyces* sp. and *Mucor* sp. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known in the art. A suitable procedure for transforming *Aspergillus* host cells, for example, is described in EP 238023.

[0082] In a yet further aspect, a method of producing an AmyE or variant thereof is provided, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant 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 the AmyE or variant thereof. 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 (ATCC). Exemplary culture media include, but are not limited to, those for fed-batch fermentations performed in a three thousand liter (3,000 L) stirred tank fermentor. The growth medium in that

case can consist of corn steep solids and soy flour as sources of organic compounds, along with inorganic salts as a source of sodium, potassium, phosphate, magnesium and sulfate, as well as trace elements. Typically, a carbohydrate source such as glucose is also part of the initial medium. Once the culture has established itself and begins growing, the carbohydrate is metered into the tank to maintain the culture as is known in the art. Samples are removed from the fermentor at regular intervals to measure enzyme titer using, for example, a colorimetric assay method. The fermentation process is halted when the enzyme production rate stops increasing according to the measurements.

[0083] An AmyE or variant thereof secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

[0084] Host cells may be cultured under suitable conditions that allow expression of the AmyE or variant thereof. Expression of the proteins 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 addition of an inducer substance, e.g., dexamethasone, IPTG, or Sepharose, to the culture medium, for example. Polypeptides can also be produced recombinantly in an *in vitro* cell-free system, such as the TnT™ (Promega) rabbit reticulocyte system.

[0085] A host for expressing AmyE or variant thereof can be cultured under aerobic conditions in the appropriate medium for the host. 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 30°C to about 75°C, depending on the needs of the host and production of the desired α-amylase variant. Culturing can occur from about 12 to about 100 hours or greater (and any hour value there between) or more particularly from 24 to 72 hours. Typically, the culture broth is at a pH of about 5.5 to about 8.0, again depending on the culture conditions needed for the host cell relative to production of the AmyE or variant thereof.

[0086] The amylolytic activity of the expressed enzyme may be determined using potato starch as substrate, for example. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

4. Purification of AmyE and Variants Thereof

[0087] Conventional methods can be used in order to prepare a purified AmyE or variant thereof. After fermentation, a fermentation broth is obtained, and the microbial cells and various suspended solids, including residual raw fermentation materials, are removed by conventional separation techniques to obtain an amylase solution. Filtration, centrifugation, microfiltration, rotary vacuum drum filtration, followed by ultra-filtration, extraction or chromatography, or the like are generally used.

[0088] It is desirable to concentrate the solution containing the expressed AmyE or variant thereof to optimize recovery, since the use of un-concentrated solutions requires increased incubation time to collect precipitates containing the purified enzyme. The solution is concentrated using conventional techniques until the desired enzyme level is obtained. Concentration of the enzyme containing solution may be achieved by any of the techniques discussed above. In one embodiment, rotary vacuum evaporation and/or ultrafiltration is used. Alternatively, ultrafiltration can be used.

[0089] By "precipitation agent" for purposes of purification is meant a compound effective to precipitate the AmyE or variant thereof from solution, whatever the nature of the precipitate may be, i.e., crystalline, amorphous, or a blend of both. Precipitation can be performed using, for example, a metal halide precipitation agent. Metal halide precipitation agents include: alkali metal chlorides, alkali metal bromides and blends of two or more of these metal halides. The metal halide may be selected from the group consisting of sodium chloride, potassium chloride, sodium bromide, potassium bromide and blends of two or more of these metal halides. Suitable metal halides include sodium chloride and potassium chloride, particularly sodium chloride, which can further be used as a preservative. The selection of conditions of the precipitation for maximum recovery, including incubation time, pH, temperature and concentration of AmyE or

variant thereof, will be readily apparent to one of ordinary skill in the art after routine testing.

[0090] Generally, at least about 5% w/v (weight/volume) to about 25% w/v of metal halide is added to the concentrated enzyme variant 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 variant 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 AmyE or variant thereof and on its concentration in solution.

[0091] Another alternative to effect precipitation of the enzyme is to use of organic compounds, which can be added to the concentrated enzyme variant solution. The organic compound precipitating agent can include: 4-hydroxybenzoic acid, alkali metal salts of 4-hydroxybenzoic acid, alkyl esters of 4-hydroxybenzoic acid, and blends of two or more of these organic compounds. The addition of said organic compound precipitation agents can take place prior to, simultaneously with or subsequent to the addition of the metal halide precipitation agent, and the addition of both precipitation agents, organic compound and metal halide, may be carried out sequentially or simultaneously. For further descriptions, *see, e.g.*, U.S. Patent No. 5,281,526 to Danisco A/S, for example.

[0092] Generally, the organic compound 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 precipitations 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. Suitable organic compounds include 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 ester 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 (methyl PARABEN) and 4-hydroxybenzoic acid propyl ester (propyl PARABEN), which are also amylase preservative agents. Addition

of the such an organic compound precipitation agent provides the advantage of high flexibility of the precipitation conditions with respect to pH, temperature, enzyme concentration, precipitation agent concentration, and time of incubation. Generally, at least 0.01% w/v of organic compound precipitation agent is added to the concentrated enzyme variant solution and usually at least 0.02% w/v. Generally, no more than 0.3% w/v of organic compound precipitation agent is added to the concentrated enzyme variant solution and usually no more than 0.2% w/v.

[0093] The concentrated enzyme solution, containing the metal halide precipitation agent and, in one aspect, the organic compound precipitation agent, is adjusted to a pH that necessarily will depend on the enzyme variant to be purified. Generally, the pH is adjusted to a level near the isoelectric point (pI) of the amylase. For example, the pH can be adjusted within a range of about 2.5 pH units below the pI to about 2.5 pH units above the pI. The pH may be adjusted accordingly if the pI of the variant differs from the wild-type pI.

[0094] 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 variant 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 than about 10 hours, and in most cases even about 6 hours.

[0095] 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, and particularly 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.

[0096] 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.

[0097] The purified enzyme may be further purified 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. Cross membrane microfiltration can be one method used. Further purification of the purified enzyme precipitate can be obtained by washing the precipitate with water. For example, the purified enzyme precipitate may be washed with water containing the metal halide precipitation agent, for example, with water containing the metal halide and the organic compound precipitation agents.

[0098] During culturing, expressed enzyme may accumulate in the culture broth. For the isolation and purification of the expressed enzyme, the culture broth may be centrifuged or filtered to eliminate cells, and the resulting cell-free liquid may be used for the purification of the enzyme. 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.

[0099] Purified enzymes are useful for all applications in which the enzyme are generally utilized. For example, they can be used in laundry detergents and spot removers, in the food industry, in starch processing and baking, and in pharmaceutical compositions as digestive aids. They can be made into a final product that is either liquid (solution, slurry) or solid (granular, powder).

[0100] Alternatively, the enzyme product can be recovered and a flocculating agent is added to the media in order to remove cells and cell debris by filtration or centrifugation without further purification of the enzyme.

[0101] The AmyE and variants thereof produced and purified by the methods described above can be used in a variety of useful industrial applications. The enzymes possess valuable properties facilitating applications related to fabric and household care (F&HC). For example, an AmyE or variant thereof can be used as a component in washing, dishwashing and hard-surface cleaning detergent compositions. AmyE or variants thereof also are useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. AmyE or variants thereof are particularly useful in starch-

conversion processes, including starch liquefaction and/or saccharification processes, as described, for example, in WO 2005/111203 and U.S. Published Application No. 2006/0014265 (Danisco A/S). These uses of AmyE or variants thereof are described in more detail below.

5 **5. Compositions and Uses of AmyE and Variants Thereof**

5.1. Starch Processing Compositions and Use

[0102] In one aspect, compositions with AmyE or variants thereof can be utilized for starch liquefaction and/or saccharification. The process may comprise hydrolysis of a slurry of gelatinized or granular starch, in particular hydrolysis of granular starch into a soluble
10 starch hydrolysate at a temperature below the initial gelatinization temperature of the granular starch. Starch processing is useful for producing sweetener, producing alcohol for fuel or drinking (i.e., potable alcohol), producing a beverage, processing cane sugar, or producing desired organic compounds, e.g., citric acid, itaconic acid, lactic acid, gluconic acid, ketones, amino acids, antibiotics, enzymes, vitamins, and hormones.
15 Conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes: a liquefaction process, a saccharification process, and an isomerization process.

[0103] 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
20 gelatinization of starch simultaneously with or followed by the addition of an AmyE or variant thereof. As used herein, the term “primary liquefaction” refers to a step of liquefaction when the slurry’s temperature is raised to or near its gelatinization temperature. Subsequent to the raising of the temperature, the slurry is sent through a heat exchanger or jet to temperatures from about 90-150°C, e.g., 100-110°C. Subsequent
25 to application to a heat exchange or jet temperature, the slurry is held for a period of 3-10 minutes at that temperature. This step of holding the slurry at 90-150°C is termed primary liquefaction.

[0104] As used herein, the term “secondary liquefaction” refers the liquefaction step subsequent to primary liquefaction (heating to 90-150°C), when the slurry is allowed to cool to room
30 temperature. This cooling step can be 30 minutes to 180 minutes, e.g. 90 minutes to 120 minutes. 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.

[0105] After the liquefaction process, the dextrins typically may be converted into dextrose by addition of a glucoamylase (e.g., AMG™) and optionally a debranching enzyme, such as an isoamylase or a pullulanase (e.g., Promozyme®). Before this step, the pH typically is reduced to a value below about 4.5, while maintaining the temperature at 95°C or more, so that the liquefying α-amylase variant activity is denatured. The temperature then is lowered to 60°C, and a glucoamylase and a debranching enzyme are added. The saccharification process proceeds typically for about 24 to about 72 hours.

[0106] An advantage of AmyE and variants thereof is the ability of AmyE to catalyze the breakdown of complex sugars, such as maltose, maltotriose, and maltoheptaose. For this reason, the reaction can be catalyzed by AmyE or a variant thereof alone, optionally without a glucoamylase. A further advantage of the present AmyE or variants thereof is that dextrins may be converted into dextrose by the action or one or more AmyE or variants thereof under the same reaction conditions that are optimal for glucoamylase. This advantageous property of AmyE and variants thereof is disclosed in co-pending application Attorney Docket Number 48452-0018-P1-US, incorporated by reference in its entirety herein. Because AmyE and variants thereof operate at the same pH and temperature as glucoamylase, AmyE and variants thereof may be added before or after additional catalysis with a glucoamylase, or by a cocktail of AmyE or a variant thereof and a glucoamylase. The delays necessitated by adjusting the pH and temperature of the reaction to accommodate the use of a glucoamylase thus are avoided.

[0107] Glucoamylases, when added, preferably are present in an amount of no more than, or even less than, 0.5 glucoamylase activity unit (AGU)/g DS (i.e., glucoamylase activity units per gram of dry solids). Glucoamylases may be added in an amount of 0.02-2.0 AGU/g DS or 0.1-1.0 AGU/g DS, e.g., 0.2 AGU/g DS. Glucoamylases are derived from a microorganism or a plant. For example, glucoamylases can be of fungal or bacterial origin. Exemplary bacterial glucoamylases are *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), *EMBO J.* 3(5): 1097-1102), or variants thereof, such as disclosed in WO 92/00381 and WO 00/04136; *A. awamori* glucoamylase (WO 84/02921); *A. oryzae* glucoamylase (*Agric. Biol. Chem.* (1991), 55(4): 941-949), or variants or fragments thereof. In one embodiment, the process also

comprises the use of a carbohydrate-binding domain of the type disclosed in WO 98/22613. Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: 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); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35: 8698-8704); and introduction of 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* (WO 99/28448), *T. leycettanus* (U.S. Patent No. RE 32,153), *T. duponti*, or *T. thermophilus* (U.S. Patent No. 4,587,215). Contemplated bacterial glucoamylases include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135138) and *C. thermohydrosulfuricum* (WO 86/01831). Suitable glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or even 90% homology to the amino acid sequence shown in SEQ ID NO: 2 in WO 00/04136. Also suitable are commercial glucoamylases, such as AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (Novozymes); OPTIDEX® 300 (Genencor Division, Danisco US Inc.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME® G900 (Enzyme Bio-Systems); and G-ZYME® G990 ZR (*A. nige*r glucoamylase and low protease content).

[0108] The AmyE or variants thereof can be used alone or can be combined with other AmyE variants, other α- or β-amylases, or other enzymes to provide a "cocktail" with a broad spectrum of activity. For example, the starch may be contacted with one or more enzyme selected from the group consisting of a fungal α-amylase (EC 3.2.1.1), a bacterial α-amylase, e.g., a *Bacillus* α-amylase or a non-*Bacillus* α-amylase, a β-amylase (EC 3.2.1.2), and/or a glucoamylase (EC 3.2.1.3). In an embodiment further another amylolytic enzyme or a debranching enzyme, such as an isoamylase (EC 3.2.1.68), or a pullulanases (EC 3.2.1.41) may be added to the AmyE or variant thereof. Isoamylase hydrolyses α-1,6-D-glucosidic branch linkages in amylopectin and β-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan and by the limited action of isoamylase on α-limit dextrins. Debranching enzymes may be added in effective amounts well known to the person skilled in the art.

[0109] β-Amylases are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-α-glucosidic linkages into amylose, amylopectin, and related glucose polymers, thereby releasing maltose. β-amylases have been isolated from various plants and microorganisms (Fogarty et al., PROGRESS IN INDUSTRIAL MICROBIOLOGY, Vol. 15, pp. 112-115, 1979). These β-amylases are characterized by having 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 A/S); and Novozym™ WBA (Novozymes A/S).

[0110] After the saccharification process, the dextrose syrup may be converted into high fructose syrup using an immobilized glucose isomerase (such as Sweetzyme®), for example. In one regard, the soluble starch hydrolysate of the process is subjected to conversion 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. Contemplated isomerases included the commercial products Sweetzyme®, IT (Novozymes A/S); G-zyme® IMGI, and G-zyme® G993, Ketomax®, G-zyme® G993, G-zyme® G993 liquid, and GenSweet® IGI.

[0111] While addition of 1 mM Ca^{2+} or more is typically required to ensure adequately high stability of the α-amylase, the free Ca^{2+} strongly inhibits the activity of the glucose isomerase. The Ca^{2+} is thus typically removed prior to isomerization, by means of an expensive unit operation, so that the level of free Ca^{2+} concentration is below 3-5 ppm. Cost savings could be obtained if such an operation were avoided.

[0112] AmyE or variants thereof advantageously require less or no added Ca^{2+} for stability. For this reason, the Ca^{2+} added to a liquefaction and/or saccharification reaction may be reduced or eliminated altogether. The removal of Ca^{2+} by ion exchange prior to contacting the reaction mixture with glucose isomerase thus may be avoided, saving time and cost and increasing the efficiency of a process of producing a high fructose syrup.

[0113] The starch to be processed may be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically, the granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana, or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley.

The starch may be a highly refined starch quality, for instance, at least 90%, at least 95%, at least 97%, or at least 99.5% pure. Alternatively, the starch can be a more crude starch containing material comprising milled whole grain, including non-starch fractions such as germ residues and fibers. The raw material, such as whole grain, is milled to open up
5 the structure and allow further processing.

[0114] Two milling processes are suitable: wet and dry milling. In dry milling, the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is usually used in the production of syrups. Both dry and wet milling are well known in the art of starch processing and also are contemplated for use
10 with the compositions and methods disclosed. The process may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch and water, where the permeate is the soluble starch hydrolysate. Another method is the process conducted in a continuous membrane reactor with ultrafiltration membranes, where the retentate is held under recirculation in presence of
15 enzymes, raw starch and water, and where the permeate is the soluble starch hydrolysate. Also contemplated is the process conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water, and where the permeate is the soluble starch hydrolysate.

20 [0115] Dry milled grain will comprise significant amounts of non-starch carbohydrate compounds, in addition to starch. When such a heterogeneous material is processed by jet cooking, often only a partial gelatinization of the starch is achieved. Accordingly, AmyE or variants thereof having a high activity towards ungelatinized starch are advantageously applied in a process comprising liquefaction and/or saccharification jet
25 cooked dry milled starch.

[0116] The starch slurry to be used in any of the above aspects may have about 20% to about 55% dry solids granular starch, about 25% to about 40% dry solids granular starch, or about 30% to about 35% dry solids granular starch. The enzyme variant converts the soluble starch into a soluble starch hydrolysate of the granular starch in the amount of at
30 least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

[0117] In another embodiment, the AmyE or variant thereof is used in a process comprising fermentation to produce a fermentation product, e.g., ethanol. Such a process for producing ethanol from starch-containing material by fermentation comprises: (i) liquefying the starch-containing material with an AmyE or variant thereof; (ii) saccharifying the liquefied mash obtained; and (iii) fermenting the material obtained in step (ii) in the presence of a fermenting organism. Optionally the process further comprises recovery of the ethanol. During the fermentation, the ethanol content reaches at least about 7%, at least about 8%, at least about 9%, at least about 10% such as at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least 15%, or at least 16% ethanol.

[0118] The saccharification and fermentation processes may be carried out as a simultaneous saccharification and fermentation (SSF) process. When fermentation is performed simultaneously with the hydrolysis, the temperature can be between 30°C and 35°C, particularly between 31°C and 34°C. The process may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid. Also contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

[0119] The soluble starch hydrolysate of the process may also be used for production of a fermentation product comprising fermenting the treated starch into a fermentation product, such as citric acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta-lactone, or sodium erythorbate.

5.2. Cleaning and Dishwashing Compositions and Use

[0120] The AmyE or variants thereof discussed herein can be formulated in detergent compositions for use in cleaning dishes or other cleaning compositions, for example. These can be gels, powders or liquids. The compositions can comprise the α-amylase variant alone, other amylolytic enzymes, other cleaning enzymes, and other components common to cleaning compositions.

[0121] Thus, a dishwashing detergent composition can comprise a surfactant. The surfactant may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent can contain 0% to about 90% by weight of a non-ionic surfactant, such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

5 **[0122]** In the detergent applications, AmyE or variants thereof are usually used in a liquid composition containing propylene glycol. The AmyE or variants thereof can be solubilized in propylene glycol, for example, by circulating in a 25% volume/volume propylene glycol solution containing 10% calcium chloride.

[0123] The dishwashing detergent composition may contain detergent builder salts of inorganic
10 and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains about 1% to about 90% of detergent builders. Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, and polyphosphates. An
15 example of phosphorus-containing organic alkaline detergent builder, when present, includes the water-soluble salts of phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates, and silicates, as well as the various types of water-insoluble crystalline or amorphous alumino silicates, of which zeolites are the best-known
20 representatives.

