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(54) **Titre : HYDROLYSE ENZYMATIQUE DE DISACCHARIDES ET D'OLIGOSACCHARIDES A L'AIDE D'ENZYMES ALPHA-GLUCOSIDASES**

(54) **Title: ENZYMATIC HYDROLYSIS OF DISACCHARIDES AND OLIGOSACCHARIDES USING ALPHA-GLUCOSIDASE ENZYMES**

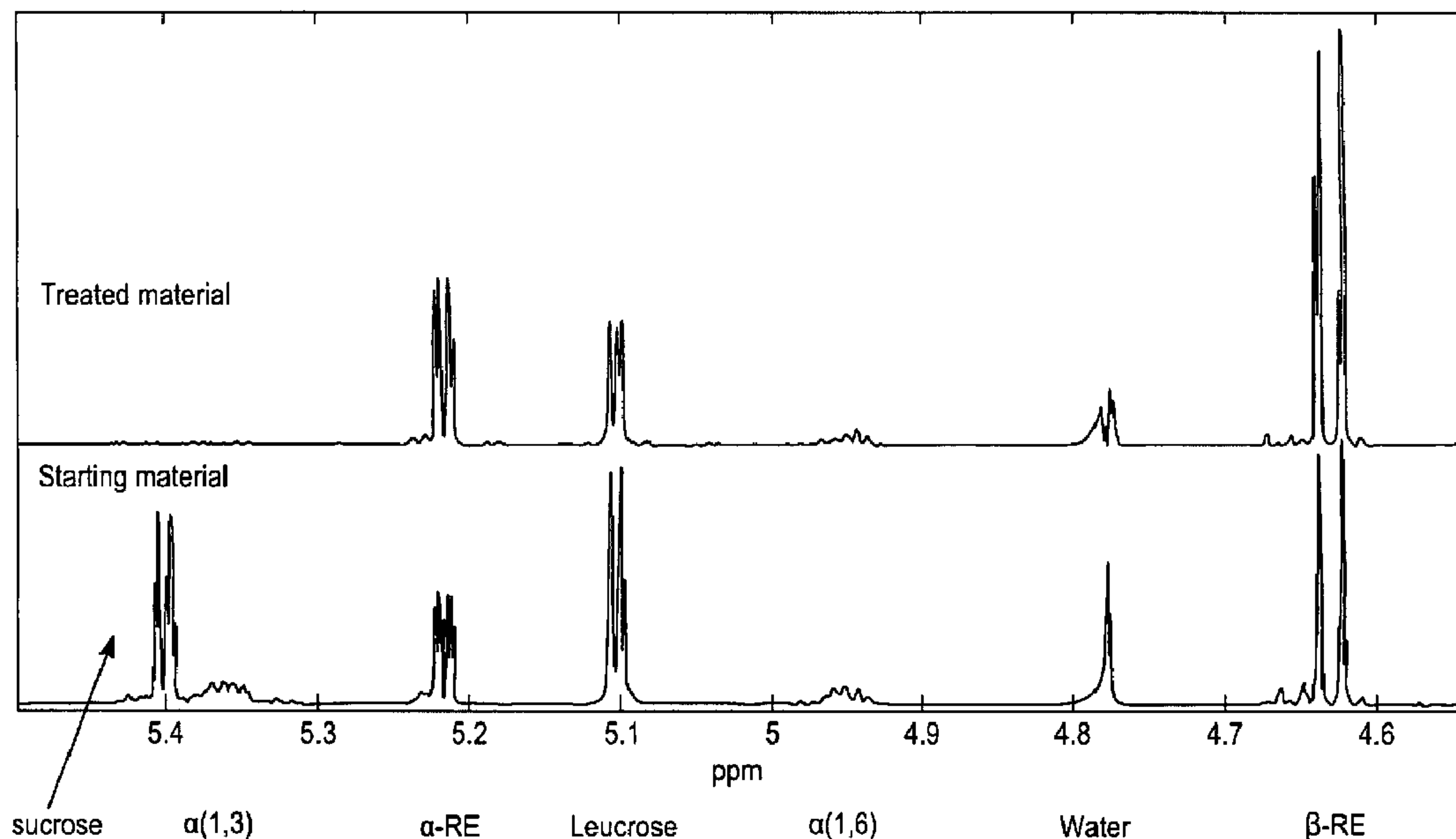


FIG. 1

(57) **Abrégé/Abstract:**

A method is disclosed for hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide (disaccharide or oligosaccharide). This method comprises contacting the saccharide with an alpha-glucosidase enzyme such as transglucosidase

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(57) **Abrégé(suite)/Abstract(continued):**

under suitable conditions, during which contacting step the enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide. This method is useful for reducing the amount of oligosaccharides in a filtrate isolated from a glucan synthesis reaction, for example.

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(54) Title: ENZYMATIC HYDROLYSIS OF DISACCHARIDES AND OLIGOSACCHARIDES USING ALPHA-GLUCOSIDASE ENZYMES

