

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
15 December 2005 (15.12.2005)

PCT

(10) International Publication Number
WO 2005/117953 A1

- (51) International Patent Classification⁷: **A61K 38/47**, C12N 9/28, 9/30, C07H 21/04
- (21) International Application Number: PCT/US2004/041276
- (22) International Filing Date: 9 December 2004 (09.12.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/575,175 27 May 2004 (27.05.2004) US
60/605,437 30 August 2004 (30.08.2004) US
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, 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, HU, IE, IS, IT, LT, LU, MC, NL, 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
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HETEROLOGOUS EXPRESSION OF AN ASPERGILLUS KAWACHI ACID-STABLE ALPHA AMYLASE

(57) Abstract: The present invention relates to the heterologous expression of an acid-stable alpha amylase having granular starch hydrolyzing activity and a granular starch hydrolyzing enzyme composition comprising the acid stable alpha amylase (asAA) having granular starch hydrolyzing activity and optionally a glucoamylase.



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HETEROLOGOUS EXPRESSION OF AN *ASPERGILLUS KAWACHI* ACID-STABLE ALPHA AMYLASE

The present application claims priority to U.S. Provisional Patent Application Serial No. 60/575,175, entitled Heterologous Expression of an *Aspergillus kawachi* Acid-Stable Alpha Amylase and Applications in Granular Starch Hydrolysis, filed May 27, 2004 and U.S. Provisional Patent Application Serial No. 60/605,437, entitled Heterologous Expression of an *Aspergillus kawachi* Acid-Stable Alpha Amylase and Applications in Granular Starch Hydrolysis, filed August 30, 2004.

FIELD OF THE INVENTION

The present invention relates to an acid-stable alpha amylase (asAA) derived from a strain of *Aspergillus kawachi*, which has granular starch hydrolyzing (GSH) activity. Further the invention relates to the heterologous expression of an asAA having GSH activity in filamentous fungal host cells and particularly in *Trichoderma* and *Aspergillus* cells and the use of asAA having GSH activity in compositions, which optionally include glucoamylases to enhance starch hydrolysis.

BACKGROUND OF THE INVENTION

Glucoamylases, and particularly glucoamylases having granular starch hydrolyzing (GSH) activity are important industrial enzymes used for producing products such as organic acids (i.e. lactic acids), amino acids (i.e. glutamic acids), alcohols (i.e. ethanol) and other compounds from starch substrates derived from grains and cereals. During microbial fermentations, and particularly during simultaneous saccharification and fermentation (SSF), it would be of benefit to reduce the amount of residual starch in the fermentation when granular starch substrates are used as a carbon feed. The present invention answers this need by providing an acid-stable alpha amylase (asAA) having granular starch hydrolyzing activity, which may be used in combination with a glucoamylase to enhance starch hydrolysis and alcohol production.

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Additionally, benefits of the present invention over prior art compositions and methods include one or more of the following: a) a reduction of thermal energy use during starch hydrolysis and end-product production; b) reduction in the requirement of high enzyme dosage; c) utilization of a continuous internal glucose feed; d) maintenance of a relatively low glucose level in the fermenter, which significantly reduces the high risk of microbial contamination and removes the catabolite repression of yeast due to high concentration of free glucose; e) reduction in formation of browning reaction products; f) reduction or removal of calcium addition, which was required during the prior art jet cooking process; g) reduction in water utilization during the fermentation process; h) use of higher solids content in the fermentation, which may result in higher end-product formation and reduced energy costs; i) reduced levels of production of certain by-products, such as glycerol; and j) decreased residual starch content of distillers dry grains plus solubles.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a fungal host cell comprising a heterologous polynucleotide that encodes an acid-stable alpha amylase (asAA) having granular starch hydrolyzing (GSH) activity which has at least 90% sequence identity to the sequence of SEQ ID NO: 3. In some embodiments, the heterologous polynucleotide will encode an asAA having GSH activity with at least 95% sequence identity to the sequence of SEQ ID NO: 3. In some embodiments, the asAA, which is expressed in the fungal host including a heterologous polynucleotide encoding the asAA, will have at least one different property compared to the corresponding asAA produced by the endogenous expression in the native fungal host. In some embodiments, the different property is the pH optimum of the asAA or the pH range for activity. In one embodiment of this aspect, the fungal host cell is a *Trichoderma* cell. In a further embodiment, the *Trichoderma* host cell is a *T. reesei* cell. In another embodiment, the fungal host cell is an *Aspergillus* cell.

In a second aspect, the invention relates to a granular starch hydrolyzing enzyme composition which comprises an acid-stable alpha amylase (asAA) having granular starch hydrolyzing (GSH) activity, wherein the asAA having GSH activity has at least 90% sequence identity to the sequence of SEQ ID NO: 3. In some embodiments, the asAA will be obtained from the expression of a heterologous polynucleotide in a fungal host cell. In further embodiments, the fungal host cell will be a *Trichoderma* or

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Aspergillus host cell. In other embodiments, the composition will further include a glucoamylase enzyme. In some preferred embodiments, the glucoamylase enzyme will be obtained from a strain of *Aspergillus* or *Rhizopus*. In other embodiments, the glucoamylase will be a glucoamylase having GSH activity and will be obtained from a strain of *Aspergillus*, *Rhizopus* or *Humicola*. In other embodiments, both the asAA and the glucoamylase will be expressed in a fungal host having a heterologous polynucleotide which expresses an asAA having GSH activity and a glucoamylase. In some embodiments, the fungal host strain will be the same and in other embodiments, the fungal host strain will be different strains. In other embodiments, the invention relates to a method of hydrolyzing granular starch using the enzyme composition of this aspect.

In a third aspect, the invention relates to a method of increasing the granular starch hydrolyzing activity of a composition comprising a glucoamylase, which comprises adding an acid-stable alpha amylase (asAA) having granular starch hydrolyzing (GSH) activity to a composition which includes a granular starch substrate and a glucoamylase to produce a soluble starch hydrolysate wherein the asAA having GSH activity has an amino acid sequence of at least 90% sequence identity to SEQ ID NO: 3 and the amount of solubilized starch is greater than a corresponding composition absent the asAA having GSH activity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides the genomic DNA sequence coding for the native *Aspergillus kawachi* acid-stable alpha-amylase, which is designated *asaA* (SEQ ID NO:1). The eight putative introns are underlined.

FIG. 2 provides the signal sequence (SEQ ID NO: 2) and mature amino acid sequence (SEQ ID NO: 3) (AsaA) for *A. kawachi* acid stable alpha-amylase (SEQ ID NO: 4). The putative signal sequence (amino acids 1-21) is underlined and bold.

FIGS. 3A-D provide the complete nucleotide sequence (SEQ ID NO: 5), 10990 bp, of plasmid pTrex3g_Akalpha (Figure 4).

FIG. 4 provides a map of pTrex3g_Akalpha, which was used for expression of the nucleic acid encoding the AsaA (*Aspergillus kawachi* asAA) and which contains EcoRI sites flanking the fungal expression vector, wherein

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- a) *cbhl* promoter is the *Trichoderma reesei* cellobiohydrolase promoter;
- b) *asaA* is the *Aspergillus kawachi* polynucleotide encoding the acid stable alpha amylase of SEQ ID NO. 4;
- c) *cbhl* terminator is the *Trichoderma reesei* cellobiohydrolase terminator;
- 5 d) *amdS* is an *Aspergillus nidulans* acetamidase nutritional marker gene; and
- e) *attB* is a Gateway cloning system (Invitrogen) lambda phage site for recombination.

FIG. 5 provides an SDS-PAGE gel indicating the expression of *asaA* from
10 *Trichoderma reesei* in a representative fermentation run for *Trichoderma reesei* clones as described in Example 5. Lane 1 represents the standard See Blue +2 marker; lane 2 represents *T. reesei* expressed AsaA after 80 hours; lane 3 represents *T. reesei* expressed AsaA after 162 hours and lane 4 represents a *T. reesei* host cell control at 162 hours in which the host cell has not been transformed with the *asaA*. An AsaA
15 protein band is clearly observed at about 90 kDa and this band is absent in the host strain control.

FIG. 6 illustrates the pH stability as % residual activity for the native *Aspergillus kawachi* (nAk-AsaA) and the expressed *A. kawachi* (rAk-AsaA) in the *T. reesei* host
20 (SEQ ID NO:3), as described in Example 6.

FIG. 7 illustrates the % (v/v) ethanol (EtOH) production from the fermentation of corn flour mash at pH 5.0 with glucoamylase (0.5 GAU/g DISTILLASE) and an alpha amylase over time, wherein Tr-AsaA (*A. kawachi* acid stable alpha amylase expressed
25 in *Trichoderma reesei*) is represented by AKAA; SPEZYME LT75 alpha amylase is represented by LT75; SPEZYME FRED is represented by FRED; SPEZYME ETHYL is represented by ETHYL; CLARLASE is represented by FA and DISTILLASE is the control. Reference is made to Example 8.

30 FIG. 8 illustrates the degradation of granular starch as glucose released after 4 hours with incubated purified DISTILLASE (GA), purified AkAA (*A. kawachi* acid stable alpha amylase expressed in *Trichoderma reesei*), and the combination of AkAA and GA at pH 5.0. Reference is made to Example 11.

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FIG. 9 illustrates SEMs of granular corn starch incubated with the enzymes described for Figure 8: purified DISTILLASE (GA), purified AkAA (*A. kawachi* acid stable alpha amylase expressed in *Trichoderma reesei*), and the combination of AkAA and GA.

5 FIG. 10 illustrates the % increase in ethanol production with AkAA for each corn mash solids (% ds) measured against mash solids (% ds).

DETAILED DESCRIPTION OF THE INVENTION

10 In some aspects, the present invention relies on routine techniques and methods used in the field of genetic engineering and molecular biology. The following resources include descriptions of general methodology useful in accordance with the invention: Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (2nd Ed., 1989); Kreigler, GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL (1990) and Ausubel
15 *et al.*, Eds. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994). These general references provide definitions and methods known to those in the art. However, it is not intended that the present invention be limited to any particular methods, protocols, and reagents described, as these may vary.

 Unless defined otherwise herein, all technical and scientific terms used herein
20 have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994) and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with general dictionaries of many of the terms used in this invention.

25 Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

 The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications, including all sequences
30 disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

 Numeric ranges are inclusive of the numbers defining the range.

 Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation,
35 respectively.

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The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole.

5 A. Definitions

As used herein the term "starch" refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein X can be any number. In particular, the term refers to any plant-based material including but not limited to grains, grasses, tubers and roots and
10 more specifically wheat, barley, corn, rye, rice, sorghum, brans, cassava, millet, potato, sweet potato, and tapioca.

The term "granular starch" refers to raw (uncooked) starch, e.g., granular starch that has not been subject to gelatinization.

The terms "granular starch hydrolyzing (GSH) enzyme" and "having granular
15 starch hydrolyzing (GSH) activity" refer to enzymes, which have the ability to hydrolyze starch in granular form.

The term "alpha-amylase (e.g., E.C. class 3.2.1.1)" refers to enzymes that catalyze the hydrolysis of alpha-1,4-glucosidic linkages. These enzymes have also been described as those effecting the exo or endohydrolysis of 1,4- α -D-glucosidic linkages in
20 polysaccharides containing 1,4- α -linked D-glucose units. Another term used to describe these enzymes is "glycogenase". Exemplary enzymes include alpha-1,4-glucan 4-glucanohydrolase.

The term "acid-stable alpha amylase ("asAA") refers to an alpha amylase that is active in the pH range of pH 3.0 to 7.0 and preferably 3.5 to 6.0.

25 The term "glucoamylase" refers to the amyloglucosidase class of enzymes (e.g., EC.3.2.1.3, glucoamylase, 1, 4-alpha-D-glucan glucohydrolase). These are exo-acting enzymes, which release glucosyl residues from the non-reducing ends of amylose and amylopectin molecules. The enzyme also hydrolyzes alpha-1, 6 and alpha -1,3 linkages although at much slower rate than alpha-1, 4 linkages.

30 The term "glycosylation" refers to the post-transcriptional modification of a protein by the addition of carbohydrate moieties, wherein the carbohydrate is either N-linked or O-linked resulting in a glucoprotein. An N-linked carbohydrate moiety of a glycoprotein is attached by a glycosidic bond to the β -amide nitrogen of an asparagine residue. An O-linked carbohydrate is attached by a glycosidic bond to a protein through the hydroxy
35 group of a serine or a threonine residue.

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The term "recombinant" when used in reference to a cell, nucleic acid, protein or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example,
5 recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The terms "protein" and "polypeptide" are used interchangeably herein. The conventional one-letter or three-letter code for amino acid residues is used herein.

10 A "signal sequence" means a sequence of amino acids bound to the N-terminal portion of a protein, which facilitates the secretion of the mature form of the protein outside the cell. The definition of a signal sequence is a functional one. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

15 The term "native acid-stable alpha amylase (n-asAA)" refers to an asAA produced from the endogenous expression of the asAA. For example, the term "n-asAA" means the endogenous expression of an acid-stable alpha amylase (i.e., SEQ ID NO: 3) from an *Aspergillus kawachi*.

The terms "recombinant acid-stable alpha amylase (r-asAA)", "recombinantly
20 expressed asAA" and "recombinantly produced asAA" refer to a mature asAA protein sequence that is produced in a host cell from the expression of a heterologous polynucleotide. For example, the term "r-asAA" means the *Aspergillus kawachi* acid-stable alpha amylase (i.e., SEQ ID NO: 3) is expressed and produced in a host in which a polynucleotide encoding the asAA has been introduced. The mature protein sequence
25 of a r-asAA excludes a signal sequence.

A "gene" refers to a DNA segment that is involved in producing a polypeptide and includes regions preceding and following the coding regions as well as intervening sequences (introns) between individual coding segments (exons).

The term "nucleic acid" encompasses DNA, RNA, single stranded or double
30 stranded and chemical modifications thereof. The terms "nucleic acid" and "polynucleotide" may be used interchangeably herein. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present invention encompasses polynucleotides, which encode a particular amino acid sequence.

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A "vector" refers to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, cassettes and the like.

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

A "promoter" is a regulatory sequence that is involved in binding RNA polymerase to initiate transcription of a gene. The promoter may be an inducible promoter or a constitutive promoter. A preferred promoter used in the invention is *Trichoderma reesei cbh1*, which is an inducible promoter.

"Under transcriptional control" is a term well understood in the art that indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably linked to an element which contributes to the initiation of, or promotes transcription.

"Under translational control" is a term well understood in the art that indicates a regulatory process that occurs after mRNA has been formed.

As used herein when describing proteins and genes that encode them, the term for the gene is italicized, (e.g., the gene that encodes *asaA* (*A. kawachi* asAA) may be denoted as *asaA*). The term for the protein is generally not italicized and the first letter is generally capitalized, (e.g., the protein encoded by the *asaA* gene may be denoted as AsaA or asaA).

The term "derived" encompasses the terms "originated from", "obtained" or "obtainable from", and "isolated from".

The term "operably linked" refers to juxtaposition wherein the elements are in an arrangement allowing them to be functionally related. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence.

The term "selective marker" refers to a gene capable of expression in a host that allows for ease of selection of those hosts containing an introduced nucleic acid or vector. Examples of selectable markers include but are not limited to antimicrobials (e.g., hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage, such as a nutritional advantage on the host cell.

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A polynucleotide or a polypeptide having a certain percent (*e.g.* 80%, 85%, 90%, 95%, or 99%) of sequence identity with another sequence means that, when aligned, that percentage of bases or amino acid residues are the same in comparing the two sequences. This alignment and the percent homology or identity can be determined
5 using any suitable software program known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18). Preferred programs include the GCG Pileup program, FASTA (Pearson *et al.* (1988) *Proc. Natl. Acad. Sci USA* 85:2444-2448), and BLAST (BLAST Manual, Altschul *et al.*, *Natl. Cent. Biotechnol. Inf.*, *Natl Lib. Med.* (NCIB NLM
10 NIH), Bethesda, MD, and Altschul *et al.*, (1997) *NAR* 25:3389-3402). Another preferred alignment program is ALIGN Plus (Scientific and Educational Software, PA), preferably using default parameters. Another sequence software program that finds use is the TFASTA Data Searching Program available in the Sequence Software Package Version 6.0 (Genetics Computer Group, University of Wisconsin, Madison, WI).

15 One skilled in the art will recognize that sequences encompassed by the invention are also defined by the ability to hybridize under stringent hybridization conditions with the exemplified *asaA* sequence (*e. g.*, SEQ ID NO:1). A nucleic acid is hybridizable to another nucleic acid sequence when a single stranded form of the nucleic acid can anneal to the other nucleic acid under appropriate conditions of temperature
20 and solution ionic strength. Hybridization and washing conditions are well known in the art (See, *e.g.*, Sambrook (1989) *supra*, particularly chapters 9 and 11). In some embodiments, stringent conditions correspond to a *T_m* of 65°C and 0.1x SSC, 0.1% SDS.

"Host strain" or "host cell" means a suitable host for an expression vector or DNA
25 construct comprising a polynucleotide encoding a granular starch hydrolyzing enzyme according to the invention. Specifically, host strains are preferably filamentous fungal cells. In a preferred embodiment of the invention, "host cell" means both the cells and protoplasts created from the cells of a filamentous fungal strain and particularly a *Trichoderma sp.* or an *Aspergillus sp.*

30 The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina (See, Alexopoulos, C. J. (1962), *INTRODUCTORY MYCOLOGY*, Wiley, New York). These fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, cellulose, and other complex polysaccharides. The filamentous fungi of the present invention are morphologically, physiologically, and genetically distinct from
35 yeasts. Vegetative growth by filamentous fungi is by hyphal elongation and carbon

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catabolism is obligatory aerobic. In the present invention, the filamentous fungal parent cell may be a cell of a species of, but not limited to, *Trichoderma*, (e.g., *Trichoderma reesei* (previously classified as *T. longibrachiatum* and currently also known as *Hypocrea jecorina*), *Trichoderma viride*, *Trichoderma koningii*, *Trichoderma harzianum*);
5 *Penicillium* sp., *Humicola* sp. (e.g., *Humicola insolens* and *Humicola grisea*); *Chrysosporium* sp. (e.g., *C. lucknowense*), *Gliocladium* sp., *Aspergillus* sp. (e.g., *A. oryzae*, *A. niger*, and *A. awamori*), *Fusarium* sp., *Neurospora* sp., *Hypocrea* sp., and *Emericella* sp. (See also, Innis et al., (1985) Sci. 228:21 -26).

As used herein, the term "*Trichoderma*" or "*Trichoderma* sp." refer to any fungal
10 genus previously or currently classified as *Trichoderma*.

The term "culturing" refers to growing a population of microbial cells under suitable conditions in a liquid or solid medium. In one embodiment, culturing refers to fermentative bioconversion of a starch substrate containing granular starch to an end-product (typically in a vessel or reactor). Fermentation is the enzymatic and anaerobic
15 breakdown of organic substances by microorganisms to produce simpler organic compounds. While fermentation occurs under anaerobic conditions it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen.