[0124] Examples of suitable organic builders include the alkali metal; ammonium and substituted ammonium; citrates; succinates; malonates; fatty acid sulphonates; carboxymethoxy succinates; ammonium polyacetates; carboxylates; polycarboxylates; aminopolycarboxylates; polyacetyl carboxylates; and polyhydroxsulphonates.

25 **[0125]** Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers, and their salts.

[0126] The cleaning composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium,
30 sodium or calcium hypochlorite, and hypobromite, as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-

bromo- and N-chloro-imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric, and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

5 **[0127]** The cleaning composition may contain oxygen bleaches, for example in the form of an inorganic persalt, optionally with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates, and perphosphates. Suitable activator materials include tetraacetylethylenediamine (TAED) 10 and glycerol triacetate. Enzymatic bleach activation systems may also be present, such as perborate or percarbonate, glycerol triacetate and perhydrolase, as disclosed in WO 2005/056783, for example.

[0128] The cleaning composition may be stabilized using conventional stabilizing agents for the enzyme(s), e.g., a polyol such as, e.g., propylene glycol, a sugar or a sugar alcohol, lactic 15 acid, boric acid, or a boric acid derivative (e.g., an aromatic borate ester). The cleaning composition may also contain other conventional detergent ingredients, e.g., deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescent agents, thickeners, and perfumes.

20 **[0129]** Finally, the AmyE or variants thereof may be used in conventional dishwashing detergents, e.g., in any of the detergents described in the following patent publications, with the consideration that the AmyE or variants thereof disclosed herein are used instead of, or in addition to, any α-amylase disclosed in the listed patents and published applications: CA 2006687, GB 2200132, GB 2234980, GB 2228945, DE 3741617, DE 25 3727911, DE 4212166, DE 4137470, DE 3833047, DE 4205071, WO 93/25651, WO 93/18129, WO 93/04153, WO 92/06157, WO 92/08777, WO 93/21299, WO 93/17089, WO 93/03129, EP 481547, EP 530870, EP 533239, EP 554943, EP 429124, EP 346137, EP 561452, EP 318204, EP 318279, EP 271155, EP 271156, EP 346136, EP 518719, EP 518720, EP 518721, EP 516553, EP 561446, EP 516554, EP 516555, EP 530635, EP 30 414197, and U.S. Patent Nos. 5,112,518; 5,141,664; and 5,240,632.

5.3. Laundry Detergent Compositions and Use

[0130] According to the embodiment, one or more AmyE or variant thereof may be a component of a detergent composition. 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 Patent No. 1,483,591. 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 well known in the art. Protected enzymes may be prepared according to the method disclosed in US 5,879,920 (Danisco A/S) or EP 238216, for example. Polyols have long been recognized as stabilizers of proteins as well as for improving the solubility of proteins. See, e.g., Kaushik et al., J. Biol. Chem. 278: 26458-65 (2003) and references cited therein; and M. Conti et al., J. Chromatography 757: 237-245 (1997).

[0131] The detergent composition may be in any convenient form, e.g., as gels, powders, granules, pastes, or liquids. 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.

[0132] 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; α-olefinsulfonate; 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 in WO 92/06154, for example.

[0133] The detergent composition may additionally comprise one or more other enzymes, such as lipase, cutinase, protease, cellulase, peroxidase, and/or laccase in any combination.

[0134] 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. Enzymes may be used in any composition compatible with the stability of the enzyme. Enzymes can be protected against generally deleterious components by known forms of encapsulation, as by granulation or sequestration in hydro gels, for example. Enzymes and specifically α-amylases either with or without the starch binding domains are not limited to laundry and dishwashing applications, but may bind use in surface cleaners and ethanol production from starch or biomass.

[0135] 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.

[0136] The detergent may contain a bleaching system, which may comprise a H_2O_2 source such as perborate or percarbonate optionally combined with a peracid-forming bleach activator, such as TAED or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxy acids of the amide, imide, or sulfone type, for example. The bleaching system can also be an enzymatic bleaching system where a perhydrolase activates peroxide, such as that described in WO 2005/056783.

[0137] 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 an aromatic borate ester; and the composition may be formulated as described in WO 92/19709 and WO 92/19708, for example.

[0138] The detergent may also contain other conventional detergent ingredients such as fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, or perfume, for example. The pH (measured in aqueous solution at use concentration) is usually neutral or alkaline, e.g., pH about 7.0 to about 11.0.

[0139] The α-amylase variant may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition, the α-amylase variant may be added in an amount corresponding to 0.00001-1.0 mg (calculated as pure enzyme protein) of α-amylase variant per liter of wash liquor. Particular forms of detergent compositions comprising the α-amylase variants can be formulated to include:

[0140] (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, about 2 to about 6%; zeolite (e.g., $NaAlSiO_4$) 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., $NaBO_3 \cdot H_2O$) 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%.

[0141] (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, about 1% to about 4%; zeolite (e.g., $NaAlSiO_4$) 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%.

[0142] (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, about 3% to about 9%; zeolite (as $NaAlSiO_4$) about 23% to about 33%; sodium sulfate (e.g., Na_2SO_4) 0% to about 4%; sodium perborate (e.g., $NaBO_3 \cdot H_2O$) 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%.

[0143] (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, about 1% to about 5%; zeolite (e.g., $NaAlSiO_4$) 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%.

[0144] (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%.

[0145] (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 $NaAlSiO_4$) 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 (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%.

[0146] (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, about 1% to about 4%; zeolite (e.g., $NaAlSiO_4$) about 20% to about 40%; sodium sulfate (e.g., Na_2SO_4) about 2% to about 8%; sodium perborate (e.g., $NaBO_3 \cdot H_2O$) 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%.

[0147] (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, about 1% to about 4%; zeolite (e.g., $NaAlSiO_4$) about 30% to about 50%; sodium sulfate (e.g., Na_2SO_4) about 3% to about 11%; sodium citrate (e.g., $C_6H_5Na_3O_7$) 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%.

[0148] (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., $NaAlSiO_4$) 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., $NaBO_3 \cdot H_2O$) 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%; 5 enzymes (calculated as pure enzyme protein) 0.0001-0.1%; and minor ingredients (e.g., optical brightener, perfume) 0-5%.

[0149] (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} 10 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) 15 0.0001-0.1%; and minor ingredients (e.g., polymers, dispersants, perfume, optical brighteners) 0-5%.

[0150] (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 20 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 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%.

25 **[0151]** (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, about 5% to 30 about 15%; sodium sulfate (e.g., Na_2SO_4) 0% to about 5%; zeolite ($NaAlSiO_4$) about 15% to about 28%; sodium perborate (e.g., $NaBO_3 \cdot H_2O$) 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%.

[0152] (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.

5 **[0153]** (14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/L comprising (C_{12}-C_{18}) alkyl sulfate about 9% to about 15%; alcohol ethoxylate about 3% to about 6%; polyhydroxy alkyl fatty acid amide about 1% to about 5%; zeolite (e.g., $NaAlSiO_4$) about 10% to about 20%; layered disilicate (e.g., SK56 from Hoechst) about 10% to about 20%; sodium carbonate (e.g., Na_2CO_3) about 3% to about 12%;
10 soluble silicate, 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 **[0154]** (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, 0% to about 4%; sodium percarbonate about 13% to about 22%; TAED 1-8%;
20 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%.

[0155] (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
25 specified bleach systems.

[0156] (17) Detergent compositions as described supra in 1), 3), 7), 9), and 12), wherein perborate is replaced by percarbonate.

[0157] (18) Detergent compositions as described supra in 1), 3), 7), 9), 12), 14), and 15), which additionally contains a manganese catalyst.

[0158] (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.

5 **[0159]** In another embodiment, the 2,6-β-D-fructan hydrolase can be incorporated in detergent compositions and used for removal/cleaning of biofilm present on household and/or industrial textile/laundry.

[0160] The detergent composition may for example be formulated as a hand or machine laundry detergent composition, including a laundry additive composition suitable for pre-
10 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 hand or machine dishwashing operations.

[0161] In a specific aspect, the detergent composition can comprise 2,6-β-D-fructan hydrolase, one or more α-amylase variants, and one or more other cleaning enzymes, such as a
15 protease, a lipase, a cutinase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, a laccase, and/or a peroxidase, and/or combinations thereof. In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (e.g., pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in
20 effective amounts.

[0162] Proteases: suitable proteases include those of animal, vegetable or microbial origin. Chemically modified or protein engineered mutants are also suitable. The protease may be a serine protease or a metalloprotease, e.g., an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived
25 from Bacillus sp., e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309 (see, e.g., U.S. Patent No. 6,287,841), 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 and
30 WO 98/20115. Suitable commercially available protease enzymes include Alcalase®, Savinase®, Primase™, Duralase™, Esperase®, and Kannase™ (Novo Nordisk A/S);

Maxatase®, Maxacal™, Maxapem™, Properase™, Purafect®, Purafect OxP™, FN2™, and FN3™ (Danisco A/S).

[0163] Lipases: suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include, but are not limited to, lipases from Humicola (synonym Thermomyces), e.g. H. lanuginosa (T. lanuginosus) (see, e.g., EP 258068 and EP 305216) and 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 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).

[0164] Polyesterases: Suitable polyesterases include, but are not limited to, those described in WO 01/34899 (Danisco A/S) and WO 01/14629 (Danisco A/S), and can be included in any combination with other enzymes discussed herein.

[0165] Amylases: The compositions can be combined with other α-amylases, such as a non-variant α-amylase. These can include commercially available amylases, such as but not limited to Duramyl®, Termamyl™, Fungamyl® and BAN™ (Novo Nordisk A/S), Rapidase®, and Purastar® (Danisco A/S).

[0166] 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 in U.S. Patent Nos. 4,435,307; 5,648,263; 5,691,178; 5,776,757; and WO 89/09259, for example. Exemplary cellulases contemplated for use are those

having color care benefit for the textile. Examples of such cellulases are cellulases described in EP 0495257; EP 531 372; WO 99/25846 (Danisco A/S), WO 96/34108 (Danisco A/S), WO 96/11262; WO 96/29397; and WO 98/08940, for example. Other examples are cellulase variants, such as those described in WO 94/07998; WO 98/12307;
5 WO 95/24471; PCT/DK98/00299; EP 531 315; 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); Clazinase™ and Puradax® HA (Danisco A/S); and KAC-500(B)™ (Kao Corporation).

[0167] Peroxidases/Oxidases: Suitable peroxidases/oxidases contemplated for use in the
10 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 Guardzyme™ (Novo Nordisk A/S), for example.

15 **[0168]** 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 as a granulate, liquid, slurry, etc. Suitable granulate detergent additive formulations include non-dusting granulates.

20 **[0169]** Non-dusting granulates may be produced, e.g., as disclosed in U.S. Patent Nos. 4,106,991 and 4,661,452 and optionally may 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
25 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 GB 1483591, for example. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or
30 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.

[0170] The detergent composition may be in any convenient form, e.g., a bar, tablet, gel, powder, granule, paste, or 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 30% or less water are also contemplated. The detergent composition comprises one or more surfactants, which may be non-ionic, including semi-polar, anionic, cationic, or zwitterionic, or any combination thereof. The surfactants are typically present at a level of from 0.1% to 60% by weight.

[0171] When included therein the detergent typically will 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.

[0172] 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").

[0173] 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).

[0174] The detergent may comprise one or more polymers. Examples are carboxymethyl-cellulose (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.

[0175] The detergent may contain a bleaching system that may comprise a source of H_2O_2, such as perborate or percarbonate, which may be combined with a peracid-forming bleach activator (e.g., tetraacetylethylenediamine or nonanoyloxybenzenesulfonate). 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.

[0176] The enzyme(s) of the detergent composition may be stabilized using conventional stabilizing agents, e.g., polyol (e.g., propylene glycol or glycerol), a sugar or sugar alcohol, lactic acid, boric acid, a boric acid derivative (e.g., an aromatic borate ester), or a phenyl boronic acid derivative (e.g., 4-formylphenyl boronic acid). The composition
5 may be formulated as described in WO 92/19709 and WO 92/19708.

[0177] 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, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

10 **[0178]** It is contemplated that in the detergent compositions, 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, particularly about 0.05 to about 5.0 mg of enzyme protein per liter of wash liquor, or even more particularly in 0.1 to about 1.0 mg of enzyme protein per liter of wash liquor.

15 **[0179]** A representative assay that may be used to test the efficacy of a cleaning composition comprising AmyE or a variant thereof includes a swatch test. 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. Alternatively, the material can be paper, such as filter paper or nitrocellulose, or a piece
20 of a hard material, such as ceramic, metal, or glass. For α-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. In one embodiment, the AmyE or variant thereof is tested in a BMI (blood/milk/ink) assay.

[0180] A "smaller swatch" is a piece of the swatch that has been cut with a single hole punch
25 device, or a custom manufactured 96-hole punch device, where the pattern of the multi-hole punch is matched to standard 96-well microtiter plates, or 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 also can be
30 made by applying a stain to a small piece of material. For example, the smaller swatch can be a piece of fabric with a stain 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 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 either metal, plastic, glass, ceramic, or other 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. In this case, supernatant can be examined for released soil either by direct absorbance measurement or after a secondary color development reaction. Analysis of the released soil might also be taken by mass spectral analysis.

[0181] In one embodiment, a treatment protocol provides control over degree of fixation of a stain. As a result, it is possible to produce swatches that, for example, release varying amounts of stain when washed in the absence of the enzyme being tested. The use of fixed swatches leads to a dramatic improvement of the signal-to-noise ratio in the wash assays. Furthermore, by varying the degree of fixation, one can generate stains that give optimum results under the various cleaning conditions.

[0182] Swatches having stains of known "strength" on various types of material are commercially available (EMPA, St. Gallen, Switzerland; wfk--Testgewebe GmbH, Krefeld Germany; or Center for Test Materials, Vlaardingen, The Netherlands) and/or can be made by the practitioner (Morris and Prato, Textile Research Journal 52(4): 280-286 (1982)). Swatches can comprise, for example, a cotton-containing fabric containing a stain made by blood/milk/ink (BMI), spinach, grass, or chocolate/milk/soot. A BMI stain can be fixed to cotton with 0.0003% to 0.3% hydrogen peroxide, for example. Other combinations include grass or spinach fixed with 0.001% to 1% glutaraldehyde, gelatin and Coomassie stain fixed with 0.001% to 1% glutaraldehyde, or chocolate, milk and soot fixed with 0.001% to 1% glutaraldehyde.

[0183] The swatch can also be agitated during incubation with the enzyme and/or detergent formulation. Wash performance data is dependent on the orientation of the swatches in the wells (horizontal versus vertical), particularly in the 96-well plate. This would indicate that mixing was insufficient during the incubation period. Although there are a number of ways to ensure sufficient agitation during incubation, a plate holder in which

the microtiter plate is sandwiched between two plates of aluminum can be constructed. This can be as simple as placing, for example, an adhesive plate sealer over the wells then clamping the two aluminum plates to the 96-well plate with any type of appropriate, commercially available clamps. It can then be mounted in a commercial incubator
5 shaker. Setting the shaker to about 400 rpm results in very efficient mixing, while leakage or cross-contamination is efficiently prevented by the holder.

[0184] Trinitrobenzenesulfonic acid (TNBS) can be used to quantify the concentration of amino groups in the wash liquor. This can serve as a measure of the amount of protein that was removed from the swatch (see, e.g., Cayot and Tainturier, Anal. Biochem. 249: 184-200
10 (1997)). However, if a detergent or an enzyme sample leads to the formation of unusually small peptide fragments (for example, from the presence of peptidases in the sample), then one will obtain a larger TNBS signal, i.e., more “noise.”

[0185] Another means of measuring wash performance of blood/milk/ink that is based on ink release that can be quantified by measuring the absorbance of the wash liquor. The
15 absorbance can be measured at any wavelength between 350 and 800 nm. In one embodiment, the wavelength is measured at 410 nm or 620 nm. The wash liquor can also be examined to determine the wash performance on stains containing grass, spinach, gelatin or Coomassie stain. Suitable wavelengths for these stains include and 670 nm for spinach or grass and 620 nm for gelatin or Coomassie. For example, an aliquot of the
20 wash liquor (typically 100-150 μL from a 96-well microplate, for example) is removed and placed in a cuvette or multiwell microplate. This is then placed in a spectrophotometer and the absorbance is read at an appropriate wavelength. The system also can be used to determine a suitable enzyme and/or detergent composition for dish washing, for example, using a blood/milk/ink stain on a suitable substrate, such as cloth,
25 plastic or ceramic.

[0186] In one aspect, a BMI stain is fixed to cotton by applying 0.3% hydrogen peroxide to the BMI/cotton swatch for 30 minutes at 25°C or by applying 0.03% hydrogen peroxide to the BMI/cotton swatch for 30 minutes at 60°C. Smaller swatches of approximately 0.25" are cut from the BMI/cotton swatch and placed in the wells of a 96-well microtiter plate.
30 Into each well, a known mixture of a detergent composition and an enzyme, such as a variant protein, is placed. After placing an adhesive plate sealer onto the top of the microtiter plate, the microtiter plate is clamped to an aluminum plate and agitated on an

orbital shaker at approximately 250 rpm for about 10 to 60 minutes. At the end of this time, the supernatants are transferred to wells in a new microtiter plate and the absorbance of the ink at 620 nm is measured. This can be similarly tests with spinach stains or grass stains fixed to cotton by applying 0.01% glutaraldehyde to the
5 spinach/cotton swatch or grass/cotton swatch for 30 minutes at 25°C. The same can be done with chocolate, milk, and/or soot stains.

5.4. Textile Desizing Compositions and Use

[0187] Also contemplated are compositions and methods of treating fabrics (e.g., to desize a textile) using one or more AmyE or variant thereof. The AmyE or variants thereof can
10 be used in any fabric-treating method, which are well known in the art (*see*, *e.g.*, U.S. Patent No. 6,077,316). For example, in one aspect, the feel and appearance of a fabric is improved by a method comprising contacting the fabric with an enzyme variant in a solution. In one aspect, the fabric is treated with the solution under pressure.

[0188] In one aspect, the enzymes are applied during or after the weaving of textiles, or during
15 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 in order to increase their tensile strength and to prevent breaking. The AmyE or variants thereof can be applied to remove these sizing starch or starch derivatives.
20 After the textiles have been woven, a fabric can proceed to a desizing stage. This can be followed by one or more additional fabric processing steps. Desizing is the act of removing size from textiles. After weaving, the size coating should be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. Also provided is a method of desizing comprising enzymatic hydrolysis of the size by
25 the action of an enzyme variant.