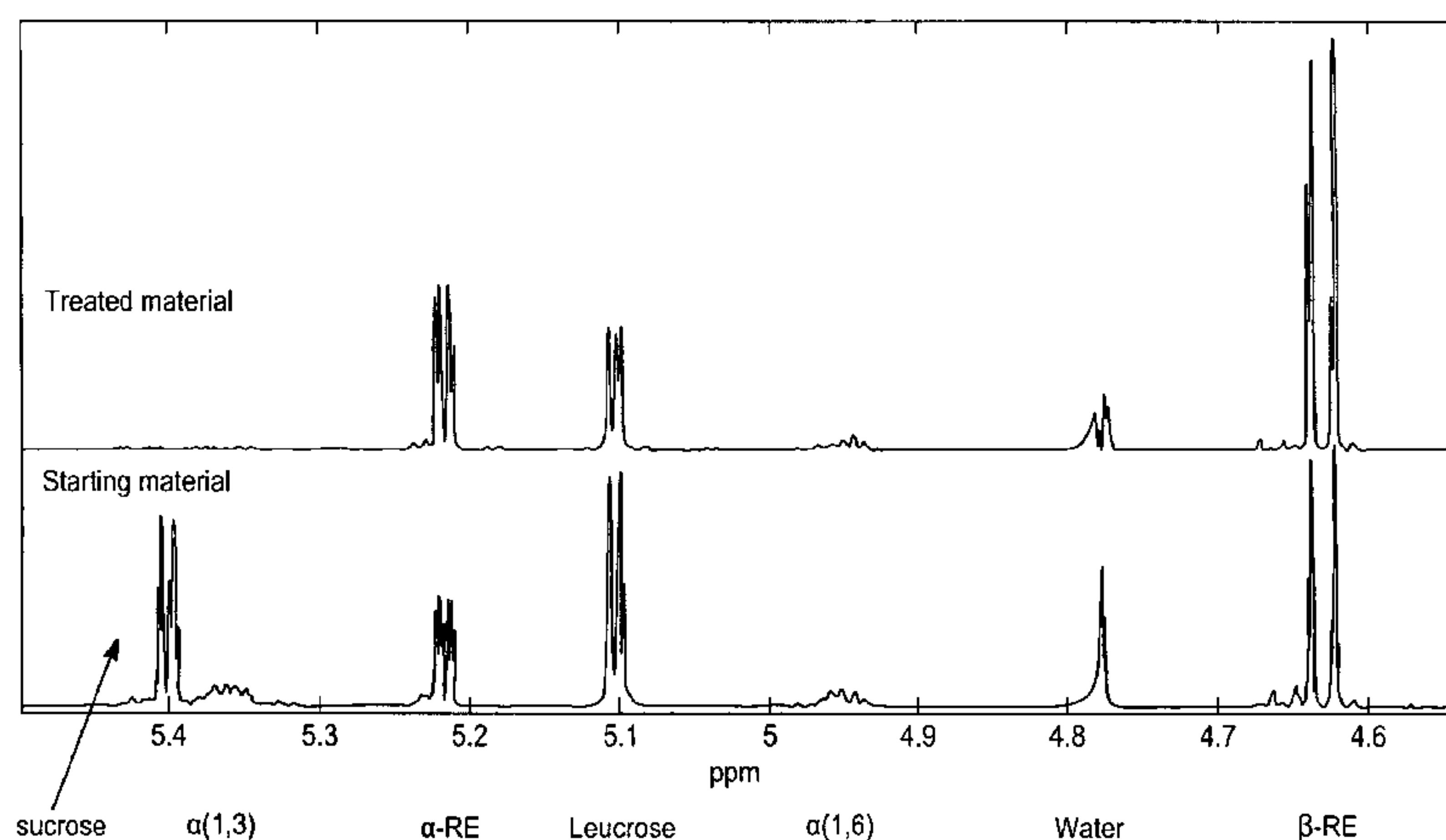


FIG. 1

(57) Abstract: A method is disclosed for hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide (disaccharide or oligosaccharide). This method comprises contacting the saccharide with an alpha-glucosidase enzyme such as transglucosidase under suitable conditions, during which contacting step the enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide. This method is useful for reducing the amount of oligosaccharides in a filtrate isolated from a glucan synthesis reaction, for example.

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TITLEENZYMATIC HYDROLYSIS OF DISACCHARIDES AND OLIGOSACCHARIDES
USING ALPHA-GLUCOSIDASE ENZYMES

This application claims the benefit of U.S. Provisional Application Nos.
5 61/945,233 (filed February 27, 2014), 61/945,241 (filed February 27, 2014),
62/004,290 (filed May 29, 2014), 62/004,308 (filed May 29, 2014), 62/004,312
(filed May 29, 2014), 62/004,300 (filed May 29, 2014), 62/004,314 (filed May 29,
2014), and 62/004,305 (filed May 29, 2014), all of which are incorporated herein
by reference in their entireties.

FIELD OF INVENTION

10 The invention is in the field of enzymatic hydrolysis of small sugar
polymers. Specifically, this invention pertains to hydrolyzing disaccharides and
oligosaccharides comprising one or more alpha-1,3 or alpha-1,6 glucosyl-glucose
linkages with an alpha-glucosidase enzyme.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

15 The official copy of the sequence listing is submitted electronically via
EFS-Web as an ASCII formatted sequence listing with a file named
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having a size of 266 kilobytes and is filed concurrently with the specification. The
20 sequence listing contained in this ASCII-formatted document is part of the
specification and is herein incorporated by reference in its entirety.

BACKGROUND

25 Transglucosidases (EC.2.4.1.24, 1,4-alpha-glucan 6-alpha-
glucosyltransferase) are D-glucosyltransferase enzymes that catalyze both
hydrolytic and transfer reactions on incubation with alpha-D-gluco-
oligosaccharides (1951, Pazur and French, *J. Amer. Chem. Soc.* 73:3536).
Maltose is the most preferred substrate for transglucosylation reactions with this
enzyme. Transfer occurs most frequently to HO-6, producing isomaltose from D-
glucose, or panose (6-O-alpha-glucosyl maltose) from maltose.

30 Transglucosidase can also transfer a glucosyl residue to the HO-2 or HO-3 of

another D-glucosyl unit to form Kojibiose or Nigerose. This enzyme can further transfer a D-glucosyl unit back to HO-4 to reform maltose.

As a result of transglucosylation reactions with transglucosidase, malto-oligosaccharide residues are converted to isomalto-oligosaccharides (IMO) containing a higher proportion of glucosyl residues linked by alpha-D-1,6 glycosidic linkages from the non-reducing end. IMO sugars are used in many food and beverage formulations in Asia. Brier et al. (U.S. Patent Appl. Publ. No. 2003/0167929) disclosed using transglucosidase to produce IMO from barley wort.

Poulose et al. (U.S. Patent Appl. Publ. No. 2008/0229514) disclosed using transglucosidase to degrade polysaccharides such as xanthan and guar gums. Xanthan gum comprises a cellulosic backbone in which alternate glucoses are 1,3-linked to branches containing mannose and glucuronic acid. The backbone of guar gum comprises beta-1,4-linked mannose residues to which galactose residues are alpha-1,6-linked at every other mannose.