The phrase "simultaneous saccharification and fermentation (SSF)" refers to a
20 process in the production of biochemicals in which a microbial organism, such as an ethanol producing microorganism and at least one enzyme such as an α -AA are in the same process step. In one embodiment of the present invention, SSF refers to the contemporaneous hydrolysis of granular starch substrates to saccharides including glucose and the fermentation of the saccharides into alcohol in the same reactor vessel.

25 The term "contacting" refers to the placing of the respective enzyme(s) in sufficiently close proximity to the respective substrate to enable the enzyme(s) to convert the substrate to the end-product. Those skilled in the art will recognize that mixing solutions of the enzyme with the respective substrates can effect contacting.

The term "enzymatic conversion" in general refers to the modification of a
30 substrate by enzyme action. The term as used herein also refers to the modification of a granular starch substrate by the action of an enzyme.

As used herein the term "saccharification" refers to enzymatic conversion of starch to glucose.

The term "gelatinization" means solubilization of a starch molecule by cooking to
35 form a viscous suspension.

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The term "liquefaction" refers to the stage in starch conversion in which gelatinized starch is hydrolyzed to give low molecular weight soluble dextrans.

The term "degree of polymerization (DP)" refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Examples of DP2 are the disaccharides, such as maltose and sucrose. A DP>3 denotes polymers with a degree of polymerization of greater than 3.

The terms "end-product" or "desired end-product" refer to any carbon-source derived molecule product which is enzymatically converted from the granular starch substrate.

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

The term "mash" refers to a mixture of a fermentable carbon source (carbohydrate) in water used to produce a fermented product, such as an alcohol. In some embodiments, the term "beer" and "mash" are used interchangeability.

As used herein "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol. The ethanologenic microorganisms are ethanologenic by virtue of their ability to express one or more enzymes that individually or together convert sugar to ethanol.

As used herein the term "ethanol producer" or ethanol producing microorganism" refers to any organism or cell that is capable of producing ethanol from a hexose or pentose. Generally, ethanol-producing cells contain an alcohol dehydrogenase and a pyruvate decarboxylase. Examples of ethanol producing microorganisms include fungal microorganisms such as yeast. A preferred yeast includes strains of *Sacchromyces*, particularly, *S. cerevisiae*.

The term "heterologous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell. In some embodiments, the protein is a commercially important industrial protein. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes.

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

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The terms "recovered", "isolated", and "separated" as used herein refer to a compound, protein, cell, nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the terms "transformed", "stably transformed" and "transgenic" used in reference to a cell means the cell has a non-native (e.g., heterologous) nucleic acid sequence integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid sequence may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

As used herein the term "specific activity" means an enzyme unit defined as the number of moles of substrate converted to product by an enzyme preparation per unit time under specific conditions. Specific activity is expressed as units (U)/mg of protein.

As used herein the term "enzyme unit" refers to the amount of enzyme that produces 1 micromole of product per minute under the specified conditions of the assay. For example, in one embodiment, the term "glucoamylase activity unit" (GAU) is defined as the amount of enzyme required to produce 1 g of glucose per hour from soluble starch substrate (4% ds) under assay conditions of 60°C and pH 4.2.

In another embodiment, a granular starch hydrolyzing enzyme unit (GSHE U) is defined as being the amount of GSHE required to produce 1 mg of glucose per minute from granular starch under assay conditions of, for example 25°C at pH 5.0. In a preferred embodiment, a GSHE U is defined as being the amount of a GSHE required to produce 1 mg glucose/min from a granular starch substrate at 50°C at pH 4.5.

The term "yield" refers to the amount of end-product or desired end-products produced using the methods of the present invention. In some preferred embodiments, the yield is greater than that produced using methods known in the art. In some embodiments, the term refers to the volume of the end product and in other embodiment the term refers to the concentration of the end product.

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"ATCC" refers to American Type Culture Collection located at Manassas, VA 20108 (ATCC; <www.atcc.org>).

"NRRL" refers to the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (and previously known as USDA Northern Regional Research Laboratory), Peoria, ILL.

"A", "an" and "the" include plural references unless the context clearly dictates otherwise.

As used herein the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

B. Preferred Embodiments

Acid-stable alpha amylases (asAA) having granular starch hydrolyzing (GSH) activity -

In one embodiment an asAA having GSH activity is obtained from a strain of *Aspergillus*, e.g., *A. oryzae*, *A. kawachi*, *A. niger*, and *A. awamori*.

In a particularly preferred embodiment, the asAA having GSH activity comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity with the amino acid sequence set forth in SEQ ID NO: 3. In another embodiment, the asAA having GSH activity comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 3. In a further embodiment, the asAA having GSH activity comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 3. The asAA may also comprise an amino acid sequence having at least 98% sequence identity with SEQ ID NO: 3. In a further embodiment, the asAA having GSH activity comprises the amino acid sequence of SEQ ID NO: 3.

In other embodiments, the asAA comprising the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 95% sequence identity with SEQ ID NO: 3 is encoded by a polynucleotide having at least 70%, at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity to the sequence of SEQ ID NO: 1. In a particularly preferred embodiment, the nucleic acid sequence encoding the asAA of SEQ ID NO: 3 (AsaA) is the nucleic acid sequence of SEQ ID NO: 1.

Glucoamylases -

Glucoamylases (GA) may be derived from bacteria, plants and fungi sources. Preferred glucoamylases useful in the compositions and methods of the invention are produced by several strains of filamentous fungi and yeast. In particular, glucoamylases
5 secreted from strains of *Aspergillus* and *Trichoderma* are commercially important. Sources of these glucoamylases include: *Aspergillus niger* G1 and G2 glucoamylase and variants thereof (Boel et al., (1984) *EMBO J.* 3:1097 - 1102; WO 92/00381 and WO 00/04136); *Aspergillus awamori* glucoamylases (WO 84/02921); *Aspergillus oryzae* glucoamylase and variants thereof (Hata et al., (1991) *Agric. Biol. Chem.* 55:941 - 949)
10 and *Aspergillus shirousami*. (See Chen et al., (1996) *Prot. Eng.* 9:499 - 505; Chen et al. (1995) *Prot. Eng.* 8:575-582; and Chen et al., (1994) *Biochem J.* 302:275-281).

Glucoamylases are also obtained from strains of *Talaromyces* such as those derived from *T. emersonii*, *T. leycettanus*, *T. duponti* and *T. thermophilus* (WO 99/28488; USP No. RE: 32,153; USP No. 4,587,215); strains of *Rhizopus*, such as *R. niveus* and *R.*
15 *oryzae*; strains of *Mucor* and *Humicola*, such as *H. grisea* (See, Boel et al., (1984) *EMBO J.* 3:1097-1102; WO 92/00381; WO 00/04136; Chen et al., (1996) *Prot. Eng.* 9:499-505; Taylor et al., (1978) *Carbohydrate Res.* 61:301-308; US Pat. No. 4,514,496; US Pat. No. 4,092,434; and Jensen et al., (1988) *Can. J. Microbiol.* 34:218 - 223).

Enzymes having glucoamylase activity used commercially are produced for
20 example, from *Aspergillus niger* (trade name DISTILLASE, OPTIDEX L-400 and G ZYME G990 4X from Genencor International Inc.) or *Rhizopus* species (trade name CU.CONC from Shin Nihon Chemicals, Japan). Also the commercial digestive enzyme, trade name GLUCZYME from Amano Pharmaceuticals, Japan (Takahashi et al., (1985) *J. Biochem.* 98:663-671). Additional enzymes include three forms of glucoamylase
25 (E.C.3.2.1.3) of a *Rhizopus* sp., namely "Gluc1" (MW 74,000), "Gluc2" (MW 58,600) and "Gluc3" (MW 61,400). Gluc1 finds particular use in the present invention.

A particular group of glucoamylase (GA) enzymes are known as granular starch hydrolyzing enzyme(s) GSHE (See e.g., Tosi et al., (1993) *Can. J. Microbiol.* 39:846 - 855). GA-GSHEs not only have glucoamylase activity, but also are able to hydrolyze
30 granular (raw) starch. GA-GSHEs have been recovered from fungal cells such as *Humicola* sp., *Aspergillus* sp. and *Rhizopus* sp. A *Rhizopus oryzae* GA-GSHE has been described in Ashikari et al., (1986) *Agric. Biol. Chem.* 50:957-964 and USP 4,863,864. Also reference is made to *Rhizopus niveus*. A *Humicola grisea* GA-GSHE is described by Allison et al., (1992) *Curr. Genet.* 21:225-229 and European Patent No., 171218. The
35 gene encoding this enzyme is also known in the art as "gla1". An *Aspergillus awamori*

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var. *kawachi* GA-GSHE is described by Hayashida *et al.*, (1989) *Agric. Biol. Chem* 53:923-929. An *Aspergillus shirousami* GA-GSHE is described by Shibuya *et al.*, (1990) *Agric. Biol. Chem.* 54:1905-1914. One particular GA-GSHE preparation for use in the present invention includes enzyme preparations sold under the designation "M1" available from Biocon India, Ltd, India. The activity of a GA-GSHE preparation may be defined in terms of the glucoamylase activity.

In one embodiment, a GA-GSHE enzyme may be derived from a strain of *Humicola grisea*, particularly a strain of *H. grisea* var. *thermoidea* (See, US Pat. No. 4,618,579). In some preferred embodiments, the *Humicola grisea* GA-GSHE enzyme is recovered from fungi including ATCC 16453, NRRL (USDA Northern Regional Research Laboratory, Peoria, ILL) 15219, NRRL 15220, NRRL 15221, NRRL 15222, NRRL 15223, NRRL 15224 and NRRL 15225, as well as genetically altered strains thereof. These species produce enzymatic glucoamylase preparations that are immunologically the same (See, EP 0 171 218).

Recombinantly expressed enzymes -

In some embodiments of the invention, microorganisms are genetically engineered to express heterologous asAA having GSH activity and microorganisms may also be engineered to express heterologous glucoamylases. Preferred host cells are filamentous fungal cells. In a preferred embodiment, the filamentous fungal host is a strain of an *Aspergillus* sp, a *Trichoderma* sp, a *Fusarium* sp and a *Penicillium* sp. Particularly preferred fungal host cells include *A. nidulans*, *A. awamori*, *A. oryzae*, *A. aculeatus*, *A. niger*, *A. japonicus*, *T. reesei*, *T. viride*, *F. oxysporum*, and *F. solani*. *Aspergillus* strains are disclosed in Ward *et al.* (1993) *Appl. Microbiol. Biotechnol.* 39:738-743 and Goedegebuur *et al.*, (2002) *Curr Gene* 41:89 – 98. In a most preferred embodiment, the host is a strain of *Trichoderma*, and particularly a strain of *T. reesei*. Strains of *T. reesei* are known and nonlimiting examples include ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56765, ATCC No. 56767 and NRRL 15709. In some preferred embodiments, the host strain is a derivative of RL-P37. RL-P37 is disclosed in Sheir-Neiss *et al.* (1984) *Appl. Microbiol. Biotechnology* 20:46 – 53.

In some preferred embodiments, a *Trichoderma* host cell or an *Aspergillus* host cell is genetically engineered to express an asAA having GSH activity characterized by an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98% and 99% identity with SEQ ID NO: 3. In some embodiments, the polynucleotide encoding an

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asAA will have a nucleic acid sequence of SEQ ID NO: 1 or a nucleic acid sequence having at least 70% sequence identity with SEQ ID NO: 1.

In some embodiments, the asAA produced in a host cell engineered to include a heterologous polynucleotide encoding an asAA having GSH activity will have different, such as improved properties compared to the asAA produced by the endogenous expression of the asAA having GSH activity in a native host. These properties may include for example, increased enzyme activity, increased enzyme stability at lower pH levels or increased specific activity. In some embodiments, a heterologously produced asAA having GSH activity according to the invention will exhibit a maximum pH activity within a pH range of 3.0 to 6.0; a pH range of 3.0 to 5.0; a pH range of 3.5 to 5.0 and also within a pH range of 3.5 to 4.5. In other embodiments, a heterologously produced asAA will have a greater stability or residual activity at a pH level of 3.0, 3.5, 4.0, 4.5 and/or 5.0 compared to a corresponding asAA endogenously produced from a native host under essentially the same conditions. In some embodiments the level of enzyme stability for a heterologously produced asAA will be at least 1.0X, 2.0X or 2.5 times greater at a specific pH level compared to an endogenously expressed asAA at the same pH level.

In other embodiments, the host strain which is genetically engineered to express an asAA having GSH activity may also be genetically engineered to express a heterologous GA.

A host strain useful in the invention may have been previously manipulated through genetic engineering. In some embodiments, various native genes of the fungal host cell will have been inactivated. These genes include, for example genes encoding cellulolytic enzymes, such as endoglucanases (EG) and exocellobiohydrolases (CBH) (e.g. *cbh1*, *cbh2*, *egl1*, *egl2* and *egl3*). US Patent No. 5,650,322 discloses derivative strains of RL-P37 having deletions in the *cbh1* gene and the *cbh2* gene.

Vectors -

While the description below refers specifically to asAA, one skilled in the art will readily understand that the same or similar methods apply to DNA constructs and vectors useful for introduction of a polynucleotide encoding GA into a host cell.

According to the invention, a DNA construct comprising nucleic acid encoding an asAA encompassed by the invention is constructed to transfer an asAA into a host cell. In one embodiment, the DNA construct is transferred to a host cell by an expression

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vector which comprises regulatory sequences operably linked to an asAA coding sequence.

The vector may be any vector which when introduced into a fungal host cell is integrated into the host cell genome and is replicated. Reference is made to the Fungal
5 Genetics Stock Center Catalogue of Strains (FGSC, < www.fgsc.net>) for a list of vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook *et al.*, (1989) *supra*, Ausubel (1987) *supra*, van den Hondel *et al.* (1991) in Bennett and Lasure (Eds.) MORE GENE MANIPULATIONS IN FUNGI, Academic Press pp. 396-428 and U.S. Patent No. 5,874,276. Particularly useful vectors include
10 pFB6, pBR322, PUC18, pUC100 and pENTR/D.

In preferred embodiments, nucleic acid encoding an asAA encompassed by the invention is operably linked to a suitable promoter, which shows transcriptional activity in the fungal host cell. The promoter may be derived from genes encoding proteins either homologous or heterologous to the host cell. Preferably, the promoter is useful in a
15 *Trichoderma* host. Suitable nonlimiting examples of promoters include *cbh1*, *cbh2*, *egl1*, *egl2*. In one embodiment, the promoter is one that is native to the host cell. For example, when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In a preferred embodiment, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235. An "inducible promoter" is a
20 promoter that is active under environmental or developmental regulation. In another embodiment, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from *A. awamori* and *A. niger* glucoamylase genes (Nunberg *et al.*, (1984) *Mol. Cell Biol.* 4:2306-2315 and Boel *et al.*, (1984) *EMBO J.* 3:1581-1585). Also, the promoters of the *T. reesei xln1* gene and the
25 *cellobiohydrolase 1* gene may be useful (EPA 137280A1).

In some preferred embodiments, the asAA coding sequence is operably linked to a signal sequence. The DNA encoding the signal sequence is preferably that which is naturally associated with the asAA gene to be expressed. Preferably, the signal sequence is encoded by an *Aspergillus kawachi* *asaA* gene that encodes an Ak-asaA.
30 More preferably the signal sequence has at least 90%, at least 95%, at least 97%, and at least 99% sequence identity to the signal sequence of SEQ ID NO: 2. In additional embodiments, a signal sequence and a promoter sequence comprising a DNA construct or vector to be introduced into a fungal host cell are derived from the same source. For example, in some embodiments, the signal sequence is the *cdh1* signal sequence which
35 is operably linked to a *cdh1* promoter.

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In some embodiments, the expression vector also includes a termination sequence. In one embodiment, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is homologous to the host cell. A particularly suitable terminator sequence is *cbh1* derived from a *Trichoderma* strain and particularly *T. reesei*. Other useful fungal terminators include the terminator from *A. niger* or *A. awamori* glucoamylase gene (Nunberg *et al.* (1984) *supra*, and Boel *et al.*, (1984) *supra*).

In some embodiments, an expression vector includes a selectable marker. Examples of preferred selectable markers include ones which confer antimicrobial resistance (e.g., hygromycin and phleomycin). Nutritional selective markers also find use in the present invention including those markers known in the art as *amdS* *argB* and *pyr4*. Markers useful in vector systems for transformation of *Trichoderma* are known in the art (See, e.g., Finkelstein, chapter 6 in BIOTECHNOLOGY OF FILAMENTOUS FUNGI, Finkelstein *et al.* Eds. Butterworth-Heinemann, Boston, MA (1992), Chap. 6.; and Kinghorn *et al.* (1992) APPLIED MOLECULAR GENETICS OF FILAMENTOUS FUNGI, Blackie Academic and Professional, Chapman and Hall, London). In a preferred embodiment, the selective marker is the *amdS* gene, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of *A. nidulans* *amdS* gene as a selective marker is described in Kelley *et al.*, (1985) *EMBO J.* 4:475 - 479 and Penttilä *et al.*, (1987) *Gene* 61:155-164.

An expression vector comprising a DNA construct with a polynucleotide encoding an asAA may be any vector which is capable of replicating autonomously in a given fungal host organism or of integrating into the DNA of the host. In some embodiments, the expression vector is a plasmid. In preferred embodiments, two types of expression vectors for obtaining expression of genes are contemplated.

The first expression vector comprises DNA sequences in which the promoter, asAA coding region, and terminator all originate from the gene to be expressed. In some embodiments, gene truncation is obtained by deleting undesired DNA sequences (e.g., DNA encoding unwanted domains) to leave the domain to be expressed under control of its own transcriptional and translational regulatory sequences.

The second type of expression vector is preassembled and contains sequences required for high-level transcription and a selectable marker. In some embodiments, the coding region for an asAA gene or part thereof is inserted into this general-purpose expression vector such that it is under the transcriptional control of the expression

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construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh1* promoter.

Methods used to ligate the DNA construct comprising a polynucleotide encoding an asAA, a promoter, a terminator and other sequences and to insert them into a suitable vector are well known in the art. Linking is generally accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide linkers are used in accordance with conventional practice. (See, Sambrook (1989) *supra*, and Bennett and Lasure, MORE GENE MANIPULATIONS IN FUNGI, Academic Press, San Diego (1991) pp 70 – 76.). Additionally, vectors can be constructed using known recombination techniques (e.g., Invitrogen Life Technologies, Gateway Technology).

