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(54) **Titre : COMPOSITIONS POUR LA PRODUCTION DE SIROPS DE GLUCOSE**
(54) **Title: COMPOSITIONS FOR PRODUCING GLUCOSE SYRUPS**

(57) **Abrégé/Abstract:**
The present invention relates to a method of making glucose syrup from liquefied starch comprising, (a) contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the ratio of pullulanase dose expressed as NPUN/gDS, to alpha-amylase dose expressed as FAU(A)/gDS is at least60, particularly at least 75, particularly at least 100, more particularly at least 150, more particularly at least 200, more particularly at least 250, more particularly at least 300, more particularly at least 400, more particularly at least 500, more particularly at least 600, more particularly at least 800 or if no alpha-amylase is present the pullulanse is present in a dose of at least 0.5, particularly at least 0.75, particularly at least 1.0, particularly at least 1.5 NPUN/gDS, and (b) saccharifyingthe liquefied starch.

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(54) **Title**: COMPOSITIONS FOR PRODUCING GLUCOSE SYRUPS

(57) **Abstract**: The present invention relates to a method of making glucose syrup from liquefied starch comprising, (a) contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the ratio of pullulanase dose expressed as NPUN/gDS, to alpha-amylase dose expressed as FAU(A)/gDS is at least60, particularly at least 75, particularly at least 100, more particularly at least 150, more particularly at least 200, more particularly at least 250, more particularly at least 300, more particularly at least 400, more particularly at least 500, more particularly at least 600, more particularly at least 800 or if no alpha-amylase is present the pullulanse is present in a dose of at least 0.5, particularly at least 0.75, particularly at least 1.0, particularly at least 1.5 NPUN/gDS, and (b) saccharifyingthe liquefied starch.

COMPOSITIONS FOR PRODUCING GLUCOSE SYRUPS

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form.

Background of the Invention

Field of the Invention

The present invention relates to compositions comprising an alpha-amylase, a pullulanase and a glucoamylase. Furthermore the present invention relates to methods of producing glucose syrup comprising high %DX.

Description of the Related Art

Starch usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages. Amylopectin is partially degraded by alpha-amylase, which hydrolyzes the 1,4-alpha-glucosidic linkages to produce branched and linear oligosaccharides.

Alpha-amylases are used commercially for a variety of purposes such as in the initial stages of starch processing (e.g., liquefaction). Prolonged degradation of amylopectin by alpha-amylase results in the formation of so-called alpha-limit dextrins that are not susceptible to further hydrolysis by the alpha-amylase. Alpha-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

Branched oligosaccharides can be hydrolyzed into linear oligosaccharides by a debranching enzyme. The remaining branched oligosaccharides can be depolymerized to D-glucose by glucoamylase, which hydrolyzes linear oligosaccharides into D-glucose.

Debranching enzymes which can attack amylopectin are divided into two classes: isoamylases (E.C. 3.2.1.68) and pullulanases (E.C. 3.2.1.41), respectively. Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by their limited action on alpha-limit dextrins.

It is well-known in the art to add isoamylases or pullulanases in starch conversion processes. Pullulanase is a starch debranching enzyme having pullulan 6-glucano-hydrolase activity (EC3.2.1.41) that catalyzes the hydrolysis the α-1,6-glycosidic bonds in pullulan, releasing maltotriose with reducing carbohydrate ends. Usually pullulanase is used in combination with an alpha amylase and/or a glucoamylase.

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Pullulanases are known in the art. US 6,074,854 and US 5,817,498 disclose a pullulanase from *Bacillus deramificans*. WO2009/075682 disclose a pullulanase derived from *Bacillus acidopullulyticus*.

Glucoamylase (1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.3) is an enzyme, which catalyzes the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules. Glucoamylases are produced by several filamentous fungi and yeast, with those from *Aspergillus, Talaromyces, Penicillium, and Trametes* being particularly commercially important.

Commercially, glucoamylases are used to convert starchy material, which is already partially hydrolyzed by an alpha-amylase and e.g., a pullulanase, to glucose in the form of syrup.

Before the enzymatic treatment the starch material, such as whole grains, may be reduced in particle size, *e.g.*, by milling, in order to open up the structure and allowing for further processing. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolyzate is used in the production of, *e.g.*, syrups. Both dry and wet milling is well known in the art of starch processing and may be used in a process of the invention.

After milling, typically the starch material is liquefied. Liquefaction is carried out in the presence of an alpha-amylase, preferably a bacterial alpha-amylase and/or acid fungal alpha-amylase.

During liquefaction, the long-chained starch is degraded into branched and linear shorter units (maltodextrins) by an alpha-amylase. Liquefaction may be carried out as a three-step hot slurry process. The liquefaction process is carried out at between 70-95°C, such as 80-90°C, such as around 85°C, for about 10 minutes to 5 hours, typically for 1-2 hours. After such treatment, the liquefied starch will typically have a "dextrose equivalent" (DE) of 10-15.

Generally liquefaction and liquefaction conditions are well known in the art.

For the production of glucose syrup the liquefied starch material is saccharified. In a typical saccharification process, maltodextrins produced during liquefaction are converted into dextrose by adding a glucoamylase and a debranching enzyme, such as an isoamylase (U.S. Patent No. 4,335,208) or a pullulanase. The temperature is lowered to 60°C, prior to the addition of the glucoamylase and debranching enzyme. The saccharification process proceeds for 24-72 hours. Prior to addition of the saccharifying enzymes, the pH is reduced to below 4.5, while maintaining a high temperature (above 95°C), to inactivate the liquefying alpha-amylase.

For the production of syrup enzyme compositions used should at least comprise a glucoamylase and a pullulanase, however, often alpha-amylase activity will also be present, e.g. when using *Aspergillus niger* glucoamylase the *A. niger* alpha-amylase from the production host will also be present in the composition. It has surprisingly been found that in order to reach high

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%DX values of the syrup, e.g., above 95%, the level of alpha-amylase present in the composition should be carefully controlled.

The present invention provides compositions and methods for producing high glucose syrups having a %DX of around 96%.

Applications for higher DX syrups are: production of DMH (dextrose monohydrate), fermentation chemicals such as organic acids (such as citric acid, lactic acid, etc.) or amino acids (such as L-lysine, L-threonine, L-tryptophane, monosodium glutamate and L-cysteine), High fructose corn syrups, crystalline fructose and other specialty syrups.

Summary of the Invention

In a first aspect, the present invention relates to a composition comprising an alpha-amylase, a pullulanase and a glucoamylase enzyme, wherein the ratio of pullulanase dose expressed as NPUN/g, to alpha-amylase dose expressed as FAU(A)/g or a KNU/g is at least 60.

In a second aspect, the present invention relates to a method of making glucose syrup from liquefied starch comprising, (a) contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the ratio of pullulanase dose expressed as NPUN/gDS, to alpha-amylase dose expressed as FAU(A)/gDS or as KNU/gDS is at least 60, particularly at least 75, particularly at least 100, more particularly at least 150, more particularly at least 200, more particularly at least 250, more particularly at least 300, more particularly at least 400, more particularly at least 500, more particularly at least 600, more particularly at least 800 or if no alpha-amylase present the pullulanase is present in a dose of at least 0.5, particularly at least 0.75, particularly at least 1.0, particularly at least 1.5 NPUN/gDS, and (b) saccharifying the liquefied starch.

Definitions

Alpha-amylase: Alpha-amylases (1,4-alpha-D-glucan glucanohydrolase, E.C. 3.2.1.1) are a group of enzymes which catalyze the hydrolysis of starch and other linear and branched 1,4 glucosidic oligo- and polysaccharides. Alpha-amylases used according to the present invention may be obtained from fungal or bacterial sources. For purposes of the present invention, fungal alpha amylase activity can be determined as FAU(A) using the alpha amylase assay described in the Materials and Methods. Activity of bacterial alpha-amylases can be determined as Kilo Novo alpha-amylase Units (KNU) according to the procedure described in the paragraph “Kilo Novo alpha-amylase Units (KNU)” below.