[0189] The AmyE or variants thereof 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. The AmyE or variants thereof also can be used in compositions and methods for producing a stonewashed look on indigo-
30 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. The α-amylase variant 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.

5.5. Compositions and Methods for Baking and Food Preparation

[0190] The presently disclosed AmyE or variant thereof also may be used in compositions and methods for baking and food preparation. For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of α-amylase activity in the flour. A level of activity that is too high may result in a product that is sticky and/or doughy and unmarketable; but flour with insufficient α-amylase activity may not contain enough sugar for proper yeast function, resulting in dry, crumbly bread. Accordingly, an AmyE or variant thereof, by itself or in combination with another α-amylase(s), may be added to the flour to augment the level of endogenous α-amylase activity in flour. The AmyE or variant thereof typically has a temperature optimum in the presence of starch in the ranges of 30-90°C, 50-80°C, 55-75°C, or 60-70°C, for example. The temperature optimum may be measured in a 1% solution of soluble starch at pH 5.5.

[0191] In addition to the use of grains and other plant products in baking, grains such as barley, oats, wheat, as well as plant components, such as corn, hops, and rice are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, i.e., partially germinated, resulting in an increase in the levels of enzymes, including α-amylase. For successful brewing, adequate levels of α-amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation. An AmyE or variant thereof, by itself or in combination with another α-amylase(s), accordingly may be added to the components used for brewing.

[0192] 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.

[0193] As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed or coarsely ground forms. The methods described herein may be used to determine α-amylase activity levels in both flours and stock.

[0194] An AmyE or variant thereof 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., 1-10 mg/kg. Additional anti-staling amylases that can be used in combination with an α-amylase variant polypeptide include an endo-amylase, e.g., a bacterial endo-amylase from *Bacillus*. The additional amylase can be a maltogenic α-amylase (EC 3.2.1.133), e.g., from *Bacillus*. Novamyl® is a suitable maltogenic α-amylase from *B. stearothermophilus* strain NCIB 11837 and is described in Christophersen et al., *Starch*, 50(1): 39-45 (1997). Other examples of anti-staling endo-amylases include bacterial α-amylases derived from *Bacillus*, such as *B. licheniformis* or *B. amyloliquefaciens*. The anti-staling amylase may be an exo-amylase, such as β-amylase, e.g., from plant sources, such as soy bean, or from microbial sources, such as *Bacillus*.

[0195] The baking composition comprising an AmyE or variant thereof further can comprise a phospholipase. The phospholipase may have A_1 or A_2 activity to remove fatty acid from the phospholipids, forming a lyso-phospholipid. It may or may not have lipase activity, i.e., activity on triglycerides. 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, such as the genus or species *Aspergillus*, *A. niger*; *Dictyostelium*, *D. discoideum*; *Mucor*, *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, *N. crassa*; *Rhizomucor*, *R. pusillus*; *Rhizopus*, *R. arrhizus*, *R. japonicus*, *R.*

stolonifer; *Sclerotinia*, *S. libertiana*; *Trichophyton*, *T. rubrum*; *Whetzelinia*, *W. sclerotiorum*; *Bacillus*, *B. megaterium*, *B. subtilis*; *Citrobacter*, *C. freundii*; *Enterobacter*, *E. aerogenes*, *E. cloacae*; *Edwardsiella*, *E. tarda*; *Etwinia*, *E. herbicola*; *Escherichia*, *E. coli*; *Klebsiella*, *K. pneumoniae*; *Proteus*, *P. vulgaris*; *Providencia*, *P. stuartii*; *Salmonella*, *S. typhimurium*; *Serratia*, *S. liquefasciens*, *S. marcescens*; *Shigella*, *S. flexneri*; *Streptomyces*, *S. violeceoruber*; *Yersinia*, *Y. enterocolitica*; *Fusarium*, *F. oxysporum*, strain DSM 2672), for example.

[0196] A 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 Lipase Unit (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.

[0197] 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*.

[0198] The dough may also comprise other conventional dough ingredients, e.g., proteins, such as milk powder, gluten, and soy; eggs (either 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, polyoxyetliylene stearates, or lysolecithin. In particular, the dough can be made without addition of emulsifiers.

[0199] 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 amylo-glucosidase, 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 hemicellulase, in particular a pentosanase such as xylanase, a protease, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, for example, a glucanotranseferase, 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 lipoxygenase, an L-amino acid oxidase or a carbohydrate oxidase. The additional enzyme 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.

[0200] The xylanase is typically of microbial origin, e.g., derived from a bacterium or fungus, such as a strain of *Aspergillus*, in particular of *A. aculeatus, A. niger* (e.g., WO 91/19782), *A. awamori* (e.g., WO 91/18977), or *A. tubigensis* (e.g., WO 92/01793); from a strain of *Trichoderma*, e.g., *T. reesei*, or from a strain of *Humicola*, e.g., *H. insolens* (e.g., WO 92/17573). Pentopan® and Novozym 384® 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 (available from Grindsted Products, Denmark) and Amylase® H or Amylase® P (available from Gist-Brocades, The Netherlands). The glucose oxidase may be a fungal glucose oxidase, in particular an *Aspergillus niger* glucose oxidase (such as Gluzyme®). An exemplary protease is Neutrase®. An exemplary lipase can be derived from strains of *Thermomyces* (*Humicola*), *Rhizomucor*, *Candida*, *Aspergillus*, *Rhizopus*, or *Pseudomonas*, in particular from *Thermomyces lanuginosus* (*Humicola lanuginosa*), *Rhizomucor miehei*, *Candida antarctica*, *Aspergillus niger*, *Rhizopus delemar* or *Rhizopus arrhizus* or *Pseudomonas cepacia.* In specific embodiments, the lipase may be Lipase A or Lipase B derived from *Candida antarctica* as described in WO 88/02775, for example, or the lipase may be derived from

Rhizomucor miehei as described in EP 238,023, for example, or *Humicola lanuginosa*, described in EP 305,216, for example, or *Pseudomonas cepacia* as described in EP 214,761 and WO 89/01032, for example.

[0201] 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, French baguette-type bread, pita bread, tortillas, cakes, pancakes, biscuits, cookies, pie crusts, crisp bread, steamed bread, pizza and the like.

[0202] In another embodiment, an AmyE or variant thereof may be used in a pre-mix, comprising flour together with an anti-staling amylase, a phospholipase and 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. In one aspect, the AmyE or variant thereof is a component of an enzyme preparation comprising an anti-staling amylase and a phospholipase, for use as a baking additive.

[0203] 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 AmyE or variant thereof 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.

[0204] Another aspect contemplates the enveloping of particles comprising an AmyE or variant thereof, i.e., α-amylase particles. To prepare the enveloped α-amylase particles, the enzyme is contacted with a food grade lipid in sufficient quantity so as to suspend all of the α-amylase particles. Food grade lipids, as used herein, may be any naturally organic compound that is insoluble in water but is soluble in non-polar organic solvents such as hydrocarbon or diethyl ether. Suitable food grade lipids include, but are not limited to, triglycerides either in the form of fats or oils which 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
5 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.

[0205] The food grade lipid, particularly in the liquid form, is contacted with a powdered form of the α-amylase particles in such a fashion that the lipid material covers at least a
10 portion of the surface of at least a majority, e.g., 100% of the α-amylase particles. Thus, each α-amylase particle is individually enveloped in a lipid. For example, all or substantially all of the α-amylase particles are provided with a thin, continuous, enveloping film of lipid. This can be accomplished by first pouring a quantity of lipid into a container, and then slurrying the α-amylase particles so that the lipid thoroughly
15 wets the surface of each α-amylase particle. After a short period of stirring, the enveloped α-amylase particles, carrying a substantial amount of the lipids on their surfaces, are recovered. The thickness of the coating so applied to the particles of α-amylase can be controlled by selection of the type of lipid used and by repeating the operation in order to build up a thicker film, when desired.

20 **[0206]** The storing, handling and incorporation of the loaded delivery vehicle can be accomplished by means of a packaged mix. The packaged mix can comprise the enveloped α-amylase. However, the packaged mix may further contain additional ingredients as required by the manufacturer or baker. After the enveloped α-amylase has been incorporated into the dough, the baker continues through the normal production
25 process for that product.

[0207] The advantages of enveloping the α-amylase particles are two-fold. First, the food grade lipid protects the enzyme from thermal denaturation during the baking process for those enzymes that are heat labile. Consequently, while the α-amylase is stabilized and protected during the proving and baking stages, it is released from the protective coating
30 in the final baked good product, where it hydrolyzes the glucosidic linkages in polyglucans. The loaded delivery vehicle also provides a sustained release of the active enzyme into the baked good. That is, following the baking process, active α-amylase is

continually released from the protective coating at a rate that counteracts, and therefore reduces the rate of, staling mechanisms.

[0208] In general, the amount of lipid applied to the α-amylase particles can vary from a few percent of the total weight of the α-amylase to many times that weight, depending upon the nature of the lipid, the manner in which it is applied to the α-amylase particles, the composition of the dough mixture to be treated, and the severity of the dough-mixing operation involved.

[0209] The loaded delivery vehicle, i.e., the lipid-enveloped enzyme, is added to the ingredients used to prepare a baked good in an effective amount to extend the shelf-life of the baked good. The baker computes the amount of enveloped α-amylase, prepared as discussed above, that will be required to achieve the desired anti-staling effect. The amount of the enveloped α-amylase required is calculated based on the concentration of enzyme enveloped and on the proportion of α-amylase to flour specified. A wide range of concentrations has been found to be effective, although, as has been discussed, observable improvements in anti-staling do not correspond linearly with the α-amylase concentration, but above certain minimal levels, large increases in α-amylase concentration produce little additional improvement. The α-amylase concentration actually used in a particular bakery production could be much higher than the minimum necessary in order 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.

[0210] A method of preparing a baked good may comprise: (a) preparing lipid-coated α-amylase particles, wherein substantially 100 percent of the α-amylase particles are coated; (b) mixing a dough containing flour; (c) adding the lipid-coated α-amylase to the dough before the mixing is complete and terminating the mixing before the lipid coating is removed from the α-amylase; (d) proofing the dough; and (e) baking the dough to provide the baked good, wherein the α-amylase is inactive during the mixing, proofing and baking stages and is active in the baked good.

[0211] The enveloped α-amylase can be added to the dough during the mix cycle, e.g., near the end of the mix cycle. The enveloped α-amylase is added at a point in the mixing stage that allows sufficient distribution of the enveloped α-amylase throughout the dough;

however, the mixing stage is terminated before the protective coating becomes stripped from the α-amylase particle(s). Depending on the type and volume of dough, and mixer action and speed, anywhere from one to six minutes or more might be required to mix the enveloped α-amylase into the dough, but two to four minutes is average. Thus, several variables may determine the precise procedure. First, the quantity of enveloped α-amylase should have a total volume sufficient to allow the enveloped α-amylase to be spread throughout the dough mix. If the preparation of enveloped α-amylase is highly concentrated, additional oil may need to be added to the pre-mix before the enveloped α-amylase is added to the dough. Recipes and production processes may require specific modifications; however, good results generally can be achieved when 25% of the oil specified in a bread dough formula is held out of the dough and is used as a carrier for a concentrated enveloped α-amylase when added near the end of the mix cycle. In bread or other baked goods, recipes which have extremely low fat content (such as French-style breads), it has been found that an enveloped α-amylase mixture of approximately 1% of the dry flour weight is sufficient to admix the enveloped α-amylase properly with the dough, but the range of percentages that may work is extremely wide and depends on the formula, finished product, and production methodology requirements of the individual baker. Second, the enveloped α-amylase suspension should be added to the mix with enough time remaining in the mix cycle for complete mixture into the dough, but not so early that excessive mechanical action will strip the protective lipid coating from a large proportion of the enveloped α-amylase particles.

[0212] In another embodiment, bacterial α-amylase (BAA) is added to the lipid-coated particles comprising an AmyE or variant thereof. BAA reduces bread to a gummy mass due to its excessive thermostability and retained activity in the fully baked loaf of bread; however, when BAA is incorporated into the lipid-coated particles, substantial additional anti-staling protection is obtained, even at very low BAA dosage levels. For example, BAA dosages of 150 RAU (Reference Amylase Units) per 100 pounds of flour have been found to be effective. In one embodiment, between about 50 to 2000 RAU of BAA is added to the lipid-coated enzyme product. This low BAA dosage level, combined with the ability of the protective coating to keep enzyme in the fully-baked loaf from free contact with the starches (except when water vapor randomly releases the enzyme from its coating), helps to achieve very high levels of anti-staling activity without the negative side-effects of BAA.

[0213] It will be apparent to those skilled in the art that various modifications and variation can be made to the compositions and methods of using same without departing from the spirit or scope of the intended use. Thus, it is the modifications and variations provided they come within the scope of the appended claims and their equivalents.

5 **[0214]** All references cited herein are incorporated by reference in their entirety for all purposes.

EXAMPLES

Example 1

1.1. Plasmid Construction

10 **[0215]** Nucleic acids encoding the AmyE of SEQ ID NO: 1 or a C-terminal truncated AmyE variant, AmyE-tr (SEQ ID NO: 2), were cloned into the *B. subtilis* pHPLT expression vector, disclosed in U.S. Patent No. 5,024,943. FIG. 2 depicts the vector comprising a nucleic acid encoding AmyE-tr.

[0216] Referring to FIG. 2, the pHPLT vector contains the *B. licheniformis* LAT promoter
15 ("Plat"), a sequence encoding the LAT signal peptide ("preLAT"), followed by PstI and HpaI restriction sites for cloning. Additional plasmid elements from plasmid pUB110 disclosed in McKenzie *et al.*, *Plasmid* 15(2): 93-103 (1986): "ori-pUB" is the origin of replication from pUB110; "reppUB" is the replicase gene from pUB110, "neo" is the neomycin/kanamycin resistance gene from pUB110; "bleo" is the bleomycin resistance
20 marker, "Tlat" is the transcriptional terminator from *B. licheniformis* amylase.

[0217] Plasmid constructs for the expression of AmyE and AmyE-tr were assembled using the AmyE-encoding sequence described by Yang *et al*, "Nucleotide sequence of the amylase gene from *Bacillus subtilis*," *Nucl. Acids Res.* 11(2): 237-49 (1983). Plasmid pME629.5 contains the nucleic acid encoding the full-length AmyE of SEQ ID NO: 1. The gene
25 has a three base deletion in the sequence encoding the starch binding domain, compared to the sequence described by Yang *et al.*

[0218] Plasmid pME630.7 contains the truncated AmyE sequence, AmyE-tr, and is shown in FIG. 2. AmyE-tr is truncated at D425 of SEQ ID NO: 1. AmyE-tr was designed from a crystal structure of an AmyE variant that lacks the starch binding domain, disclosed in
30 Fujimoto *et al.*, "Crystal structure of a catalytic-site mutant alpha-amylase from *Bacillus*

subtilis complexed with maltopentaose," *J. Mol. Biol.* 277: 393-407(1998). *See* RCSB Protein Data Bank© Accession No. 1BAG, "Alpha-Amylase From *Bacillus Subtilis* Complexed With Maltopentaose."

[0219] For expression plasmid construction, the nucleic acid encoding AmyE was PCR-amplified using Herculase® (Stratagene, California). The PCR products were purified using a column provided in a Qiagen QIAquik™ PCR purification kit (Qiagen, Valencia, California), and resuspended in 50 μL of Milli-Q™-purified water. 50 μL of the purified DNA was digested sequentially with HpaI (Roche) and PstI (Roche), and the resultant DNA resuspended in 30 μL of Milli-Q™-purified water. 10-20 ng/μL DNA was cloned into plasmid pHPLT using PstI and HpaI cloning sites. The ligation mixtures were directly transformed into competent *B. subtilis* cells (genotype: D*aprE*, D*nprE*, *degUHy32 oppA*, D*spoIIE3501*, *amyE*::*xylRPxylAcomK-phleo*). SC6.1 *B. subtilis* cells have a competency gene (comK) which is placed under a xylose-inducible promoter. Competency for DNA binding and uptake is induced by the addition of xylose. Because the AmyE gene in the parent plasmid has two PstI sites, a PCR fusion reaction was carried out to remove these sites before cloning. PCR fusion was done after two separate PCR reactions. The following primers were used for making the pHPLT construct using HpaI and PstI sites:

SEQ ID NO: 18: Primer PSTAMYE-F '
CTTCTTGCTGCCTCATTCTGCAGCTTCAGCACTTACAGCACCGTCGATCAAAA GCGGAAC 3'
SEQ ID NO: 19: Primer AMYENOPST-R '
CTGGAGGCACTATCCTGAAGGATTTCTCCGTATTGGAACTCTGCTGATGTATT TGTG
SEQ ID NO: 20: Primer AMYENOPST-F '
CACAAATACATCAGCAGAGTTCCAATACGGAGAAATCCTTCAGGATAGTGCC TCCAG
SEQ ID NO: 21: Primer HPAIAMYE-R '
CAGGAAATCCGTCCTCTGTTAACTCAATGGGGAAGAGAACCGCTTAAGCCCG AGTC

SEQ ID NO: 22: Primer HPAIAMYE-R '
CAGGAAATCCGTCCTCTGTTAACTCAATCAGGATAAAGCACAGCTACAGACC
TGG
SEQ ID NO: 23: Primer AMYE SEQ-F '
5 TACACAAGTACAGTCCTATCTG 3'
SEQ ID NO: 24: Primer AMYE SEQ-F '
CATCCTCTGTCTCTATCAATAC 3'

[0220] The plasmids pME629.5 and pME630.7 express AmyE with a 31 residue signal
10 sequence, which is cleaved post-translationally. The subsequent 10 N-terminal amino
acids are processed separately as proposed by Yang *et al.* (1983).

1.2. Protein Expression

[0221] Transformants for AmyE full-length and truncated clones were selected on LA with
10 µg/mL neomycin, 1% insoluble starch and incubated overnight at 37°C.
15 Transformants showing a clearing (or halo) around the colony were selected, and vials
were made for further studies. Precultures of the transformants were grown for 8h in LB
with 10 µg/mL neomycin. Then, 30 µL of this pre-culture were added into a 250 mL
flask filled with 30 mL of cultivation media (described below) supplemented with 10
µg/mL neomycin and 5 mM $CaCl_2$. The cultivation media was an enriched semi-defined
20 media based on MOPs buffer, with urea as the major nitrogen source, glucose as the
main carbon source, and supplemented with 1% soytone for robust cell growth. The
shake flasks were incubated for 60-65 hours at 37°C, with mixing at 250 rpm. Cultures
were harvested by centrifugation at 5000 rpm for 20 minutes in conical tubes. Since
both AmyE full-length and AmyE truncated proteins expressed at high levels, the culture
25 supernatants were used for assays without further purification.