Lantero et al. (U.S. Patent No. 5770437) disclosed using a transglucosidase to degrade sucrose, melezitose and trehalulose. These sugars comprise glucose linked to fructose via 1,2- (sucrose), 1,3- (melezitose), or 1,1- (trehalulose) linkages.

Although various hydrolytic activities of transglucosidase have been disclosed, this type of enzyme is generally considered to be an alpha-glucosidase, given its ability to hydrolyze alpha-linkages between two glucosyl residues. For example, transglucosidase is associated with having maltase activity (hydrolysis of the alpha-1,4 glycosidic link between the two glucosyl residues of maltose), which is a type of alpha-glucosidase activity.

Notwithstanding the foregoing disclosures, surprisingly, it has now been found that alpha-glucosidases such as transglucosidase (EC 2.4.1.24) can hydrolyze alpha-1,3 and alpha-1,6 glycosidic linkages of glucosyl-glucose. Alpha-glucosidase enzymes are disclosed herein as being useful for degrading disaccharides and oligosaccharides containing glucosyl-alpha-1,3-glucose and glucosyl-alpha-1,6-glucose.

SUMMARY OF INVENTION

In one embodiment, the invention concerns a method of hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide comprising at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein the
5 saccharide is a disaccharide or oligosaccharide, and wherein the method comprises: contacting the saccharide with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide, and wherein the amount of the saccharide is reduced compared to the amount of the
10 saccharide that was present prior to the contacting step.

In another embodiment, the alpha-glucosidase enzyme of the hydrolysis method is immobilized.

In another embodiment, the saccharide of the hydrolysis method has a degree of polymerization before hydrolysis of 3 to 7. In another embodiment, the
15 concentration of the saccharide after the contacting step is less than 50% of the concentration of the saccharide that was present prior to the contacting step.

In another embodiment, the suitable conditions of the hydrolysis method comprise (i) a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction; wherein the saccharide is a byproduct of the glucan
20 synthesis reaction. The glucan synthesis reaction produces at least one insoluble alpha-glucan product in another embodiment. In another embodiment, the fraction is a filtrate of the glucan synthesis reaction. In another embodiment, the glucan synthesis reaction produces at least one soluble alpha-glucan product that is (i) a product of a glucosyltransferase, or (ii) a product of the concerted
25 action of both a glucosyltransferase and an alpha-glucanohydrolase capable of hydrolyzing glucan polymers having one or more alpha-1,3-glycosidic linkages or one or more alpha-1,6-glycosidic linkages. The fraction is a chromatographic fraction of the glucan synthesis reaction in another embodiment in which the glucan synthesis reaction produces at least one soluble alpha-glucan product.

In another embodiment, the alpha-glucosidase enzyme is a transglucosidase. In another embodiment, the transglucosidase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:1

5 In another embodiment, the invention concerns a composition produced by contacting a saccharide with an alpha-glucosidase enzyme, wherein the saccharide is a disaccharide or oligosaccharide and comprises at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide, and wherein the composition comprises a reduced amount of
10 the saccharide compared to the amount of the saccharide that was present prior to the contacting step.

In another embodiment, the saccharide of the composition has a degree of polymerization before hydrolysis of 3 to 7. The concentration of the saccharide after the contacting step is less than 50% of the concentration of the saccharide
15 that was present prior to the contacting step, for example.

In another embodiment, the saccharide of the composition is in (i) a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction; wherein the saccharide is a byproduct of the glucan synthesis reaction. In another embodiment, the fraction is a filtrate of the glucan synthesis reaction
20 or a chromatographic fraction of the glucan synthesis reaction.

In another embodiment, the invention concerns a method of enriching fructose present in a fraction of a glucan synthesis reaction, comprising: (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase
25 enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the fraction; and (b) separating fructose from the hydrolyzed fraction of step (a) to obtain a composition having a higher concentration of fructose compared to the fructose concentration of the fraction of step (a).

30 In another embodiment, the invention concerns a fermentation method comprising: (a) contacting a fraction obtained from a glucan synthesis reaction

with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the fraction; (b) fermenting the fraction of step (a) with a microbe to yield a product, wherein the fermenting can be performed after step (a) or simultaneously with step (a); and (c) optionally, isolating the product of (b); wherein the yield of the product of (b) is increased compared to the product yield of fermenting a fraction of the glucan synthesis reaction that has not been contacted with the enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCES

Figure 1: ¹H NMR spectra of glucan reaction filtrate material before (starting material) and after (treated material) hydrolysis treatment with NOVO 188 enzyme (see Examples 2-3).

Figure 2: ¹H NMR spectra of glucan reaction filtrate material before (starting material) and after (treated material) hydrolysis treatment with TG L-2000 transglucosidase (see Examples 2-3).

Table 1. Summary of Nucleic Acid and Protein Sequence Identification Numbers

Description	Nucleic acid SEQ ID NO.	Protein SEQ ID NO.
"TG L-2000", <i>A. niger</i> transglucosidase (mature form without signal peptide)		1 (965 aa)
"GC 321 Glucoamylase", <i>T. reesei</i> glucoamylase (TrGA) (mature form without signal peptide)		2 (599 aa)
"gtfJ", <i>Streptococcus salivarius</i> glucosyltransferase. The first 42 amino acids of the protein are deleted compared to GENBANK Identification No. 47527; a start methionine is included.		3 (1477 aa)
"Aclglu1", <i>Aspergillus clavatus</i> alpha-glucosidase, full-length precursor form including signal peptide.	4 (3147 bases)	5 (990 aa)
"Aclglu1", <i>Aspergillus clavatus</i> alpha-glucosidase, mature form lacking signal peptide.		6 (971 aa)
"Nfiglu1", <i>Neosartorya fischeri</i> alpha-	7 (3158 bases)	8 (988 aa)