Acid alpha-Amylase Units (FAU(A)): Acid alpha-amylase activity may be measured in FAU(A) (Acid Fungal Alpha-amylase Units). 1 FAU(A) is defined as the amount of enzyme which

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degrades 5.260 mg starch dry matter per hour under the standard conditions specified in the table "First reaction, starch degradation" below.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrins and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

FAU(A), the acid alpha-amylase activity is determined in accordance with the following description. The principle of the reaction is based on the two steps. In the first step, the enzyme acid alpha-amylase hydrolyzes starch into different oligosaccharides. In the second step, iodine forms a blue complex with starch but not with its degradation products. The intensity of color is therefore directly proportional to the concentration of starch. The activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

First reaction, starch degradation	
Substrate	Starch, approx. 0.3g/L
Buffer	Citrate, approx. 0.05M
	CaCl2, 1.85mM
pH	2.50 ± 0.05
Incubation temperature	37°C
Reaction time	180 seconds
Enzyme working range	0.01-0.04 FAU(A)/mL

Second reaction, starch-iodine complex	
Iodine	0.0432g/L
Incubation temperature	37°C
Reaction time	60 seconds
Wavelength	600nm

Kilo Novo alpha-amylase Units (KNU)

Bacterial alpha-amylase activity may be determined using potato starch as substrate. The method is based on breakdown of starch in solution by amylase and the fact that starch gives a blue-black colour in presence of iodine. As the enzyme reaction proceeds, aliquots of the reaction

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are withdrawn and analyzed for their starch content by mixing with an iodine solution. As starch is broken down, the blue-black colour in the presence of iodine fades and gradually turns into a reddish-brown colour. This is compared with a coloured glass standard. The end point is reached when the colour matches the glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions as defined in the "KNU" table below (i.e., at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5260 mg/hour starch dry substance; e.g. Merck Amylum solubile.

KNU	
Temperature	37± 0.05°C
pH	5.6
Substrate concentration	4.63 mg dry weight / mL
Reaction time	7 – 20 min, up to 1 hr
Ca^{2+} concentration	approx. 0.0003 M for reaction mix containing 2 mL sample solution

Pullulanase: The term "pullulanase" means a starch debranching enzyme having pullulan 6-glucano-hydrolase activity (EC3.2.1.41) that catalyzes the hydrolyses the α-1,6-glycosidic bonds in pullulan, releasing maltotriose with reducing carbohydrate ends. For purposes of the present invention, pullulanase activity is determined as NPUN according to the procedure described in the Materials and Methods and in the following paragraph.

Pullulanase activity (NPUN): The NPUN (New Pullulanase Unit Novozymes) is a unit of endopullulanase activity measured in the following procedure.

1 NPUN = One pullulanase unit (NPUN) is defined as the enzyme amount, which releases reducing ends equivalent to 0.35µmol glucose per minute under the standard conditions specified in the table "First reaction, pullulan degradation" below.

In the first reaction, the substrate is equally present in both sample main and sample blank. However, the reaction of sample main is performed at pH 5.0, while there is no reaction in the sample blank at pH 9.6, where neither pullulanases nor amyloglucosidases (glucoamylase) are enzymatically active.

First reaction, pullulan degradation	
Substrate	BH4 reduced pullulan, 5.3g/L
Buffer (main)	Acetate, approx. 0.1M
	EDTA, 5.3mM
	Acarbose, 0.018% (if sample contains glucoamylase)

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pH (main)	5.0
Buffer (blank)	CHES, 42mM
	acetate, 17mM
	EDTA, 5.3mM
pH (blank)	9.6
Incubation temperature	50°C
Reaction time	540 seconds
Enzyme working range	0.03-0.15 NPUN/mL

In the second reaction, the pH is adjusted to approx. 9.6 and the glucose in samples is phosphorylated to non-reducing D-glucose-6-phosphate by glucokinase, which has optimal activity and stability in this range and is specific to glucose at pH 9 (ref. Goward, Biochem. J. 1986, 237, pp 415-420). This step depends on identical pH in sample main and sample blank to remove equal amounts of glucose in both.

Second reaction, background glucose elimination	
Substrate	glucose in sample, after first reaction
Buffer	CHES, 58mM (main) or 76mM (blank)
	acetate, 43mM (main) or 7.2mM (blank)
	EDTA, 2.2mM
	ATP, 1.11mg/ml
	MgCl2, 4.4mM
Glucokinase	0.11 U/ml
pH	approx. 9.6
Incubation temperature	50°C
Reaction time	720 seconds

Glucoamylase: The term glucoamylase (1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.3) is defined as an enzyme, which catalyzes the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules. For purposes of the present invention, glucoamylase activity is determined as AGU according to the procedure described in the Materials and Methods and in the following paragraph.

Glucoamylase activity (AGU): The Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute in a 0.1 M acetate buffer at an incubation temperature 37°C, a pH of 4.3, a maltose starting concentration of 100 mM, and a

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reaction time of 6 minutes, thereby generating alpha-D-glucose. The definition applies to an enzyme working range of 0.5-4.0 AGU/mL.

After incubation, the reaction may be stopped with NaOH and the amounts of glucose measured using the following two-step color reaction method: Glucose is phosphorylated by ATP, in a reaction catalyzed by hexokinase. The glucose-6-phosphate formed is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this same reaction, an equimolar amount of NAD+ is reduced to NADH with a resulting increase in absorbance at 340 nm.

Reaction conditions are as specified in the table below:

Color reaction	
Tris	approx. 35 mM
ATP	0.7 mM
NAD^+	0.7 mM
Mg^{2+}	1.8 mM
Hexokinase	> 850 U/L
Glucose-6-P-DH	> 850 U/L
pH	approx. 7.8
Temperature	37.0 °C ± 1.0 °C
Reaction time	420 sec
Wavelength	340 nm

Degree of polymerization (DP): DP refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are monosaccharides, such as glucose and fructose. DP2 are disaccharides, such as maltose and sucrose.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. According to some embodiments, the mature polypeptide of SEQ ID NO: 6 consists essentially of amino acids 18 to 573 of SEQ ID NO: 6, the mature polypeptide of SEQ ID NO: 7 consists essentially of amino acids 18 to 573 of SEQ ID NO: 7, the mature polypeptide of SEQ ID NO: 8 consists essentially of amino acids 18 to 573 of SEQ ID NO: 8, the mature polypeptide of SEQ ID NO: 5 consists essentially of amino acids 18 to 573 of SEQ ID NO: 9, the mature polypeptide of SEQ ID NO: 10 consists essentially of

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amino acids 18 to 573 of SEQ ID NO: 10, the mature polypeptide of SEQ ID NO: 11 consists essentially of amino acids 18 to 573 of SEQ ID NO: 11, the mature polypeptide of SEQ ID NO: 12 consists essentially of amino acids 18 to 573 of SEQ ID NO: 12, the mature polypeptide of SEQ ID NO: 13 consists essentially of amino acids 18 to 576 of SEQ ID NO: 13, the mature polypeptide of SEQ ID NO: 14 consists essentially of amino acids 18 to 576 of SEQ ID NO: 14.

In particular, the mature polypeptide of SEQ ID NO: 6 may consist of amino acids 18 to 573 of SEQ ID NO: 6.

The mature polypeptide of SEQ ID NO: 7 may consist of amino acids 18 to 573 of SEQ ID NO: 7.