Example 2

[0222] The following assays were used in the examples described below. Any deviations from
the protocols provided below are indicated in the examples. In these experiments, a
spectrophotometer was used to measure the absorbance of the products formed after the
30 completion of the reactions.

2.1. Bradford Assay for Protein Content Determination in 96-well Microtiter Plate

[0223] Protein concentration in sample supernatants was determined using the Bradford QuickStart™ Dye Reagent (Bio-Rad, California). Samples were obtained by filtration of broths from cultures grown in microtiter plates (MTPs) for 3 days at 37°C with shaking at 280 rpm and humidified aeration. A 10 µL sample of the culture filtrate was combined with 200 µL Bradford QuickStart™ Dye Reagent in a well of a second MTP. After thorough mixing, the MTP's were incubated for at least 10 minutes at room temperature. Air bubbles were removed and the OD (optical density) was measured at 595 nm. To determine the protein concentration, the background reading (from uninoculated wells) was subtracted from the sample readings.

2.2. Conventional Ethanol Fermentation

[0224] Two batches of liquifact (31% DS) obtained from Illinois River Energy, containing 400 ppm urea were adjusted to pH 4.3 and pH 5.8 (using 5N H_2SO_4). 100 g substrate was added to a 125 mL Erlenmeyer flask. AmyE-tr and Spezyme® Xtra amylase were dosed at 0.20 mg/g DS. Fermentations were inoculated with 0.2 ml of 10% (w/v) Red Star Ethanol Red yeast pre-hydrated ~45 min in DI water. Flasks were incubated at 32ºC with stir bars at 320 rpm for a 48h fermentation.

2.3. Ethanol fermentation on whole ground corn

[0225] Two batches of 32% DS corn flour substrate with 400 ppm urea were prepared at pH 4.3 and pH 5.8 (adjusted with 5N H_2SO_4). 100 g substrate was added to a 125 ml Erlenmeyer flask. Full length AmyE (SEQ ID NO: 1) and AmyE-tr (SEQ ID NO: 2) were dosed at 0.20 mg/g DS, *A. kawachii* α-amylase (AkAA; SEQ ID NO: 6) was dosed at 1.5 SSU/g DS. The amino acid sequence of AkAA is disclosed in SEQ ID NO: 4 of U.S. Patent No. 7,332,319. The ability of AmyE and AmyE-tr to hydrolyze whole ground corn was also compared to a mixture of *T. reesei* glucoamylase (TrGA; SEQ ID NO: 7) dosed at 0.5 GAU/g plus *A. kawachii* α-amylase dosed at 1.5 SSU/g DS. The amino acid sequence of TrGA was disclosed in SEQ ID NO: 3 of WO 2006/060062. Fermentations were inoculated with 0.2 ml of 10% (w/v) Red Star Ethanol Red yeast prehydrated ~45 min in DI water. Flasks were incubated at 32ºC with stir bars at 300 rpm for 72h fermentation.

2.4. Glucose formation determination by HPLC measurement

Hydrolysis of maltose and maltoheptaose

[0226] 0.5% maltose or maltoheptaose solutions were prepared in 50 mM sodium acetate, pH 4.5 or 5.6, or in 50 mM malic acid pH 5.6, as specified for each experiment. All enzyme samples were initially diluted to 1 mg/mL. Reaction mixtures were prepared by diluting the enzyme using the appropriate substrate solutions to give a final enzyme concentration of 1 ppm, then 200 μL aliquots were transferred to sterile screw top tubes and place in a 37ºC incubator. The reactions were stopped at the indicated times by diluting 10-fold into 10mM sodium hydroxide.

Hydrolysis of insoluble starch

[0227] For measuring the hydrolysis of insoluble granular starch, purified Amy E (24.5g/L) was diluted to a final concentration of 20.4 ppm in malic acid buffer, pH 5.6. The protein was then added to a 5% corn flour solution prepared in malic acid buffer, pH 5.6, to a final concentration of 1 ppm, and the mixture was incubated in a shaker at 32ºC. Samples were periodically removed and diluted 10 fold into 50 mM NaOH to quench the reaction.

HPLC Detection method

[0228] The formation of glucose and other breakdown products of the substrates were analyzed by HPLC using an Agilent 1100 LC system equipped with a Dionex PA-1 column and electrochemical detector. 10 μL samples were injected and a gradient of NaOH and sodium acetate was applied at 1.0 mL/min at 25ºC. The distribution of saccharides was determined from previously run standards. Elution profiles were obtained over 45 minutes. Quantitation of glucose produced (reported as g/L) was obtained using authenticated glucose reference standard (Sigma, MO) to convert peak area for the sugars to actual sugar concentrations.

Example 3

[0229] The performance of truncated AmyE in conventional ethanol fermentation was tested on Illinois River Energy liquefact (31% DS), using the conventional ethanol fermentation assay described in Example 2.2. The performance of AmyE-tr (SEQ ID NO: 2) was compared to Spezyme® Xtra amylase (AmyR; SEQ ID NO: 5) at pH 4.3 and pH 5.8. Fermentations were carried out for 48h. AmyE-tr and Spezyme® Xtra amylase were dosed at 0.2 mg/g DS. As shown in FIG. 3, the final ethanol yield produced by AmyE-tr at pH 5.8 is 12.0% (v/v). AmyE-tr at pH 4.3 yielded a final ethanol yield of 7.3% (v/v). Final ethanol yields in the presence of Spezyme® Xtra amylase were 2.7% (v/v) at pH

4.3 and 3.9% (v/v) at pH 5.8. AmyE-tr thus produces significantly more ethanol in conventional ethanol fermentation of liquefact than Spezyme® Xtra amylase. This example also demonstrates that AmyE-tr produces more ethanol at pH 5.8 than at pH 4.3.

Example 4

5 **[0230]** The ability of AmyE (SEQ ID NO: 1) and AmyE-tr (SEQ ID NO: 2) to catalyze the hydrolysis of insoluble granular (uncooked) starch into ethanol at pH 4.3 and pH 5.8 was compared, using the ethanol fermentation on whole ground corn assay described in Example 2.3. The ethanol forming performance of AmyE and AmyE-tr was compared to *A. kawachii* α-amylase (AkAA, SEQ ID NO: 6), dosed at 1.5 SSU/g, a mixture of *T.* 10 *reesei* glucoamylase (TrGA; SEQ ID NO: 7) dosed at 0.5 GAU/g plus *A. kawachii* α-amylase dosed at 1.5 SSU/g DS. Both AmyE full-length and truncated AmyE were dosed at 0.2 mg/g DS.

[0231] FIG. 4 shows the final ethanol yield produced by the enzymes at pH 4.3 and pH 5.8. When tested at pH 5.8, both AmyE (—●—) and AmyE-tr (—■—) performed 15 comparably to the TrGA/AkAA (—▲—), with AmyE actually surpassing the ethanol yields observed for TrGA/AkAA . AmyE (—○—) and AmyE-tr (—□—) produced ethanol at pH 4.3, but the yield was not as high as obtained with TrGA/AkAA (—Δ—). In comparison, AkAA performed poorly at both pHs tested (—♦◊—). This example demonstrates that AmyE can completely replace glucoamylase in a saccharification 20 reaction at around pH 5.8. It also demonstrates that AmyE can replace glucoamylase partially or completely in a saccharification reaction at pH 4.3.

Example 5

[0232] The ability of AmyE to convert maltose to glucose at pH 4.5 and 5.6 (using sodium acetate buffer) was tested, using the glucose formation assay described in Example 2.4. 25 The reactions were analyzed after 2, 5, and 8 days. As shown in FIG. 5, AmyE (SEQ ID NO: 1), AmyE-tr (SEQ ID NO: 2), and Amy 31A (SEQ ID NO: 3) effectively converted maltose to glucose, whereas *Geobacillus stearothermophilus* α-amylase, AmyS (SEQ ID NO: 4, shown with a 34 amino acid leader sequence), showed only a minimal amount of glucose formation under these conditions.

Example 6

[0233] The ability of AmyE (SEQ ID NO: 1) and AmyE-tr (SEQ ID NO: 2) to catalyze the hydrolysis of DP7 or an insoluble, uncooked granular starch was tested. The HPLC method used for detection of saccharides produced from insoluble starch is described in Example 2.4. Degradation products were quantified by HPLC analysis at various times after the reaction was initiated.

[0234] FIG. 6 depicts hydrolysis products obtained after incubating a 0.5% maltoheptaose substrate in the presence of 1 ppm AmyE-tr for 72 hours. As can be seen in the bottom panel of FIG. 6, AmyE-tr converts nearly all of the DP7 substrate to glucose by 72 hours. The results demonstrate that AmyE is capable of degrading a DP7 substrate to glucose efficiently.

[0235] By comparison, the degradation of a DP7 substrate by 1 ppm of either AmyS (SEQ ID NO: 4) or SPEZYME® FRED ("Fred"; SEQ ID NO: 8) is depicted in FIG. 7 and FIG. 8, respectively. Samples from reactions were analyzed using the HPLC procedure set forth in Example 2.4 above. The panels in FIG. 7 from top to bottom represent the reaction products at 0 hours, 2 hours, 4 hours and 24 hours after addition of AmyS. The panels in FIG. 8 from top to bottom represent the reaction products at 0 hours, 1 hours, 2 hours and 3 hours after addition of SPEZYME® FRED. The results show that a considerable portion of the DP7 substrate remains at a degree of polymerization of DP2 or greater in the presence of AmyS or SPEZYME® FRED at the times indicated.

[0236] FIG. 9 depicts the results of incubating a 5% corn flour solution with 1 ppm AmyE (SEQ ID NO: 1) at 32°C, according to the procedure set forth in Example 2.4. The results show that AmyE by itself can convert insoluble granular starch efficiently to gluocse.

SEQUENCE LISTING

SEQ ID NO: 1: Full length *Bacillus subtilis* AmyE amino acid sequence. The native signal sequence is not shown.

```
  1 LTAPSIKSGT ILHAWNWSFN TLKHNMKDIH DAGYTAIQTS PINQVKEGNQ
 51 GDKSMSNWYW LYQPTSYQIG NRYLGTEQEF KEMCAAAEEY GIKVIVDAVI
101 NHTTSDYAAI SNEVKSIPNW THGNTQIKNW SDRWDVTQNS LLGLYDWNTQ
151 NTQVQSYLKR FLDRALNDGA DGFRFDAAKH IELPDDGSYG SQFWPNITNT
201 SAEFQYGEIL QDSASRDAAY ANYMDVTASN YGHSIRSALK NRNLGVSNIS
251 HYASDVSADK LVTWVESHDT YANDDEESTW MSDDDIRLGW AVIASRSGST
301 PLFFSRPEGG GNGVRFPGKS QIGDRGSALF EDQAITAVNR FHNVMAGQPE
351 ELSNPNGNNQ IFMNQRGSHG VVLANAGSSS VSINTATKLP DGRYDNKAGA
401 GSFQVNDGKL TGTINARSVA VLYPDDIAKA PHVFLENYKT GVTHSFNDQL
451 TITLRADANT TKAVYQINNG PETAFKDGDQ FTIGKGDPFG KTYTIMLKGT
501 NSDGVTRTEK YSFVKRDPAS AKTIGYQNPN HWSQVNAYIY KHDGSRVIEL
551 TGSWPGKPMT KNADGIYTLT LPADTDTTNA KVIFNNGSAQ VPGQNQPGFD
601 YVLNGLYNDS GLSGSLPH
```

SEQ ID NO: 2: Truncated *Bacillus subtilis* AmyE (AmyE-tr) amino acid sequence. The native signal sequence is not shown.

```
  1 LTAPSIKSGT ILHAWNWSFN TLKHNMKDIH DAGYTAIQTS PINQVKEGNQ
 51 GDKSMSNWYW LYQPTSYQIG NRYLGTEQEF KEMCAAAEEY GIKVIVDAVI
101 NHTTSDYAAI SNEVKSIPNW THGNTQIKNW SDRWDVTQNS LLGLYDWNTQ
151 NTQVQSYLKR FLDRALNDGA DGFRFDAAKH IELPDDGSYG SQFWPNITNT
201 SAEFQYGEIL QDSASRDAAY ANYMDVTASN YGHSIRSALK NRNLGVSNIS
251 HYASDVSADK LVTWVESHDT YANDDEESTW MSDDDIRLGW AVIASRSGST
301 PLFFSRPEGG GNGVRFPGKS QIGDRGSALF EDQAITAVNR FHNVMAGQPE
351 ELSNPNGNNQ IFMNQRGSHG VVLANAGSSS VSINTATKLP DGRYDNKAGA
401 GSFQVNDGKL TGTINARSVA VLYPD
```

SEQ ID NO: 3: *Bacillus subtilis* α-amylase variant Amy31A amino acid sequence (UniProtKB/TrEMBL Accession No. O82953). The native signal sequence is shown in **bold**.

1 **MFEKRFKTSL LPLFAGFLLL FHLVLSGPAA ANAETANKSN K**VTASSVKNG
51 TILHAWNWSF NTLTQNMKDI RDAGYAAIQT SPINQVKEGN QGDKSMSNWY
101 WLYQPTSYQI GNRYLGTEQE FKDMCAAAEK YGVKVIVDAV VNHTTSDYGA
151 ISDEIKRIPN WTHGNTQIKN WSDRWDITQN ALLGLYDWNT QNTEVQAYLK
201 GFLERALNDG ADGFRYDAAK HIELPDDGNY GSQFWPNITN TSAEFQYGEI
251 LQDSASRDTA YANYMNVTAS NYGHSIRSAL KNRILSVSNI SHYASDVSAD
301 KLVTWVESHD TYANDDEEST WMSDDDIRLG WAVIGSRSGS TPLFFSRPEG
351 GGNGVRFPGK SQIGDRGSAL FKDQAITAVN QFHNEMAGQP EELSNPNGNN
401 QIFMNQRGSK GVVLANAGSS SVTINTSTKL PDGRYDNRAG AGSFQVANGK
451 LTGTINARSA AVLYPDDIGN APHVFLENYQ TEAVHSFNDQ LTVTLRANAK
501 TTKAVYQINN GQETAFKDGD RLTIGKEDPI GTTYNVKLTG TNGEGASRTQ
551 EYTFVKKDPS QTNIIGYQNP DHWGNVNAYI YKHDGGGAIE LTGSWPGKAM
601 TKNADGIYTL TLPANADTAD AKVIFNNGSA QVPGQNHPGF DYVQNGLYNN
651 SGLNGYLPH

SEQ ID NO: 4: Truncated *Geobacillus stearothermophilus* α-amylase (AmyS, a/k/a "Ethyl3") protein sequence. The signal sequence is shown in **bold**.

1 **MLTFHRIIRK GWMFLLAFLL TASLFCPTGQ HAKA**AAPFNG TMMQYFEWYL

51 PDDGTLWTKV ANEANNLSSL GITALWLPPA YKGTSRSDVG YGVYDLYDLG
101 EFNQKGTVRT KYGTKAQYLQ AIQAAHAAGM QVYADVVFDH KGGADGTEWV
151 DAVEVNPSDR NQEISGTYQI QAWTKFDFPG RGNTYSSFKW RWYHFDGVDW
201 DESRKLSRIY KFIGKAWDWE VDTENGNYDY LMYADLDMDH PEVVTELKNW
5 251 GKWYVNTTNI DGFRLDAVKH IKFSFFPDWL SYVRSQTGKP LFTVGEYWSY
301 DINKLHNYIT KTNGTMSLFD APLHNKFYTA SKSGGAFDMR TLMTNTLMKD
351 QPTLAVTFVD NHDTEPGQAL QSWVDPWFKP LAYAFILTRQ EGYPCVFYGD
401 YYGIPQYNIP SLKSKIDPLL IARRDYAYGT QHDYLDHSDI IGWTREGVTE
451 KPGSGLAALI TDGPGGSKWM YVGKQHAGKV FYDLTGNRSD TVTINSDGWG
10 501 EFKVNGGSVS VWVPRKTT

SEQ ID NO: 5: *Geobacillis stearothermophilus* α-amylase (AmyR; Spezyme® Xtra amylase) amino acid sequence.

1 AAPFNGTMMQ YFEWYLPDDG TLWTKVANEA NNLSSLGITA LWLPPAYKGT
51 SRSDVGYGVY DLYDLGEFNQ KGTVRTKYGT KAQYLQAIQA AHAAGMQVYA
15 101 DVVFDHKGGA DGTEWVDAVE VNPSDRNQEI SGTYQIQAWT KFDFPGRGNT
151 YSSFKWRWYH FDGVDWDESR KLSRIYKFRG IGKAWDWEVD TENGNYDYLM
201 YADLDMDHPE VVTELKNWGK WYVNTTNIDG FRLDAVKHIK FSFFPDWLSY
251 VRSQTGKPLF TVGEYWSYDI NKLHNYITKT NGTMSLFDAP LHNKFYTASK
301 SGGAFDMRTL MTNTLMKDQP TLAVTFVDNH DTEPGQALQS WVDPWFKPLA
20 351 YAFILTRQEG YPCVFYGDYY GIPQYNIPSL KSKIDPLLIA RRDYAYGTQH
401 DYLDHSDIIG WTREGVTEKP GSGLAALITD GPGGSKWMYV GKQHAGKVFY
451 DLTGNRSDTV TINSDGWGEF KVNGGSVSVW VPRKTT

SEQ ID NO: 6: *Aspegillus kawachi* α-amylase (AkAA) amino acid sequence.