Where it is desired to obtain a fungal host cell having one or more inactivated genes known methods may be used (e.g. methods disclosed in U.S. Patent No. 5,246,853, U.S. Patent No. 5,475,101 and WO92/06209). Gene inactivation may be accomplished by complete or partial deletion, by insertional inactivation or by any other means which renders a gene nonfunctional for its intended purpose (such that the gene is prevented from expression of a functional protein). Any gene from a *Trichoderma sp* or other filamentous fungal host, which has been cloned can be deleted, for example *cbh1*, *cbh2*, *egl1* and *egl2* genes. In some embodiments, gene deletion may be accomplished by inserting a form of the desired gene to be inactivated into a plasmid by methods known in the art. The deletion plasmid is then cut at an appropriate restriction enzyme site(s), internal to the desired gene coding region, and the gene coding sequence or part thereof is replaced with a selectable marker. Flanking DNA sequences from the locus of the gene to be deleted (preferably between about 0.5 to 2.0kb) remain on either side of the marker gene. An appropriate deletion plasmid will generally have unique restriction enzyme sites present therein to enable the fragment containing the deleted gene, including the flanking DNA sequences and the selectable markers gene to be removed as a single linear piece.

Transformation, expression and culture of host cells -

Introduction of a DNA construct or vector into a host cell includes techniques such as transformation; electroporation; nuclear microinjection; transduction; transfection, (e.g., lipofection mediated and DEAE-Dextrin mediated transfection); incubation with calcium phosphate DNA precipitate; high velocity bombardment with DNA-coated microprojectiles; and protoplast fusion. General transformation techniques are known in the art (See, e.g., Ausubel et al., (1987), *supra*, chapter 9; and Sambrook

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(1989) *supra*, and Campbell *et al.*, (1989) *Curr. Genet.* 16:53-56). The expression of heterologous protein in *Trichoderma* is described in USP 6,022,725; U.S. Pat. No. 6,268,328; Harkki *et al.* (1991); *Enzyme Microb. Technol.* 13:227-233; Harkki *et al.*, (1989) *Bio Technol.* 7:596-603; EP 244,234; EP 215,594; and Nevalainen *et al.*, "The
5 *Molecular Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*", in MOLECULAR INDUSTRIAL MYCOLOGY, Eds. Leong and Berka, Marcel Dekker Inc., NY (1992) pp. 129 - 148). Reference is also made to Cao *et al.*, (2000) *Sci.* 9:991 - 1001 for transformation of *Aspergillus* strains.

Preferably, genetically stable transformants are constructed with vector systems
10 whereby the nucleic acid encoding an asAA is stably integrated into a host strain chromosome. Transformants are then purified by known techniques.

In one nonlimiting example, stable transformants including an *amdS* marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium
15 containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on solid non-selective medium (*i.e.*, medium that lacks acetamide), harvesting spores from this culture medium and determining the percentage of these spores which subsequently germinate and grow on selective medium containing acetamide. Alternatively, other methods known in the art may be used to select
20 transformants.

In one specific embodiment, the preparation of *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelia. (See, Campbell *et al.*, (1989) *Curr. Genet.* 16:53-56). In some embodiments, the mycelia are obtained from germinated vegetative spores. The mycelia are treated with an enzyme
25 that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M. It is preferable to use about a 1.2 M solution of sorbitol in the suspension medium.

Uptake of DNA into the host *Trichoderma sp.* strain is dependent upon the
30 calcium ion concentration. Generally, between about 10 mM CaCl₂ and 50 mM CaCl₂ is used in an uptake solution. Besides the need for the calcium ion in the uptake solution, other compounds generally included are a buffering system such as TE buffer (10 Mm Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG). It is believed that the polyethylene glycol acts to
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fuse the cell membranes, thus permitting the contents of the medium to be delivered into the cytoplasm of the *Trichoderma sp.* strain and the plasmid DNA is transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA integrated into the host chromosome.

5 Usually a suspension containing the *Trichoderma sp.* protoplasts or cells that have been subjected to a permeability treatment at a density of 10^5 to 10^7 /mL, preferably 2×10^6 /mL are used in transformation. A volume of 100 μ L of these protoplasts or cells in an appropriate solution (e.g., 1.2 M sorbitol; 50 mM CaCl_2) are mixed with the desired DNA. Generally a high concentration of PEG is added to the uptake solution. From 0.1
10 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. However, it is preferable to add about 0.25 volumes to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride and the like may also be added to the uptake solution and aid in transformation. Similar procedures are available for other fungal host cells. (See, e.g., U.S. Patent Nos. 6,022,725 and 6,268,328, both
15 of which are incorporated by reference).

Generally, the mixture is then incubated at approximately 0°C for a period of between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired gene or DNA sequence. The 25% PEG 4000 is generally added in volumes of 5 to 15 times the volume of the transformation mixture;
20 however, greater and lesser volumes may be suitable. The 25% PEG 4000 is preferably about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture is then incubated either at room temperature or on ice before the addition of a sorbitol and CaCl_2 solution. The protoplast suspension is then further added to molten aliquots of a growth medium. This growth medium permits the growth
25 of transformants only.

Generally, cells are cultured in a standard medium containing physiological salts and nutrients (See, e.g., Pourquie, J. *et al.*, BIOCHEMISTRY AND GENETICS OF CELLULOSE DEGRADATION, eds. Aubert, J. P. *et al.*, Academic Press, pp. 71-86, 1988 and Ilmen, M. *et al.*, (1997) *Appl. Environ. Microbiol.* 63:1298-1306). Common commercially prepared
30 media (e.g., Yeast Malt Extract (YM) broth, Luria Bertani (LB) broth and Sabouraud Dextrose (SD) broth) also find use in the present invention.

Culture conditions are also standard, (e.g., cultures are incubated at approximately 28°C in appropriate medium in shake cultures or fermenters until desired levels of asAA expression are achieved). Preferred culture conditions for a given
35 filamentous fungus are known in the art and may be found in the scientific literature

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and/or from the source of the fungi such as the American Type Culture Collection and Fungal Genetics Stock Center.

After fungal growth has been established, the cells are exposed to conditions effective to cause or permit the expression of an asAA as defined herein. In cases where
5 an asAA having GSH activity coding sequence is under the control of an inducible promoter, the inducing agent (e.g., a sugar, metal salt or antimicrobial), is added to the medium at a concentration effective to induce asAA expression.

Identification of asAA Activity -

10 In order to evaluate the expression of an asAA having GSH activity by a cell line that has been transformed with a heterologous polynucleotide encoding an asAA encompassed by the invention, assays can be carried out at the protein level, the RNA level or by use of functional bioassays particular to alpha amylase activity and/or production. In general assays employed include, Northern blotting, dot blotting (DNA or
15 RNA analysis), RT-PCR (reverse transcriptase polymerase chain reaction), or *in situ* hybridization, using an appropriately labeled probe (based on the nucleic acid coding sequence) and conventional Southern blotting and autoradiography.

In addition, the production and/or expression of an asAA having GSH activity may be measured in a sample directly, for example, by assays directly measuring
20 reducing sugars such as glucose in the culture media and by assays for measuring glucoamylase activity, expression and/or production. Substrates useful for assaying GSH activity include granular starch substrates. For example, glucose concentration may be determined by any convenient method such as by using glucose reagent kit No 15-UV (Sigma Chemical Co.) or an instrument such as Technicon Autoanalyzer. Also
25 reference is made to glucose oxidase kits and glucose hexose kits commercially available from Instrumentation Lab. (Lexington, MA).

In addition, gene expression may be evaluated by immunological methods, such as immunohistochemical staining of cells, tissue sections or immunoassay of tissue culture medium, e.g., by Western blot or ELISA. Such immunoassays can be used to
30 qualitatively and quantitatively evaluate expression of an asAA. The details of such methods are known to those of skill in the art and many reagents for practicing such methods are commercially available.

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35 culture medium, e.g., by Western blot or ELISA. Such immunoassays can be used to

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qualitatively and quantitatively evaluate expression of a asAA. The details of such methods are known to those of skill in the art and many reagents for practicing such methods are commercially available.

Alpha amylase activity may be measured by using the DNS method as described
5 in Miller, G. L. (1959) *Anal. Chem.* 31:426 - 428. Glucoamylase activity may be assayed by the 3,5-dinitrosalicylic acid (DNS) method (See, Goto et al., (1994) *Biosci. Biotechnol. Biochem.* 58:49 - 54).

In some embodiments of the invention, the asAA having GSH activity expressed
10 by a *Trichoderma* or *Aspergillus* host will be greater than 1 gram protein per liter (g/L), greater than 2 g/L, greater than 5 g/L, greater than 10 g/L, greater than 20 g/L, greater than 25 g/L, greater than 30 g/L, greater than 50 g/L and also greater than 100 g/L of culture media.

Methods for Purifying asAA -

15 In general, an asaA (including n-asAA or r-asAA) produced in cell culture is secreted into the medium and may be purified or isolated, e.g., by removing unwanted components from the cell culture medium. In some cases, an AsaA may be produced in a cellular form necessitating recovery from a cell lysate. In such cases the enzyme is purified from the cells in which it was produced using techniques routinely employed by
20 those of skill in the art. Examples include, but are not limited to, affinity chromatography (Tilbeurgh *et al.*, (1984) *FEBS Lett.* 16:215); ion-exchange chromatographic methods (Goyal *et al.*, (1991) *Biores. Technol.* 36:37; Fliess *et al.*, (1983) *Eur. J. Appl. Microbiol. Biotechnol.* 17:314; Bhikhabhai *et al.*, (1984) *J. Appl. Biochem.* 6:336; and Ellouz *et al.*, (1987) *Chromatography* 396:307), including ion-exchange using materials with high
25 resolution power (Medve *et al.*, (1998) *J. Chromatography A* 808:153; hydrophobic interaction chromatography (Tomaz and Queiroz, (1999) *J. Chromatography A* 865:123; two-phase partitioning (Brumbauer, *et al.*, (1999) *Bioseparation* 7:287); ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation;
30 and gel filtration using, e.g., Sephadex G-75.

Fermentations -

In some embodiments of the present invention, fungal cells expressing a
heterologous asAA are grown under batch or continuous fermentation conditions. A
35 classical batch fermentation is a closed system, wherein the composition of the medium

is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies
5 as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is
10 diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch
15 systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and fed-
20 batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase
25 growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. In other
30 systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well

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as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

Compositions -

5 A particularly useful enzyme composition according to the invention is a granular starch hydrolyzing enzyme composition which includes an asAA having at least 90%, at least 95% or at least 98% sequence identity to SEQ ID NO: 3, wherein the asAA is obtained from the heterologous expression of asAA.

Another particularly useful enzyme composition according to the invention is a
10 granular starch hydrolyzing enzyme composition which includes a mixture of glucoamylase and an asAA having at least 90%, at least 95% or at least 98% sequence identity to SEQ ID NO: 3, wherein the asAA is obtained from the heterologous expression of asAA. In some embodiments, the GA is obtained from an *Aspergillus* strain, e.g., DISTALLASE®. In other embodiments, the GA is obtained from a *Rhizopus*
15 or *Humicola* strain.

In some embodiments, the asAA is available as a cell free filtrate (for example wherein the asAA is isolated from a culture medium), and in other embodiments, the asAA is available in a culture medium containing the fungal host cells which express and secrete the asAA having GSH activity.

20 As understood by those in the art, the quantity of asAA having GSH activity used in the compositions and methods of the present invention will depend on the enzymatic activity of the asAA.

In some embodiments, the range of asAA present in the enzyme compositions is from 0.01 to 15.0 SSU. In other embodiments, the amount of glucoamylase useful in a
25 mixture is in the range of 0.1 to 10.0 GAU/g ds.

A particularly useful enzymatic composition includes a mixture of an asAA having at least 95% sequence identity to SEQ ID NO: 3 and a GA having 0.1 to 10 GAU/g ds. Another particularly useful enzymatic composition includes a mixture of an asAA having
30 at least 98% sequence identity to SEQ ID NO: 3 and a GA having 0.1 to 10 GAU/g ds.

In some embodiments, the ratio of asAA having GSH activity (SSU) to GA activity (GAU) will be in the range of 15:1 to 1:15. In further embodiments, the ratio (SSU to GAU) will be in the range of about 10:1 to 1:10; about 10:1 to 1:5; about 5:1 to 1:5, about 4:1 to 1:4; about 3:1 to 1:3; about 2:1 to 1:4 and also about 2:1 to 1:2. In some preferred
35 embodiments, the ratio of SSU to GAU will be between about 4:1 to 2:1. In other

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embodiments, the asAA having GSH activity and the GA are present in a ratio such that the hydrolysis of granular starch in a substrate is greater than the additive effect of the enzymes when supplied at the same levels under the same conditions. In some cases the hydrolysis will be at least 1.5 times greater, at least 2.0 times greater and also at
5 least 3.0 times greater. The exact amounts of the components encompassed by the compositions of the invention will depend on the combination of enzymes.

In general, asAA having GSH activity will be mixed with a slurry of a granular starch substrate in an amount of about 0.01 to 15.0 SSU per gram of dry solids content of the slurry. In some embodiments, the asAA having GSH activity is added in an
10 amount of about 0.01 to 10.0 SSU, about 0.01 to 5.0 SSU; about 0.05 to 10.0 SSU; about 0.05 to 5.0 SSU; about 0.1 to 10.0 SSU; about 0.1 to 5.0 SSU; about 0.1 to 2.0 SSU; about 0.25 to 2.5 SSU; about 0.5 to 5.0 SSU; about 0.5 to 2.5 SSU; and also about 0.5 to 1.5 SSU per gram of dry solids content of the slurry.

As understood by those in the art, the quantity of glucoamylase used in the
15 method and compositions of the present invention depends on the enzymatic activity of the glucoamylase. Generally, an amount of between 0.001 and 10.0 GAU of glucoamylase per gram (ds) slurry adjusted to 20 – 45% dry solids may be added. In some embodiments, the glucoamylase is added in an amount between 0.01 and 10 GAU; between 0.01 and 5.0 GAU; between 0.05 and 5.0 GAU; between 0.1 and 10.0
20 GAU; between 0.1 and 5.0 GAU; between 0.1 and 2.0 GAU; between 0.25 and 1.5 GAU of glucoamylase per gram (ds) slurry. In one preferred embodiment, the dosage range for glucoamylase will be from 0.1 to 2.0 GAU/g (ds) slurry.

Additional enzymes may be included in the compositions and methods encompassed by the invention. These additional enzymes, which find use in the present
25 invention include debranching enzymes such as pullulanases (E.C. 3:2.1.41) and isoamylases (E.C. 3.2.1.68). Such enzymes hydrolyze alpha-1,6-glucosidic bonds. Thus, during the hydrolysis of the starch, debranching enzymes remove successive glucose units from the non-reducing ends of the starch.

Another enzyme that may be used in the compositions of the invention are beta-
30 amylases (E.C. 3.2.1.2). These are exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers. The enzymes are characterized as having an optimum pH range from 4.5 to 7.0 and optimum temperature range from 40°C to 65°C. Commercial beta-amylases are available from Genencor International Inc.

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Further additional enzymes which may be used are proteases, such as fungal and bacterial proteases. Fungal proteases include for example, those obtained from *Aspergillus*, *Mucor* and *Rhizopus*, such as *A. niger*, *A. awamori*, *A. oryzae* and *M. miehei*. Other enzymes include but are not limited to cellulases, hemicellulases, lipases and cutinases.

The effective amount of these enzymes to be included in the methods of the invention can be readily determined by one skilled in the art.

In some embodiments, an antimicrobial may be added to the compositions and fermentation medium of the invention. Antimicrobials are compounds that kill or inhibit the growth of microorganisms.

In one embodiment, an enzyme composition comprising either an *asaA* alone or in combination with a glucoamylase will be used for SSF. In some embodiments, the enzyme composition will be contemporaneously added to a slurry of a granular starch substrate and the mixture will be fermented in a single step. The slurry may have about 10 – 50% ds; about 10 – 45%; about 15 – 40%; about 20 – 40%; about 25 – 40%; or about 25 – 35% ds.

A granular starch substrate may be obtained from any plant part including stems, grains, roots and tubers. Particularly preferred plant sources include corn; wheat; rye; sorghum; rice; millet; barley; cassava; legumes, such as beans and peas; potatoes; sweet potatoes; bananas; and tapioca.

Specifically contemplated starch substrates are cornstarch and wheat starch. The starch from a grain may be ground or whole and includes corn solids, such as kernels, bran and/or cobs. The starch may be highly refined raw starch or feedstock from starch refinery processes. Those of general skill in the art are well aware of available methods which may be used to prepare granular starch substrates for use in the methods encompassed by the invention. Some of these methods include dry milling of whole cereal grains using hammer mills and roller mills and wet milling.

Various starches are commercially available. For example, cornstarches are available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starches are available from Sigma; sweet potato starches are available from Wako Pure Chemical Industry Co. (Japan); and potato starch is available from Nakaari Chemical Pharmaceutical Co. (Japan).

Various references have reported on the amount of starch found in cereal grains and reference is made to The Alcohol Textbook, 3rd Ed. K. Jacques et al., Eds. 1999, Nottingham University Press. For example, corn contains about 60 – 68% starch; barley

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contains about 55 – 65% starch; millet contains about 75 – 80% starch; wheat contains about 60 – 65% starch; and polished rice contains 70 – 72% starch.

In some embodiments, a granular starch substrate is slurried (generally with water) and the slurry comprises i) about 10 to about 55% dry solids content (ds); ii) about 15 to about 50% dry solids content; iii) about 20 to about 45% dry solids content; iv) about 25 to about 45% dry solids content; v) about 30 to about 45% dry solids content; vi) about 30 to about 40% dry solids content; and also vii) about 30 to 35% dry solids content. The granular starch slurry is contacted with an enzyme composition according to the invention at a temperature below the gelatinization temperature of the starch in the granular starch substrate to yield glucose.

The exact temperature used in accordance with the methods of the invention depends upon the specific starch substrate used. General starch gelatinization temperature ranges are disclosed in Swinkels pages 32 – 38 in STARCH CONVERSION TECHNOLOGY, eds Van Beynum et al., (1985) Marcel Dekker Inc., NY and THE ALCOHOL TEXTBOOK, A REFERENCE FOR THE BEVERAGE, FUEL AND INDUSTRIAL ALCOHOL INDUSTRIES, 3rd Ed., eds Jacques et al., 1999, Nottingham University Press, UK. In some embodiments the temperature will be least about 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, and 65°C. In other embodiments, the temperature will be between about 30 – 65°C, about 35 – 65°C, about 40 – 65°C, and about 45 – 65°C. In other embodiments, the temperature will be between about 25 – 45°C, about 25 – 40°C and about 30 – 35°C. In preferred embodiments, the starch substrate is never subjected to the thermal conditions used for liquefactions.

In some embodiments, the residence time of the method is from about 2 to 300 hrs, but more typically from 2 to 120 hours. In some embodiments, the process is conducted from about 5 to 100 hours. In other embodiments, the process is conducted from about 5 to 80 hours. In still other embodiments, the process is conducted for at least 5 hours but less than 100 hours. In other embodiments, the process is conducted for at least about 10 hours but less than about 100 hours.

In some embodiments, at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 94%, 95%, 96%, 97%, 98% and 99% of the dry solids of the granular starch is hydrolyzed. In some embodiments, the granular starch substrate is completely hydrolyzed. In some embodiments, at least 90% of the granular starch is hydrolyzed in 100 hours. In certain embodiments, at least 90% of the granular starch substrate is hydrolyzed in a time period of 24 hours. In other embodiments, at least 95% of the granular starch substrate is hydrolyzed in a time period of 24 hours.