The mature polypeptide of SEQ ID NO: 8 may consist of amino acids 18 to 573 of SEQ ID NO: 8.

The mature polypeptide of SEQ ID NO: 9 may consist of amino acids 18 to 573 of SEQ ID NO: 9.

The mature polypeptide of SEQ ID NO: 10 may consist of amino acids 18 to 573 of SEQ ID NO: 10.

The mature polypeptide of SEQ ID NO: 11 may consist of amino acids 18 to 573 of SEQ ID NO: 11.

The mature polypeptide of SEQ ID NO: 12 may consist of amino acids 18 to 573 of SEQ ID NO: 12.

The mature polypeptide of SEQ ID NO: 13 may consist of amino acids 18 to 576 of SEQ ID NO: 13.

The mature polypeptide of SEQ ID NO: 14 may consist of amino acids 18 to 576 of SEQ ID NO: 14.

In further embodiments, the mature polypeptide of SEQ ID NO: 6 consists of amino acids 18 to 573 of SEQ ID NO: 6, the mature polypeptide of SEQ ID NO: 7 consists of amino acids 18 to 573 of SEQ ID NO: 7, the mature polypeptide of SEQ ID NO: 8 consists of amino acids 18 to 573 of SEQ ID NO: 8, the mature polypeptide of SEQ ID NO: 9 consists of amino acids 18 to 573 of SEQ ID NO: 9, the mature polypeptide of SEQ ID NO: 10 consists of amino acids 18 to 573 of SEQ ID NO: 10, the mature polypeptide of SEQ ID NO: 11 consists of amino acids 18 to 573 of SEQ ID NO: 11, the mature polypeptide of SEQ ID NO: 12 consists of amino acids 18 to 573 of SEQ ID NO: 12, the mature polypeptide of SEQ ID NO: 13 consists of amino acids 18 to 576 of SEQ ID NO: 13, and the mature polypeptide of SEQ ID NO: 14 consists of amino acids 18 to 576 of SEQ ID NO: 14.

The prediction of mature polypeptide sequences may be based on the SignalP program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6) that predicts amino acids 1 to 17 of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID

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NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14 are a signal peptide. The sequence defined by amino acids 19 to 474 (particularly 19 to 471) of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, or SEQ ID NO: 12 or amino acids 19 to 471 of SEQ ID NO: 13 or of SEQ ID NO: 14 is the catalytic domain. The sequence defined by amino acids 480 to 573 of SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12 or amino acids 483 to 576 of SEQ ID NO: 13 or SEQ ID NO: 14 is a starch binding domain.

According to other embodiments, the mature polypeptide of SEQ ID NO: 18 is defined by amino acids 22 to 450 of SEQ ID NO: 18.

In further embodiments, the mature peptide of SEQ ID NO: 19 is defined by amino acids 22-471of SEQ ID NO: 19, whereas amino acids 1-21 are a signal peptide.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Detailed Description of the Invention

The present invention relates to methods and compositions for producing high glucose syrup. In particular glucose syrups having %DX (%DP1) values above 95%.

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It has surprisingly been found that in order to reach high %DX values of the syrup, e.g., above 95%, the level of alpha-amylase present in the composition should be carefully controlled.

The methods and compositions according to the invention provides glucose syrups with higher %DX when alpha-amylase levels are optimized so that at a given ratio of NPUN to AGU the NPUN/FAU(A) or NPUN/KNU is adjusted to be at least 60, particularly at least 80. The high %DX syrups can be obtained at industrially relevant substrate concentrations – typically more than 30% initial dry solids content (DS).

In one embodiment, the present invention relates to a method of making glucose syrup from liquefied starch comprising, (a) contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the ratio of pullulanase dose expressed as NPUN/gDS, to alpha-amylase dose expressed as FAU(A)/gDS or KNU/gDS is at least 60, particularly at least 75, particularly at least 100, more particularly at least 150, more particularly at least 200, more particularly at least 250, more particularly at least 300, more particularly at least 400, more particularly at least 500, more particularly at least 600, more particularly at least 800 or if no alpha-amylase present the pullulanase is present in a dose of at least 0.5, particularly at least 0.75, particularly at least 1.0, particularly at least 1.5 NPUN/gDS, and (b) saccharifying the liquefied starch.

In some embodiments, the method comprises contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the ratio of pullulanase dose expressed as NPUN/gDS, to alpha-amylase dose expressed as FAU(A)/gDS or KNU/gDS is within the range of 100-700, such as within the range of 200-600, such as within the range of 300-500, such as within the range of 350-500, such as within the range of 375-475, or such as within the range of 400-450.

According to other embodiments, the method comprises contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the dose of alpha amylase is within the range of 0-0.008 FAU(A)/gDS and the dose of pullulanase is within the range of 0.5-1.5 NPUN/gDS.

In other embodiments, the dose of alpha amylase is within the range of 0-0.007 FAU(A)/g DS and the dose of pullulanase is within the range of 0.6-1.4 NPUN/g DS.In other embodiments the dose of alpha amylase is within the range of 0-0.006 FAU(A)/gDS and the dose of pullulanase is within the range of 0.7-1.3 NPUN/gDS.

In other embodiments, the dose of alpha amylase is within the range of 0-0.005 FAU(A)/gDS and the dose of pullulanase is within the range of 0.75-1.25 NPUN/gDS.

In other embodiments, the dose of alpha amylase is within the range of 0-0.004 FAU(A)/gDS and the dose of pullulanase is within the range of 0.8-1.2 NPUN/gDS.

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In other embodiments, the dose of alpha amylase is within the range of 0-0.003 FAU(A)/gDS and the dose of pullulanase is within the range of 0.9-1.1 NPUN/gDS.

In other embodiments, the dose of alpha amylase is within the range of 0-0.0025 FAU(A)/g DS and the dose of pullulanase is within the range of 0.95-1 NPUN/g DS.

The ratio between pullulanase expressed as NPUN/gDS and glucoamylase expressed as AGU/gDS may in particular be within the range of 2-15, such as within the range of 2-10, within the range of 2-5, within the range of 3-5 or within the range of 3.5-4.

According to other embodiments, the method comprises contacting the liquefied starch with a glucoamylase, a pullulanase, and optionally an alpha-amylase wherein the dose of pullulanase is within the range of 0.5-1.5 NPUN/gDS and the dose of glucoamylase is within the range of 0.125-0.375 AGU/gDS.

In other embodiments, the dose of pullulanase is within the range of 0.6-1.4 NPUN/gDS and the dose of glucoamylase is within the range of 0.15-0.35 AGU/gDS.

In other embodiments, the dose of pullulanase is within the range of 0.7-1.3 NPUN/gDS and the dose of glucoamylase is within the range of 0.175-0.325 AGU/gDS.

In other embodiments, the dose of pullulanase is within the range of 0.8-1.2 NPUN/gDS and the dose of glucoamylase is within the range of 0.2-0.3 AGU/gDS.

At low levels of glucoamylase longer saccharification times may be needed. In one embodiment, the glucoamylase dose, expressed as AGU/gDS, is at least 0.1, particularly at least 0.15, particularly at least 0.18, particularly at least 0.2, more particularly at least 0.22, more particularly at least 0.23, more particularly at least 0.25, even more particularly at least 0.28.

Using the method and compositions according to the invention very high %DX values can be obtained. In a particular embodiment, the glucose syrup comprises a DP1 (%DX) of at least 95.8 %, particularly at least 95.9%, particularly at least 96%, more particularly at least 96.1%.

Saccharification times may vary depending on enzyme dose. In one particular embodiment the saccharification time is at least 24 hrs, at least 30 hrs, at least 36 hrs, at least 48 hrs, at least 54 hrs, at least 60 hrs, at least 72 hrs.