*1 MRVSTSSIAL AVSLFGKLAL G*LSAAEWRTQ SIYFLLTDRF GRTDNSTTAT
25 51 CNTGDQIYCG GSWQGIINHL DYIQGMGFTA IWISPITEQL PQDTSDGEAY
101 HGYWQQKIYN VNSNFGTADD LKSLSDALHA RGMYLMVDVV PNHMGYAGNG
151 NDVDYSVFDP FDSSSYFHPY CLITDWDNLT MVQDCWEGDT IVSLPDLNTT
201 ETAVRTIWYD WVADLVSNYS VDGLRIDSVE EVEPDFFPGY QEAAGVYCVG
251 EVDNGNPALD CPYQKYLDGV LNYPIYWQLL YAFESSSGSI SNLYNMIKSV
30 301 ASDCSDPTLL GNFIENHDNP RFASYTSDYS QAKNVLSYIF LSDGIPIVYA
351 GEEQHYSGGD VPYNREATWL SGYDTSAELY TWIATTNAIR KLAISADSDY
401 ITYANDPIYT DSNTIAMRKG TSGSQIITVL SNKGSSGSSY TLTLSGSGYT
451 SGTKLIEAYT CTSVTVDSNG DIPVPMASGL PRVLLPASVV DSSSLCGGSG
501 N**TTTTTTAAT STSKATTSSS SSSAAATTSS SCTATSTT**LP ITFEELVTTT
35 551 YGEEVYLSGS ISQLGEWDTS DAVKLSADDY TSSNPEWSVT VSLPVGTTFE
601 YKFIKVDEGG SVTWESDPNR EYTVPECGSG SGETVVDTWR

SEQ ID NO: 7: *Trichoderma reesei* glucoamylase (TrGA) amino acid sequence (SEQ ID NO: 3 of WO 2006/060062). The pro-sequence is italicized.

1 *MHVLSTAVLL GSVAVQKVLG RPGSSGLSDV TKR*SVDDFIS TETPIALNNL
40 51 LCNVGPDGCR AFGTSAGAVI ASPSTIDPDY YYMWTRDSAL VFKNLIDRFT
100 ETYDAGLQRR IEQYITAQVT LQGLSNPSGS LADGSGLGEP KFELTLKPFT
151 GNWGRPQRDG PALRAIALIG YSKWLINNNY QSTVSNVIWP IVRNDLNYVA
201 QYWNQTGFDL WEEVNGSSFF TVANQHRALV EGATLAATLG QSGSAYSSVA
251 PQVLCFLQRF WVSSGGYVDS NINTNEGRTG KDVNSVLTSI HTFDPNLGCD
45 301 AGTFQPCSDK ALSNLKVVVD SFRSIYGVNK GIPAGAAVAI GRYAEDVYYN
351 GNPWYLATFA AAEQLYDAIY VWKKTGSITV TATSLAFFQE LVPGVTAGTY
401 SSSSSTFTNI INAVSTYADG FLSEAAKYVP ADGSLAEQFD RNSGTPLSAL
451 HLTWSYASFL TATARRAGIV PPSWANSSAS TIPSTCSGAS VVGSYSRPTA

501 TSFPPSQTPK PGVPSGTPYT PLPCATPTSV AVTFHELVST QFGQTVKVAG
551 NAAALGNWST SAAVALDAVN YADNHPLWIG TVNLEAGDVV EYKYINVGQD
601 GSVTWESDPN HTYTVPAVAC VTQVVKEDTW QS

SEQ ID NO: 8: SPEZYME® FRED α-amylase amino acid sequence.

5 1 ANLNGTLMQY FEWYTPNDGQ HWKRLQNDSA YLAEHGITAV WIPPAYKGTS
51 QADVGYGAYD LYDLGEFHQK GTVRTKYGTK GELQSAIKSL HSRDINVYGD
101 VVINHKGGAD ATEDVTAVEV DPADRNRVIS GEYLIKAWTH FHFPGRGSTY
151 SDFKWHWYHF DGTDWDESRK LNRIYKFQGK AWDWEVSSEN GNYDYLMYAD
201 IDYDHPDVVA EIKRWGTWYA NELQLDGFRL DAVKHIKFSF LRDWVNHVRE
10 251 KTGKEMFTVA EYWQNDLGAL ENYLNKTNFN HSVFDVPLHY QFHAASTQGG
301 GYDMRKLLNG TVVSKHPLKS VTFVDNHDTQ PGQSLESTVQ TWFKPLAYAF
351 ILTRESGYPQ VFYGDMYGTK GDSQREIPAL KHKIEPILKA RKQYAYGAQH
401 DYFDHHDIVG WTREGDSSVA NSGLAALITD GPGGAKRMYV GRQNAGETWH
451 DITGNRSEPV VINSEGWGEF HVNGGSVSIY VQR

15 **SEQ ID NO: 9:** Nucleotide sequence encoding the AmyE of SEQ ID NO: 1.

CTTACAGCACCGTCGATCAAAAGCGGAACCATTCTTCATGCATGGAATTGGTCGTTCAATACGT
TAAAACACAATATGAAGGATATTCATGATGCAGGATATACAGCCATTCAGACATCTCCGATTAA
CCAAGTAAAGGAAGGGAATCAAGGAGATAAAAGCATGTCGAACTGGTACTGGCTGTATCAGCCG
ACATCGTATCAAATTGGCAACCGTTACTTAGGTACTGAACAAGAATTTAAAGAAATGTGTGCAG
20 CCGCTGAAGAATATGGCATAAAGGTCATTGTTGACGCGGTCATCAATCATACCACCAGTGATTA
TGCCGCGATTTCCAATGAGGTTAAGAGTATTCCAAACTGGACACATGGAAACACACAAATTAAA
AACTGGTCTGATCGATGGGATGTCACGCAGAATTCATTGCTCGGGCTGTATGACTGGAATACAC
AAAATACACAAGTACAGTCCTATCTGAAACGGTTCTTAGACAGGGCATTGAATGACGGGGCAGA
CGGTTTTCGATTTGATGCCGCCAAACATATAGAGCTTCCAGATGATGGCAGTTACGGCAGTCAA
25 TTTTGGCCGAATATCACAAATACATCAGCAGAGTTCCAATACGGAGAAATCCTTCAGGATAGTG
CCTCCAGAGATGCTGCATATGCGAATTATATGGATGTGACAGCGTCTAACTATGGGCATTCCAT
AAGGTCCGCTTTAAAGAATCGTAATCTGGGCGTGTCGAATATCTCCCACTATGCATCTGATGTG
TCTGCGGACAAGCTAGTGACATGGGTAGAGTCGCATGATACGTATGCCAATGATGATGAAGAGT
CGACATGGATGAGCGATGATGATATCCGTTTAGGCTGGGCGGTGATAGCTTCTCGTTCAGGCAG
30 TACGCCTCTTTTCTTTTCCAGACCTGAGGGAGGCGGAAATGGTGTGAGGTTCCCGGGGAAAAGC
CAAATAGGCGATCGCGGGAGTGCTTTATTGAAGATCAGGCTATCACTGCGGTCAATAGATTTC
ACAATGTGATGGCTGGACAGCCTGAGGAACTCTCGAACCCGAATGGAAACAACCAGATATTTAT
GAATCAGCGCGGCTCACATGGCGTTGTGCTGGCAAATGCAGGTTCATCCTCTGTCTCTATCAAT
ACGGCAACAAAATTGCCTGATGGCAGGTATGACAATAAAGCTGGAGCGGGTTCATTTCAAGTGA
35 ACGATGGTAAACTGACAGGCACGATCAATGCCAGGTCTGTAGCTGTGCTTTATCCTGATGATAT
TGCAAAAGCGCCTCATGTTTTCCTTGAGAATTACAAAACAGGTGTAACACATTCTTTCAATGAT
CAACTGACGATTACCTTGCGTGCAGATGCGAATACAACAAAAGCCGTTTATCAAATCAATAATG
GACCAGAGACGGCGTTTAAGGATGGAGATCAATTCACAATCGGAAAAGGAGATCCATTTGGCAA
AACATACACCATCATGTTAAAAGGAACGAACAGTGATGGTGTAACGAGGACCGAGAAATACAGT
40 TTTGTTAAAAGAGATCCAGCGTCGGCCAAAACCATCGGCTATCAAAATCCGAATCATTGGAGCC
AGGTAAATGCTTATATCTATAAACATGATGGGAGCCGAGTAATTGAATTGACCGGATCTTGGCC
TGGAAAACCAATGACTAAAAATGCAGACGGAATTTACACGCTGACGCTGCCTGCGGACACGGAT
ACAACCAACGCAAAAGTGATTTTTAATAATGGCAGCGCCCAAGTGCCCGGTCAGAATCAGCCTG
GCTTTGATTACGTGCTAAATGGTTTATATAATGACTCGGGCTTAAGCGGTTCTCTTCCCCAT

45 **SEQ ID NO: 10:** Nucleotide sequence encoding AmyE-tr (SEQ ID NO: 2).

CTTACAGCACCGTCGATCAAAAGCGGAACCATTCTTCATGCATGGAATTGGTCGTTCAATACGT
TAAAACACAATATGAAGGATATTCATGATGCAGGATATACAGCCATTCAGACATCTCCGATTAA
CCAAGTAAAGGAAGGGAATCAAGGAGATAAAAGCATGTCGAACTGGTACTGGCTGTATCAGCCG
ACATCGTATCAAATTGGCAACCGTTACTTAGGTACTGAACAAGAATTTAAAGAAATGTGTGCAG
50 CCGCTGAAGAATATGGCATAAAGGTCATTGTTGACGCGGTCATCAATCATACCACCAGTGATTA
TGCCGCGATTTCCAATGAGGTTAAGAGTATTCCAAACTGGACACATGGAAACACACAAATTAAA

AACTGGTCTGATCGATGGGATGTCACGCAGAATTCATTGCTCGGGCTGTATGACTGGAATACAC
AAAATACACAAGTACAGTCCTATCTGAAACGGTTCTTAGACAGGGCATTGAATGACGGGGCAGA
CGGTTTTCGATTTGATGCCGCCAAACATATAGAGCTTCCAGATGATGGCAGTTACGGCAGTCAA
TTTTGGCCGAATATCACAAATACATCAGCAGAGTTCCAATACGGAGAAATCCTTCAGGATAGTG
5 CCTCCAGAGATGCTGCATATGCGAATTATATGGATGTGACAGCGTCTAACTATGGGCATTCCAT
AAGGTCCGCTTTAAAGAATCGTAATCTGGGCGTGTCGAATATCTCCCACTATGCATCTGATGTG
TCTGCGGACAAGCTAGTGACATGGGTAGAGTCGCATGATACGTATGCCAATGATGATGAAGAGT
CGACATGGATGAGCGATGATGATATCCGTTTAGGCTGGGCGGTGATAGCTTCTCGTTCAGGCAG
TACGCCTCTTTTCTTTTCCAGACCTGAGGGAGGCGGAAATGGTGTGAGGTTCCCGGGGAAAAGC
10 CAAATAGGCGATCGCGGGAGTGCTTTATTTGAAGATCAGGCTATCACTGCGGTCAATAGATTTC
ACAATGTGATGGCTGGACAGCCTGAGGAACTCTCGAACCCGAATGGAAACAACCAGATATTTAT
GAATCAGCGCGGCTCACATGGCGTTGTGCTGGCAAATGCAGGTTCATCCTCTGTCTCTATCAAT
ACGGCAACAAAATTGCCTGATGGCAGGTATGACAATAAAGCTGGAGCGGGTTCATTTCAAGTGA
ACGATGGTAAACTGACAGGCACGATCAATGCCAGGTCTGTAGCTGTGCTTTATCCTGAT

15 **SEQ ID NO: 11:** Nucleotide sequence encoding *B. subtilis* Amy31A (SEQ ID NO: 3).

TCTGTTAAAAACGGCACTATTCTGCATGCATGGAACTGGAGCTTTAACACGCTGACCCAGAACA
TGAAAGATATTCGTGACGCGGGCTATGCTGCGATCCAAACCAGCCCTATCAACCAGGTCAAAGA
AGGCAACCAAGGCGACAAATCCATGTCCAACTGGTACTGGCTGTATCAACCGACGTCCTATCAG
ATTGGCAACCGTTATCTGGGCACGGAGCAAGAGTTCAAAGACATGTGTGCTGCGGCTGAGAAAT
20 ATGGTGTGAAAGTTATCGTGGACGCTGTGGTAAACCACACGACCTCTGATTATGGTGCTATTAG
CGACGAGATTAAACGTATTCCAAATTGGACCCATGGTAATACCCAGATCAAAAATTGGAGCGAC
CGCTGGGACATTACCCAGAATGCGCTGCTGGGTCTGTATGACTGGAACACGCAAAACACCGAAG
TACAGGCATATCTGAAGGGCTTCCTGGAACGCGCTCTGAACGATGGTGCTGATGGTTTTCGCTA
CGACGCCGCAAAGCATATTGAGCTGCCGGATGACGGCAACTACGGTTCCCAATTCTGGCCGAAC
25 ATCACCAACACCTCTGCCGAATTCCAGTACGGCGAGATCCTGCAAGACTCCGCGAGCCGTGACA
CCGCTTATGCCAACTATATGAACGTAACTGCCTCTAACTATGGCCATTCCATTCGTTCTGCGCT
GAAAAATCGTATCCTGTCCGTGTCCAATATCTCCCACTATGCATCCGACGTTTCTGCTGACAAA
CTGGTAACTTGGGTCGAGTCTCACGACACCTATGCAAATGATGACGAGGAGAGCACCTGGATGA
GCGATGATGATATTCGTCTGGGTTGGGCGGTTATTGGTTCTCGCTCTGGTTCTACTCCGCTGTT
30 CTTTAGCCGTCCGGAAGGTGGCGGCAATGGCGTTCGTTTCCCGGGTAAATCTCAAATTGGTGAT
CGTGGCTCTGCACTGTTTAAAGATCAAGCTATTACGGCGGTGAATCAGTTCCATAATGAGATGG
CAGGTCAACCTGAAGAACTGTCCAATCCAAACGGTAACAACCAAATCTTCATGAACCAGCGTGG
CAGCAAAGGCGTCGTCCTGGCGAACGCCGGTAGCTCTTCTGTTACCATCAACACGTCTACCAAA
CTGCCAGACGGCCGCTATGATAACCGTGCGGGTGCTGGTTCCTTTCAGGTAGCCAACGGCAAGC
35 TGACGGGCACCATCAACGCTCGTTCTGCTGCTGTTCTGTACCCGGACGACATTGGCAACGCTCC
GCACGTGTTCCTGGAGAATTACCAGACCGAAGCGGTACATAGCTTTAATGACCAGCTGACCGTC
ACTCTGCGTGCCAACGCAAAAACCACGAAAGCAGTCTATCAGATCAATAATGGTCAAGAAACTG
CTTTCAAGGATGGCGACCGTCTGACTATTGGTAAGGAGGACCCGATTGGCACCACTTATAACGT
TAAACTGACTGGCACCAATGGCGAGGGCGCTAGCCGCACTCAAGAGTATACGTTCGTAAAGAAA
40 GACCCGTCTCAAACCAACATCATCGGTTACCAGAATCCTGACCACTGGGGTAATGTGAACGCTT
ACATCTATAAACATGATGGTGGCGGTGCTATCGAACTGACCGGCTCTTGGCCAGGTAAAGCCAT
GACGAAAAACGCGGATGGCATCTATACCCTGACCCTGCCGGCCAATGCGGATACCGCAGATGCG
AAGGTTATCTTCAATAACGGCTCCGCGCAGGTTCCGGGCCAAAACCATCCGGGCTTTGACTACG
TACAAAATGGTCTGTATAACAACTCTGGCCTGAACGGTTACCTGCCGCAC

45 **SEQ ID NO: 12:** Nucleotide sequence encoding *Geobacillus stearothermophilus* AmyS (SEQ ID NO: 4).

GCCGCACCGTTTAACGGTACCATGATGCAGTATTTTGAATGGTACTTGCCGGATGATGGCACGT
TATGGACCAAAGTGGCCAATGAAGCCAACAACTTATCCAGCCTTGGCATCACCGCTCTTTGGCT
GCCGCCCGCTTACAAAGGAACAAGCCGCAGCGACGTAGGGTACGGAGTATACGACTTGTATGAC
50 CTCGGCGAATTCAATCAAAAAGGGACCGTCCGCACAAAATATGGAACAAAAGCTCAATATCTTC
AAGCCATTCAAGCCGCCCACGCCGCTGGAATGCAAGTGTACGCCGATGTCGTGTTCGACCATAA
AGGCGGCGCTGACGGCACGGAATGGGTGGACGCCGTCGAAGTCAATCCGTCCGACCGCAACCAA

GAAATCTCGGGCACCTATCAAATCCAAGCATGGACGAAATTTGATTTTCCCGGGCGGGGCAACA
CCTACTCCAGCTTTAAGTGGCGCTGGTACCATTTTGACGGCGTTGACTGGGACGAAAGCCGAAA
ATTAAGCCGCATTTACAAATTCATCGGCAAAGCGTGGGATTGGGAAGTAGACACAGAAAACGGA
AACTATGACTACTTAATGTATGCCGACCTTGATATGGATCATCCCGAAGTCGTGACCGAGCTGA
AAAACTGGGGGAAATGGTATGTCAACACAACGAACATTGATGGGTTCCGGCTTGATGCCGTCAA
GCATATTAAGTTCAGTTTTTTTCCTGATTGGTTGTCGTATGTGCGTTCTCAGACTGGCAAGCCG
CTATTTACCGTCGGGGAATATTGGAGCTATGACATCAACAAGTTGCACAATTACATTACGAAAA
CAAACGGAACGATGTCTTTGTTTGATGCCCCGTTACACAACAAATTTTATACCGCTTCCAAATC
AGGGGGCGCATTTGATATGCGCACGTTAATGACCAATACTCTCATGAAAGATCAACCGACATTG
GCCGTCACCTTCGTTGATAATCATGACACCGAACCCGGCCAAGCGCTGCAGTCATGGGTCGACC
CATGGTTCAAACCGTTGGCTTACGCCTTTATTCTAACTCGGCAGGAAGGATACCCGTGCGTCTT
TTATGGTGACTATTATGGCATTCCACAATATAACATTCCTTCGCTGAAAAGCAAAATCGATCCG
CTCCTCATCGCGCGCAGGGATTATGCTTACGGAACGCAACATGATTATCTTGATCACTCCGACA
TCATCGGGTGGACAAGGGAAGGGGTCACTGAAAAACCAGGATCCGGGCTGGCCGCACTGATCAC
CGATGGGCCGGGAGGAAGCAAATGGATGTACGTTGGCAAACAACACGCTGGAAAAGTGTTCTAT
GACCTTACCGGCAACCGGAGTGACACCGTCACCATCAACAGTGATGGATGGGGGGAATTCAAAG
TCAATGGCGGTTCGGTTTCGGTTTGGGTTCCTAGAAAAACGACC

SEQ ID NO: 13: Nucleotide sequence for Spezyme® Xtra amylase gene (SEQ ID NO: 5).