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The yield of glucose (percent of the total solubilized dry solids) may be at least about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% and 98%. However, in a preferred embodiment, the glucose is continually produced and substantially all of the glucose is used in the process to produce an end-product, such as ethanol. (Reference is made to MOLECULAR STRUCTURE AND FUNCTION OF FOOD CARBOHYDRATE, ED. G.G. BIRCH ET AL, APPLIED SCIENCE PUBLISHERS, LONDON).

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

In the disclosure and experimental section which follows, the following abbreviations apply:

asAA having GSH activity (an acid-stable alpha amylase having granular starch hydrolyzing activity); asaA (an acid-stable alpha amylase having granular starch hydrolyzing activity as illustrated in SEQ ID NO: 3 and which has been obtained from the endogenous expression of an asAA in *Aspergillus kawachi*); Tr-asaA (the expression of the *A. kawachi* acid-stable alpha amylase expressed in a *Trichoderma reesei* host); AkAA (the acid stable alpha amylase having SEQ ID NO: 3 and sometimes used interchangeability with asaA); and other abbreviations including GA (glucoamylase); wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per minute); H₂O (water); dH₂O (deionized water); dIH₂O (deionized water, Milli-Q filtration); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); g or gm (grams); µg (micrograms); mg (milligrams); µL (microliters); ml and mL (milliliters); mm (millimeters); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); PAGE (polyacrylamide gel electrophoresis); DO (dissolved oxygen); phthalate buffer (sodium phthalate in water, 20 mM, pH 5.0); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); w/w (weight to weight); v/v (volume to volume); Genencor (Genencor International, Inc., Palo Alto, CA); DDGS (Distilleries Dry Grain plus Solids); MT (Metric ton); and EtOH (ethanol).

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The following assays and methods are used in the examples provided below:

Glucoamylase assay: Glucoamylase activity was measured using a well-known assay which is based on the ability of glucoamylase to catalyze the hydrolysis of p-nitrophenyl-alpha-D-glucopyranoside (PNPG) to glucose and p-nitrophenol. At an alkaline pH, the nitrophenol; forms a yellow color that is proportional to glucoamylase activity and is monitored at 400nm and compared against an enzyme standard measured as a GAU.

One "Glucoamylase Activity Unit" (GAU) is defined as the amount of enzyme that will produce 1 gm of reducing sugar, calculated as glucose per hour from a soluble starch substrate (4%ds) at pH 4.2 and 60°C.

The measurement of acid-stable alpha amylase activity is based on the degree of hydrolysis of soluble potato starch substrate (4% ds) by an aliquot of the enzyme sample at pH 4.5, 50°C. The reducing sugar content is measured using the DNS method as described in Miller, G. L. (1959) Anal. Chem. 31:426 - 428. One unit of the enzyme activity (SSU, soluble starch unit) is equivalent to the reducing power of 1mg of glucose released per minute at the specific incubation conditions.

Determination of total starch content: The enzyme-enzyme starch liquefaction and saccharification process was used to determine the total starch content. In a typical analysis, 2 g of the dry sample was taken in a 100 ml Kohlrausch flask and 45 ml of MOPS buffer, pH 7.0 was added. The slurry was well stirred for 30 min. SPEZYME FRED (1:50 diluted in water), 1.0 ml was added and heated to boiling for 3 - 5 min. The flask was placed in an autoclave maintained at 121°C for 15 min. After autoclaving the flask was placed in a water bath at 95°C and 1 ml of 1:50 dilutes SPEZYME FRED was added and incubated for 45 min. The pH was adjusted to pH 4.2 and the temperature was reduced to 60°C. This was followed by addition of 20 ml acetate buffer, pH 4.2. Saccharification was carried out by adding 1.0 ml of 1:100 diluted OPTIDEX L-400 (Glucoamylase from Genencor International Inc.) and the incubation was continued for 18 hr at 60°C. The enzyme reaction was terminated by heating at 95°C for 10 min. The total sugar composition was determined by HPLC analysis using glucose as a standard. The soluble starch hydrolysate from water extraction of a sample at room temperature without enzymatic treatment was subtracted from the total sugar.

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Residual starch iodine test: A sample of the beer (fermentation broth) was centrifuged in 2 ml plastic centrifuge tubes. The supernatant was decanted and the tube containing the pellet was placed in an ice bath. Several drops of 0.025N iodine solution (0.1N iodine from VWR Cat. No. VW3207-1 diluted 4X) was added to the pellet and mixed. A positive (+) starch shows a range of color from blue to purple and the intensity of color is directly proportional to the concentration of starch. A negative result (-) remains yellowish.

Total protein analysis: The total nitrogen (N) in the sample preparations was determined using the Kjeldhal method (American Assoc. Cereal Chemists (AACC), (1983), Methods 22B60 8th Ed. St Paul, MN). Protein content was calculated by 6.25 X total N.

Ethanol and carbohydrate determinations:

Ethanol and carbohydrate composition of the samples were determined using the HPLC method as described herein:

- a) a 1.5 mL Eppendorf centrifuge tube was filled with fermentor beer and cooled on ice for 10 min;
- b) the sample tube was centrifuged for 1 min in Eppendorf table top centrifuge;
- c) a 0.5 mL sample of the supernatant was transferred to a test tube containing 0.05 mL of Kill solution (1.1N H₂SO₄) and allowed to stand for 5 min;
- d) 5.0 mL of water is added to the test tube sample and then filtered into a HPLC vial through 0.45 µm Nylon Syringe Filter; and
- e) run on HPLC.

HPLC conditions:

a) Ethanol System:

Column: Phenomenex Rezex Organic Acid Column (RHM-Monosaccharide) #00H-0132-KO (Equivalent to Bio-Rad 87H)
Column Temperature: 60°C
Mobile Phase: 0.01 N H₂SO₄
Flow Rate: 0.6 mL/min
Detector: RI
Injection Volume: 20 µL

b) Carbohydrate System:

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Column: Phenomenex Rezex Carbohydrate (RCM-Monosaccharide)

#00H-0130-KO (Equivalent to Bio-Rad 87H)

Column Temperature: 70°C

Mobile Phase: Nanopure DI H₂O

5 Flow Rate: 0.8 mL/min

Detector: RI

Injection Volume: 10 µL (3% DS material)

The column separates based on the molecular weight of the saccharides, which are designated as DP1 (monosaccharides); DP2 (disaccharides); DP3 (trisaccharides) and
10 DP+4 (oligosaccharide sugars having a degree of polymerization greater than 3).

Preparation of asaA used in examples 7 - 12 was as follows. At the end of the fermentation of the *T. reesei* which expresses asaA (prepared according to examples 2 and 3), the biomass was separated by centrifugation and the clear culture filtrate was
15 concentrated using a 10,000 molecular weight cut-off ultrafiltration membrane. This ultrafiltrated concentrate having (90 SSU/g) was used.

Preparation of glucoamylase used in examples 7 - 12 was as follows: A selected fungal strain of *Aspergillus niger* as described in USP 3,249,514 was used. After fermentation,
20 fungal mycelia were separated using conventional separation methods including filtration and centrifugation. The clear filtrate was concentrated by ultrafiltration at 5°C to a specified activity.

25

EXAMPLE 1

Cloning the *Aspergillus kawachi* acid-stable alpha-amylase gene.

Genomic DNA was extracted from an overnight culture of *A. kawachi* mycelia. The FastDNA Kit (QbioGene, Carlsbad, CA) SPINTM protocol was used according to the
30 manufacturer's instructions for fungi. For homogenization, the sample was processed for 30 sec at speed 4.0 on a FastPrep Instrument. PCR primers were designed, based on the asaA sequence of A. Kaneko, et al. (Kaneko et al., (1996), *J. Ferm Bioeng* 81:292-298). The forward primer contained a motif for directional cloning into the pENTR/D vector (Invitrogen).

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The sequence of the alpha6 primer was CACCATGAGAGTGTCTGACTTCAAG (SEQ ID NO. 6) and the sequence of the Akaa3 primer was CTACCTCCACGTATCAACCAC (SEQ ID NO. 7).

The 2.36 kb PCR product was purified by gel extraction (Gel Purification kit, Qiagen) and cloned into pENTR/D, according to the Invitrogen Gateway system protocol. The vector was then transformed into chemically competent Top10 *E.coli* (Invitrogen) with kanamycin selection. Plasmid DNA from several clones was digested with restriction enzymes to confirm the correct size insert. The alpha-amylase gene insert was sequenced (Sequetech, Mountain View, CA) from several clones (SEQ ID NO:1). Plasmid DNA from one clone, pENTR/D_Akaa#11, was added to the LR clonase reaction (Invitrogen Gateway system) with pTrex3g/*amdS* destination vector DNA. Recombination, in the LR-clonase reaction, replaced the CmR and *ccdB* genes of the destination vector with the *A. kawachi asaA* from the pENTR/D vector. This recombination directionally inserted *asaA* between the *cbhl* promoter and terminator of the destination vector. Recombination site sequences of 48 and 50 bp remained upstream and downstream, respectively, of the alpha amylase. An aliquot of the LR clonase reaction was transformed into chemically competent Top10 *E.coli* and grown overnight with carbenicillin selection. Plasmid DNA from several clones was restriction digested to confirm the correct insert size. Plasmid DNA from clone, pTrex3g_Akalpha#1 (Figure 4) was digested with *EcoRI* to release the expression cassette including the *cbhl* promoter:*asaA*:*cbhl* terminator:*amdS*. This 7.8 kb cassette was purified by agarose extraction using standard techniques and transformed into a strain of *T. reesei* derived from the publicly available strain QM6a, as further described below.

EXAMPLE 2

Biolistic transformation of *T. reesei*.

A *Trichoderma reesei* spore suspension was spread onto the center ~6 cm diameter of an MABA transformation plate (150 μ l of a 5×10^7 - 5×10^8 spore/ml suspension). The plate was then air dried in a biological hood. The stopping screens (BioRad 165-2336) and macrocarrier holders (BioRad 1652322) were soaked in 70% ethanol and air dried. DriRite desiccant was placed in small Petri dishes (6 cm Pyrex) and overlaid with Whatman filter paper. The macrocarrier holder containing the

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macrocarrier (BioRad 165-2335) was placed flatly on top of filter paper and the Petri dish lid replaced.

A tungsten particle suspension was prepared by adding 60 mg tungsten M-10 particles (microcarrier, 0.7 micron, BioRad #1652266) to an Eppendorf tube. 1 ml
5 ethanol (100%) was added. The tungsten was vortexed in the ethanol solution and allowed to soak for 15 minutes. The Eppendorf tube was microfuged briefly at maximum speed to pellet the tungsten. The ethanol was decanted and washed three times with sterile distilled water. After the water wash was decanted the third time, the tungsten was resuspended in 1 ml of sterile 50% glycerol. The tungsten was prepared fresh at
10 least every two weeks.

The transformation reaction was prepared by adding 25 μ l of suspended tungsten to a 1.5 ml Eppendorf tube for each transformation. Subsequent additions were made in order, 0.5-5 μ l DNA (XbaI-digested expression cassette), 25 μ l 2.5M CaCl_2 , 10 μ l 0.1 M spermidine. The reaction was vortexed continuously for 5-10 minutes,
15 keeping the tungsten suspended. The Eppendorf tube was then microfuged briefly and decanted. The tungsten pellet was washed with 200 μ l of 70% ethanol, microfuged briefly to pellet and decanted. The pellet was washed with 200 μ l of 100% ethanol, microfuged briefly to pellet, and decanted. The tungsten pellet was resuspended in 24 μ l 100% ethanol. The Eppendorf tube was placed in an ultrasonic water bath for 15
20 seconds and 8 μ l aliquots were transferred onto the center of the desiccated macrocarriers. The macrocarriers were left to dry in the desiccated Petri dishes.

A He tank was turned on to 1500 psi. 1100 psi rupture discs (BioRad 165-2329) were used in the Model PDS-1000/He Biolistic Particle Delivery System (BioRad). When the tungsten solution was dry, a stopping screen and the macrocarrier holder were
25 inserted into the PDS-1000. An MABA plate, containing the target *T. reesei* spores, was placed 6 cm below the stopping screen. A vacuum of 29 inches Hg was pulled on the chamber and held. The He Biolistic Particle Delivery System was fired. The chamber was vented and the MABA plate removed for incubation at 28°C until colonies appeared (5 days).

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With reference to this Example 2 the following solutions were prepared,

<u>Modified <i>amdS</i> Biolistic agar (MABA)</u>		<u>per liter</u>
Part I, make in 500 ml dH ₂ O		
5	1000x salts	1 ml
	Noble agar	20 g
	pH to 6.0, autoclave	
Part II, make in 500 ml dH ₂ O		
	Acetamide	0.6 g
10	CsCl	1.68 g
	Glucose	20 g
	KH ₂ PO ₄	15 g
	MgSO ₄ ·7H ₂ O	0.6 g
	CaCl ₂ ·2H ₂ O	0.6 g
15	pH to 4.5, 0.2 micron filter sterilize; leave in 50°C oven to warm, add to agar, mix, pour plates. Stored at room temperature.	
	<u>1000x Salts</u>	<u>per liter</u>
20	FeSO ₄ ·7H ₂ O	5 g
	MnSO ₄ ·H ₂ O	1.6 g
	ZnSO ₄ ·7H ₂ O	1.4 g
	CoCl ₂ ·6H ₂ O	1 g
	Bring up to 1L dH ₂ O.	
25	0.2 micron filter sterilize	

EXAMPLE 3

PEG-Mediated protoplast fusion transformation of *T. reesei*.

30

A 1 -2 cm² agar plug of a sporulated mycelia (grown on potato dextrose agar (PDA), Difco for 5 days at 30°C) was inoculated into 50ml of YEG (5g/L yeast extract plus 20g/L glucose) broth in a 250 ml, 4-baffle shake flask and incubated at 30-37°C for 16-20 hours at 200 rpm. The mycelia were recovered by transferring the shake flask contents into 50ml conical tubes and spinning at 2500 rpm for 10 minutes. The supernatant was discarded and the mycelial pellet was transferred into a 250 ml, 0.22 m CA or PES Corning filter bottle containing 40 ml of filtered β-D-glucanase solution. The solution was incubated at 30°C, 200 rpm, for 2 hours to generate protoplasts. Protoplasts were harvested by filtration, through sterile Miracloth (CalBiochem, La Jolla, CA), into a 50ml conical tube. The protoplasts were pelleted by centrifugation at 2000 rpm for 5 minutes and the supernatant discarded. Protoplast pellets were washed once with 50ml of 1.2 M sorbitol; centrifuged (2000 rpm, 5 min.) and supernatant was discarded. The pellet was washed with 25ml of sorbitol/CaCl₂. A haemocytometer was used to count the protoplasts and then pelleted by centrifugation at 2000 rpm for 5 min.

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The supernatant was discarded and protoplast pellets resuspended in a volume of sorbitol/CaCl₂ sufficient to generate a protoplast solution with a protoplast concentration of 1.25×10^8 /ml.

For each transformation, an aliquot of 20 µg of expression vector DNA (in a volume no greater than 20 µl) was transferred into 15ml conical tubes, on ice. Protoplast suspension (200µl) and 50µl PEG solution was added to each tube. This was mixed gently and incubated on ice for 20 min. PEG (2 ml) solution was added to each transformation tube and incubated at room temperature for 5 minutes. 4 ml sorbitol/CaCl₂ solution was added to each tube (total volume 6.2 ml) and mixed gently. Then 2 ml of the transformation mixture was added to each of 3 molten (50°C) top agar tubes. Each top agar mixture was poured onto a separate transformation plate and incubated at 30°C for four to seven days.

For transformation with *amdS* selection, acetamide/sorbitol plates and top agar were used. Selection plates were the same as transformation plates, but without sorbitol. Putative transformants were purified by transferring isolated colonies to fresh selective media containing acetamide.

Media and solutions were prepared as follows.

- 1) 40 ml β-D-glucanase solution- dissolved 600 mg β-D-glucanase (InterSpex Products Inc., San Mateo, CA) and 400mg MgSO₄ · 7H₂O in 40 ml 1.2M sorbitol.
- 2) 200 ml PEG mix- Dissolved 50g PEG 4000 (BDH Laboratory Supplies Poole, England) and 1.47g CaCl₂ · 2H₂O in 200 ml dH₂O. Prepared fresh monthly.
- 3) Sorbitol/ CaCl₂ solution- Dissolved 50mM CaCl₂ in 1.2M sorbitol.
- 4) Acetamide/sorbitol agar-
Part 1 – Dissolved 0.6g acetamide (Aldrich, 99% sublime.), 1.68g CsCl, 20g glucose, 20g KH₂PO₄, 0.6g MgSO₄ · 7H₂O, 0.6g CaCl₂ · 2H₂O, 1 ml 1000 x salts (see below) in 200 ml dH₂O, adjusted to pH 5.5, brought volume up to 300 mls with dH₂O, and filter sterilized.
Part II – 20g Noble agar and 218g sorbitol were added to a 1L cylinder, brought to volume (700mls) with dH₂O, and autoclaved.
Part II was added to Part I for a final volume of 1L.
- 5) 1000 x Salts – Combined 5g FeSO₄ · 7H₂O, 1.6g MnSO₄ · H₂O, 1.4g ZnSO₄ · 7H₂O, 1g CoCl₂ · 6H₂O and brought the volume up to 1L with dH₂O. Filter sterilized.

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EXAMPLE 4PEG-Mediated protoplast fusion transformation of *Aspergillus niger*.

5 A 2 cm² agar plug was inoculated from a sporulated *A. niger* plate, into 50ml of YEG (5g/L yeast extract plus 20g/L glucose) broth in a 250 ml, 4-baffle shake flask. The agar plug was incubated at 30°-37°C for 16-20 hours at 200 rpm, mycelia was harvested through a sterile Miracloth filter and washed with Solution A. Washed mycelia was aseptically transferred into 40 ml of protoplasting solution and incubated at 30°C, 200
10 rpm, for 1-2 hours, protoplasting progress was monitored microscopically. The protoplasting reaction was filtered through sterile Miracloth, into two 50 ml sterile disposable centrifuge tubes and the volume brought up to 45 mls each with Solution B. The protoplasts were centrifuged at 2500 rpm for 5 minutes to obtain pellets and the supernatant was discarded. The pellet was washed twice more with 20 ml volumes of
15 Solution B. The pellet was resuspended in 10 ml Solution B and protoplasts counted using a haemocytometer. Protoplasts were again centrifuged and the supernatant discarded. Protoplasts were resuspended, in Solution B to yield $\sim 1 \times 10^7 / 100 \mu\text{l}$. On ice, 100 μl protoplast solution was added to pre-chilled 15 ml tubes, one tube per transformation. 10 μg DNA was added in a volume not exceeding 10 μl . Solution C
20 (12.5 μl) was added, mixed gently, and incubated on ice for 20 minutes.