In the process according to the invention, the starch hydrolysis/saccharification may in particular take place at a pH which is within the range of 3.5-5.0, such as at pH in the range of 4.0-4.5, and at a temperature, which is within the range of 59-70°C, such as in the range of 59-65°C or such as in the range of 59-62°C.

The liquefied starch used as substrate for the saccharification process according to the invention may be a starch slurry or partially hydrolysed starch (liquefact or maltodextrin). In particular, the in starch slurry or partly hydrolysed starch may have a Dextrose equivalent (DE) in the range of 5-42, such as in the range of 5-30, in the range of 8-18 or such as in the range of 9-14.

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The starch may be from any source, in particular from corn, wheat or tapioca. The starch slurry or partially hydrolysed starch may have residual alpha amylase activity from the liquefaction process present or it may have been deactivated, such as by reducing the pH to below 4.5, while maintaining a high temperature (above 95°C), to inactivate the liquefying alpha-amylase.

The conductivity of said starch slurry or partially hydrolysed starch may in particular be within the range of 0-500 microS/cm. According to some embodiments the calcium content corresponds to 0-200 ppm free calcium.

In the process according to the invention, the starch hydrolysis/saccharification may in particular take place at a pH which is within the range of 3.5-5.0, such as at pH in the range of 4.0-4.7, and at a temperature, which is within the range of 58-70°C, such as in the range of 58-65°C, in the range of 59-65°C or such as in the range of 59-62°C.

The composition comprising liquefied starch provided as a starting material; i.e. composition comprising liquefied starch provided the in step i) of the process may contain from 25-45% dry solids (%DS), such as from 25-40% DS.

The method according to the invention is applicable at industry relevant substrate doses. In one embodiment the initial dry solids content (DS) in the liquefied starch substrate is at least 25%, particularly at least 30%, more particularly at least 35%, even more particularly at least 40%.

Enzyme Compositions

The present invention also relates to compositions comprising a glucoamylase, a pullulanase and an alpha-amylase.

In a particular embodiment, the composition comprises an alpha-amylase, a pullulanase and a glucoamylase enzyme, wherein the ratio of pullulanase dose expressed as NPUN/g, to alpha-amylase dose expressed as FAU(A)/g or as KNU/g is at least 60.

More particularly, the composition comprises an alpha-amylase, a pullulanase and a glucoamylase enzyme, wherein the ratio of pullulanase dose expressed as NPUN/g, to alpha-amylase dose expressed as FAU(A)/g or as KNU/g is at least 75, particularly at least 100, more particularly at least 150, more particularly at least 200, more particularly at least 250, more particularly at least 300, more particularly at least 400, more particularly at least 500, more particularly at least 600, more particularly at least 800.

In another embodiment, the ratio of pullulanase dose expressed as NPUN/g, to alpha-amylase dose expressed as FAU(A)/g or as KNU/g is in the range from 60 - 1000, more particularly 70 - 800, more particularly 80 - 600, more particularly 90 - 500, more particularly 100 - 400.

The ratio of pullulanase dose expressed as NPUN/g, to alpha-amylase dose expressed as FAU(A)/g or KNU/g may in particular be within the range of 100-700, such as within the range

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of 200-600, such as within the range of 300-500, such as within the range of 350-500, such as within the range of 375-475, or such as within the range of 400-450.

In a further aspect, the invention relates to a composition, wherein the ratio between pullulanase expressed as NPUN/g and glucoamylase expressed as AGU/g is at least 2, particularly at least 2.5, particularly at least 3, more particularly at least 3.5, more particularly at least 5, more particularly at least 10, even more particularly at least 15.

The ratio between pullulanase expressed as NPUN/g and glucoamylase expressed as AGU/g may in particular be within the range of 2-15, such as within the range of 2-10, within the range of 2-5, within the range of 3-5 or within the range of 3.5-4.

Pullulanase

Any pullulanase may be used in a process of the present invention. In an embodiment, the pullulanase is a pullulanase from *Bacillus deramificans* ,e.g., disclosed in US 6,074,854 and US 5,817,498, or a pullulanase derived from *Bacillus acidopullulyticus, e.g.,* disclosed in WO2009/075682 (SEQ ID 4; GENESEQP: AXB71624).

Commercially available pullulanases include Promozyme D2 available from Novozymes A/S, Bagsvaerd, Denmark), Novozym 26062 (Novozymes) and Optimax L 1000 (DuPont-Genencor)

Glucoamylase

A glucoamylase used according to the invention may be derived from any suitable source, *e.g.*, derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al., 1984, *EMBO J.* 3 (5): 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921, *A. oryzae* glucoamylase (*Agric. Biol. Chem.*, 1991, 55 (4): 941-949), or variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al., 1996, *Prot. Eng.* 9: 499-505); D257E and D293E/Q (Chen et al., 1995, *Prot. Eng.* 8: 575-582); N182 (Chen et al., 1994, *Biochem. J.* 301: 275-281); disulphide bonds, A246C (Fierobe et al., 1996, *Biochemistry* 35: 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al., 1997, *Protein Eng.* 10: 1199-1204.

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see U.S. Patent No. 4,727,026 and Nagasaka et al., 1998, "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, *Appl Microbiol Biotechnol* 50:323-330), *Talaromyces* glucoamylases, in particular derived from *Talaromyces*

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emersonii (WO 99/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), *Talaromyces duponti*, and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215).

Contemplated fungal glucoamylases include *Trametes cingulata*, disclosed in WO 2006/069289.

In an embodiment, the glucoamylase is derived from a strain of the genus *Pycnoporus*, in particular a strain of *Pycnoporus* as described in WO 2011/066576 (SEQ ID NOs 2, 4 or 6), or from a strain of the genus *Gloephyllum*, in particular a strain of *Gloephyllum* as described in WO 2011/068803 (SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16) or a strain of the genus *Nigrofomes*, in particular a strain of *Nigrofomes* sp. disclosed in WO 2012/064351 (SEQ ID NO: 2) or a strain of *Penicillium*, in particular *Penicillium oxalicum* disclosed in WO2011/127802 (SEQ ID NO: 2) or WO2013/036526. Contemplated are also glucoamylases which exhibit a high identity to any of the above-mentioned glucoamylases, i.e., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as 100% identity to any one of the mature parts of the enzyme sequences mentioned above.

In an embodiment, the glucoamylase is derived from a strain of the genus *Trichoderma,* in particular as described in WO2009/048487, WO2009/048488, WO2008/045489, WO2011/022465, WO2012/001139.

Commercially available glucoamylase compositions include AMG 200L; AMG 300L; SAN™ SUPER, SAN™ EXTRA L, SPIRIZYME™ PLUS, SPIRIZYME™ FUEL, SPIRIZYME™ B4U, SPIRIZYME ULTRA™, SPIRIZYME EXCEL™ and AMG™ E (from Novozymes A/S, Denmark); OPTIDEX™ 300, GC480™ and GC147™ (from Genencor Int., USA); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900, G-ZYME™ and G990 ZR (from DuPont-Genencor)

Alpha-amylase

Fungal alpha-amylases include alpha-amylases derived from a strain of the genus *Aspergillus*, such as, *Aspergillus oryzae, Aspergillus niger* and *Aspergillus kawachii* alpha-amylases.

A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase which is derived from a strain of *Aspergillus oryzae*. According to the present invention, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874.

Another preferred acidic alpha-amylase is derived from a strain *Aspergillus niger*. In a preferred embodiment, the acid fungal alpha-amylase is the one from *A. niger* disclosed as

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"AMYA_ASPNG" in the Swiss-prot/TeEMBL database under the primary accession no. P56271 and described in WO 89/01969 (Example 3).