GCCGCACCGTTTAACGGTACCATGATGCAGTATTTTGAATGGTACTTGCCGGATGATGGCACGT
TATGGACCAAAGTGGCCAATGAAGCCAACAACTTATCCAGCCTTGGCATCACCGCTCTTTGGCT
GCCGCCCGCTTACAAAGGAACAAGCCGCAGCGACGTAGGGTACGGAGTATACGACTTGTATGAC
CTCGGCGAATTCAATCAAAAAGGGACCGTCCGCACAAAATATGGAACAAAAGCTCAATATCTTC
AAGCCATTCAAGCCGCCCACGCCGCTGGAATGCAAGTGTACGCCGATGTCGTGTTCGACCATAA
AGGCGGCGCTGACGGCACGGAATGGGTGGACGCCGTCGAAGTCAATCCGTCCGACCGCAACCAA
GAAATCTCGGGCACCTATCAAATCCAAGCATGGACGAAATTTGATTTTCCCGGGCGGGGCAACA
CCTACTCCAGCTTTAAGTGGCGCTGGTACCATTTTGACGGCGTTGATTGGGACGAAAGCCGAAA
ATTAAGCCGCATTTACAAATTCAGGGGCATCGGCAAAGCGTGGGATTGGGAAGTAGACACAGAA
AACGGAAACTATGACTACTTAATGTATGCCGACCTTGATATGGATCATCCCGAAGTCGTGACCG
AGCTGAAAAACTGGGGGAAATGGTATGTCAACACAACGAACATTGATGGGTTCCGGCTTGATGC
CGTCAAGCATATTAAGTTCAGTTTTTTTCCTGATTGGTTGTCGTATGTGCGTTCTCAGACTGGC
AAGCCGCTATTTACCGTCGGGGAATATTGGAGCTATGACATCAACAAGTTGCACAATTACATTA
CGAAAACAAACGGAACGATGTCTTTGTTTGATGCCCCGTTACACAACAAATTTTATACCGCTTC
CAAATCAGGGGGCGCATTTGATATGCGCACGTTAATGACCAATACTCTCATGAAAGATCAACCG
ACATTGGCCGTCACCTTCGTTGATAATCATGACACCGAACCCGGCCAAGCGCTTCAGTCATGGG
TCGACCCATGGTTCAAACCGTTGGCTTACGCCTTTATTCTAACTCGGCAGGAAGGATACCCGTG
CGTCTTTTATGGTGACTATTATGGCATTCCACAATATAACATTCCTTCGCTGAAAAGCAAAATC
GATCCGCTCCTCATCGCGCGCAGGGATTATGCTTACGGAACGCAACATGATTATCTTGATCACT
CCGACATCATCGGGTGGACAAGGGAAGGGGTCACTGAAAAACCAGGATCCGGGCTGGCCGCACT
GATCACCGATGGGCCGGGAGGAAGCAAATGGATGTACGTTGGCAAACAACACGCTGGAAAAGTG
TTCTATGACCTTACCGGCAACCGGAGTGACACCGTCACCATCAACAGTGATGGATGGGGGGAAT
TCAAAGTCAATGGCGGTTCGGTTTCGGTTTGGGTTCCTAGAAAAACGACC

SEQ ID NO: 14: Nucleotide sequence for *Aspergillus kawachii* alpha amylase (AkAA) gene (SEQ ID NO: 6).

ATGAGAGTGTCGACTTCAAGTATTGCCCTTGCTGTGTCCCTTTTTGGGAAGCTGGCCCTTGGGC
TGTCAGCTGCAGAATGGCGCACTCAATCCATCTACTTCCTTTTGACGGATCGGTTCGGTAGGAC
GGACAATTCGACTACAGCTACGTGCAATACGGGTGACCAAATCTACTGTGGTGGAAGTTGGCAA
GGAATTATCAACCATCTGGACTATATCCAGGGCATGGGATTCACAGCTATCTGGATCTCGCCTA
TCACTGAGCAGCTACCCCAGGATACTTCGGATGGTGAAGCCTACCATGGATACTGGCAGCAGAA
GATATACAATGTGAACTCCAACTTCGGCACGGCAGATGATCTGAAGTCCCTCTCCGATGCTCTT

CACGCCCGCGGAATGTACCTCATGGTCGACGTCGTCCCTAACCACATGGGCTACGCAGGTAACG
GCAACGATGTGGATTACAGCGTCTTCGACCCCTTCGACTCCTCCTCCTACTTCCATCCATACTG
CCTCATCACAGATTGGGACAACTTGACCATGGTCCAAGACTGTTGGGAGGGTGACACCATCGTG
TCTCTGCCAGATCTGAACACCACGGAAACCGCCGTGAGAACCATTTGGTACGATTGGGTAGCCG
5 ACCTGGTATCCAACTACTCAGTCGACGGCCTCCGTATCGACAGTGTCGAAGAAGTCGAACCCGA
CTTCTTCCCGGGCTACCAAGAAGCAGCAGGAGTCTACTGCGTCGGTGAAGTCGACAACGGCAAC
CCTGCTCTCGACTGCCCATACCAAAAATATCTAGATGGTGTTCTCAACTATCCCATCTACTGGC
AACTCCTCTACGCCTTTGAATCCTCCAGCGGCAGCATCAGCAACCTCTACAACATGATCAAATC
CGTCGCCAGCGACTGCTCCGATCCGACCCTCCTGGGCAACTTTATCGAAAACCACGACAACCCC
10 CGCTTCGCCTCCTACACATCCGACTACTCCCAAGCCAAAAACGTCCTCAGCTACATCTTCCTCT
CCGACGGCATCCCCATCGTCTACGCCGGCGAAGAACAGCACTACTCCGGCGGCGACGTGCCCTA
CAACCGCGAAGCTACCTGGCTATCAGGCTACGACACCTCCGCGGAGCTCTACACCTGGATAGCC
ACCACAAACGCGATCCGGAAACTAGCTATCTCAGCAGACTCGGACTACATTACTTACGCGAACG
ACCCAATCTACACAGACAGCAACACCATCGCGATGCGCAAAGGCACCTCCGGCTCCCAAATCAT
15 CACCGTCCTCTCCAACAAAGGCTCCTCCGGAAGCAGCTACACCCTCACCCTCAGCGGAAGCGGC
TACACGTCCGGCACGAAGCTCATCGAAGCGTACACCTGCACGTCCGTGACGGTGGACTCGAACG
GGGATATCCCTGTGCCGATGGCTTCGGGATTACCTAGAGTTCTCCTCCCTGCTTCGGTGGTTGA
TAGTTCTTCGCTTTGTGGGGGGAGTGGTAACACAACCACGACCACAACTGCTGCTACCTCCACA
TCCAAAGCCACCACCTCCTCTTCTTCTTCTTCTGCTGCTGCTACTACTTCTTCATCATGCACCG
20 CAACAAGCACCACCCTCCCCATCACCTTCGAAGAACTCGTCACCACTACCTACGGGGAAGAAGT
CTACCTCAGCGGATCTATCTCCCAGCTCGGAGAGTGGGATACGAGTGACGCGGTGAAGTTGTCC
GCGGATGATTATACCTCGAGTAACCCCGAGTGGTCTGTTACTGTGTCGTTGCCGGTGGGGACGA
CCTTCGAGTATAAGTTTATTAAGGTCGATGAGGGTGGAAGTGTGACTTGGGAAAGTGATCCGAA
TAGGGAGTATACTGTGCCTGAATGTGGGAGTGGGAGTGGGGAGACGGTGGTTGATACGTGGAGG
25 TAG

SEQ ID NO: 15: Nucleotide sequence for *Trichoderma reesei* glucoamylase gene (SEQ ID NO: 7).

```
   1 ATGCACGTCC TGTCGACTGC GGTGCTGCTC GGCTCCGTTG CCGTTCAAAA GGTCCTGGGA
  61 AGACCAGGAT CAAGCGGTCT GTCCGACGTC ACCAAGAGGT CTGTTGACGA CTTCATCAGC
 121 ACCGAGACGC CTATTGCACT GAACAATCTT CTTTGCAATG TTGGTCCTGA TGGATGCCGT
 181 GCATTCGGCA CATCAGCTGG TGCGGTGATT GCATCTCCCA GCACAATTGA CCCGGACTAC
 241 TATTACATGT GGACGCGAGA TAGCGCTCTT GTCTTCAAGA ACCTCATCGA CCGCTTCACC
 301 GAAACGTACG ATGCGGGCCT GCAGCGCCGC ATCGAGCAGT ACATTACTGC CCAGGTCACT
 361 CTCCAGGGCC TCTCTAACCC CTCGGGCTCC CTCGCGGACG GCTCTGGTCT CGGCGAGCCC
 421 AAGTTTGAGT TGACCCTGAA GCCTTTCACC GGCAACTGGG GTCGACCGCA GCGGGATGGC
 481 CCAGCTCTGC GAGCCATTGC CTTGATTGGA TACTCAAAGT GGCTCATCAA CAACAACTAT
 541 CAGTCGACTG TGTCCAACGT CATCTGGCCT ATTGTGCGCA ACGACCTCAA CTATGTTGCC
 601 CAGTACTGGA ACCAAACCGG CTTTGACCTC TGGGAAGAAG TCAATGGGAG CTCATTCTTT
 661 ACTGTTGCCA ACCAGCACCG AGCACTTGTC GAGGGCGCCA CTCTTGCTGC CACTCTTGGC
 721 CAGTCGGGAA GCGCTTATTC ATCTGTTGCT CCCCAGGTTT TGTGCTTTCT CCAACGATTC
 781 TGGGTGTCGT CTGGTGGATA CGTCGACTCC AACATCAACA CCAACGAGGG CAGGACTGGC
 841 AAGGATGTCA ACTCCGTCCT GACTTCCATC CACACCTTCG ATCCCAACCT TGGCTGTGAC
 901 GCAGGCACCT TCCAGCCATG CAGTGACAAA GCGCTCTCCA ACCTCAAGGT TGTTGTCGAC
 961 TCCTTCCGCT CCATCTACGG CGTGAACAAG GGCATTCCTG CCGGTGCTGC CGTCGCCATT
1021 GGCCGGTATG CAGAGGATGT GTACTACAAC GGCAACCCTT GGTATCTTGC TACATTTGCT
1081 GCTGCCGAGC AGCTGTACGA TGCCATCTAC GTCTGGAAGA AGACGGGCTC CATCACGGTG
1141 ACCGCCACCT CCCTGGCCTT CTTCCAGGAG CTTGTTCCTG GCGTGACGGC CGGGACCTAC
1201 TCCAGCAGCT CTTCGACCTT TACCAACATC ATCAACGCCG TCTCGACATA CGCCGATGGC
1261 TTCCTCAGCG AGGCTGCCAA GTACGTCCCC GCCGACGGTT CGCTGGCCGA GCAGTTTGAC
1321 CGCAACAGCG GCACTCCGCT GTCTGCGCTT CACCTGACGT GGTCGTACGC CTCGTTCTTG
1381 ACAGCCACGG CCCGTCGGGC TGGCATCGTG CCCCCCTCGT GGGCCAACAG CAGCGCTAGC
1441 ACGATCCCCT CGACGTGCTC CGGCGCGTCC GTGGTCGGAT CCTACTCGCG TCCCACCGCC
1501 ACGTCATTCC CTCCGTCGCA GACGCCCAAG CCTGGCGTGC CTTCCGGTAC TCCCTACACG
```

1561 CCCCTGCCCT GCGCGACCCC AACCTCCGTG GCCGTCACCT TCCACGAGCT CGTGTCGACA
1621 CAGTTTGGCC AGACGGTCAA GGTGGCGGGC AACGCCGCGG CCCTGGGCAA CTGGAGCACG
1681 AGCGCCGCCG TGGCTCTGGA CGCCGTCAAC TATGCCGATA ACCACCCCCT GTGGATTGGG
1741 ACGGTCAACC TCGAGGCTGG AGACGTCGTG GAGTACAAGT ACATCAATGT GGGCCAAGAT
5 1801 GGCTCCGTGA CCTGGGAGAG TGATCCCAAC CACACTTACA CGGTTCCTGC GGTGGCTTGT
1861 GTGACGCAGG TTGTCAAGGA GGACACCTGG CAGTCGTAA

SEQ ID NO: 16: Nucleotide sequence for AmyL gene (SEQ ID NO: 8).

ACAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACACGCCCAATGACGGCCAACATT
10 GGAAGCGTCTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCC
CCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTA
GGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTG
CGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGG
CGGCGCTGATGCGACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTA
15 ATTTCCGGAGAATACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACAT
ACAGCGATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCT
GAACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAGTGAAAACGGCAAC
TATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGATGTCGTAGCAGAAATTAAGA
GATGGGGCACTTGGTATGCCAATGAGCTCCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACA
20 CATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATG
TTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAA
ATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAGGG
AGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTGAAATCG
GTTACATTTGTCGATAACCATGATACACAGCCGGGGCAGTCGCTTGAGTCGACTGTCCAAACAT
25 GGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATACCCTCAGGTTTTCTA
CGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAATT
GAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACC
ATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATT
AATAACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGTGAGACA
30 TGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGT
TTCACGTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGA

SEQ ID NO: 17: Native signal sequence of the AmyE of SEQ ID NO: 1.

MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASAETANKSNE

SEQ ID NO: 18: Primer PSTAMYE-F '

35 CTTCTTGCTGCCTCATTCTGCAGCTTCAGCACTTACAGCACCGTCGATCAAAAGCGG
AAC '

SEQ ID NO: 19: Primer AMYENOPST-R '

CTGGAGGCACTATCCTGAAGGATTTCTCCGTATTGGAACTCTGCTGATGTATTTGTG '

SEQ ID NO: 20: Primer AMYENOPST-F '

40 CACAAATACATCAGCAGAGTTCCAATACGGAGAAATCCTTCAGGATAGTGCCTCCAG '

SEQ ID NO: 21: Primer HPAIAMYE-R '

CAGGAAATCCGTCCTCTGTTAACTCAATGGGGAAGAGAACCGCTTAAGCCCGAGTC '

SEQ ID NO: 22: Primer HPAIAMYE-R '

CAGGAAATCCGTCCTCTGTTAACTCAATCAGGATAAAGCACAGCTACAGACCTGG '

5 **SEQ ID NO: 23:** Primer AMYE SEQ-F '

TACACAAGTACAGTCCTATCTG '

SEQ ID NO: 24: Primer AMYE SEQ-F '

CATCCTCTGTCTCTATCAATAC

What Is Claimed Is:

1. A method of using a Bacillus subtilis α-amylase (AmyE) to produce glucose from an oligosaccharide or starch substrate solution, comprising:

(i) contacting AmyE with the oligosaccharide or polysaccharide substrate; and

5 (ii) converting the substrate solution to a solution comprising glucose, wherein the AmyE comprises an amino acid sequence with at least about 85% sequence identity to the AmyE of SEQ ID NO: 1.

2. The method of claim 1, wherein the oligosaccharide solution comprises predominantly maltoheptaose (DP7) or higher oligosaccharides.

10 3. The method of claim 1, wherein the starch solution is uncooked corn starch.

4. The method of claim 1, wherein the pH of the substrate solution during said converting is about pH 5.6 to about pH 5.8.

5. The method of claim 1, where said converting does not comprise contacting the substrate solution with a glucoamylase.

15 6. The method of claim 1, wherein step (i) further comprises contacting the starch substrate with a glucoamylase.

7. The method of claim 6, wherein the glucoamylase is added to a concentration of less than about 0.5 GAU/g ds.

8. The method of claim 7, wherein the glucoamylase is added to a concentration of less
20 than about 0.02 GAU/g ds.

9. The method of claim 1, wherein the solution comprising glucose contains at least about 0.2 g/L glucose.

10. The method of claim 9, wherein the solution comprising glucose contains at least about 0.4 g/L glucose.

25 11. The method of claim 10, wherein the solution comprising glucose contains at least about 1.4 g/L glucose.

12. The method of claim 1, wherein the AmyE is selected from the group consisting of the

AmyE comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, NCBI Accession No. ABW75769, NCBI Accession No. ABK54355, NCBI Accession No. AAF14358, NCBI Accession No. AAT01440, NCBI Accession No. AAZ30064, NCBI Accession No. NP_388186, NCBI Accession No. AAQ83841, and NCBI Accession No.
5 BAA31528.

13. The method of claim 1, further comprising (iii) fermenting the solution comprising glucose to produce ethanol.

14. The method of claim 13, wherein the ethanol concentration is at least about 6% v/v ethanol.

10 15. The method of claim 14, wherein the ethanol concentration is at least about 14 % v/v ethanol.

16. The method of claim 1, further comprising (iii) contacting the solution comprising glucose with a glucose isomerase to produce high fructose corn syrup.

17. A method of desizing textiles comprising (i) contacting a textile with AmyE and (ii)
15 desizing the textile, wherein the AmyE comprises an amino acid sequence with at least about 85% sequence identity to the AmyE of SEQ ID NO: 1.

18. A method of washing an item, comprising

(i) contacting an item to be washed with a detergent composition comprising AmyE and

(ii) washing the item, wherein the AmyE comprises an amino acid sequence with at least
20 about 85% sequence identity to the AmyE of SEQ ID NO: 1.

19. The method of claim 18, wherein the item to be washed is dishware or clothing.

20. The method of claim 18, wherein the detergent composition is a non-dusting granulate or a stabilized liquid.

21. The method of claim 18, wherein the detergent composition further comprises a
25 cellulase, a protease, an amylase, or a combination thereof.

22. The method of claim 21, wherein the amylase is an α-amylase, a β-amylase, or a glucoamylase.

23. The method of claim 18, wherein the detergent composition further comprises a lipase, a peroxidase, a mannanase, a pectate lyase, or a combination thereof.

24. The method of claim 18, wherein the detergent composition is a manual or automatic dishwashing detergent composition.