glucosidase, full-length precursor form including signal peptide.		
"Nfiglu1", <i>Neosartorya fischeri</i> alpha-glucosidase, mature form lacking signal peptide.		9 (969 aa)
"Ncrglu1", <i>Neurospora crassa</i> alpha-glucosidase, full-length precursor form including signal peptide.	10 (3385 bases)	11 (1044 aa)
"Ncrglu1", <i>Neurospora crassa</i> alpha-glucosidase, mature form lacking signal peptide.		12 (1022 aa)
"TauSec098", <i>Rasamsonia composticola</i> alpha-glucosidase, full-length precursor form including signal peptide.	13 (3293 bases)	14 (1035 aa)
"TauSec098", <i>Rasamsonia composticola</i> alpha-glucosidase, mature form lacking signal peptide.		15 (1013 aa)
"TauSec099", <i>Rasamsonia composticola</i> alpha-glucosidase, full-length precursor form including signal peptide.	16 (3162 bases)	17 (990 aa)
"TauSec099", <i>Rasamsonia composticola</i> alpha-glucosidase, mature form lacking signal peptide.		18 (973 aa)
"BloGlu1", <i>Bifidobacterium longum</i> (subsp. longum JDM301) alpha-glucosidase (wild type).	19 (1815 bases)	20 (604 aa)
"BloGlu1", <i>Bifidobacterium longum</i> (subsp. longum JDM301) alpha-glucosidase, codon-optimized sequence.	21 (1812 bases)	
"BloGlu2", <i>Bifidobacterium longum</i> alpha-glucosidase (wild type).		22 (604 aa)
"BloGlu2", <i>Bifidobacterium longum</i> alpha-glucosidase, codon-optimized sequence encoding amino acid sequence.	23 (1812 bases)	24 (604 aa)
"BloGlu3", <i>Bifidobacterium longum</i> (subsp. F8) alpha-glucosidase (wild type)	25 (1815 bases)	26 (604 aa)
"BloGlu3", <i>Bifidobacterium longum</i> (subsp. F8) alpha-glucosidase, codon-optimized sequence encoding amino acid sequence.	27 (1812 bases)	
"BpsGlu1", <i>Bifidobacterium</i>		28 (585 aa)

<i>pseudolongum</i> alpha-glucosidase (wild type).		
"BpsGlu1", <i>Bifidobacterium pseudolongum</i> alpha-glucosidase, codon-optimized sequence encoding amino acid sequence.	29 (1755 bases)	30 (586 aa)
"BthGlu1", <i>Bifidobacterium thermophilum</i> RBL67 alpha-glucosidase (wild type).	31 (1806 bases)	32 (601 aa)
"BthGlu1", <i>Bifidobacterium thermophilum</i> RBL67 alpha-glucosidase, codon-optimized sequence.	33 (1803 bases)	
"BbrGlu2", <i>Bifidobacterium breve</i> alpha-glucosidase (wild type).		34 (662 aa)
"BbrGlu2", <i>Bifidobacterium breve</i> alpha-glucosidase, codon-optimized sequence encoding amino acid sequence.	35 (1812 bases)	36 (604 aa)
"BbrGlu5", <i>Bifidobacterium breve</i> ACS-071-V-Sch8b alpha-glucosidase (wild type).	37 (1821 bases)	38 (606 aa)
"BbrGlu5", <i>Bifidobacterium breve</i> ACS-071-V-Sch8b alpha-glucosidase, codon-optimized sequence.	39 (1818 bases)	
"Gtf-S", <i>Streptococcus</i> sp. C150 glucosyltransferase, GENBANK GI No. 321278321.		40 (1570 aa)
"GTF0459", <i>Streptococcus</i> sp. C150 glucosyltransferase, N-terminal-truncated version of GENBANK GI No. 321278321.	41 (4179 bases)	42 (1392 aa)
"Gtf-C", <i>Streptococcus mutans</i> MT-4239 glucosyltransferase, GENBANK GI No. 3130088.		43 (1455 aa)
"GTF0088BsT1", <i>Streptococcus mutans</i> MT-4239 glucosyltransferase, N- and C-terminal-truncated version of GENBANK GI No. 3130088.	44 (2715 bases)	45 (904 aa)
"MUT3325", <i>Penicillium marneffei</i> ATCC 18224 mutanase, GENBANK GI No. 212533325.	46 (1308 bases)	47 (435 aa)

DETAILED DESCRIPTION OF THE INVENTION

The disclosures of all cited patent and non-patent literature are incorporated herein by reference in their entirety.

As used herein, the term “invention” or “disclosed invention” is not meant
5 to be limiting, but applies generally to any of the inventions defined in the claims or described herein. These terms are used interchangeably herein.

The terms “saccharide”, “saccharide molecule” and “carbohydrate” are used interchangeably herein and refer to a disaccharide or oligosaccharide, unless otherwise noted. A “disaccharide” herein refers to a carbohydrate having
10 two monosaccharides joined by a glycosidic linkage. An “oligosaccharide” herein refers to a carbohydrate that consists of 2 to 9 monosaccharides, for example, joined by glycosidic linkages. An oligosaccharide can also be referred to herein as an “oligomer”. Monosaccharides that are comprised within a disaccharide or oligosaccharide can be referred to as “monosaccharide units” or “monomeric
15 units”, for example. Preferred monosaccharides herein are fructose and glucose.