Other contemplated wild-type alpha-amylases include those derived from a strain of the genera *Rhizomucor* and *Meripilus,* preferably a strain of *Rhizomucor pusillus* (WO 2004/055178) or *Meripilus giganteus*.

In a preferred embodiment, the alpha-amylase is derived from *Aspergillus kawachii* and disclosed by Kaneko et al., 1996, *J. Ferment. Bioeng.* 81: 292-298, "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from *Aspergillus kawachii*"; and further as EMBL:#AB008370.

The fungal alpha-amylase may also be a wild-type enzyme comprising a starch-binding domain (SBD) and an alpha-amylase catalytic domain (*i.e.*, non-hybrid), or a variant thereof. In an embodiment the wild-type alpha-amylase is derived from a strain of *Aspergillus kawachii*.

Fungal Hybrid Alpha-Amylase

In a preferred embodiment, the alpha amylase is a fungal acid alpha-amylase is a hybrid alpha-amylase. Preferred examples of fungal hybrid alpha-amylases include the ones disclosed in WO 2005/003311 or U.S. application publication no. 2005/0054071 (Novozymes) or U.S. application no. 60/638,614 (Novozymes). A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CD) and a carbohydrate-binding domain/module (CBM), such as a starch binding domain, and optional a linker.

Specific examples of contemplated hybrid alpha-amylases include those disclosed in Table 1 to 5 of the examples in U.S. application no. 60/638,614, including Fungamyl variant with catalytic domain JA118 and *Athelia rolfsii* SBD (SEQ ID NO:100 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Athelia rolfsii* AMG linker and SBD (SEQ ID NO:101 in US 60/638,614), *Rhizomucor pusillus* alpha-amylase with *Aspergillus niger* glucoamylase linker and SBD (which is disclosed in Table 5 as a combination of amino acid sequences SEQ ID NO: 20, SEQ ID NO: 72 and SEQ ID NO: 96 in U.S. application no. 11/316,535) or as V039 in Table 5 in WO 2006/069290, and *Meripilus giganteus* alpha-amylase with *Athelia rolfsii* glucoamylase linker and SBD (SEQ ID NO: 102 in U.S. application no. 60/638,614). Other specifically contemplated hybrid alpha-amylases are any of the ones listed in Tables 3, 4, 5, and 6 in Example 4 in U.S. application no. 11/316,535 and WO 2006/069290.

Other specific examples of contemplated hybrid alpha-amylases include those disclosed in U.S. application publication no. 2005/0054071, including those disclosed in Table 3 on page 15, such as *Aspergillus niger* alpha-amylase with *Aspergillus kawachii* linker and starch binding domain.

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Contemplated are also alpha-amylases which exhibit a high identity to any of above mention alpha-amylases, *i.e.*, more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature enzyme sequences.

Bacterial Alpha-amylase

Bacterial alpha-amylases useful in the processes according to the invention include alpha-amylases derived from a strain of the genus *Bacillus,* such as *Bacillus licheniformis and Bacillus stearothermophilus.*

Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE™ (DSM), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X, LIQUOZYME™ SC and SAN™ SUPER, SAN™ EXTRA L (Novozymes A/S) and CLARASE™ L-40,000, DEX-LO™, SPEZYME™ FRED, SPEZYME™ AA, SPEZYME™ ALPHA, SPEZYME™ DELTA AA, GC358, GC980, SPEZYME™ CL and SPEZYME™ RSL (DuPont-Genencor), FUELZYME™ (from Verenium Corp, USA).

In a particular embodiment, the composition according to the invention comprises an alpha amylase, a glucoamylase (AMG), and a pullulanase, and wherein the alpha amylase is selected from *Aspergillus niger* or *Rhizomucor pusillus* alpha amylases, or variants thereof, the glucoamylase is selected from *Aspergillus niger, Talaromyces emersonii, Trametes cingulata* or *Gloeophyllum trabeum* glucoamylases, or variants thereof, and the pullulanase is selected from *Bacillus deramificans* or *Bacillus acidopullulyticus* pullulanases, or hybrids and/or variants thereof.

In further particular embodiments, the composition according to the invention comprises a glucoamylase which comprises/consists essentially of/consists of an amino acid sequence selected from the group consisting of:

i) The amino acid sequence set forth in any one of SEQ ID NO: 1, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 or a mature polypeptide thereof;

ii) A subsequence of the amino acid sequence set forth in any one of SEQ ID NOs: 1, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 or of said mature polypeptide thereof;

iii) An amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in i) and ii).

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When the glucoamylase comprises a subsequence as defined in ii) or an amino acid sequence as defined in iii), it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the respective amino acid defined in i) of which it is a subsequence or variant, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)".

In other embodiments, the composition according to the invention comprises an alpha-amylase, which comprises/consists essentially of/consists of an amino acid sequence selected from the group consisting of:

i) The amino acid sequence set forth in any one of SEQ ID NOs: 2, 5, 19, 20, 21, 22, 23, 24 and 25 or a mature polypeptide thereof;

ii) A subsequence of the amino acid sequence set forth in any one of SEQ ID NOs: 2, 5, 19, 20, 21, 22, 23, 24 and 25 or of said mature polypeptide thereof;

iii) A variant amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in i) and ii).

When the alpha-amylase defined above is a subsequence or a variant, it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the respective alpha-amylase selected from SEQ ID NOs: 2, 5, 19, 20, 21, 22, 23, 24 and 25 or the mature polypeptide thereof, of which it is a subsequence or variant, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))" or "Kilo Novo alpha-amylase Units (KNU)": In the present context an alpha-amylase comprising an amino acid sequences selected from the group consisting of SEQ ID NOs: 2, 5 and 19 or the mature polypeptide thereof, is considered to be a fungal alpha amylase and activity is tested as provided in relation to the above definition of "Acid alpha-Amylase Units (FAU(A))". An alpha-amylase comprising an amino acid sequences selected from the group consisting of SEQ ID NOs: 20-25 or the mature polypeptide thereof is considered to be a bacterial alpha amylase and activity is tested as provided in relation to the above definition of "Kilo Novo alpha-amylase Units (KNU)"

In particular embodiments, the alpha-amylase comprising or consisting of the amino acid sequence defined in iii) is a variant of an alpha-amylase comprising or consisting of the amino sequence defined in SEQ ID NO: 20 or a mature polypeptide thereof, wherein the following mutations have been made: I181*/G182*/N193F (using the amino acid numbering in SEQ ID NO: 20).

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According to other embodiments, the alpha-amylase comprising or consisting of the amino acid sequence defined in iii) is a variant of an alpha-amylase comprising or consisting of the amino sequence defined in SEQ ID NO: 23 or a mature polypeptide thereof, wherein the following mutations have been made: H156Y+A181T+N190F+A209V+Q264S (using the amino acid numbering in SEQ ID NO: 21).

In even further embodiments, the alpha-amylase comprising or consisting of the amino acid sequence defined in iii) is a variant of an alpha-amylase comprising or consisting of the amino sequence defined in SEQ ID NO: 23 or a mature polypeptide thereof, wherein the following mutations have been made: G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S (using the numbering in SEQ ID NO: 21).

In still other embodiments, the composition according to the invention comprises a pullulanase, which comprises/consists essentially of/consists of an amino acid sequence selected from the group consisting of:

i) The amino acid sequence set forth in any one of SEQ ID NOs: 3, 16, 17 and 18 or a mature polypeptide thereof;

ii) A subsequence of the amino acid sequence set forth in any one of SEQ ID NOs: 3, 16, 17 and 18 or of said mature polypeptide thereof;

iii) An amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in i) and ii).