25. The method of claim 24, wherein the detergent composition further comprises a protease, a lipase, a peroxidase, an amylase, a cellulase, a mannanase, a pectate lyase, or a combination
5 thereof.

```
AmyE_FL    1 LTAPSIKSGTILHAWNWSFNTLKHNMKDIHDAGYTAIQTSPINQVKEGNQGDKSMSNWYW
Amy31A    42 VTASSVKNGTILHAWNWSFNTLTQNMKDIRDAGYAAIQTSPINQVKEGNQGDKSMSNWYW

AmyE_FL   61 LYQPTSYQIGNRYLGTEQEFKEMCAAAEEYGIKVIVDAVINHTTSDYAAISNEVKSIPNW
Amy31A   102 LYQPTSYQIGNRYLGTEQEFKDMCAAAEKYGVKVIVDAVVNHTTSDYGAISDEIKRIPNW

AmyE_FL  121 THGNTQIKNWSDRWDVTQNSLLGLYDWNTQNTQVQSYLKRFLDRALNDGADGFRFDAAKH
Amy31A   162 THGNTQIKNWSDRWDITQNALLGLYDWNTQNTEVQAYLKGFLERALNDGADGFRYDAAKH

AmyE_FL  181 IELPDDGSYGSQFWPNITNTSAEFQYGEILQDSASRDAAYANYMDVTASNYGHSIRSALK
Amy31A   222 IELPDDGNYGSQFWPNITNTSAEFQYGEILQDSASRDTAYANYMNVTASNYGHSIRSALK

AmyE_FL  241 NRNLGVSNISHYASDVSADKLVTWVESHDTYANDDEESTWMSDDDIRLGWAVIASRSGST
Amy31A   282 NRILSVSNISHYASDVSADKLVTWVESHDTYANDDEESTWMSDDDIRLGWAVIGSRSGST

AmyE_FL  301 PLFFSRPEGGGNGVRFPGKSQIGDRGSALFEDQAITAVNRFHNVMAGQPEELSNPNGNNQ
Amy31A   342 PLFFSRPEGGGNGVRFPGKSQIGDRGSALFKDQAITAVNQFHNEMAGQPEELSNPNGNNQ

AmyE_FL  361 IFMNQRGSHGVVLANAGSSSVSINTATKLPDGRYDNKAGAGSFQVNDGKLTGTINARSVA
Amy31A   402 IFMNQRGSKGVVLANAGSSSVTINTSTKLPDGRYDNRAGAGSFQVANGKLTGTINARSAA

AmyE_FL  421 VLYPDDIAKAPHVFLENYKTGVTHSFNDQLTITLRADANTTKAVYQINNGPETAFKDGDQ
Amy31A   462 VLYPDDIGNAPHVFLENYQTEAVHSFNDQLTVTLRANAKTTKAVYQINNGQETAFKDGDR

AmyE_FL  481 FTIGKGDPFGKTYTIMLKGTNSDGVTRTEKYSFVKRDPASAKTIGYQNPNHWSQVNAYIY
Amy31A   522 LTIGKEDPIGTTYNVKLTGTNGEGASRTQEYTFVKKDPSQTNIIGYQNPDHWGNVNAYIY

AmyE_FL  541 KHDGSRVIELTGSWPGKPMTKNADGIYTLTLPADTDTTNAKVIFNNGSAQVPGQNQPGFD
Amy31A   582 KHDGGGAIELTGSWPGKAMTKNADGIYTLTLPANADTADAKVIFNNGSAQVPGQNHPGFD

AmyE_FL  601 YVLNGLYNDSGLSGSLPH
Amy31A   642 YVQNGLYNNSGLNGYLPH
```

AmyE_FL 601 (SEQ ID NO: 1)
Amy31A 642 (SEQ ID NO: 25; note that this is equivalent to SEQ ID NO: 3 without the signal sequence)