MMS top agar (3 tubes of 10 ml each, per transformation) was melted and maintained at 55°C. Protoplasts were removed from the ice and Solution C (1 ml) and Solution B (2 ml) were added to each tube and the tubes were mixed gently. 1 ml of the protoplast mixture was added to each of the 3 top agar tubes and the top agar was
25 poured onto MMS plates. This was repeated for each transformation and plates were incubated for 4-7 days at 30°C.

Solution A (per 500 ml) - 0.44 g K₂HPO₄; 0.34 g KH₂PO₄; 48.156 g anhydrous MgSO₄ (FW 120.37); and dH₂O added for a final volume of 500 ml, pH 5.5. Filter sterilized and
30 store at room temperature.

Protoplasting solution - Dissolved 180 units beta-D-glucanase (InterSpex Products, Inc) in 40 ml Solution A. Filter sterilized, 0.2 micron.

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Solution B (per 500 ml) - 5ml 1M Tris, pH 7.5; 2.77g CaCl₂ (FW 110.99); 109.32g Sorbitol (FW 182.2; 1.2M); and dH₂O added for a final volume of 500 ml.

Filter sterilized and store at room temperature.

- 5 Solution C (per 500 ml) - 250g PEG 4000; 2.77g CaCl₂; 5 ml 1M Tris, pH 7.5; and dH₂O added for a final volume of 500 ml. Filter sterilized.

MMS Agar * - Dissolved in 1L dH₂O,

- 6 g/L NaNO₃; 0.52 g/L KCl; 1.52 g/L KH₂PO₄; 218.5 g/L D-Sorbitol; 1 ml/L Trace
10 elements (see below); 10 g/L agar (low melt agarose in the top agar).
Autoclave. Post-sterilization, aseptically added 10 ml 50% glucose and 1.25 ml 20% MgSO₄·7H₂O.

*For *amdS* selection, replace the nitrate in the MMS with 0.59 g/L acetamide and 3.4 g/L CsCl.

15

Trace Elements Solution

Dissolve in 250 ml dH₂O,

- 1 g/L FeSO₄·7H₂O
8.8 g/L ZnSO₄·7H₂O
20 0.4 g/L CuSO₄·5H₂O
0.15 g/L MnSO₄·4H₂O
0.1 g/L Na₂B₄O₇·10H₂O
50 mg/L (NH₄)₆Mo₇O₂₄·4H₂O
Mix and added 0.2 ml concentrated HCl to dissolve. Brought volume up to 1L with
25 dH₂O. Filter sterilized.

EXAMPLE 5

- .. Fermentation of *T. reesei* transformed with the *asaA* gene and assay of activity in *T.*
30 *reesei* clones.

- In general, the fermentation protocol as described in Foreman *et al.* (Foreman *et al.* (2003) *J. Biol. Chem* 278:31988-31997) was followed. More specifically, duplicate fermentations were run for each of the strains displayed in Figure 5. 0.8 L of Vogels
35 minimal medium (Davis *et al.*, (1970) METHODS IN ENZYMOLOGY 17A, pg 79 - 143 and Davis, Rowland, NEUROSPORA, CONTRIBUTIONS OF A MODEL ORGANISM, Oxford

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University Press, (2000)) containing 5% glucose was inoculated with 1.5 ml frozen spore suspension. After 48 hours, each culture was transferred to 6.2L of the same medium in a 14L Biolafitte fermenter. The fermenter was run at 25°C, 750 RPM and 8 standard liters per minute airflow. One hour after the initial glucose was exhausted, a
5 25% (w/w) lactose feed was started and fed in a carbon limiting fashion to prevent lactose accumulation. The concentrations of glucose and lactose were monitored using a glucose oxidase assay kit or a glucose hexokinase assay kit with beta-galactosidase added to cleave lactose, respectively (Instrumentation Laboratory Co., Lexington, MA). Samples were obtained at regular intervals to monitor the progress of the fermentation.
10 Collected samples were spun in a 50ml centrifuge tube at 3/4 speed in an International Equipment Company (Needham Heights, MA) clinical centrifuge. Sample supernatants were run of 4 - 12% BIS-TRIS SDS -PAGE gels, under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer (Figure 5).

15

EXAMPLE 6

Comparison of pH stability of native and recombinant *A. kawachi* acid-stable alpha amylase having GSH activity (asaA).

20 Samples of recombinantly produced asaA (Tr-asaA) as described above and samples of native asaA were diluted to equal protein concentrations with 20 mM acetate buffer at pH 4.5. Reactions were run in 100 mM citrate/NaOH buffers at 50°C for 30 minutes at pH levels 3 to 7. 1.0 mL of the reaction was added to 5 mL of 10% corn starch (Cargill Foods, MN) in 100 mM acetate, pH 4.5 in sample tubes. The tubes were
25 shaken at 50°C for 20 minutes. Then 0.5 mL 2.0% NaOH was added. Tubes were spun and 0.5 mL of the supernatant were assayed for reducing sugars using the Dinitro Salicylic Acid (DNS) Assay (Goto et al., (1994) *supra*). The results are depicted in Figure 6. The r-asaA exhibited 100% residual activity at pH 3.9. In comparison the n-asaA exhibited 100% residual activity at pH 4.5.

30

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EXAMPLE 7

Effect of Tr-asaA concentrations during simultaneous saccharification and fermentation (SSF) of non-cooked whole ground corn substrate.

5

Tr-asaA was evaluated at two levels of glucoamylase (GA) from a cell free culture filtrate (0.5 and 1.0 GAU/g). Thirty six percent corn flour slurry was prepared containing dry corn steep (2.5 % of corn flour). The pH of the slurry was adjusted to 4.8 with dilute sulfuric acid. The total dry solids of the slurry were 33.85 %. Fermentations were carried out in 125 ml flasks containing 100 gm of mash (slurry). The desired levels of enzymes were added then 3 ml of propagated yeast slurry was added to start the fermentation. The yeast inoculum was prepared by adding 0.26 gm of dry Fali yeast to 100 gm of mash containing GA activity at 0.9 GAU/g of raw material solids. This slurry was placed in a 32°C water bath and gently mixed for about 17 hours. At various time intervals samples of the fermentation (beer) were taken for HPLC analysis. After 72 hours, the fermentations were terminated and the beer was dried at 60°C to obtain the distillers dry grains plus solubles (DDGS).

10

The starch content of the DDGS was determined and the insoluble solids of the beer after terminating the fermentation were spot checked for starch by the addition of Iodine. The enzymes used in this study were *A. niger* GA. Table 1 summarizes ethanol levels, iodine stain of the mash solids and % starch of the DDGS.

15

TABLE 1

20

Effect of asaA During Conversion of Granular Corn Starch to Ethanol under Yeast Fermentation Conditions

GA GAU/g	asaA SSU/g	% v/v EtOH 24 hr	% v/v EtOH 50 hr	% v/v EtOH 72 hr	% starch DDGS	Iodine
0.5		7.7	11.4	13.7	27.4	+
0.5	0.25	9.2	14.7	16.9	7.7	+
0.5	0.50	9.6	15.4	17.0	5.7	+/-
0.5	1.0	10.0	16.2	17.3	4.1	+/-
0.5	2.0	10.9	16.5	17.5	2.8	-
0.5	3.0	11.2	16.8	17.5	1.6	-
0.5	4.0	11.2	16.9	17.4	1.7	-
0.5	5.0	11.2	17.0	17.7	1.5	-
1.0		9.3	14.4	16.2	13.0	+
1.0	0.25	11.6	17.1	17.8	3.6	+/-
1.0	0.5	12.1	16.8	17.9	2.6	-

25

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1.0	1.0	12.7	17.2	17.7	2.2	-
1.0	2.0	12.7	17.6	17.8	1.6	-
1.0	3.0	12.9	17.5	17.8	1.1	-
1.0	4.0	13.2	17.5	17.9	0.8	-
1.0	5.0	13.3	17.2	17.9	1.1	-
2.0		11.2	15.5	16.9	9.6	+
3.0		11.4	15.9	17.2	5.8	+

The results as illustrated in Table 1 demonstrate Tr-asaA enhanced the hydrolysis of granular corn starch by glucoamylase.

EXAMPLE 8

5

Conversion of granular starch substrates by glucoamylase and alpha amylases

Commercial alpha amylases from different sources were compared with Tr-asaA under the simultaneous saccharification and fermentation conditions in the presence of glucoamylase at 0.5 GAU/g of ds. The activity of the commercial alpha amylases was
10 determined using the soluble starch substrate (SSU) method assay as described earlier.

TABLE 2

Alpha Amylase	Microbial Strain	SSU/ml
Tr-asaA	<i>A. kawachi</i> asAA expressed in <i>T. reesei</i>	90
SPEZYME LT AA	<i>Bacillus amyloliquefaciens</i>	2,759
SPEZYME FRED	<i>Bacillus licheniformis</i> **	4,842
SPEZYME Ethyl	<i>Bacillus stercorophilus</i> **	22,082
CLARASE L	<i>Aspergillus oryzae</i>	23,087

** denotes a recombinant strain

15

Ethanol fermentation was carried out using whole ground corn as described in Example 7. Alpha amylases from the sources listed in Table 2 were added at 1.0 SSU/gram of ground corn and glucoamylase at 0.5GAU/g. The samples were taken during the course of the fermentation and analyzed for ethanol content (Figure 7). After
20 the fermentation, the insoluble solids (DDGS) were separated and the residual starch content of the corn mash at pH 5.0 was determined. The results are summarized in Table 3.

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TABLE 3
Ave % v/v EtOH

GAU/g	Alpha Amylase at (1.0 SSU/g)	22 hr	46 hr	72 hr	Residual % starch in DDGS 72hr
0.5	-	7.77	11.56	14.44	29.8
0.5	SPEZYME LT-AA	7.72	11.56	14.78	30.8
0.5	SPEZYME FRED	7.84	11.77	14.59	30.8
0.5	SPEZYME Ethyl	7.94	11.82	14.57	29.1
0.5	CLARASE L	7.94	11.72	14.62	30.8
0.5	Tr-asaA	9.57	15.75	18.44	9.0

The results in Table 3 clearly show that Tr-asaA is very effective in aiding
5 glucoamylase to hydrolyze granular starch under the ethanol fermentation conditions
using yeast. Additionally, as observed from the table, % ethanol produced in the
fermentation (18.44) is greater and % residual starch in DDGS (9.0) is significantly lower
using the enzyme combination of the present invention.

10

EXAMPLE 9

Evaluation of whole ground wheat in the ethanol fermentation using Tr- asaA.

To a 36% slurry of whole ground wheat, dry corn steep liquor was added at 2.5%
based on the weight of the whole ground wheat. The fermentations were carried out in
15 125 ml flasks containing 100 gm of mash. The pH of the slurry was adjusted to 4.8 with
dilute sulfuric acid. The mash was diluted to a final concentration of 33.85% ds.

Glucoamylase (0.5 GAU/g ground wheat) and asaA (1.0 SSU/g whole ground
wheat) were added to the mash. This was followed by adding 3.0 ml of propagated yeast
to start the fermentation. Yeast inoculum was prepared by adding 0.26 gm of dry Fali
20 yeast to 100 gm of mash. The fermentations were run in a 32°C water bath while gently
stirred. At various time intervals samples of the fermentation broth (beer) were taken,
centrifuged for HPLC analysis of sugar composition and ethanol (Table 4)

TABLE 4

Whole ground wheat granular starch in the yeast fermentation for ethanol production

5

GA GAU/g	Tr-asaA SSU/g	Hrs	% w/v DP>2	% w/v DP-2	% w/v DP-1	% w/v Lactic	% w/v Glycerol	% v/v Ethanol
		0	0.98	0.97	2.00	0.22	0.12	0.00
0	0	24	1.33	0.00	0.02	1.09	0.15	2.42
0	0	48	1.17	0.00	0.00	1.39	0.13	2.38
0	0	72	1.08	0.00	0.01	1.38	0.13	2.16
0	0.1	24	1.30	0.00	0.02	1.06	0.16	4.49
0	0.1	48	1.16	0.00	0.02	1.51	0.16	2.28
0	0.1	72	1.23	0.00	0.01	1.83	0.15	2.91
0	0.25	24	1.28	0.03	0.02	1.06	0.17	2.94
0	0.25	48	1.05	0.00	0.02	1.41	0.15	2.73
0	0.25	72	1.24	0.00	0.03	1.92	0.17	3.06
0	0.5	24	1.25	0.00	0.01	1.02	0.13	3.03
0	0.5	48	1.22	0.00	0.02	1.60	0.18	3.27
0	0.5	72	1.26	0.00	0.03	1.90	0.18	3.24
0	0.75	24	1.29	0.03	0.02	1.06	0.16	3.21
0	0.75	48	1.29	0.00	0.03	1.62	0.10	3.57
0	0.75	72	1.34	0.00	0.03	1.90	0.18	3.60
0	1.0	24	1.29	0.04	0.02	1.04	0.16	3.43
0	1.0	48	1.32	0.04	0.01	1.55	0.18	4.02
0	1.0	72	1.46	0.09	0.04	1.84	1.21	4.15
0.2	0	24	1.18	0.00	0.00	1.04	0.18	3.34
0.2	0	48	1.16	0.00	0.02	1.67	0.19	4.14
0.2	0	72	1.16	0.00	0.02	1.92	0.19	4.78
0.2	0.1	24	1.20	0.00	0.03	1.05	0.20	3.64
0.2	0.1	48	1.12	0.00	0.02	1.59	0.20	4.60
0.2	0.1	72	1.14	0.00	0.03	1.86	0.21	5.58
0.2	0.25	24	1.16	0.00	0.03	1.02	0.21	3.80

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0.2	0.25	48	1.14	0.00	0.03	1.57	0.22	5.13
0.2	0.25	72	1.06	0.03	0.03	1.71	0.21	5.90
0.2	0.5	24	1.20	0.00	0.03	1.03	0.22	4.04
0.2	0.5	48	1.14	0.00	0.01	1.54	0.22	5.61
0.2	0.5	72	1.13	0.03	0.04	1.74	0.23	6.65
0.2	0.75	24	1.16	0.00	0.03	1.03	0.22	4.13
0.2	0.75	48	1.24	0.00	0.04	1.54	0.24	5.68
0.2	0.75	72	1.10	0.00	0.01	1.63	0.23	7.14
0.2	1.0	24	1.00	0.00	0.03	0.96	0.17	4.14
0.2	1.0	48	1.16	0.00	0.04	1.50	0.25	5.90
0.2	1.0	72	1.21	0.03	0.04	1.68	0.23	6.76
0.5	0	24	1.07	0.00	0.03	0.98	0.24	4.50
0.5	0	48	1.01	0.00	0.02	1.41	0.25	6.29
0.5	0	72	1.10	0.03	0.15	1.55	0.25	7.49
0.5	0.1	24	1.12	0.00	0.04	0.94	0.24	4.62
0.5	0.1	48	1.12	0.00	0.03	1.34	0.27	6.92
0.5	0.1	72	1.09	0.03	0.03	1.46	0.27	8.45
0.5	0.25	24	1.17	0.00	0.05	0.97	0.27	5.01
0.5	0.25	48	1.20	0.00	0.04	1.28	0.28	7.18
0.5	0.25	72	1.06	0.03	0.03	1.34	0.27	8.78
0.5	0.5	24	1.16	0.00	0.05	0.91	0.26	5.29
0.5	0.5	48	1.11	0.00	0.04	1.18	0.28	7.71
0.5	0.5	72	1.07	0.03	0.04	1.23	0.28	9.47
0.5	0.75	24	1.15	0.00	0.05	0.90	0.28	5.33
0.5	0.75	48	1.11	0.03	0.06	1.16	0.30	8.08
0.5	0.75	72	1.06	0.04	0.05	1.17	0.30	9.91
0.5	1.0	24	1.12	0.00	0.06	0.89	0.29	5.52
0.5	1.0	48	1.12	0.00	0.05	1.14	0.32	8.39

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EXAMPLE 10

Effect of substrate treatment on the ethanol yield and composition of
Distilleries Dry Grain Solids, (DDGS).

5

Whole ground corn substrate was subjected to a conventional dry milling process for fuel alcohol fermentation using a hammer mill to reduce particle size. Three different mashes were prepared.

Treatment 1 (Trt 1) is a high temperature treatment, which involved a batch
10 liquefaction of a 36% ds corn flour slurry containing 0.9% dry corn steep (DCS) with 3.5U/g SPEZYME ETHYL at pH 5.6 by jet cooking according to the prior art procedures. The slurry was place in a 90°C bath for 1.5 hours, mixed and then cooled to 30°C with a pH adjustment to 5.0 with dilute sulfuric acid. The slurry was further diluted with water to 32.71% ds.

15 Treatment 2 (Trt 2) is a low temperature treatment. The mash was prepared by incubating a 36% corn flour slurry containing 0.9% DCS with the pH adjusted to 5.0 with dilute sulfuric acid at 60°C for three hours. Prior to incubation 0.05 GAU/g of glucoamylase was added.

Treatment 3 (Trt 3) is a room temperature treatment - a corn slurry was obtained
20 at room temperature prior to use in the fermentation with 0.5 GAU glucoamylase/g of corn and 1.0 SSU/g corn of Tr-asaA.

Yeast fermentaiton was then carried out on each treatment as described in example 7.

After the fermentation, ethanol yield was determined and the insoluble solids
25 from each treatment were separated by centrifugation, dried at 60°C and the total carbohydrate content and nitrogen content were determined. The results are illustrated in Table 5, wherein Trt 3 is a process encompassed by the invention.

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TABLE 5

Comparison of ethanol yield and the composition of DDGS of different treatments of whole ground corn substrate under ethanol fermentation using yeast

5

Corn Mash Treatment	Kgs DDGS /MT corn	%Residual starch content in DDGS	%Total Protein in DDGS	Ethanol L/MT corn
Trt 1 High Temperature	326	4.8	27.5	402
Trt 2 Low Temperature	299	3.8	29.5	429
Trt 3 No heat treatment GA +Tr-asaA	274	3.5	31.6	438

As observed from the results illustrated in Table 5, the % residual starch in DDGS treated according to the process of the present invention (Trt 3) was less than the % residual starch obtained from the prior art treatment (Trt 1) or the low temperature treatment (Trt 2). The values were 3.5% (Trt 3) as opposed to 4.8% or 3.8% for Trt1 and Trt 2. The total protein content of the DDGS and the amount of ethanol produced was higher from Trt 3 according to the invention as compared to the prior art treatment (Trt 1).

15

EXAMPLE 11

Incubation of granular corn starch with purified *Aspergillus kawachi* alpha amylase and purified *Aspergillus niger* glucoamylase.

Enzyme Purification:

20

Aspergillus niger glucoamylase (GA) and *Aspergillus kawachi* alpha amylase (Akaa) were both purified from culture filtrate using a preparative high pressure liquid chromatographic (HPLC) method using an AKTA (Amersham Pharmacia, Biotech., NJ).

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In a typical experiment, both crude enzyme samples were desalted with 10 mM MES buffer (pH 5.75) to bring down the conductivity using a spin column (Bio-Rad, CA). The samples were brought up to 2M NH_4SO_4 . The sample was loaded on to a Q-Sepharose column (Amersham, Biosciences, NJ) and eluted with 20 mM MES buffer (pH 5.75) using a gradient of 1.5 M KCl. The fractions with corresponding activity were pooled together and concentrated for further experiments.