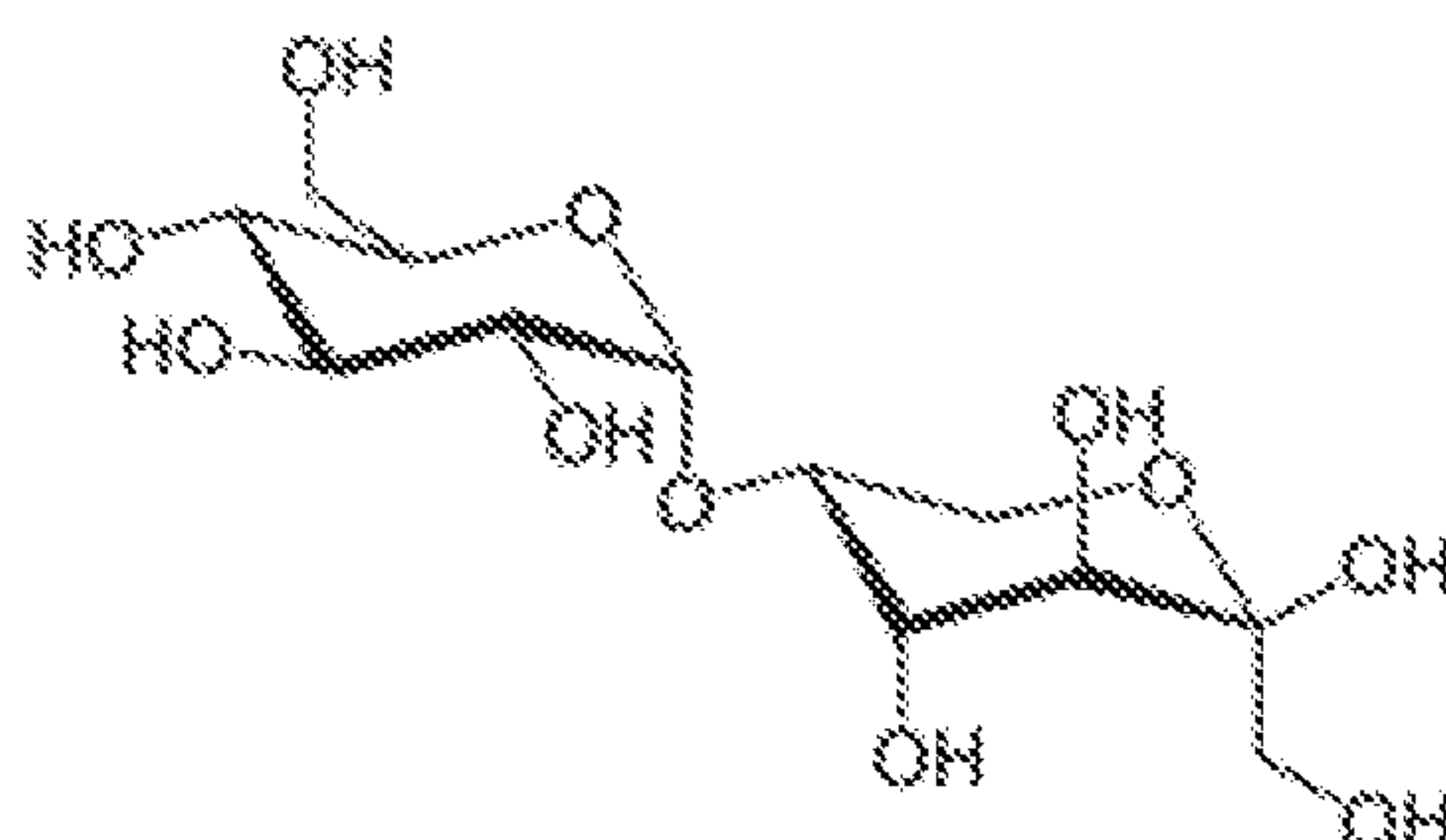
The terms “glycosidic linkage” and “glycosidic bond” are used interchangeably herein and refer to the type of covalent bond that joins a carbohydrate molecule to another carbohydrate molecule.

The terms “alpha-1,3 glucosyl-glucose linkage”, “alpha-1,3 glucose-
20 glucose linkage” and “glucose-alpha 1,3-glucose” herein refers to an alpha-1,3-glycosidic linkage between two alpha-D-glucose molecules. The terms “alpha-1,6 glucosyl-glucose linkage”, “alpha-1,6 glucose-glucose linkage” and “glucose-alpha 1,6-glucose” herein refers to an alpha-1,6-glycosidic linkage between two alpha-D-glucose molecules. Alpha-1,3 glucosyl-glucose linkage(s) and/or alpha-
25 1,6 glucosyl-glucose linkage(s) herein are comprised within a disaccharide or oligosaccharide in certain embodiments.

The terms “alpha-1,5 glucosyl-fructose linkage”, “alpha-1,5 glucose-fructose linkage” and “glucose-alpha-1,5-fructose” herein refers to an alpha-1,5-glycosidic linkage between an alpha-D-glucose molecule and a fructose
30 molecule. An alpha-1,5 glucosyl-fructose linkage herein is comprised within a disaccharide or oligosaccharide in certain embodiments.

"Alpha-D-glucose" herein can also be referred to as "glucose".

A disaccharide containing an alpha-1,5 glucosyl-fructose linkage is referred to herein as leucrose. The terms "leucrose" and "D-glucopyranosyl-alpha(1-5)-D-fructopyranose" are used interchangeably herein. Leucrose has the following structure:



The terms "alpha-glucosidase", "alpha-1,4-glucosidase", and "alpha-D-glucoside glucohydrolase" are used interchangeably herein. Alpha-glucosidases (EC 3.2.1.20) ("EC" refers to Enzyme Commission number) have previously been recognized as enzymes that catalyze hydrolytic release of terminal, non-reducing (1,4)-linked alpha-D-glucose residues from oligosaccharide (e.g., disaccharide) and polysaccharide substrates. Alpha-glucosidases are now disclosed herein to also have hydrolytic activity toward alpha-1,5 glucosyl-fructose linkages, and hydrolytic activity toward alpha-1,3 and alpha-1,6 glucosyl-glucose linkages.

Transglucosidase and glucoamylase enzymes are examples of alpha-glucosidases with such activity.

The terms "transglucosidase" (TG), "transglucosidase enzyme", and "1,4-alpha-glucan 6-alpha-glucosyltransferase" are used interchangeably herein.

Transglucosidases (EC 2.4.1.24) have previously been recognized as D-glucosyltransferase enzymes that catalyze both hydrolytic and transfer reactions on incubation with certain alpha-D-gluco-oligosaccharides. Transglucosidases are now disclosed herein to also have hydrolytic activity toward alpha-1,5 glucosyl-fructose linkages, and hydrolytic activity toward alpha-1,3 and alpha-1,6 glucosyl-glucose linkages.