When the pullulanase comprises a subsequence as defined in ii) or a variant amino acid sequence as defined in iii), it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the pullulanase activity of the respective amino acid defined in i) of which it is a subsequence or variant, when tested as set forth above in relation to the definition of “Pullulanase activity (NPUN)”.

Within the scope of the present invention are embodiments wherein the glucoamylase comprises or consists of an amino acid sequence selected from the group consisting of:

i) The amino acid sequence set forth in SEQ ID NO: 4 or a mature polypeptide thereof;

ii) a subsequence of the amino acid sequence set forth in SEQ ID NO: 4 or of said mature polypeptide thereof; and

iii) a variant amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%,

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such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in i) and ii);

wherein the pullulanase comprises or consists of an amino acid sequence selected from the group consisting of:

iv) The amino acid sequence set forth in any one of SEQ ID NOs: 3, 16, 17 and 18 or a mature polypeptide thereof;

v) A subsequence of the amino acid sequence set forth in any one of SEQ ID NOs: 3, 16, 17 and 18 or of said mature polypeptide thereof; and

vi) A variant amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in iv) and v); and

wherein the alpha-amylase comprises or consists of an amino acid sequence selected from the group consisting of:

vii) The amino acid sequence set forth in SEQ ID NO: 5 or a mature polypeptide thereof;

viii) A subsequence of the amino acid sequence set forth in SEQ ID NO: 5 or of said mature polypeptide thereof; and

ix) A variant amino acid sequence, which has at least 70%, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, such as at least 99.5% sequence identity to any one of the amino acids sequences set forth in vii) and viii).

When said glucoamylase is a subsequence or a variant amino acid sequence as defined above, it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the respective amino acid sequence (e.g. the amino acid sequence set forth in SEQ ID NO: 4 or a mature polypeptide thereof) of which it is a subsequence or variant, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)".

When said pullulanase is a subsequence or a variant amino acid sequence as defined above, it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the pullulanase activity of the respective amino acid sequence (e.g. the amino acid sequence set forth in any one of SEQ ID NOs: 3, 16, 17 and 18 or a mature polypeptide

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thereof) of which it is a subsequence or variant, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

When the alpha-amylase defined above is a subsequence or a variant, it preferably has at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the respective alpha-amylase selected from SEQ ID NOs: 5 or of the mature polypeptide thereof, of which it is a subsequence or variant, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))".

In a particular embodiment, the glucoamylase is selected from the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the polypeptide of SEQ ID NO: 1 or the mature polypeptide thereof, of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has glucoamylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)".

In another particular embodiment, the glucoamylase is selected from the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 or the mature polypeptide thereof, of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has glucoamylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)".

In a particular embodiment, the alpha-amylase is selected from the alpha-amylase disclosed in SEQ ID NO: 2 or an alpha-amylase having a sequence identity to the polypeptide of SEQ ID NO: 2 or the mature polypeptide thereof, of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has alpha-amylase activity; e.g. e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))". In another particular embodiment the alpha-amylase is selected from the alpha-amylases disclosed in SEQ ID NO: 5 or an alpha-amylase having a sequence identity to the polypeptide of SEQ ID NO: 5 or the mature polypeptide thereof, of at least 70%, at least 75%, at

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least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has alpha-amylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))".

In a particular embodiment, the pullulanase is selected from the pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the polypeptide of SEQ ID NO: 3 or the mature polypeptide thereof, of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has pullulanase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

In a further specific embodiment, the composition comprises

i) a glucoamylase is selected from the glucoamylases disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 1 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has glucoamylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the glucoamylases disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has alpha-amylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))"; and

iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature

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polypeptide of SEQ ID NO: 3 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has pullulanase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 1 or the mature polypeptide thereof, of at least 90% which has glucoamylase activity; e.g. at least 90% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 2 or the mature polypeptide thereof of at least 90%, which has alpha-amylase activity; e.g. at least 90% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))"; and

iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 or the mature polypeptide thereof of at least 90%, which has pullulanase activity; e.g. at least 90% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the glucoamylase of SEQ ID NO: 1 or the mature polypeptide thereof of at least 95% which has glucoamylase activity; e.g. at least 95% of the glucoamylase activity of the glucoamylases disclosed in SEQ ID NO: 1 or the mature polypeptide thereof,

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when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 95%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))";

iii) and a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 95%, which has pullulanase activity; e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 1 of at least 97% which has glucoamylase activity; e.g. at least 95% of the glucoamylase activity of the glucoamylases disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 97%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))";

iii) and a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 97%, which has pullulanase activity; e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylases disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the

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mature polypeptide of SEQ ID NO: 1 of at least 99% which has glucoamylase activity; e.g. at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 1 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 99%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 2 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))";

iii) and a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 99%, which has pullulanase activity; e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

In a further specific embodiment, the composition comprises:

i) a glucoamylase is selected from the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has glucoamylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has alpha-amylase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))", and

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iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which has pullulanase activity; e.g. at least 75%, such as at least 80%, at least 85%, at least 90% or such as at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 90% which has glucoamylase activity e.g. at least 90% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 90%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))"; and

iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 90%, which has pullulanase activity e.g. at least 90% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 95% which has glucoamylase activity e.g. at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

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ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 95%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))"; and

iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 95%, which has pullulanase activity; e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 97% which has glucoamylase activity e.g. at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 97%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))"; and

iii) a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 97%, which has pullulanase activity e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

Particularly, the composition may comprise:

i) a glucoamylase selected from the glucoamylases disclosed in SEQ ID NO: 4 or the mature polypeptide thereof, and a glucoamylase having a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 99% which has glucoamylase activity e.g. at least 95% of the glucoamylase activity of the glucoamylase disclosed in SEQ

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ID NO: 4 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Glucoamylase activity (AGU)";

ii) an alpha-amylase selected from the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, and an alpha-amylase having a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 99%, which has alpha-amylase activity; e.g. at least 95% of the alpha-amylase activity of the alpha-amylase disclosed in SEQ ID NO: 5 or the mature polypeptide thereof, when tested as set forth above in relation to the definition of "Acid alpha-Amylase Units (FAU(A))";

iii) and a pullulanase selected from a pullulanase disclosed in SEQ ID NO: 3 or the mature polypeptide thereof, and a pullulanase having a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 99%, which has pullulanase activity; e.g. at least 95% of the pullulanase activity of the amino acid sequence set forth in SEQ ID NO: 3 or a mature polypeptide thereof, when tested as set forth above in relation to the definition of "Pullulanase activity (NPUN)".

The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. The compositions may be stabilized in accordance with methods known in the art.

Uses

The composition according to the invention may be used in a saccharification process to produce glucose syrup. Therefore in a further aspect, the invention relates to a method of making glucose syrup from liquefied starch comprising, contacting the liquefied starch with a composition according to the invention.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Material and Methods

Glucoamylase activity

Glucoamylase activity (AGU)

The Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions (37°C, pH 4.3, substrate: maltose 100 mM, buffer: acetate 0.1 M, reaction time 6 minutes as set out in the glucoamylase incubation below), thereby generating glucose.

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glucoamylase incubation:	
Substrate:	maltose 100 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	6 minutes
Enzyme working range:	0.5-4.0 AGU/mL

The analysis principle is described by 3 reaction steps:

Step 1 is an enzyme reaction:

Glucoamylase (AMG), EC 3.2.1.3 (exo-alpha-1,4-glucan-glucohydrolase), hydrolyzes maltose to form alpha-D-glucose. After incubation, the reaction is stopped with NaOH.

Steps 2 and 3 result in an endpoint reaction:

Glucose is phosphorylated by ATP, in a reaction catalyzed by hexokinase. The glucose-6-phosphate formed is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this same reaction, an equimolar amount of NAD+ is reduced to NADH with a resulting increase in absorbance at 340 nm. An autoanalyzer system such as Konelab 30 Analyzer (Thermo Fisher Scientific) may be used.