FIG. 1

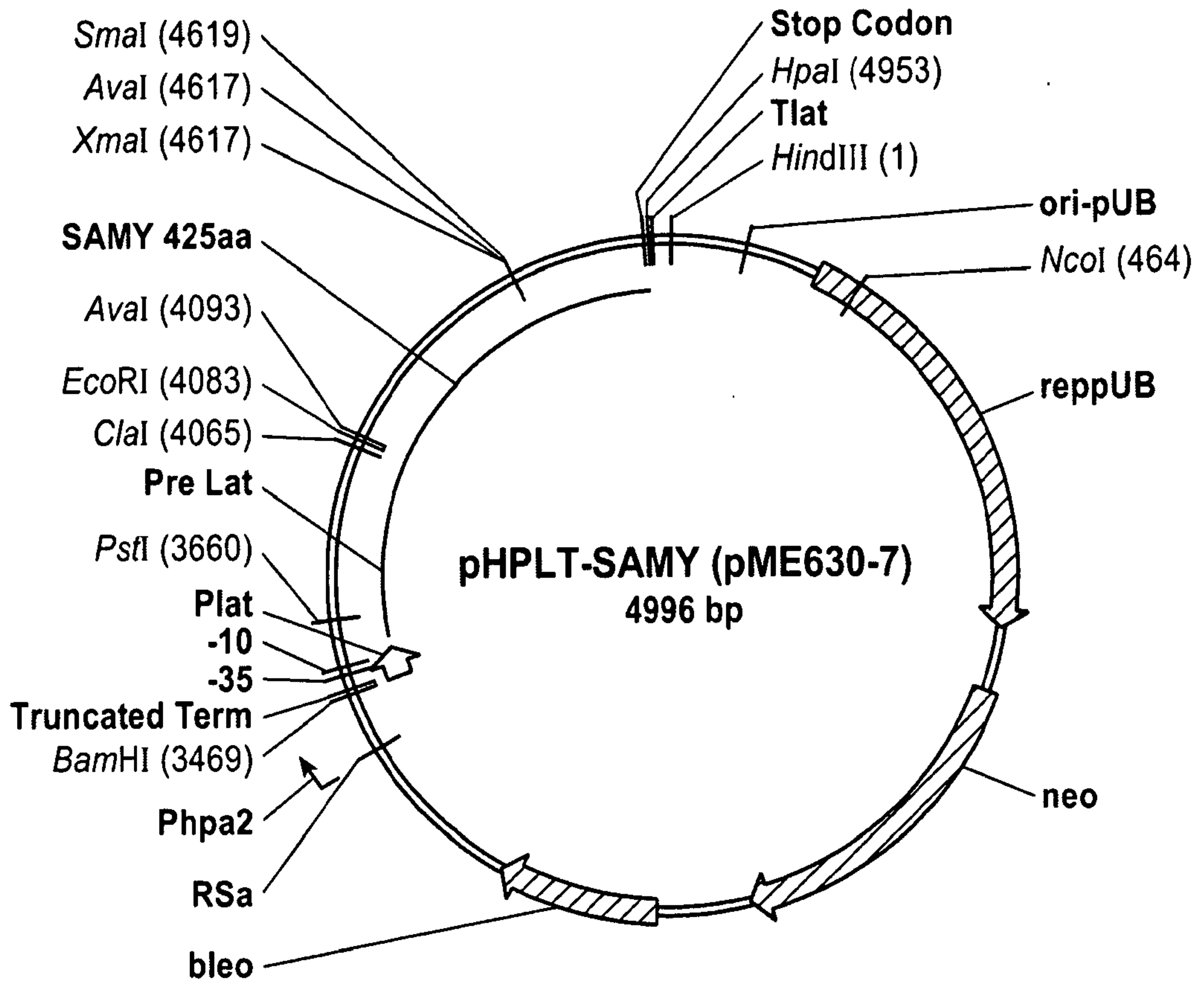


FIG. 2

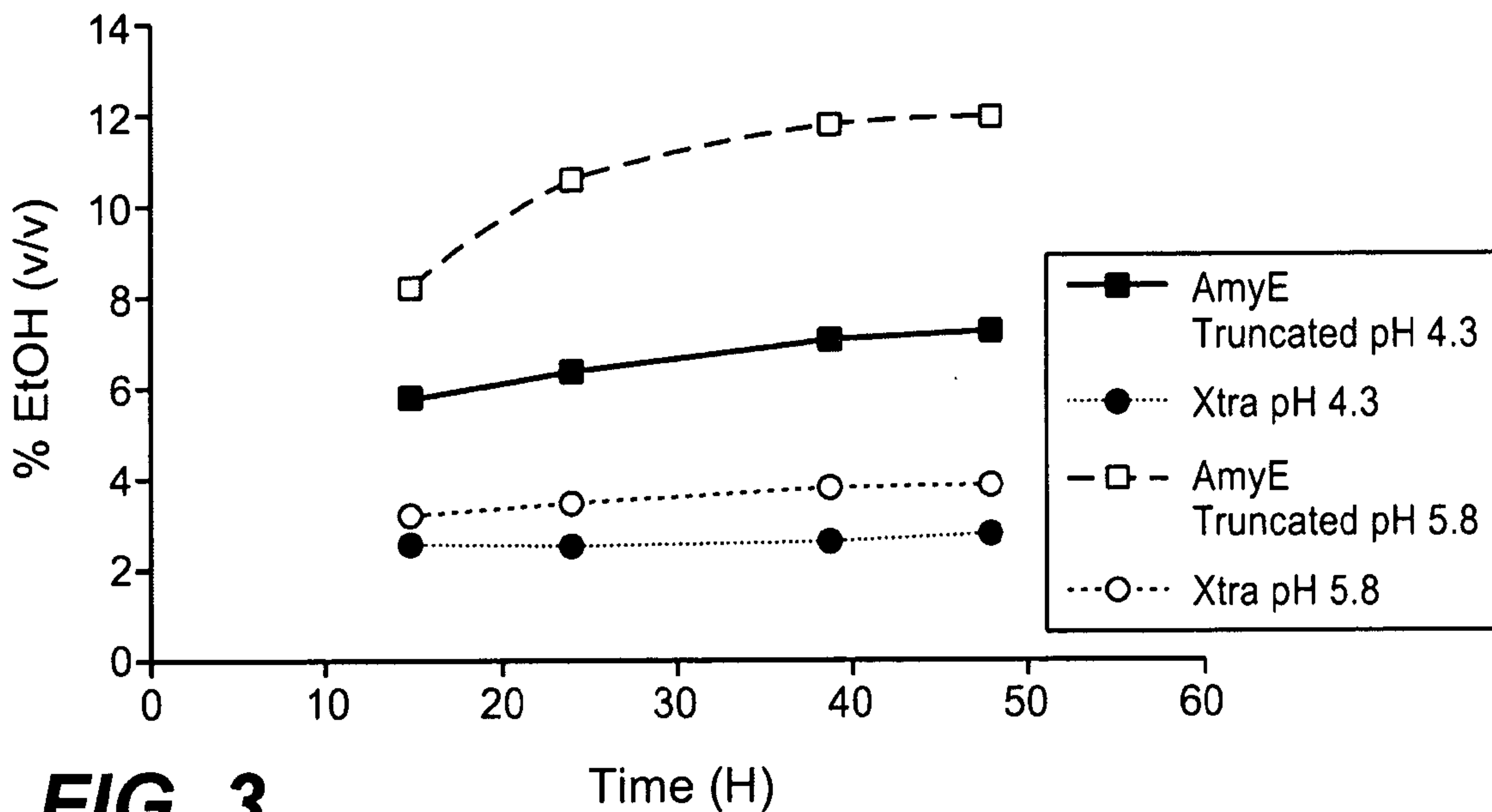


FIG. 3

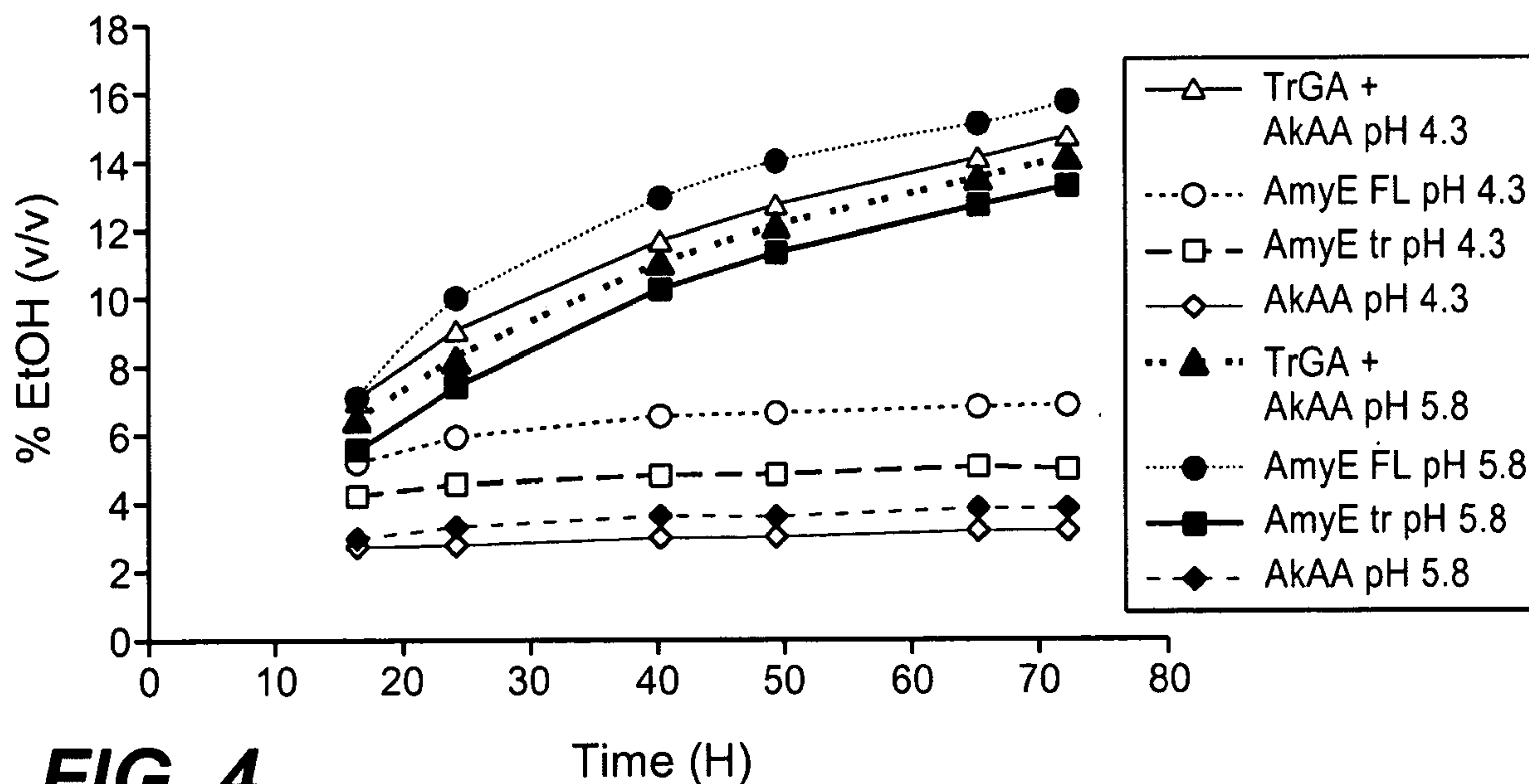


FIG. 4

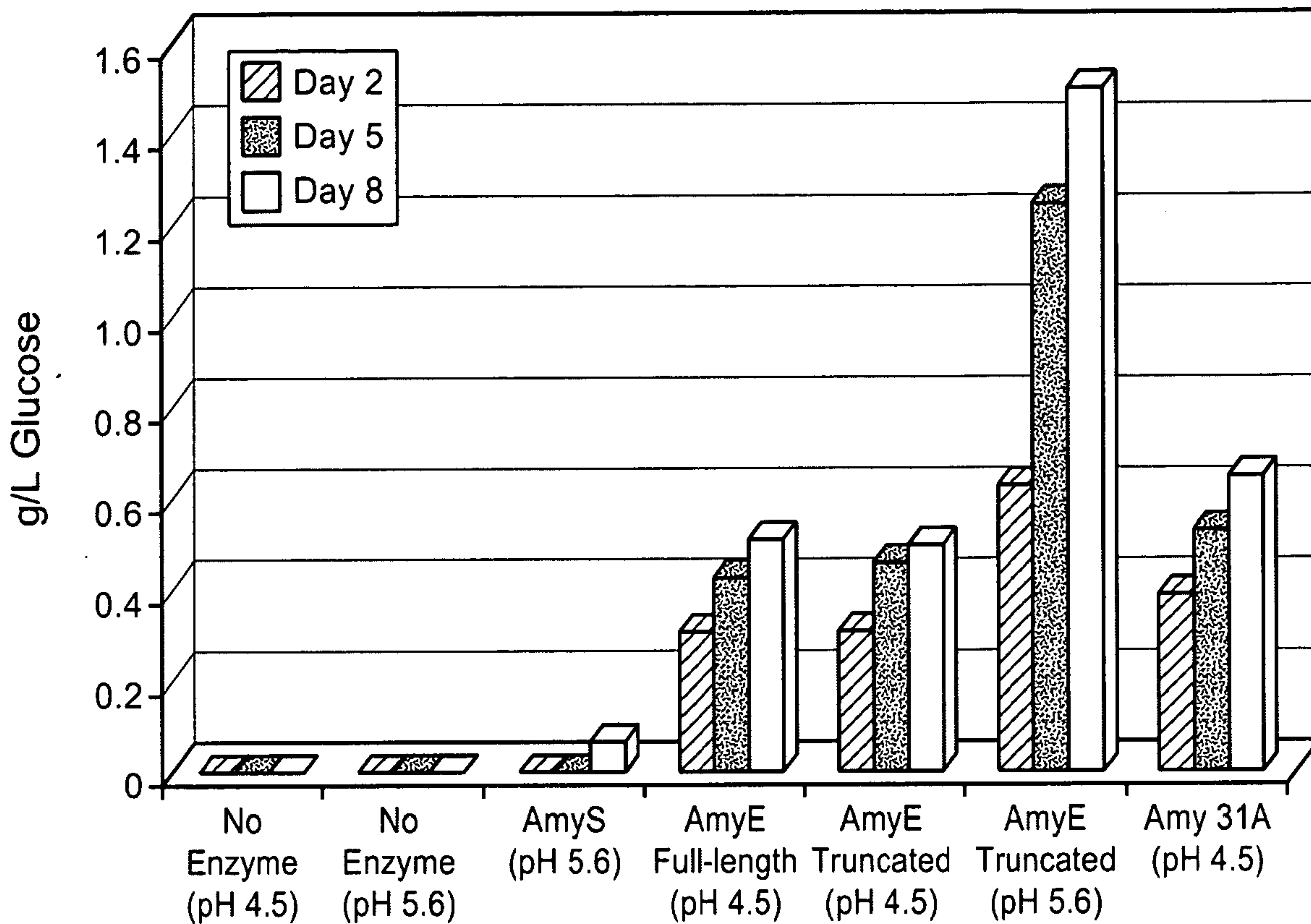


FIG. 5

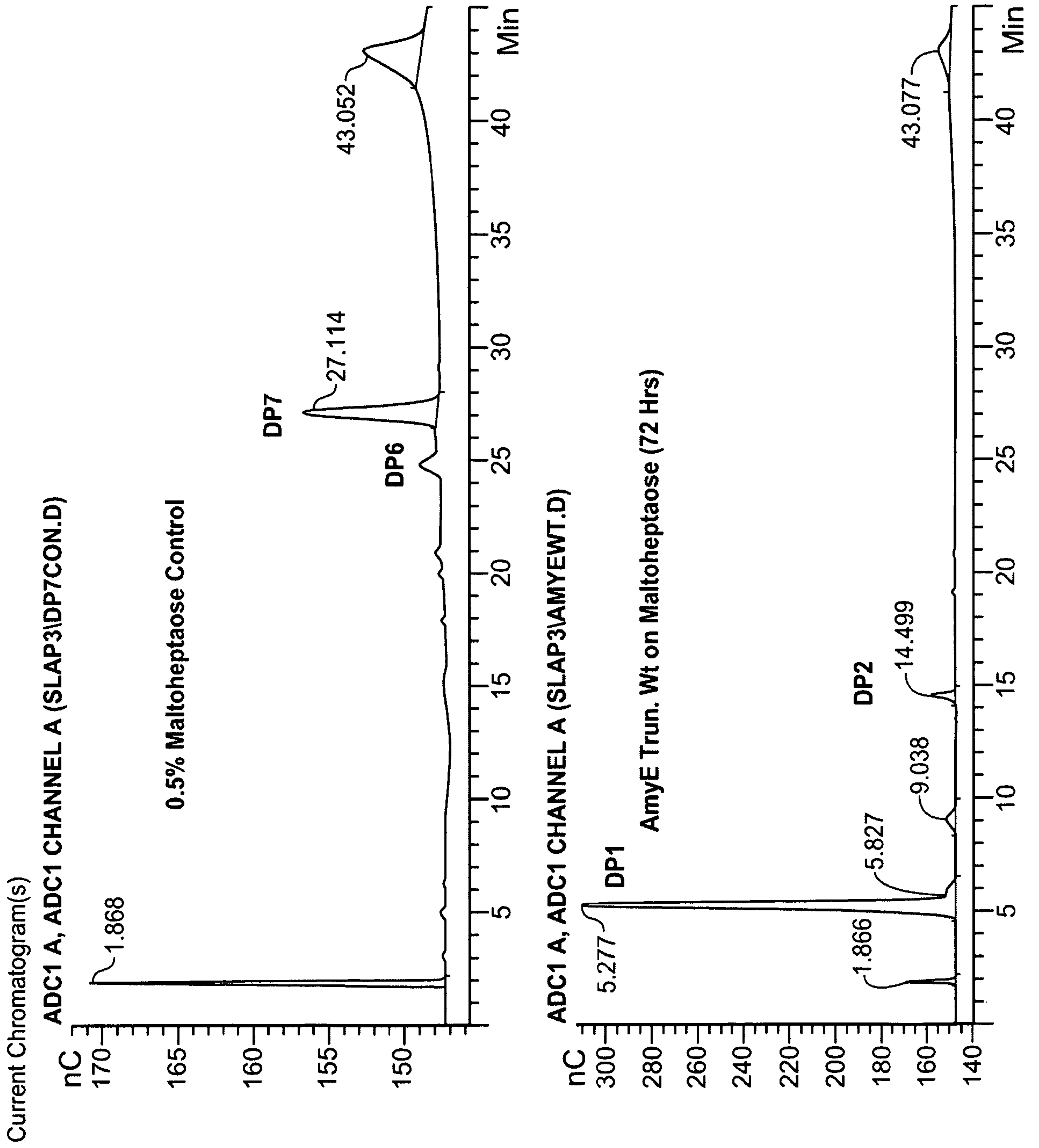


FIG. 6

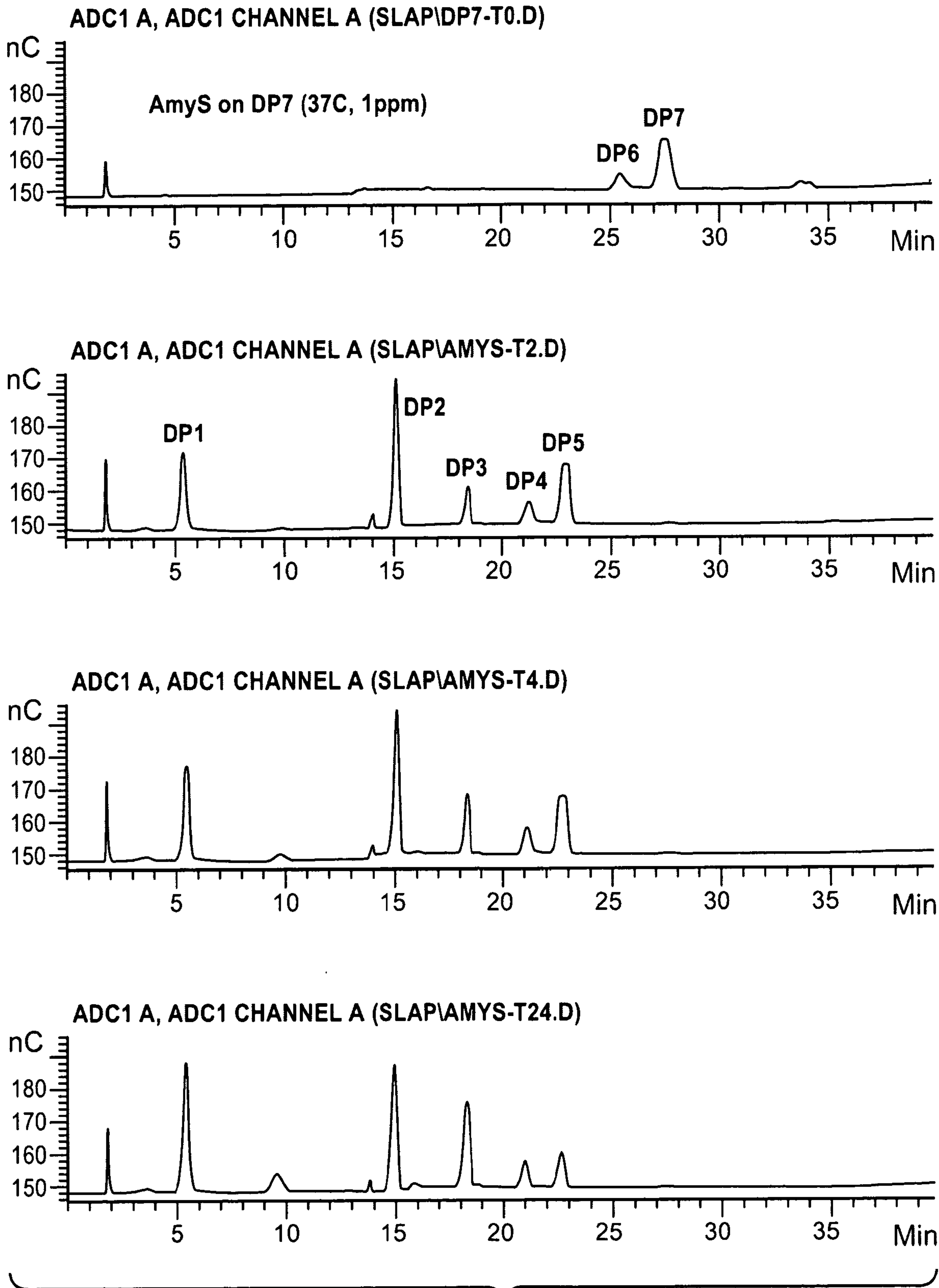


FIG. 7

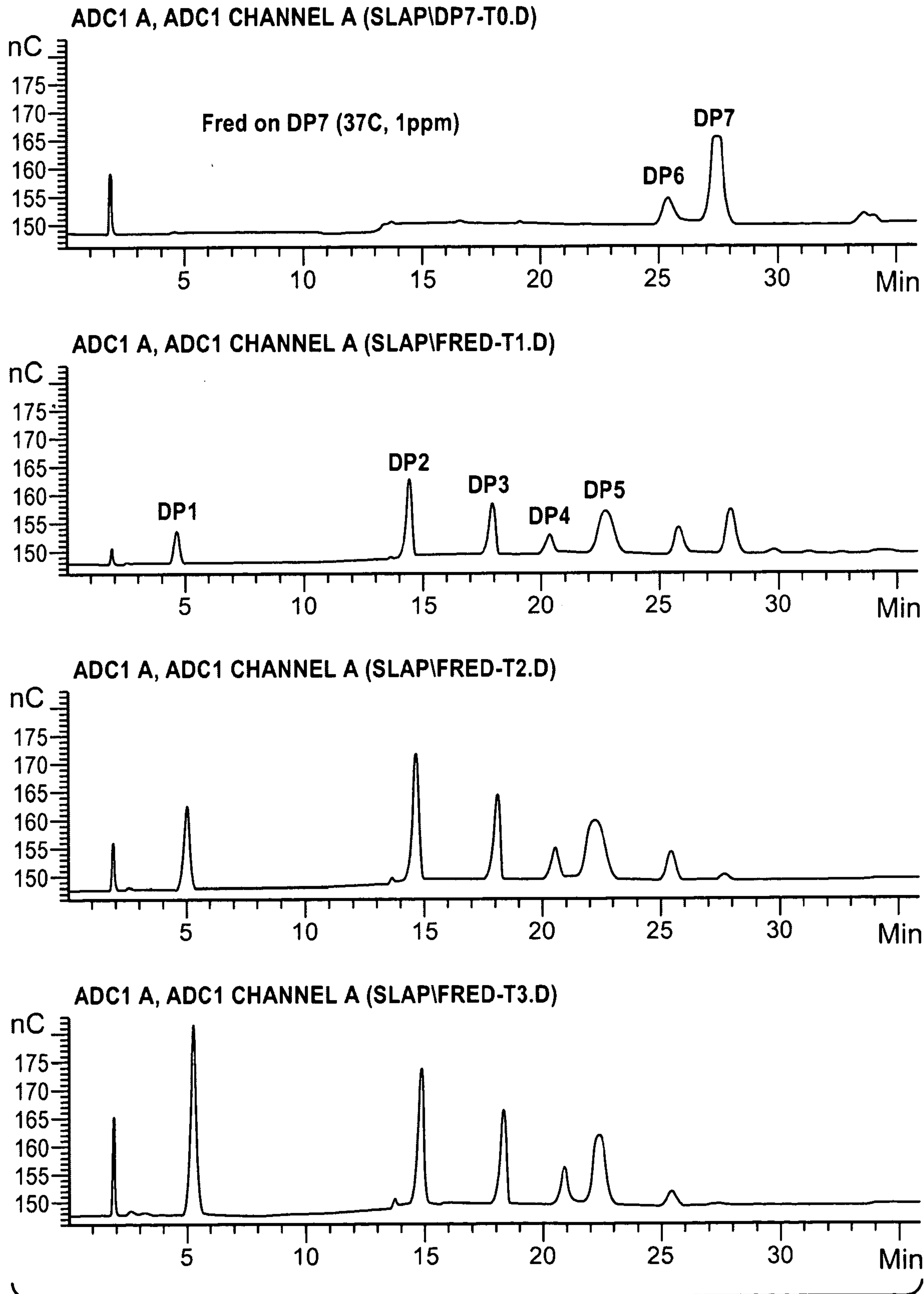


FIG. 8

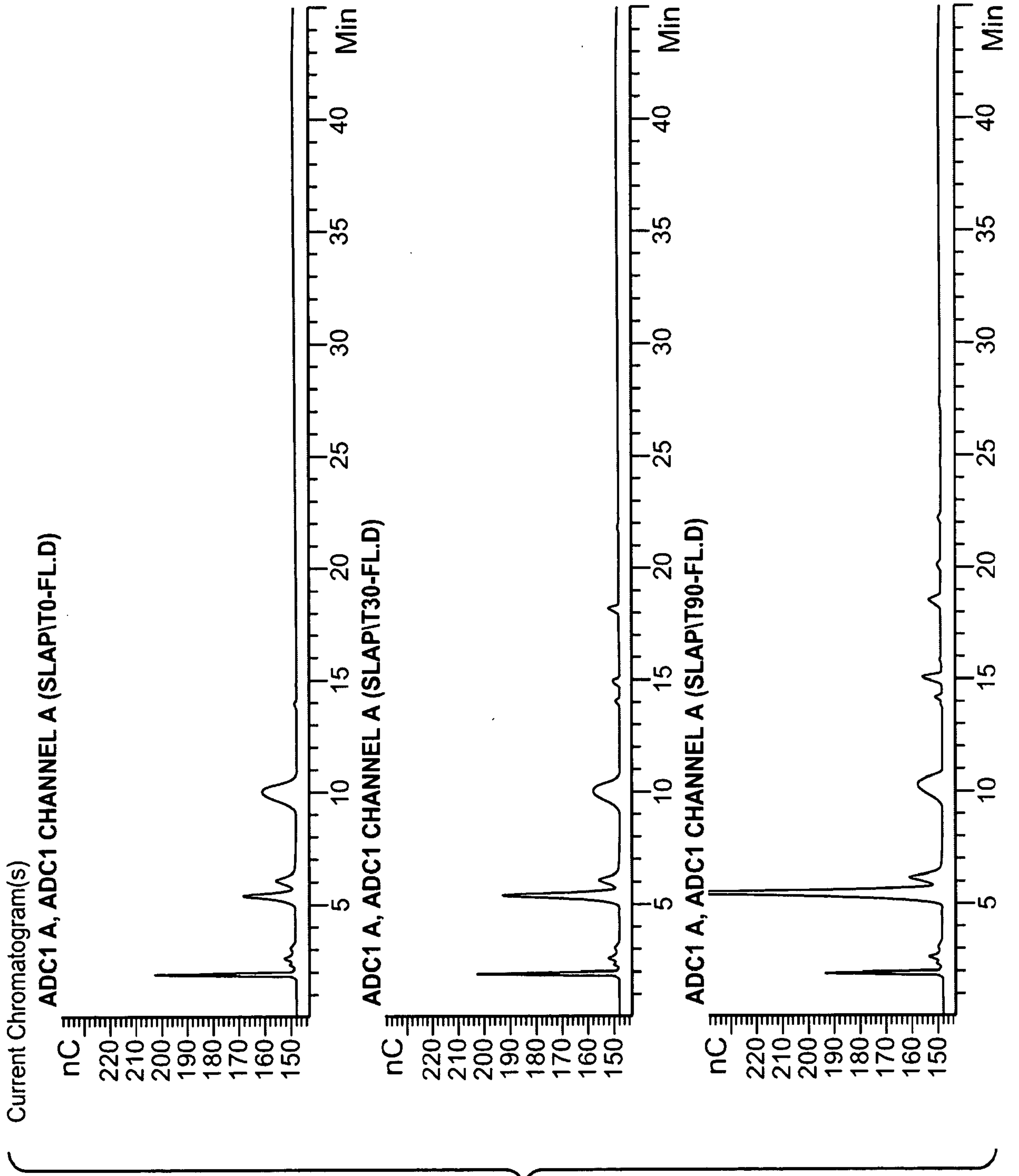


FIG. 9

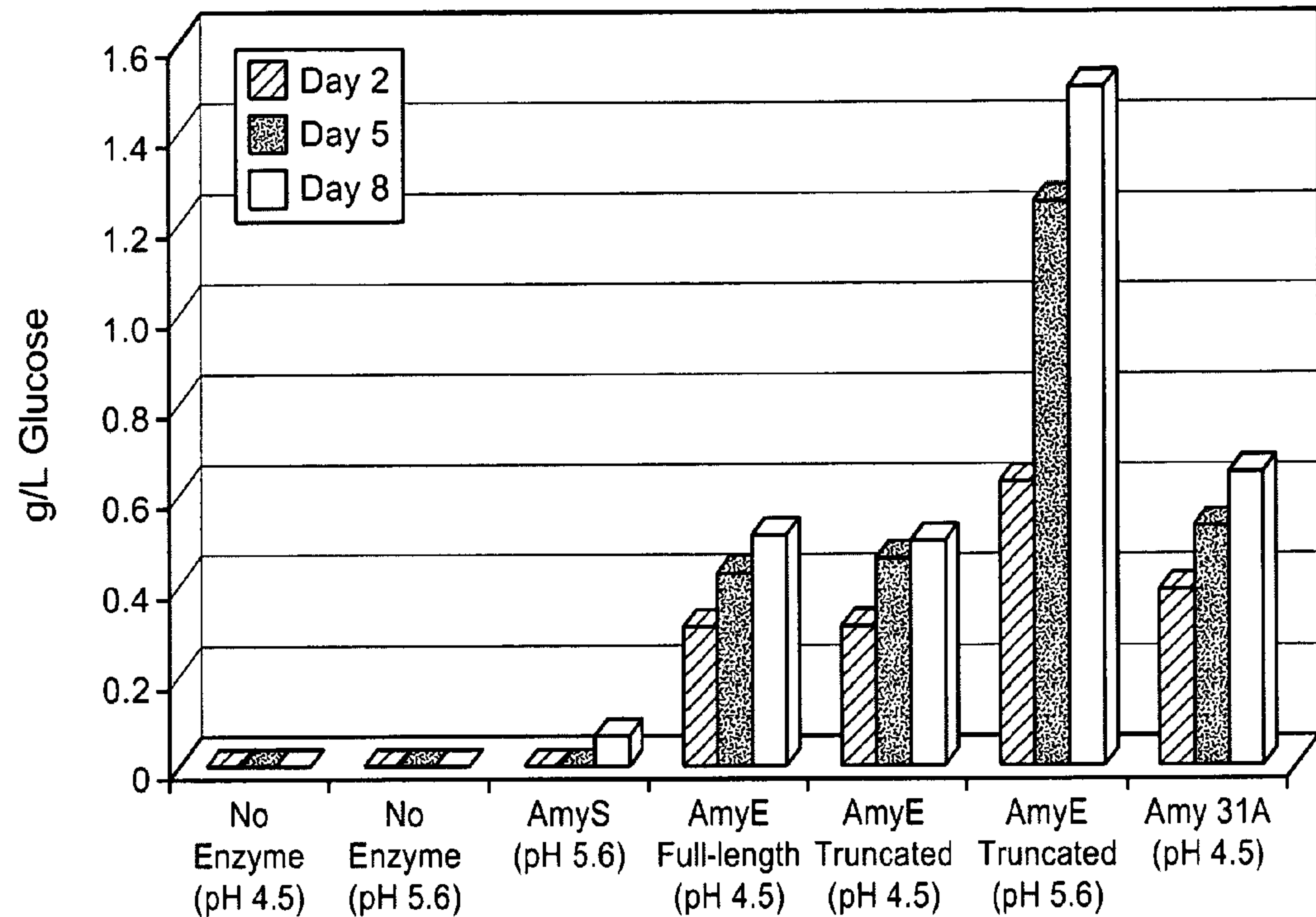


FIG. 5