The terms "glucoamylase" (GA), "glucoamylase enzyme", and "alpha-1,4-glucan glucohydrolase" are used interchangeably herein. Glucoamylases (EC 3.2.1.3) have previously been recognized as exo-acting enzymes that catalyze

hydrolysis of both alpha-1,4 and alpha-1,6 glycosidic linkages from non-reducing ends of glucose-containing di-, oligo- and poly-saccharides. Glucoamylases are now disclosed herein to also have hydrolytic activity toward alpha-1,5 glucosyl-fructose linkages.

5 Enzymatic hydrolysis is a process in which an enzyme facilitates the cleavage of bonds in molecules with the addition of the elements of water. "Hydrolyzing", "hydrolysis of", or "hydrolytic activity toward" an alpha-1,3 or alpha 1,6 glucosyl-glucose linkage herein refers to enzymatic hydrolysis of the alpha-1,3 or alpha-1,6 glycosidic linkage between two glucose molecules by an alpha-
10 glucosidase such as a transglucosidase. Such hydrolysis occurs when contacting a disaccharide or oligosaccharide containing an alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkage with an alpha-glucosidase herein under suitable conditions. Thus, a "hydrolysis reaction" herein comprises at least (i) a disaccharide or oligosaccharide containing an alpha-1,3 and/or alpha-1,6
15 glucosyl-glucose linkage(s), and (ii) an alpha-glucosidase.

The term "saccharification" herein refers to a process of breaking a saccharide (disaccharide or oligosaccharide) into its monosaccharide components. A saccharide can be saccharified in a hydrolysis reaction herein.

20 "Suitable conditions" for contacting a saccharide (disaccharide or oligosaccharide) comprising at least one alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkage with an alpha-glucosidase herein refer to those conditions (e.g., temperature, pH, time) that support the hydrolysis of one or more alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in the saccharide by the alpha-glucosidase. Suitable conditions can comprise "aqueous conditions", for
25 example, comprising at least 20 wt% water. Aqueous conditions may characterize a solution or mixture. The solution or mixture in which a saccharide comprising at least one alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkage is contacted with an alpha-glucosidase can be referred to as an alpha-glucosidase reaction, for example (e.g., a transglucosidase or glucoamylase reaction).

30 An "immobilized" enzyme herein refers to an enzyme that is attached to an inert, insoluble material. Methods for preparing immobilized enzymes are

disclosed, for example, in U.S. Patent No. 5541097, which is incorporated herein by reference.

The terms "glucan" and "glucan polymer" are used interchangeably herein and refer to a polysaccharide of glucose monomers linked by glycosidic bonds.

5 An "alpha-glucan" herein refers to a glucan polymer comprising at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% alpha-glycosidic linkages.

An "insoluble glucan" herein refers to a glucan polymer that is not soluble in aqueous conditions. An example of insoluble glucan herein is poly alpha-1,3-
10 glucan with a DP of at least 8 or 9. A glucosyltransferase reaction in certain embodiments as presently disclosed produces at least one insoluble glucan product.

The terms "soluble glucan", "soluble alpha-glucan", "soluble fiber", "soluble glucan fiber", "soluble dietary fiber" and the like are used interchangeably herein
15 to refer to a glucan polymer that is soluble in aqueous conditions. Examples of soluble glucan herein are certain oligosaccharides, such as poly alpha-1,3-glucan with a DP less than 8, and certain oligosaccharides disclosed in the Examples provided below. A glucosyltransferase reaction in certain
20 embodiments as presently disclosed produces at least one soluble glucan product. Another set of features that characterizes soluble alpha-glucan compounds in certain embodiments herein is that they are (i) water-soluble glucose oligomers having a degree of polymerization of 3 or more, (ii) digestion-resistant (i.e., exhibit very slow or no digestibility) with little or no absorption in
25 the human small intestine, and (iii) at least partially fermentable in the lower gastrointestinal tract. Digestibility of a soluble glucan fiber composition can be measured using AOAC method 2009.01, for example.

The terms "poly alpha-1,3-glucan" and "alpha-1,3-glucan polymer" are used interchangeably herein. Poly alpha-1,3-glucan is a polymer comprising glucose monomeric units linked together by glycosidic linkages, wherein at least
30 about 50% of the glycosidic linkages are alpha-1,3-glycosidic linkages. The term "alpha-1,3-glycosidic linkage" as used herein refers to the type of covalent bond

that joins alpha-D-glucose molecules to each other through carbons 1 and 3 on adjacent alpha-D-glucose rings.

The “molecular weight” of a glucan herein can be represented as number-average molecular weight (M_n) or as weight-average molecular weight (M_w).

5 Alternatively, molecular weight can be represented as Daltons, grams/mole, DP_w (weight average degree of polymerization), or DP_n (number average degree of polymerization). Various means are known in the art for calculating these molecular weight measurements such as with high-pressure liquid chromatography (HPLC), size exclusion chromatography (SEC), or gel
10 permeation chromatography (GPC).

The terms “glucosyltransferase enzyme”, “gtf enzyme”, “gtf enzyme catalyst”, “gtf”, “glucansucrase” and the like are used interchangeably herein. The activity of a gtf enzyme herein catalyzes the reaction of sucrose substrate to make the products glucan and fructose. Other products (byproducts) of a gtf
15 reaction can include glucose (results from when glucose is hydrolyzed from the glucosyl-gtf enzyme intermediate complex), various soluble oligosaccharides (e.g., DP2-DP7), and leucrose (results from when glucose of the glucosyl-gtf enzyme intermediate complex is linked to fructose). Wild type forms of glucosyltransferase enzymes generally contain (in the N-terminal to C-terminal
20 direction) a signal peptide, a variable domain, a catalytic domain, and a glucan-binding domain. A glucosyltransferase herein is classified under the glycoside hydrolase family 70 (GH70) according to the CAZy (Carbohydrate-Active EnZymes) database (Cantarel et al., *Nucleic Acids Res.* 37:D233-238, 2009).