Incubation with granular corn starch with purified enzymes:

The purified enzyme preparations were added to a 4.0% granular corn starch (Cargill, Minneapolis, MN) in 0.1 M acetate buffer (pH 4.5) as follows for Scanning Electron Microscopic (SEM) analysis.

Purified GA at 0.5 GAU/g corn starch; purified AkAA at 1.0 SSU/g starch; and GA and AkAA combined were incubated at 32°C with gentle stirring. Aliquot samples (0.75 ml) were taken at intervals of 2, 4 and 8 hours, centrifuged and the soluble sugar determined by the method described in the examples above. The pellet was resuspended in distilled water (5 ml) and centrifuged. The pellet was suspended again in 5 ml of absolute ethanol (99%), stirred for uniform mixing and centrifuged. The alcohol treated pellet was air-dried in the tube and used for SEM analysis.

SEM analysis:

Approximately 200 μl dry volume was transferred to a 1.5 ml Ependorf tube and 0.8 ml absolute ethanol was added. The components were vortexed to make a suspension of starch particles. A few drops of suspension was placed on a freshly cleaned glass cover slip and allowed to air dry. The cover slip was mounted on specimen stubs with carbon adhesive tabs, painted around the circumference with colloidal silver adhesive (Electron Microscopy Sciences, Ft. Washington, PA) and coated with a thin layer of gold in a ScanCoat Six Sputter Coater (Edwards High Vacuum Intl. Crawley, UK). Scanning electron microscopy was done at 5kv in the secondary electron imaging mode using a Quanta 200 FEG scanning electron microscope (FEI Inc., Hillsboro, Ore) at instrumental magnification of 1,000 and 5,000 X. Eight to ten images were made from different areas on each sample stub. The effect of individual and combined enzyme treatments on the granular corn starch is illustrated in Figures 8 and 9.

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Hydrolysis and microscopic examination:

Reducing sugar (mg/ml reducing equivalents), measured as glucose released after 4 hours as a result of granular starch hydrolysis by AkAA and GA is illustrated in Figure 8. Degradation of granular starch with glucoamylase alone was 4.9 mg/ml; degradation of granular starch with AkAA alone was only 0.3 mg/ml. However, degradation of granular starch with the combination of GA and AkAA was 12.1 mg/ml. The degradation value for the enzymes combined illustrates a synergistic interaction, which is significantly greater than the additive value of the enzymes.

The starch granules treated as described in this example were observed with a scanning electron microscope. As shown in Figure 9, corn starch granules incubated with purified AkAA did show minor surface modification. These were observed as small pin prick holes. Corn starch granules incubated with purified GA showed many small defined deep holes. Significantly, corn starch granules incubated with the combination of GA and AkAA showed numerous wide and deep penetrating cavities. Additionally, surface erosion, which exposed the layered structure of the granular center, was observed in the granules incubated with the combination of GA and AkAA.

EXAMPLE 12

Effect of % Dry Solids Content (ds) of a Granular Corn Starch Slurry
on Ethanol Yield

Corn flour was slurried with water to obtain a 36% ds mash. A small amount of corn steep (0.2% of the slurry) was added along with 400 ppm (0.04%) urea to the mash prior to adjusting the pH to 4.5 with sulfuric acid. The dry solids content of the slurry was adjusted from 20 to 36% ds. The fermentations were carried out in 125 ml flasks containing a total of 100 g mash. The enzymes were diluted so that a constant volume of 0.5 ml was used for each enzyme. Each flask was inoculated with 3 ml of yeast that was propagated 17 hours prior to use. The yeast propagation procedure involved adding 0.5% dry Fali yeast to 25% ds mash containing 0.5 GAU/g of GA and 1.5 SSU/g AkAA and incubating while gently mixing in a 32°C water bath. At approximately 24 hour time intervals samples of beer were dried at 60°C to obtain DDGS.

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Table 6
Effect of DS Content on the Ethanol Production and
Residual Starch in DDGS at 75 hours

5

%DS	GA (0.5 GAU/g)		AkAA (1.5 SSU/g) + GA (0.5 GAU/g)	
	% v/v EtOH	DDGS % residual starch	% v/v EtOH	DDGS % residual starch
20	9.86	2.28	10.15	1.37
24	11.75	5.79	12.51	1.00
28	13.51	13.07	14.80	1.83
32	15.38	18.06	17.47	3.78
36	16.39	29.37	18.07	13.36

Almost all of the glucose (DP-1) generated during the fermentation was converted to ethanol except at the high solids (data not shown). For each % DS tested, the AkAA increased the rate and amount of ethanol produced. In all instances the % starch in the DDGS is decreased when the AkAA is used in combination with the GA and further the % starch found in DDGS from a corn starch substrate having a % ds as high as 36% is reduced by half when compared to the % starch in a DDGS without the addition of AkAA. Figure 10 shows as the % ds in the corn slurry increases, the influence of AkAA on ethanol production increases. These results demonstrate the positive effect AkAA has on hydrolyzing granular starch and particularly at high solids %.

20

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CLAIMS

1. A method for producing an acid stable alpha amylase (asAA) having granular starch hydrolyzing (GSH) activity in a filamentous fungal host cell comprising
 - a) transforming a filamentous fungal host cell with a DNA construct including a promoter having transcriptional activity in the filamentous fungal host cell operably linked to a heterologous polynucleotide encoding an asAA having GSH activity and at least 90% sequence identity to SEQ ID NO: 3,
 - b) cultivating the transformed filamentous fungal host cell in a suitable culture medium to allow expression of said asAA, and
 - c) producing the asAA.
2. The method according to claim 1 further comprising recovering the produced asAA.
3. The method according to claim 1, wherein the filamentous fungal host cell is a *Trichoderma* cell.
4. The method according to claim 3, wherein the *Trichoderma* cell is a *T. reesei* cell.
5. The method according to claim 1, wherein the filamentous fungal host cell is an *Aspergillus* cell.
6. The method according to claim 5, wherein the *Aspergillus* cell is an *A. niger* cell.
7. The method according to claim 1, wherein the asAA has at least 95% sequence identity with SEQ ID NO: 3.
8. The method according to claim 1, wherein the asAA has at least 98% sequence identity with SEQ ID NO: 3.

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9. An enzyme composition comprising the asAA produced according to claim 1.
10. The method according to claim 2, further comprising purifying the recovered asAA.
11. An enzyme composition comprising the asAA produced according to claim 10.
12. The method according to claim 1, wherein the asAA is encoded by a polynucleotide having the sequence of SEQ ID NO: 1.
13. The *Trichoderma* cell obtained according to the method of claim 3.
14. An enzyme composition comprising a) an acid stable alpha amylase (asAA) having granular starch hydrolyzing activity and having at least 90% sequence identity with the amino acid sequence of SEQ ID NO: 3 and b) a glucoamylase.
15. The enzyme composition of claim 14, wherein the ratio of asAA to glucoamylase activity is 10: 1 to 1: 10.
16. The enzyme composition of claim 15, wherein the ratio of asAA to glucoamylase activity is 5: 1 to 1: 5.
17. The enzyme composition of claim 16, wherein the ratio of asAA to glucoamylase is 4:1 to 1:2.
18. The enzyme composition of claim 14, wherein the asAA is obtained from the production of asAA in a *Trichoderma* host.
19. The enzyme composition of claim 18, wherein the *Trichoderma* host is *T. reesei*.
20. The enzyme composition of claim 14, wherein the asAA has at least 95% sequence identity with SEQ ID NO: 3.

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21. The enzyme composition of claim 20, wherein the asAA has at least 98% sequence identity with SEQ ID NO: 3.

22. The enzyme composition of claim 14, wherein the glucoamylase is produced from a strain of *Humicola*, *Rhizopus* or *Aspergillus*.

23. The enzyme composition of claim 14, wherein the glucoamylase is produced from a strain of *Aspergillus*.

24. The enzyme composition of claim 23, wherein the glucoamylase is produced from a strain of *Aspergillus niger*.

25. The enzyme composition of claim 14 further comprising a third enzyme.

26. The enzyme composition of claim 25, wherein the third enzyme is selected from the group consisting of proteases, pullulanases and cellulases.

27. The enzyme composition of claim 14, wherein the composition is used for simultaneous saccharification and fermentation.

28. The enzyme composition of claim 14, wherein the composition is contemporaneously combined with a slurry of a granular starch substrate and ethanologenic microorganisms in a fermentation under suitable fermentation conditions to produce an alcohol.

29. The enzyme composition of claim 28, wherein the granular starch substrate is obtained from corn, wheat or rice.

30. The enzyme composition of claim 29, wherein the granular starch substrate is obtained from corn.

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31. A granular starch hydrolyzing enzyme composition comprising an acid stable alpha amylase (asAA) having granular starch hydrolyzing activity and having at least 90% sequence identity with the amino acid sequence of SEQ ID NO: 3.

32. A fermentation medium comprising an acid stable alpha amylase (asAA) having granular starch hydrolyzing activity produced from a culture of *Trichoderma* cells, said *Trichoderma* cells comprising a heterologous polynucleotide encoding the asAA which has at least 90% sequence identity with SEQ ID NO: 3.

33. The fermentation medium of claim 32, wherein the *Trichoderma* cells are *T. reesei*.

34. The fermentation medium of claim 32, wherein the heterologous polynucleotide encodes an asAA having at least 95% sequence identity with SEQ ID NO: 3.

FIGURE 1A

ATGAGAGTGTGCGACTTCAAGTATTGCCCTTGCTGTGTCCCTTTTTGGGAAGCTGGCC
CTTGGGCTGTGAGCTGCAGAATGGCGCACTCAATCCATCTACTTCCTTTTGACGGAT
CGGTTCGGTAGGACGGACAATTCGACTACAGCTACGTGCAATACGGGTGACCAAGTA
TGGTATTGCTGTACTTCCGTCATTTCATCTGCTGACTTGGATAGATCTACTGTGGTGG
AAGTTGGCAAGGAATTATCAACCATGTTTCGTATCTCACTTCATACCATCCATGCTGG
GCGCTTCTGACTATTGCTCCAGCTGGACTATATCCAGGGCATGGGATTCACAGCTAT
CTGGATCTCGCCTATCACTGAGCAGCTACCCAGGATACTTCGGATGGTGAAGCCTA
CCATGGATACTGGCAGCAGAAGATGTATGCCCTCATTGCATTTCATATTTATGCTTA
CTCGCAGACTGCAGCTGACTTGGCAGATACAATGTGAACTCCAACCTTCGGCACGGCA
GATGATCTGAAGTCCCTCTCCGATGCTCTTCACGCCCCGCGAATGTACCTCATGGTC
GACGTCGTCCCTAACCACATGGTAAGTACTGCTTTACCTCTATATTAGTAAACCCAA
TGCGAACAATGACTGTATCAGGGCTACGCAGGTAACGGCAACGATGTGGATTACAGC
GTCTTCGACCCCTTCGACTCCTCCTCTACTTCCATCCATACTGCCTCATCACAGAT
TGGGACAACCTTGACCATGGTCCAAGACTGTTGGGAGGGTGACACCATCGTGTCTCTG
CCAGATCTGAACACCACGGAAACCGCCGTGAGAACCATTTGGTACGATTGGGTAGCC
GACCTGGTATCCAACCTACTCAGGTGCGACCCCAACCCACTAAAACAAGCCACATACT
AAAAAATTGCTCAGTCGACGGCCTCCGTATCGACAGTGTGGAAGAAGTCGAACCCGA
CTTCTTCCCGGGCTACCAAGAAGCAGCAGGAGTCTACTGCGTCGGTGAAGTCGACAA
CGGCAACCCTGCTCTCGACTGCCCATACCAAAAATATCTAGATGGTGTCTCAACTA
TCCCATGTACATACCCCTTCTACCTTCTCGAACCCATCACTAACTCAATTGCTGCA
GCTACTGGCAACTCCTCTACGCCTTTGAATCCTCCAGCGGCAGCATCAGCAACCTCT
ACAACATGATCAAATCCGTGCGCAGCGACTGCTCCGATCCGACCCCTCCTGGGCAACT
TTATCGAAAACCACGACAACCCCCGCTTCGCCTCGTATGTCCCTTCCATCACTGCCC
CCTTTTAAAGTAAACCCCACTGACAGGCAAAGCTACACATCCGACTACTCCCAAGCC
AAAAACGTCTCAGCTACATCTTCCTCTCCGACGGCATCCCCATCGTCTACGCCGGC
GAAGAACAGCACTACTCCGGCGGGCAGCTGCCCTACAACCGGAAGCTACCTGGCTA
TCAGGCTACGACACCTCCGCGGAGCTCTACACCTGGATAGCCACCACAAACGCGATC
CGGAAACTAGCTATCTCAGCAGACTCGGACTACATTACTTACGCGGTTTGCCCTTTC
CCTTCCCCCACCAGAGCTCAACCCCATTCCTAACAAAATATTTCAATGGTAGAA

FIGURE 1B

CGACCCAATCTACACAGACAGCAACACCATCGCGATGCGCAAAGGCACCTCCGGCTC
CCAAATCATCACCGTCCTCTCCAACAAAGGCTCCTCCGGAAGCAGCTACACCTCAC
CCTCAGCGGAAGCGGCTACACGTCCGGCACGAAGCTCATCGAAGCGTACACCTGCAC
GTCCGTGACGGTGGACTCGAACGGGGATATCCCTGTGCCGATGGCTTCGGGATTACC
TAGAGTTCTCCTCCCTGCTTCGGTGGTTGATAGTTCTTCGCTTTGTGGGGGGAGTGG
TAACACAACCACGACCACAACCTGCTGCTACCTCCACATCCAAAGCCACCACCTCCTC
TTCTTCTTCTTCTGCTGCTGCTACTACTTCTTCATCATGCACCGCAACAAGCACCAC
CCTCCCCATCACCTTCGAAGAACTCGTCACCACTACCTACGGGGAAGAAGTCTACCT
CAGCGGATCTATCTCCCAGCTCGGAGAGTGGGATACGAGTGACGCGGTGAAGTTGTC
CGCGGATGATTATACCTCGAGTAACCCCGAGTGGTCTGTTACTGTGTCGTTGCCGGT
GGGGACGACCTTCGAGTATAAGTTTATTAAGGTCGATGAGGGTGGAAGTGTGACTTG
GGAAAGTGATCCGAATAGGGAGTATACTGTGCCTGAATGTGGGAGTGGGAGTGGGGA
GACGGTGGTTGATACGTGGAGGTAG

FIGURE 2: SEQ ID NO. 4

MRVSTSSIALAVSLFGKLALGLSAAEWRTQSIYFLLTDRFGRTDNSTTA
TCNTGDQIYCGGSWQGIINHLDYIQGMGFTAIWISPIEQLPQDTS DGE
AYHGYWQQKIYNVNSNFGTADDLKSLS DALHARGMYLMVDVVPNHMGYA
GNGNDVDYSVFDPF DSSSYFHPYCLITDWDNLTMVQDCWEGDTIVSLPD
LNTTETAVRTIWYDWVADLVSNYSVDGLRIDSVEEVEP DFFPGYQEAAG
VYCVGEVDNGNPALDCPYQKYLDGVLNYPPIYWQLLYAFESSSGSISNLY
NMIKSVASDCSDPTLLGNFIENHDNPRFASYTSDYSQAKNVLSYIFLSD
GIPIVYAGEEQHYSGGDVPYNREATWLSGYD TSAELYTWIATTNAIRKL
AISADSDYITYANDPIYTDSNTIAMRKGTSGSQIITVLSNKGSSGSSYT
LTLSGSGYTSGTKLIEAYTCTSVTVDSNGDIPVPMASGLPRVLLPASVV
DSSSLCGSGNTTTTTTAATSTSKATTSSSSSSAAATTSSSCTATSTTL
PITFEELVTTYGEEVYLSGSISQLGEWDTSDAVKLSADDYTSSNPEWS
VTVSLPVGTTFEYKFIKVDEGGSVTWESDPNREYTVPECGSGSGETVVD
TWR