Color reaction	
Tris	approx. 35 mM
ATP	0.7 mM
NAD^+	0.7 mM
Mg^{2+}	1.8 mM
Hexokinase	> 850 U/L
Glucose-6-P-DH	> 850 U/L
pH	approx. 7.8
Temperature	37.0 °C ± 1.0 °C
Reaction time	420 sec
Wavelength	340 nm

Acid alpha-amylase activity

When used according to the present invention, the activity of any acid alpha-amylase may be measured in FAU(A) (Acid Fungal Alpha-amylase Units).

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Acid alpha-amylase activity (FAU(A))

Acid alpha-amylase activity may be measured in FAU(A) (Acid Fungal Alpha-amylase Units). 1 FAU(A) is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrins and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

FAU(A), the acid alpha-amylase activity is determined in accordance with the following description, and is measured relative to a Novozymes standard which is available on request from Novozymes A/S, Denmark. The principle of the reaction is based on the two steps. In the first step the enzyme acid alpha-amylase hydrolyzes starch into different oligosaccharides. In the second step iodine forms a blue complex with starch but not with its degradation products.

The intensity of color is therefore directly proportional to the concentration of starch. The activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

First reaction, starch degradation	
Substrate	Starch, approx. 0.3g/L
Buffer	Citrate, approx. 0.05M
	CaCl2, 1.85mM
pH	2.50 ± 0.05
Incubation temperature	37°C
Reaction time	180 seconds
Enzyme working range	0.01-0.04 FAU(A)/mL

Second reaction, starch-iodine complex	
Iodine	0.0432g/L
Incubation temperature	37°C
Reaction time	60 seconds
Wavelength	600nm

If further details are preferred, these can be found in EB-SM-0510.02 available on request from Novozymes A/S, Denmark.

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Pullulanase activity

Endo-pullulanases hydrolyse α-1,6-glycosidic bonds in pullulan (-BH4 reduced to reduce background reducing sugar), releasing maltotriose units with reducing carbohydrate ends. Pullulanase is a pullulan 6-glucano-hydrolase with the enzyme classification number E.C.3.2.1.41.

The NPUN (New Pullulanase Unit Novozymes) is a unit of endopullulanase activity measured in the following procedure, and is measured relative to a Novozymes standard which is available on request from Novozymes A/S, Denmark.

1 NPUN = One pullulanase unit (NPUN) is defined as the enzyme amount, which releases reducing ends equivalent to 0.35µmol glucose per minute under the standard conditions.

In the first reaction, the substrate is equally present in both sample main and sample blank. However, the reaction of sample main is performed at pH 5.0, while there is no reaction in the sample blank at pH 9.6, where neither pullulanases nor amyloglucosidases (glucoamylase) are enzymatically active.

First reaction, pullulan degradation	
Substrate	BH4 reduced pullulan, 5.3g/L
Buffer (main)	Acetate, approx. 0.1M
	EDTA, 5.3mM
	Acarbose, 0.018% (if sample contains glucoamylase)
pH (main)	5.0
Buffer (blank)	CHES, 42mM
	acetate, 17mM
	EDTA, 5.3mM
pH (blank)	9.6
Incubation temperature	50°C
Reaction time	540 seconds
Enzyme working range	0.03-0.15 NPUN/mL

In the second reaction, the pH was adjusted to approx. 9.6 and the glucose in samples is phosphorylated to non-reducing D-glucose-6-phosphate by glucokinase, which has optimal activity and stability in this range and is specific to glucose at pH 9 (ref. Goward, Biochem. J. 1986, 237, pp 415-420). This step depends on identical pH in sample main and sample blank to remove equal amounts of glucose in both.

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Second reaction, background glucose elimination	
Substrate	glucose in sample, after first reaction
Buffer	CHES, 58mM (main) or 76mM (blank) acetate, 43mM (main) or 7.2mM (blank) EDTA, 2.2mM ATP, 1.11mg/ml MgCl2, 4.4mM
Glucokinase	0.11 U/ml
pH	approx. 9.6
Incubation temperature	50°C
Reaction time	720 seconds

The second reaction is stopped by and alkaline reagent > pH 11 containing PAHBAH (p-Hydroxy benzoic acid hydrazide) and bismuth, which complexes with reducing sugars to produce color detected at 405nm. The produced color is proportional to the pullulanase activity.

Third reaction, PAHBAH-Bi reaction	
Substrate	maltotriose formed by pullulanase, after second reaction
PAHBAH	56mM
Tartrate	75mM
Bi^{3+}	6.0mM
NaOH	195mM
pH	alkaline
Incubation temperature	50°C
Reaction time	1000 seconds
Wavelength	405nm

If further details are preferred, these can be found in 2010-28835-02 available on request from Novozymes A/S, Denmark.

Example 1:

Maltodextrin which dextrose equivalent (DE) was adjusted to 11 was prepared from a conventional starch liquefaction process using corn starch and spray-dried for this experiment. The maltodextrin powder was dissolved in milliQ water and the pH was adjusted by HCl/NaOH to be 4.3 at 60°C, and then the solid was adjusted to 33% dry solid (DS) by measuring refractive index (RI) of the syrup. Saccharification was started by mixing 18 g maltodextrin solution and 2

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ml enzyme mixture containing *Aspergillus niger* glucoamylase (SEQ ID NO: 1) , pullulanase (Promozyme D2®) (SEQ ID NO: 3), and *Aspergillus niger* acid alpha-amylase (SEQ ID NO: 2) at different dosages. The samples were incubated at 60°C with stirring and were taken at different time intervals for determination of sugar component. The enzyme dosages and the DP1-DP4+ compositions at the time point that DP1 fraction is maximized were shown in Table 1.

A.niger glucoamylase (SEQ ID NO: 1)	A.niger alpha-amylase (SEQ ID NO: 2)	Promozyme D2® (SEQ ID NO: 3)		Peak time	DP1	DP2	DP3	DP4+	Comment
AGU/gDS	FAU(A)/gDS	NPUN/gDS	NPUN/FAU(A)	hr	%	%	%	%	
0.18	0.045	0.34	7.56	48	95.6	2.4	0.5	1.4	Dextrozyme DX®
0.18	0.011	0.34	30.22	72	95.3	2.8	0.4	1.5	
0.18	0.023	0.34	15.11	60	95.4	2.6	0.5	1.5	
0.18	0.045	0.34	7.56	48	95.5	2.4	0.6	1.5	
0.18	0.09	0.34	3.78	48	95.4	2.4	0.7	1.5	
0.18	0.18	0.34	1.89	48	95.4	2.4	0.7	1.5	
0.18	0.011	1.01	89.78	36	95.9	2.1	0.6	1.4	
0.18	0.023	1.01	44.89	48	95.8	2.4	0.6	1.3	
0.18	0.045	1.01	22.44	36	95.6	2.2	0.8	1.5	
0.18	0.09	1.01	11.22	60	95.5	2.6	0.6	1.3	
0.18	0.18	1.01	5.61	48	95.5	2.4	0.8	1.4	

Table 1 shows that the enzyme blends with NPUN/FAU(A) ratio higher than 44.89 showed higher DP1 fraction than Dextrozyme DX®. Even at 3-times higher NPUN activity (1.01 NPUN/gDS) than Dextrozyme DX, the peak DP1 was not as high as Dextrozyme DX at higher FAU(A) activity than 0.045.