The term “sucrose” herein refers to a non-reducing disaccharide
25 composed of an alpha-D-glucose molecule and a beta-D-fructose molecule linked by an alpha-1,2-glycosidic bond. Sucrose is known commonly as table sugar.

The terms “glucan synthesis reaction”, “glucan reaction” “gtf reaction” and the like are used interchangeably herein and refer to a reaction that is performed by a glucosyltransferase enzyme. A glucan synthesis reaction as used herein
30 generally refers to a solution comprising at least one active glucosyltransferase enzyme in a solution comprising sucrose and water, and optionally other

components. Other components that can be in a glucan synthesis reaction herein include fructose, glucose, leucrose, soluble oligosaccharides (e.g., DP2-DP7), and soluble glucan product(s), for example. Also, one or more alpha-glucanohydrolase enzymes can be comprised in a glucan synthesis reaction in some aspects. It would be understood that certain glucan products, such as poly alpha-1,3-glucan with a degree of polymerization (DP) of at least 8 or 9, are water-insoluble and thus are not dissolved in a glucan synthesis reaction, but rather may be present out of solution.

The terms "alpha-glucanohydrolase" and "glucanohydrolase" are used interchangeably herein and refer to an enzyme capable of hydrolyzing an alpha-glucan oligomer. An alpha-glucanohydrolase can be defined by its endohydrolysis activity towards certain alpha-D-glycosidic linkages. Examples of alpha-glucanohydrolase enzymes herein include dextranases (EC 3.2.1.11; capable of endohydrolyzing alpha-1,6-linked glycosidic bonds), mutanases (EC 3.2.1.59; capable of endohydrolyzing alpha-1,3-linked glycosidic bonds), and alternanases (EC 3.2.1.-; capable of endohydrolytically cleaving alternan). Various factors including, but not limited to, level of branching, the type of branching, and the relative branch length within certain alpha-glucans may adversely impact the ability of an alpha-glucanohydrolase to endohydrolyze some glycosidic linkages.

The "percent dry solids" of a glucan synthesis reaction refers to the wt% of all the sugars in a glucan synthesis reaction. The percent dry solids of a gtf reaction can be calculated, for example, based on the amount of sucrose used to prepare the reaction.

A "fraction" of a glucan synthesis reaction herein refers to a liquid solution portion of a glucan synthesis reaction. A fraction can be a portion of, or all of, the liquid solution from a glucan synthesis reaction, and has been separated from a soluble or insoluble glucan product synthesized in the reaction. A fraction can optionally be referred to as a "mother liquor" in embodiments in which the product is an insoluble (solid) glucan product. An example of a fraction is a filtrate of a glucan synthesis reaction. Since a fraction can contain dissolved sugars such as

sucrose, fructose, glucose, leucrose, soluble oligosaccharides (e.g., DP2-DP7), a fraction can also be referred to as a "mixed sugar solution" derived from a glucan synthesis reaction. A "hydrolyzed fraction" herein refers to a fraction that has been treated with an alpha-glucosidase herein to hydrolyze leucrose and/or oligosaccharides present in the fraction.

The terms "filtrate", "glucan reaction filtrate", "glucan filtrate" and the like are used interchangeably herein and refer to a fraction that has been filtered away from a solid glucan product synthesized in a glucan synthesis reaction. A "hydrolyzed filtrate" herein refers to a filtrate that has been treated with an alpha-glucosidase herein to hydrolyze leucrose and/or oligosaccharides present in the filtrate.

The terms "percent by volume", "volume percent", "vol %", "v/v %" and the like are used interchangeably herein. The percent by volume of a solute in a solution can be determined using the formula: $[(\text{volume of solute})/(\text{volume of solution})] \times 100\%$.

The terms "percent by weight", "weight percentage (wt%)", "weight-weight percentage (% w/w)" and the like are used interchangeably herein. Percent by weight refers to the percentage of a material on a mass basis as it is comprised in a composition, mixture, or solution. All percentages herein are weight percentages, unless otherwise noted.

As used herein, "polydispersity index", "PDI", "heterogeneity index", "dispersity" and the like refer to a measure of the distribution of molecular mass in a given polymer (e.g., a glucose oligomer such as a soluble alpha-glucan) sample and can be calculated by dividing the weight average molecular weight by the number average molecular weight ($PDI = M_w/M_n$).

The terms "increased", "enhanced" and "improved" are used interchangeably herein. These terms refer to a greater quantity or activity such as a quantity or activity slightly greater than the original quantity or activity, or a quantity or activity in large excess compared to the original quantity or activity, and including all quantities or activities in between. Alternatively, these terms may refer to, for example, a quantity or activity that is at least 1%, 2%, 3%, 4%,

5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% more than the quantity or activity for which the increased quantity or activity is being compared.

The terms "sequence identity" or "identity" as used herein with respect to polynucleotide or polypeptide sequences refer to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window. Thus, "percentage of sequence identity" or "percent identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity.

The Basic Local Alignment Search Tool (BLAST) algorithm, which is available online at the National Center for Biotechnology Information (NCBI) website, may be used, for example, to measure percent identity between or among two or more of the polynucleotide sequences (BLASTN algorithm) or polypeptide sequences (BLASTP algorithm) disclosed herein. Alternatively, percent identity between sequences may be performed using a Clustal algorithm (e.g., ClustalW or ClustalV). For multiple alignments using a Clustal method of alignment, the default values may correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using a Clustal method may be KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids, these parameters may be KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. Alternatively still,

percent identity between sequences may be performed using an EMBOSS algorithm (e.g., needle) with parameters such as GAP OPEN=10, GAP EXTEND=0.5, END GAP PENALTY=false, END GAP OPEN=10, END GAP EXTEND=0.5 using a BLOSUM matrix (e.g., BLOSUM62).

5 Various polypeptide amino acid sequences are disclosed herein as features of certain embodiments. Variants of these sequences that are at least about 70-85%, 85-90%, or 90%-95% identical to the sequences disclosed herein can be used. Alternatively, a variant amino acid sequence can have at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%,
10 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with a sequence disclosed herein. A variant amino acid sequence herein has the same function/activity of the disclosed sequence, or at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the function/activity of a disclosed sequence.