FIGURE 3A: SEQ ID NO. 5

CTGCAGCCACTTGCAGTCCCGTGGAATTCTCACGGTGAATGTAGGCCTTTTGTAG
GGGTAGGAATTGTCACTCAAGCACCCCAACCTCCATTACGCCTCCCCCATAGA
GTTCCCAATCAGTGAGTCATGGCACTGTTCTCAAATAGATTGGGGAGAAGTTGA
CTTCCGCCCCAGAGCTGAAGGTGCGACAACCGCATGATATAGGGTCCGGCAACGG
CAAAAAAGCACGTGGCTCACCGAAAAGCAAGATGTTTGCGATCTAACATCCAGG
AACCTGGATACATCCATCATCACGCACGACCACTTTGATCTGCTGGTAAACTCG
TATTCGCCCTAAACCGAAGTGCCTGGTAAATCTACACGTGGGCCCTTTCCGGTA
TACTGCGTGTGCTTCTCTAGGTGCCATTCTTTCCCTTCTCTAGTGTTGAATT
GTTTGTGTTGGAGTCCGAGCTGTAACCTCTGAATCTCTGGAGAATGGTGGA
CTAACGACTACCGTGACCTGCATCATGTATATAATAGTGATCCTGAGAAGGGG
GGTTTGGAGCAATGTGGGACTTTGATGGTCATCAAACAAAGAACGAAGACGCCCT
CTTTTGCAAAGTTTTGTTTCGGCTACGGTGAAGAACTGGATACTTGTGTCTCT
CTGTGTATTTTTGTGGCAACAAGAGGCCAGAGACAATCTATTCAAACACCAAGC
TTGCTCTTTTGAGCTACAAGAACCTGTGGGGTATATCTAGAGTTGTGAAGTCG
GTAATCCCGCTGTATAGTAATACGAGTCGCATCTAAATACTCCGAAGCTGCTGC
GAACCCGGAGAATCGAGATGTGCTGGAAAGCTTCTAGCGAGCGGCTAAATTAG
CATGAAAGGCTATGAGAAATTCTGGAGACGGCTTGTTGAATCATGGCGTTCCAT
TCTTCGACAAGCAAAGCGTTCCGTGCGAGTAGCAGGCACTCATTCCCGAAAAAA
CTCGGAGATTCTAAGTAGCGATGGAACCGGAATAATATAATAGGCAATACATT
GAGTTGCCTCGACGGTTGCAATGCAGGGGTACTGAGCTTGGACATAACTGTTT
CGTACCCACCTCTTCTCAACCTTTGGCGTTTCCCTGATTCAGCGTACCCGTAC
AAGTCGTAATCACTATTAACCCAGACTGACCGGACGTGTTTTGCCCTTCATTTGG
AGAAATAATGTCATTGCGATGTGTAATTTGCCTGCTTGACCGACTGGGGCTGTT
CGAAGCCCGAATGTAGGATTGTTATCCGAACCTCTGCTCGTAGAGGCATGTTGTG
AATCTGTGTGCGGGCAGGACACGCCTCGAAGGTTACCGGCAAGGGAAACCACCG
ATAGCAGTGTCTAGTAGCAACCTGTAAAGCCGCAATGCAGCATCACTGGAAAT
ACAAACCAATGGCTAAAAGTACATAAGTTAATGCCTAAAGAAGTCATATACCAGC
GGCTAATAATTGTACAATCAAGTGGCTAAACGTACCGTAATTTGCCAACGGCTT
GTGGGGTTGCAGAAGCAACGGCAAAGCCCCCACTTCCCCACGTTTGTTCCTCAC
TCAGTCCAATCTCAGCTGGTGATCCCCCAATTGGGTGCTTGTTTGTTCGGTG
AAGTGAAGAAGACAGAGGTAAGAATGTCTGACTCGGAGCGTTTTGCATACAAC
CAAGGGCAGTGATGGAAGACAGTGAAATGTTGACATTCAAGGAGTATTTAGCCA
GGGATGCTTGAGTGATCGTGTAAGGAGGTTTGTCTGCCGATACGACGAATACT
GTATAGTCACTTCTGATGAAGTGGTCCATATTGAAATGTAAGTCGGCACTGAACA
GGCAAAAGATTGAGTTGAAACTGCCTAAGATCTCGGGCCCTCGGGCCCTTCGGC
CTTTGGGTGTACATGTTTGTGCTCCGGGCAAAATGCAAAGTGTGGTAGGATCGAA
CACACTGCTGCCTTTACCAAGCAGCTGAGGGTATGTGATAGGCAAATGTTTCAGG
GGCCACTGCATGGTTTCGAATAGAAAGAGAAGCTTAGCCAAGAACAATAGCCGA
TAAAGATAGCCTCATTAAACGGAATGAGCTAGTAGGCAAAGTCAGCGAATGTGT
ATATATAAAGGTTTCGAGGTCCGTGCCTCCCTCATGCTCTCCCCATCTACTCATCA
ACTCAGATCCTCCAGGAGACTTGTACACCATCTTTTGAGGCACAGAAACCCAAT
AGTCAACCATCACAAGTTTGTACAAAAAGCAGGCTCCGCGGCCGCCCTTCA
cCATGAGAGTGTGCACTTCAAGTATTGCCCTTGCTGTGTCCCTTTTGGGAAGCT
GGCCCTTGGGCTGTCAGCTGCAGAATGGCGCACTCAATCCATCTACTTCTTTT
GACGGATCGGTTCCGTAGGACGGACAATTGACTACAGCTACGTGCAATACGG
GTGACCAAGTATGGTATTGCTGTACTTCCGTCAATCATCTGCTGACTTGGATAGA
TCTACTGTGGTGAAGTTGGCAAGGAATTATCAACCATGTTGATCTCACTTCA
TACCATCCATGCTGGGCGCTTCTGACTATTGCTCCAGCTGGACTATATCCAGGG
CATGGGATTACAGCTATCTGGATCTCGCCTATCACTGAGCAGCTACCCAGGA
TACTTCGGATGGTGAAGCCTACCATGGATACTGGCAGCAGAAGATGTATGCCCT
CATTGCATTCATATTTTATGCTTACTCGCAGACTGCAGCTGACTTGGCAGATACA
ATGTGAACTCCAACCTTCGGCACGGCAGATGATCTGAAGTCCCTCTCCGATGCTC

FIGURE 3B

TTCACGCCCCGCGGAATGTACCTCATGGTCGACGTCGTCCCTAACCACATGGTAA
GTACTGCTTTACCTCTATATTAGTAAACCCAATGCGAACAATGACTGTATCAGGG
CTACGCAGGTAACGGCAACGATGTGGATTACAGCGTCTTCGACCCCTTCGACTC
CTCCTCCTACTTCCATCCATACTGCCTCATCACAGATTGGGACAACCTTGACCATG
GTCCAAGACTGTTGGGAGGGTGACACCATCGTGTCTCTGCCAGATCTGAACAC
CACGGAACCGCCGTGAGAACCATTTGGTACGATTGGGTAGCCGACCTGGTAT
CCAACTACTCAGGTGCGACCCCAACCCACTAAAACAAGCCACATACTAAAAAT
TGCTCAGTCGACGGCCTCCGTATCGACAGTGTGGAAGAAGTCGAACCCGACTT
CTTCCCGGGCTACCAAGAAGCAGCAGGAGTCTACTGCGTCGGTGAAGTCGACA
ACGGCAACCCTGCTCTCGACTGCCCATACCAAAAATATCTAGATGGTGTCTCA
ACTATCCCATGTACATACCCCCTTCTACCTTCTCGAACCCTACTAACTCAATT
GCTGCAGCTACTGGCAACTCCTCTACGCCTTTGAATCCTCCAGCGGCAGCATCA
GCAACCTCTACAACATGATCAAATCCGTGCGCAGCGACTGCTCCGATCCGACCC
TCCTGGGCAACTTTATCGAAAACCAGACAACCCCGCTTCGCCTCGTATGTCC
CTTCCATCACTGCCCCCTTTTAAAGTAAACCCCACTGACAGGCAAAGCTACACA
TCCGACTACTCCCAAGCCAAAACGTCCTCAGCTACATCTTCTCTCCGACGGC
ATCCCCATCGTCTACGCGGCGAAGAACAGCACTACTCCGGCGGGCGACGTGCC
CTACAACCGCGAAGCTACCTGGCTATCAGGCTACGACACCTCCGCGGAGCTCT
ACACCTGGATAGCCACCACAAACGCGATCCGGAACTAGCTATCTCAGCAGACT
CGGACTACATTACTTACGCGGTTTGCCCTTTCCCTTCCCCCACCAGAGCTCA
ACCCCCATTCTAACAAAATATTTCAATGGTAGAACGACCCAATCTACACAGACAG
CAACACCATCGCGATGCGCAAAGGCACCTCCGGCTCCCAAATCATCACCGTCC
TCTCCAACAAAGGCTCCTCCGGAAGCAGCTACACCCTCACCTCAGCGGAAGC
GGCTACACGTCCGGCACGAAGCTCATCGAAGCGTACACCTGCACGTCCGTGAC
GGTGGACTCGAACGGGGATATCCCTGTGCCGATGGCTTCGGGATTACCTAGAG
TTCTCCTCCCTGCTTCGGTGGTTGATAGTTCTTCGCTTTGTGGGGGAGTGGTA
ACACAACCACGACCACAACTGCTGCTACCTCCACATCCAAAGCCACCACCTCCT
CTTCTTCTTCTTCTGCTGCTGCTACTACTTCTTCATCATGCACCGCAACAAGCAC
CACCTCCCCATCACCTTCGAAGAACTCGTCACCACTACCTACGGGGAAGAAGT
CTACCTCAGCGGATCTATCTCCAGCTCGGAGAGTGGGATACGAGTGACGCGG
TGAAGTTGTCCGCGGATGATTATACCTCGAGTAACCCCGAGTGGTCTGTTACTG
TGTCGTTGCCGGTGGGGACGACCTTCGAGTATAAGTTTATTAAGGTGATGAGG
GTGGAAGTGTGACTTGGGAAAGTGATCCGAATAGGGAGTATACTGTGCCTGAAT
GTGGGAGTGGGAGTGGGGAGACGGTGGTTGATACGTGGAGGTAGAAGGGTGG
GCGCGCCGACCCAGCTTTCTTGTAACAAGTGGTGATCGCGCCAGCTCCGTGCG
AAAGCCTGACGCACCGGTAGATTCTTGGTGAGCCCGTATCATGACGGCGGCGG
GAGCTACATGGCCCCGGGTGATTTATTTTTTTGTATCTACTTCTGACCCTTTTC
AAATATACGGTCAACTCATCTTCACTGGAGATGCGGCCTGCTTGGTATTGCGA
TGTTGTGAGCTTGGCAAATTGTGGCTTTCGAAAACACAAAACGATTCTTAGTAG
CCATGCATTTTAAGATAACGGAATAGAAGAAAGAGGAAATTAACAAAAA
AACAAACATCCCGTTCATAACCCGTAGAATCGCCGCTCTTCGTGTATCCAGTA
CCAGTTTATTTTGAATAGCTCGCCCGCTGGAGAGCATCCTGAATGCAAGTAACA
ACCGTAGAGGCTGACACGGCAGGTGTTGCTAGGGAGCGTCGTGTTCTACAAGG
CCAGACGTCTTCGCGGTTGATATATATGTATGTTTGAAGTGCAGGCTGCTCAGCG
ACGACAGTCAAGTTCGCCCTCGCTGCTTGTGCAATAATCGCAGTGGGGAAGCC
ACACCGTGACTCCCATCTTTCAGTAAAGCTCTGTTGGTGTATCAGCAATACAC
GTAATTTAACTCGTTAGCATGGGGCTGATAGCTTAATTACCGTTTACCAGTGCC
ATGGTCTGTCAGCTTTCCTTGGCCCGTAAAATTGCGCGAAGCCAGCCAATCACC
AGCTAGGCACCAGCTAAACCCTATAATTAGTCTCTTATCAACACCATCCGCTCCC
CCGGGATCAATGAGGAGAATGAGGGGGATGCGGGGCTAAAGAAGCCTACATAA
CCCTCATGCCAACTCCAGTTTACACTCGTCGAGCCAACATCCTGACTATAAGC
TAACACAGAATGCCTCAATCCTGGGAAGAACTGGCCGCTGATAAGCGCGCCCG

FIGURE 3C

CCTCGCAAAAACCATCCCTGATGAATGGAAAGTCCAGACGCTGCCTGCGGAAG
ACAGCGTTATTGATTTCCCAAAGAAATCGGGGATCCTTTCAGAGGCCGAAGTGA
AGATCACAGAGGCCTCCGCTGCAGATCTTGTGTCCAAGCTGGCGGCCGGAGAG
TTGACCTCGGTGGAAGTTACGCTAGCATTCTGTAAACGGGCAGCAATCGCCCA
GCAGTTAGTAGGGTCCCCTCTACCTCTCAGGGAGATGTAACAACGCCACCTTAT
GGGACTATCAAGCTGACGCTGGCTTCTGTGCAGACAAACTGCGCCACAGATT
CTTCCCTGACGCCGCTCTCGCGCAGGCAAGGGAAGTTCGATGAATACTACGCAA
AGCACAAGAGACCCGTTGGTCCACTCCATGGCCTCCCCATCTCTCTCAAAGACC
AGCTTCGAGTCAAGGTACACCGTTGCCCTAAGTCGTTAGATGTCCCTTTTTGT
CAGCTAACATATGCCACCAGGGCTACGAAACATCAATGGGCTACATCTCATGGC
TAAACAAGTACGACGAAGGGGACTCGGTTCTGACAACCATGCTCCGCAAAGCC
GGTGCCGTCTTCTACGTCAAGACCTCTGTCCCGCAGACCCTGATGGTCTGCGA
GACAGTCAACAACATCATCGGGCGCACCGTCAACCCACGCAACAAGAACTGGT
CGTGCGGCGGCAGTTCTGGTGGTGAGGGTGCATCGTTGGGATTCTGGTGG
CGTCATCGGTGTAGGAACGGATATCGGTGGCTCGATTGAGTGCCGGCCGCGT
TCAACTTCCTGTACGGTCTAAGGCCGAGTCAATGGGCGGCTGCCGTATGCAAAG
ATGGCGAACAGCATGGAGGGTCAGGAGACGGTGCACAGCGTTGTGCGGCCGA
TTACGCACTCTGTTGAGGGTGAGTCCTTCGCTCTTCTTCTTTCTGCTCTAT
ACCAGGCCTCCACTGTCCTCCTTCTTCTTCTTCTTCTTCTTCTTCTGCTCTAT
GTCACTGATGAAGTATGTTAGACCTCCGCTCTTCAACCAATCCGTCTCGGT
AGGAGCCATGGAATACGACTCCAAGGTCATCCCCATGCCCTGGCGCCAGTCC
GAGTCGGACATTATTGCCTCCAAGATCAAGAACGGCGGGCTCAATATCGGCTAC
TACAACCTCGACGGCAATGTCTTCCACACCTCCTATCCTGCGCGCGTGGAA
ACCACCTCGCCGCACTCGCCAAAGCCGGTCACACCGTGACCCCGTGACGCG
CATACAAGCAGCATTTTCGGCCACGATCTCATCTCCCATATCTACGCGGCTGACG
GCAGCGCCGACGTAATGCGCGATATCAGTGCATCCGGCGAGCCGGCGATTCCA
AATATCAAAGACCTACTGAACCCGAACATCAAAGCTGTTAACATGAACGAGCTCT
GGGACACGCATCTCCAGAAGTGGAATTACCAGATGGAGTACCTTGAGAAATGG
CGGGAGGCTGAAGAAAAGGCCGGGAAGGAACTGGACGCCATCATCGCGCCGA
TTACGCTACCGCTGCGGTACGGCATGACCAGTTCGGTACTATGGGTATGCC
TCTGTGATCAACCTGCTGGATTTACGAGCGTGGTTGTTCCGGTTACCTTTGCG
GATAAGAACATCGATAAGAAGAATGAGAGTTTCAAGGCGGTTAGTGAGCTTGAT
GCCCTCGTGACGGAAGAGTATGATCCGGAGGCGTACCATGGGGCACCGGTTG
CAGTGCAGGTTATCGGACGGAGACTCAGTGAAGAGAGGACGTTGGCGATTGCA
GAGGAAGTGGGGAAGTTGCTGGGAAATGTGGTGACTCCATAGCTAATAAGTGT
CAGATAGCAATTTGCACAAGAAATCAATACCAGCAACTGTAAATAAGCGCTGAA
GTGACCATGCCATGCTACGAAAGAGCAGAAAAAACCTGCCGTAGAACCGAAG
AGATATGACACGCTTCCATCTCTCAAAGGAAGAATCCCTTCAGGGTTGCGTTTC
CAGTCTAGACACGTATAACGGCACAAGTGTCTCTCACCAATGGGTTATATCTC
AAATGTGATCTAAGGATGGAAGCCGAGAATATCGATCGCGCGCAGATCCATAT
ATAGGGCCCCGGTTATAATTACCTCAGGTGACGTCCTCATGGCCATTGGAATTC
GTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCCA
CACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCTAATGAGT
GAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAA
CCTGTCTGCGCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATC
CACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAA
AGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCAGAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
GACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCT
CTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTCTCCCTTCGG

FIGURE 3D

GAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGG
TCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC
TGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGA'CACGACTTA
TCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGG
CGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAAC
AGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGG
TAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTG
CAAGCAGGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTAAAGGGATTTTGGT
CATGAGATTATCAAAAAGGATCTTACCTAGATCCTTTTAAATTAATAAATGAAGTT
TTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTA
ATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCT
GACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGTTTATCAGC
AATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAAGTGGTCTGCAACTTTAT
CCGCCTCCATCCAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTTCGC
CAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTAC
GCTCGTCGTTTGGTATGGCTTCATTACGCTCCGGTTCCTAACGATCAAGGCGAG
TTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCTCTCGA
TCGTTGTCAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTATGGCAGCAC
TGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGA
GTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAGTGCT
CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTT
GAGATCCAGTTCGATGTAACCCACTCGTGACCCAACTGATCTTCAGCATCTTTT
ACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAA
AAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCTTTTCAAT
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATG
TATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCC
ACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT
ATCACGAGGGCCCTTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGA
CACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAG
CAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGGTGTCGGGGGCTGG
CTTAACATGCGGCATCAGAGCAGATTGTAAGTGCACCATAAAAATTGTA
AACGTTAATATTTTGTAAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTT
AACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCGAG
ATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTG
GACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGGATGGCCCACTACG
TGAACCATCACCCAAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAA
TCGGAACCCCTAAAGGGAGCCCCCGATTAGAGCTTGACGGGGAAAGCCGGCGA
ACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCT
GGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATG
CGCCGCTACAGGGCGCGTACTATGGTTGCTTTGACGTATGCGGTGTGAAATAC
CGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCATTGCGCATTACAGG
CTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCA
GCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTAAACGCCAGGG
TTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGCCCAAGCTTACTAGTAC
TTCTCGAGCTCTGTACATGTCCGGTCGCGACGTACGCGTATCGATGGCGCCAG
CTGCAGGCGGCGCGC

FIGURE 4.