Example 2:

Saccharification using different enzyme blends

Maltodextrin powder from corn starch liquefaction was dissolved in water while heating to make slurry at 34.4% dry solids. The solid content of the slurry was measured using Refractive index measurement showing 1.39271. The slurry was adjusted to a pH of 4.3 using a 1M Hydrochloric acid solution. 18 gram aliquots of this slurry were added to 18 glass reaction scintillation vials with septum cap closures and were inserted in a heating block to be heated to a temperature of 61°C. Each vial was given an enzyme dosage based on the table below and

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additional water was added to each vial to reach a target dry solid of 33%. The enzyme blend comprised a glucoamylase derived from *Gloeophyllum trabeum* (SEQ ID NO: 4), an alpha-amylase derived from *Rhizomucor pusillus* (SED ID NO: 5) and a pullulanase derived from *Bacillus deramificans* (SEQ ID NO: 3). 1.5 mL samples were taken via needles through the septum from each vial at different time points (36 hour, 42 hour, 48 hour, 54 hours and 60 hours) and were deactivated at 105°C for 5 minutes. 1mL of each deactivated sample was diluted with 4mL deionized water. The diluted samples were evaluated using a HPLC method DP1-4 for measuring dextrose purity (%DP1 or %DX).

Results from Table 2 show the higher the NPUN/FAU(A) ratio, the higher the percent dextrose.

Table 2.

GA SEQ ID NO: 4 Dose (AGU/g DS)	AA SEQ ID NO: 5 Dose (FAU(A)/g DS)	PUL SEQ ID NO: 3 Dose (NPUN/g DS)	NPUN/FAU(A) ratio	%DX 36 hr	%DX 42 hr	%DX 48 hr	%DX 54 hr	%DX 60 hr
0.18	0	0.72	N/A	92.2	93.5	94.6	95.3	95.8
0.18	0	1.08	N/A	93.5	94.8	95.6	96.1	96.5
0.18	0.008	0.9	112.5	94.1	95.2	95.5	95.8	95.8
0.18	0.008	0.9	112.5	94.4	95.3	95.7	95.9	96.0
0.18	0.015	0.72	48	93.6	94.8	95.3	95.5	95.6
0.18	0.015	1.08	72	94.0	94.9	95.3	95.5	95.6
0.23	0	0.9	N/A	94.1	95.0	95.6	96.0	96.3
0.23	0.008	0.72	90	95.5	95.8	96.0	96.0	96.0
0.23	0.008	0.9	112.5	95.5	95.8	95.9	96.0	96.0
0.23	0.008	1.08	135	95.7	96.0	96.0	96.0	96.1
0.23	0.008	1.08	135	95.7	95.9	96.0	96.1	96.0
0.23	0.015	0.9	60	95.3	95.7	95.8	95.8	95.9
0.23	0.015	0.9	60	95.0	95.5	95.6	95.8	95.7
0.28	0	0.72	N/A	94.6	95.5	95.8	96.1	96.1
0.28	0	1.08	N/A	95.7	96.3	96.3	96.6	96.4
0.28	0.008	0.9	112.5	95.9	96.0	96.0	96.0	96.0
0.28	0.015	0.72	48	95.8	95.9	95.9	95.9	95.9
0.28	0.015	1.08	72	95.7	95.7	95.8	95.9	95.8

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In a different experiment, performed according to the same procedure as above, results are shown in Table 3.

Table 3.

AA SEQ ID NO: 5 AA dose (AFAU/gDS)	PUL SEQ ID NO: 3 dose (NPUN/gDS)	GA SEQ ID NO: 4 AMG dose (AGU/gDS)	NPUN/AFAU ratio	%DX at 36 hr	%DX at 42hr	%DX at 48 hr	%DX at 60 hr
0.0	0.96	0.25	N/A	96.2	96.6	96.7	96.8
0.001	0.96	0.25	960	96.8	96.7	96.7	96.6
0.002	0.96	0.25	480	96.6	96.6	96.6	96.5
0.003	0.96	0.25	320	96.3	96.4	96.5	96.3
0.005	0.96	0.25	191	96.2	96.3	96.2	96.2

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

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Claims

1. A composition comprising an alpha-amylase, a pullulanase and a glucoamylase enzyme,

wherein the alpha-amylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 2 and 5, or a mature polypeptide thereof and has alpha-amylase activity;

wherein the pullulanase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3, or a mature polypeptide thereof and has pullulanase activity; and

wherein the glucoamylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 1 and 4, or a mature polypeptide thereof and has glucoamylase activity.

2. The composition according to claim 1, wherein the alpha-amylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of any one of SEQ ID NO: 2 and 5, or a mature polypeptide thereof.

3. The composition according to claim 1, wherein the alpha-amylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of any one of SEQ ID NO: 2 and 5, or a mature polypeptide thereof.

4. The composition according to any one of claims 1 to 3, wherein the pullulanase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3, or a mature polypeptide thereof.

5. The composition according to any one of claims 1 to 3, wherein the pullulanase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3, or a mature polypeptide thereof.

6. The composition according to any one of claims 1 to 5, wherein the glucoamylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%,

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at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of any one of SEQ ID NO: 1 and 4, or a mature polypeptide thereof.

7. The composition according to any one of claims 1 to 5, wherein the glucoamylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of any one of SEQ ID NO: 1 and 4, or a mature polypeptide thereof.

8. The composition according to claim 1,

wherein the alpha-amylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 2, or a mature polypeptide thereof and has alpha-amylase activity;

wherein the pullulanase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3, or a mature polypeptide thereof and has pullulanase activity; and

wherein the glucoamylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 1, or a mature polypeptide thereof and has glucoamylase activity.

9. The composition according to claim 8, wherein the alpha-amylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 2 or a mature polypeptide thereof.

10. The composition according to claim 8, wherein the alpha-amylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 2 or a mature polypeptide thereof.

11. The composition according to any one of claims 8 to 10, wherein the pullulanase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3 or a mature polypeptide thereof.

12. The composition according to any one of claims 8 to 10, wherein the pullulanase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least

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95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3 or a mature polypeptide thereof.

13. The composition according to any one of claims 8 to 12, wherein the glucoamylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 1 or a mature polypeptide thereof.

14. The composition according to any one of claims 8 to 12, wherein the glucoamylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 1 or a mature polypeptide thereof.

15. The composition according to claim 1,

wherein the alpha-amylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 5, or a mature polypeptide thereof and has alpha-amylase activity;

wherein the pullulanase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 3, or a mature polypeptide thereof and has pullulanase activity; and

wherein the glucoamylase comprises an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 4, or a mature polypeptide thereof and has glucoamylase activity.

16. The composition according to claim 15, wherein the alpha-amylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 5 or a mature polypeptide thereof.

17. The composition according to claim 15, wherein the alpha-amylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 5 or a mature polypeptide thereof.

18. The composition according to any one of claims 15 to 17, wherein the pullulanase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%,

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at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3 or a mature polypeptide thereof.

19. The composition according to any one of claims 15 to 17, wherein the pullulanase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 3 or a mature polypeptide thereof.

20. The composition according to any one of claims 15 to 19, wherein the glucoamylase comprises an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 4 or a mature polypeptide thereof.

21. The composition according to any one of claims 15 to 19, wherein the glucoamylase consists of an amino acid sequence having at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO: 4 or a mature polypeptide thereof.

22. A method of making glucose syrup from liquefied starch comprising, contacting the liquefied starch with a composition according to any one of claims 1-21.

23. The method according to claim 22, wherein the initial dry solids content (DS) in the liquefied starch substrate is at least 25% w/v, at least 30% w/v, at least 35% w/v, or at least 40% w/v.

24. The method according to claim 22 or 23, wherein saccharification time is at least 24 hrs, at least 30 hrs, at least 36 hrs, at least 48 hrs, at least 54 hrs, at least 60 hrs, or at least 72 hrs.

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