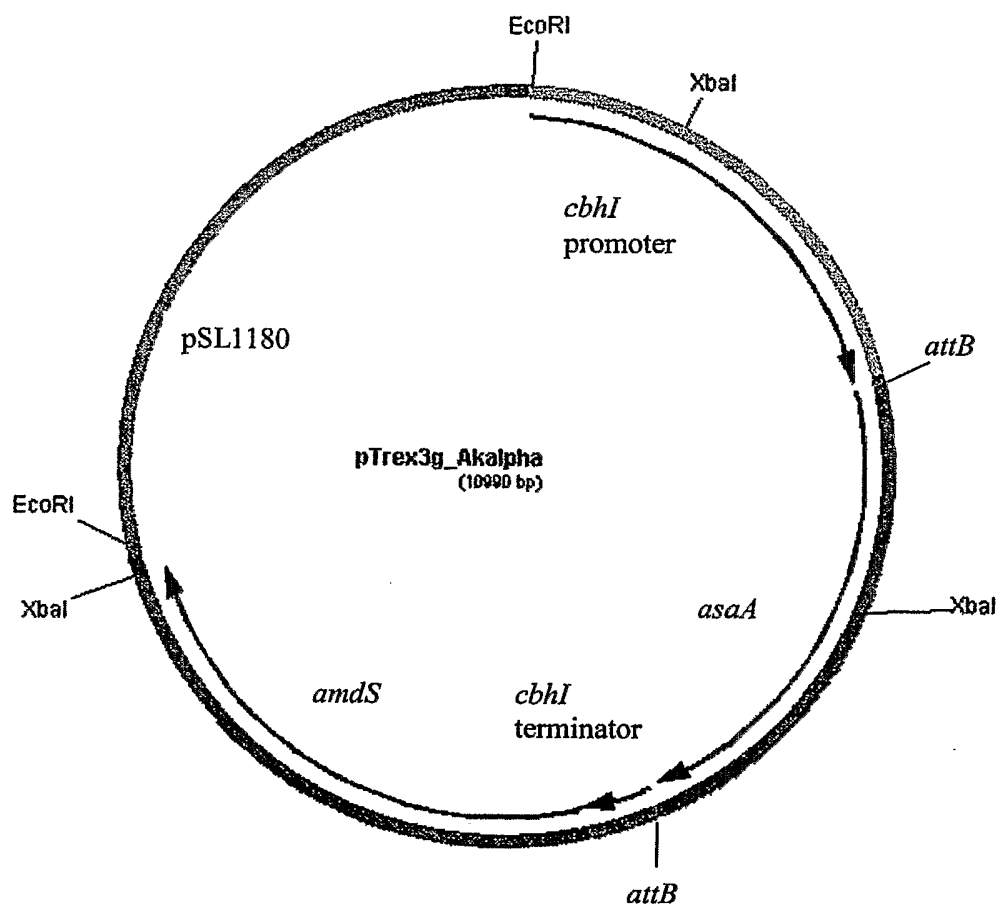
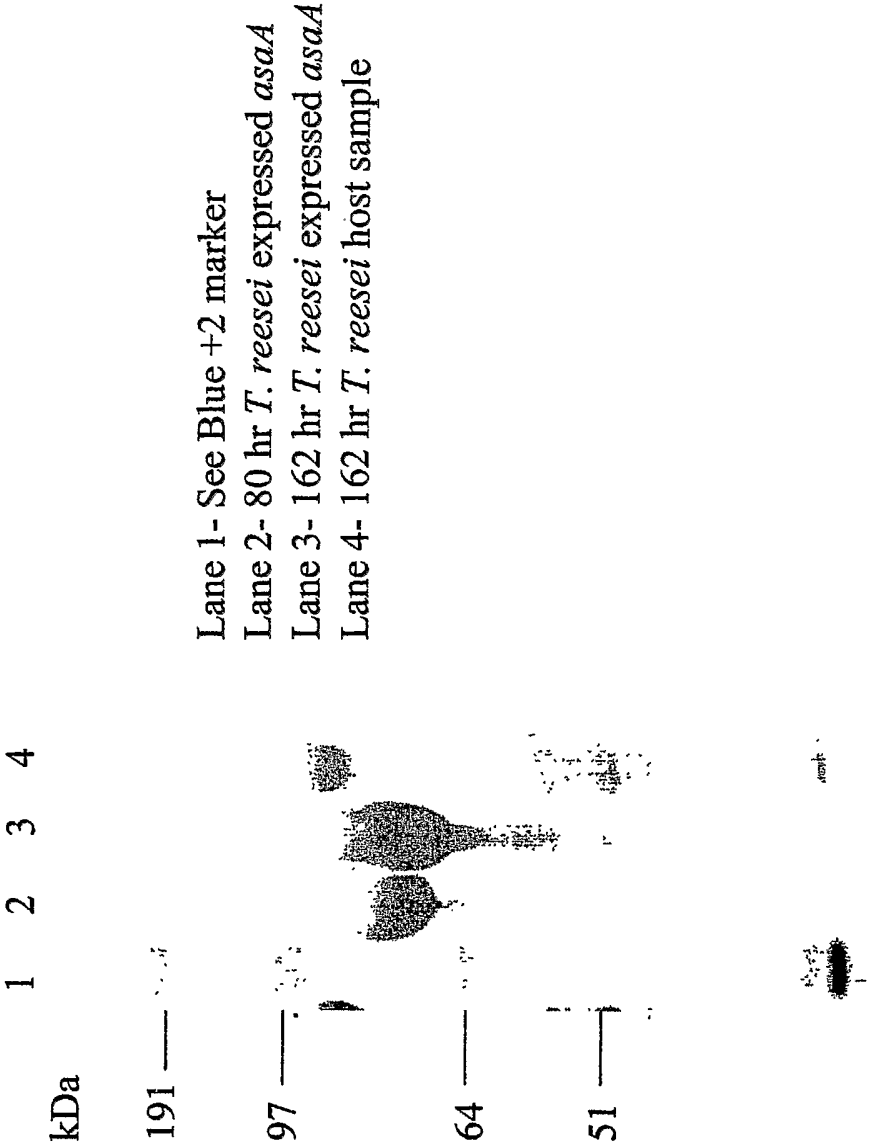


FIG. 5



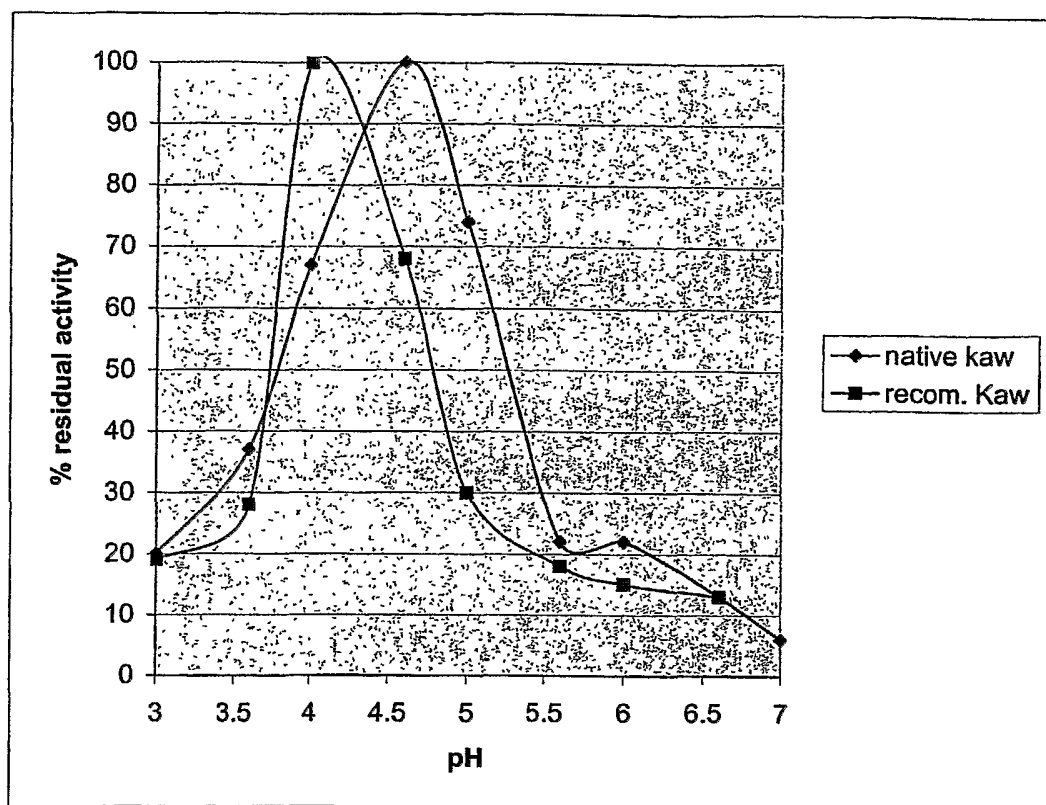


FIGURE 6

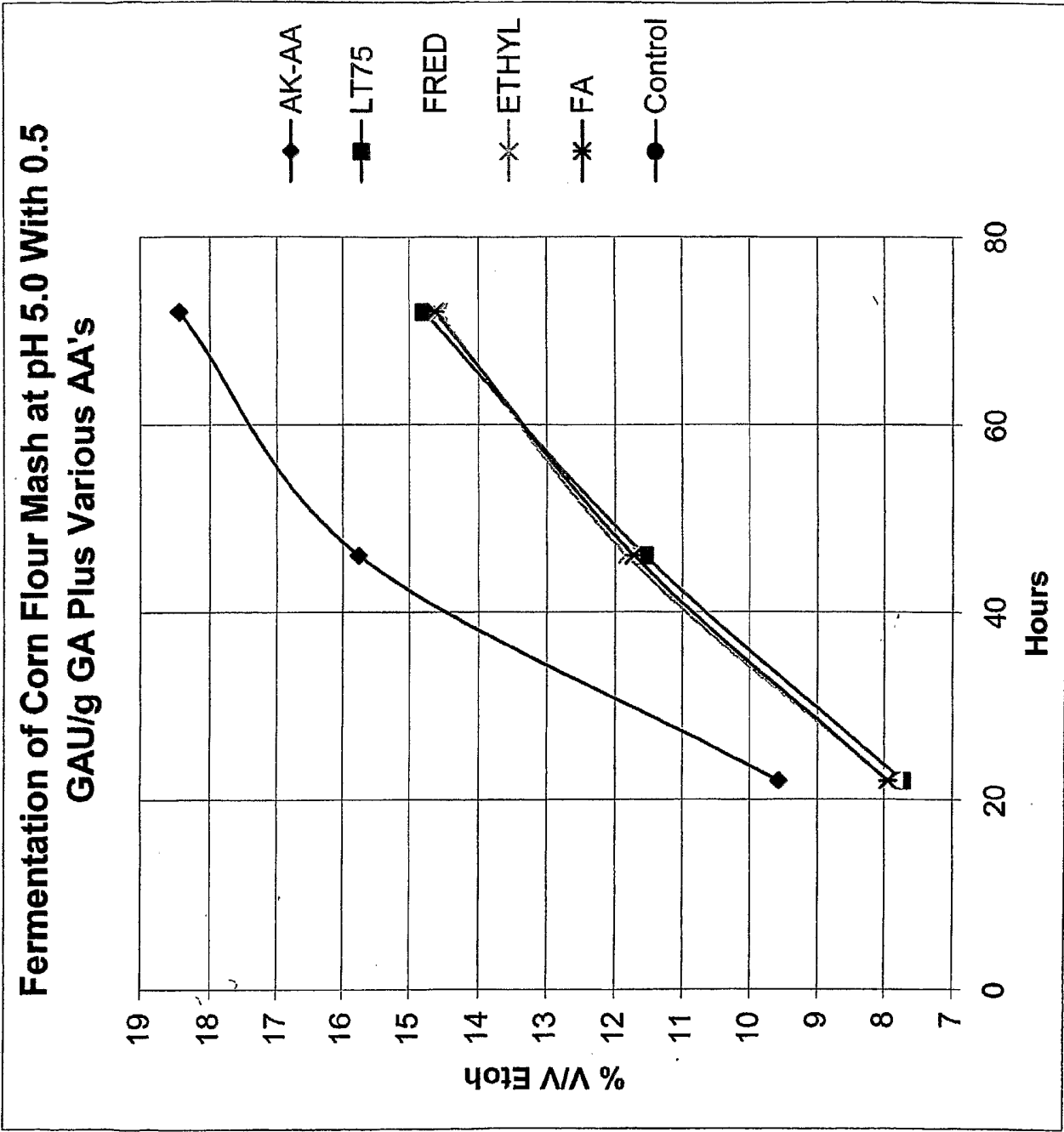


FIGURE 7

FIG. 8

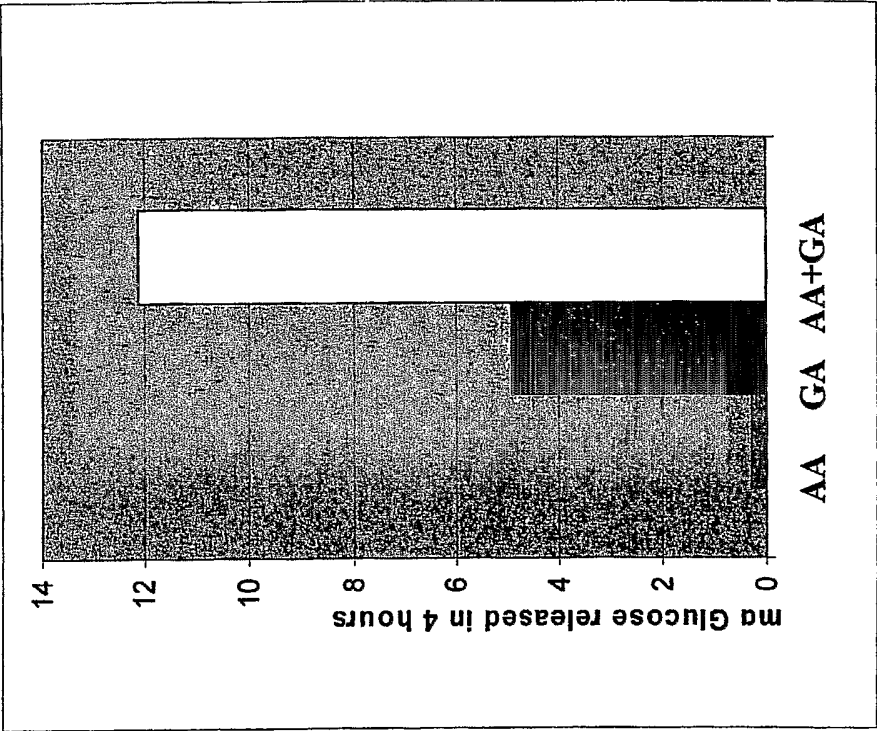


FIG. 9

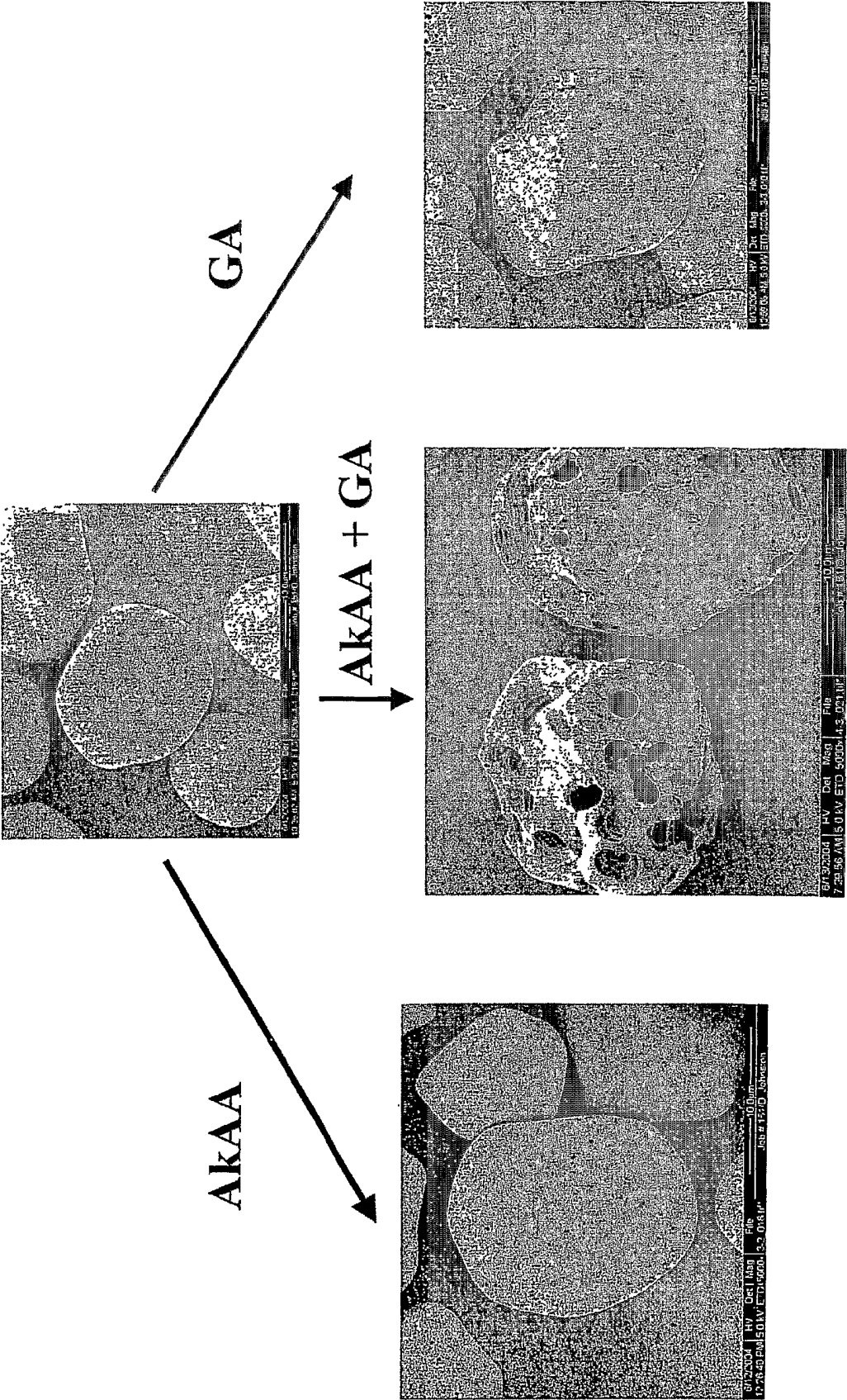
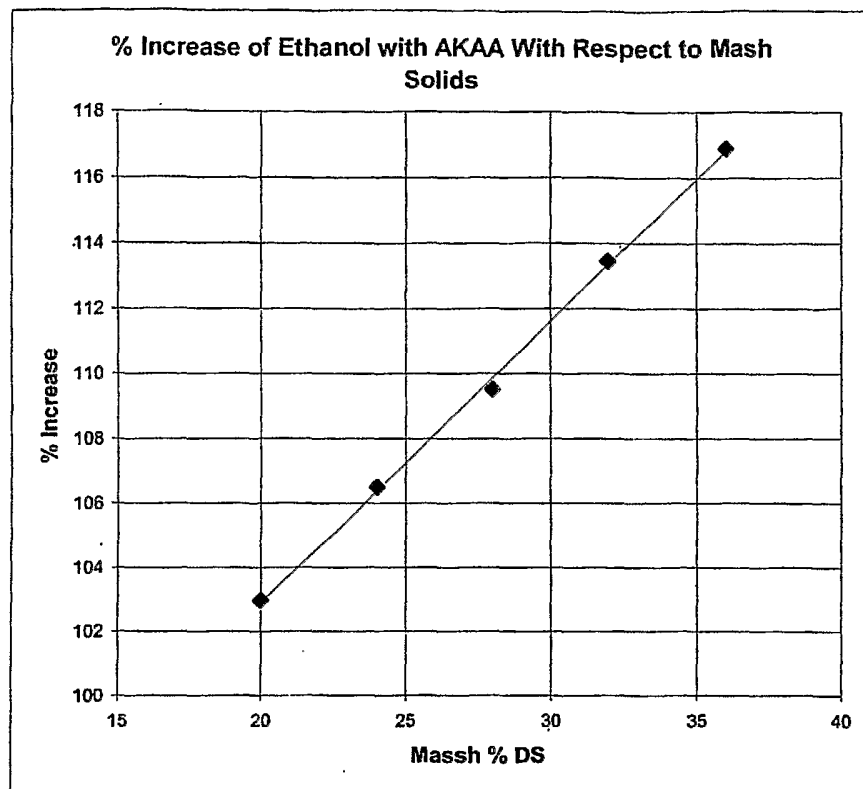


FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/41276

A. CLASSIFICATION OF SUBJECT MATTER																				
IPC(7) : A61K 38/47; C12N 9/28, 9/30; C07H 21/04																				
US CL : 424/94.61; 435/202, 203; 536/23.2																				
According to International Patent Classification (IPC) or to both national classification and IPC																				
B. FIELDS SEARCHED																				
Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/94.61; 435/202, 203; 536/23.2																				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN & WEST.																				
C. DOCUMENTS CONSIDERED TO BE RELEVANT																				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
X	KAJIWARA et al. Production of acid-stable alpha-amylase by <i>Aspergillus kawachii</i> during barley shochu-koji production. Journal of fermentation and bioengineering, 1997, Vol. 84, No. 3, pages 224-227.	9, 11, 31, 32																		
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A		1-8, 10, 12-30, 33-34																		
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Date of the actual completion of the international search 07 July 2005 (07.07.2005)		Date of mailing of the international search report 20 JUL 2005																		
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230		Authorized officer <i>Valerie Bell-Harris</i> Tekchand Sardha Telephone No. (571) 272 1600																		