15 The term "isolated" as used in certain embodiments refers to any cellular component that is completely separated from its native source (e.g., an isolated polynucleotide or polypeptide molecule). In some instances, an isolated polynucleotide or polypeptide molecule is part of a greater composition, buffer system or reagent mix. For example, the isolated polynucleotide or polypeptide
20 molecule can be comprised within a cell or organism in a heterologous manner. Another example is an isolated alpha-glucosidase (e.g., glucoamylase, transglucosidase), or glucosyltransferase enzyme. The enzyme reactions (e.g., alpha-glucosidase reaction, glucosyltransferase reaction) disclosed herein are synthetic, non-naturally occurring processes.

25

Embodiments of the disclosed invention concern a method of hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide comprising at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage. The saccharide is a
30 disaccharide or oligosaccharide. This method comprises contacting the saccharide with an alpha-glucosidase enzyme under suitable conditions. In the

contacting step, the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide. Due to this hydrolysis, the amount of the saccharide is reduced compared to the amount of the saccharide that was present prior to the contacting step. Thus, this hydrolysis method can alternatively be referred to as a method of reducing the amount of a saccharide in a composition.

Significantly, it is believed to be previously unknown that alpha-glucosidase enzymes can hydrolyze alpha-1,3 and alpha-1,6 glucosyl-glucose linkages. Alpha-glucosidase reactions following this hydrolysis method can thus be used to remove oligosaccharide byproducts containing these glucose-glucose linkages from a glucan synthesis reaction and/or a fraction obtained therefrom. Such removal represents an improvement over chemical processes of byproduct removal, such as acid hydrolysis, which can result in degradation of glucan product. Finally, a glucan reaction fraction that is treated according to the above hydrolysis method is better-suited for downstream applications such as fermentation, for example, since the level of glucose monosaccharides is increased in the fraction. Monosaccharides are generally more tractable for downstream processes compared to oligosaccharide byproducts.

An alpha-glucosidase (EC 3.2.1.20) is used in embodiments herein to hydrolyze alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in a saccharide comprising at least one of these linkages. Alpha-glucosidase enzymes have previously been recognized to catalyze hydrolytic release of terminal, non-reducing (1,4)-linked alpha-D-glucose residues from oligosaccharide (e.g., disaccharide) and polysaccharide substrates. These enzymes are now disclosed herein to also have hydrolytic activity toward alpha-1,3 and alpha-1,6 glucosyl-glucose linkages, for example.

An alpha-glucosidase can be from any source (e.g., plant, animal, microbe such as a bacteria or fungus/yeast), for example, such as those sources disclosed below from which a transglucosidase and/or glucoamylase can be derived. For example, an alpha-glucosidase can be a fungal alpha-glucosidase.

Other examples of suitable alpha-glucosidases herein include those disclosed in U.S. Patent Nos. 6355467, 5922580, 5795766, 5763252, and 8633006, which are all incorporated herein by reference.

An alpha-glucosidase enzyme in certain embodiments herein may
5 comprise the amino acid sequence of SEQ ID NO:5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, or that of DIAZYME RDF ULTRA (DuPont Industrial Biosciences). Alternatively, an alpha-glucosidase enzyme may comprise an amino acid sequence that is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:5, 6, 8, 9, 11,
10 12, 14, 15, 17, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, or to the amino acid sequence of DIAZYME RDF ULTRA, and have hydrolytic activity toward alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in saccharides. Several of the foregoing sequences, for example, are mature alpha-glucosidases that lack an N-terminal signal peptide. For such sequences, it would be understood that an
15 N-terminal start-methionine would typically be added (if necessary) (directly or via an intervening heterologous amino acid sequence such as an epitope) if expressing it without using a signal peptide (such as with an expression system where the enzyme is expressed intracellularly and obtained from a cell lysate).

A transglucosidase (EC 2.4.1.24; 1,4-alpha-glucan 6-alpha-
20 glucosyltransferase) can be used in certain embodiments herein as an alpha-glucosidase to hydrolyze alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in a saccharide comprising at least one of these linkages. This class of enzymes has previously been recognized as D-glucosyltransferase enzymes that catalyze hydrolytic and transfer reactions on incubation with certain alpha-D-gluco-
25 oligosaccharides. Transglucosidases as now disclosed herein also have hydrolytic activity toward alpha-1,3 and alpha-1,6 glucosyl-glucose linkages.

A transglucosidase enzyme herein may be derived from any microbial source, such as a bacteria or fungus. Examples of fungal transglucosidases include, but are not limited to, those of *Trichoderma* species (e.g., *T. reesei*),
30 *Aspergillus* species and *Neosartorya* species (e.g., *N. fischeri*). Examples of *Aspergillus* species from which a transglucosidase may be derived include, but

are not limited to, *A. niger*, *A. awamori*, *A. oryzae*, *A. terreus*, *A. clavatus*, *A. fumigatus* and *A. nidulans*. Other examples of transglucosidase enzymes useful herein are described in Barker et al. (1953, *J. Chem. Soc.* 3588-3593); Pazur et al. (1986, *Carbohydr. Res.* 149:137-147), Nakamura et al. (1997, *J. Biotechnol.*

5 53:75-84), and U.S. Patent Appl. Publ. No. 2008/0229514, all of which are incorporated herein by reference. Still other examples of transglucosidase enzymes useful herein are those that are thermostable; U.S. Patent No. 4689296, which is incorporated herein by reference, discloses a process for producing thermostable transglucosidase. Yet more examples of
10 transglucosidase enzymes useful herein may be any of those in the GENBANK database (NCBI), such as accession numbers: D45356 (GID:2645159, *A. niger*), BAD06006.1 (GID:4031328, *A. awamori*), BAA08125.1 (GID:1054565, *A. oryzae*), XP_001210809.1 (GID:115492363, *A. terreus*), XP_001216899.1 (GID:115433524, *A. terreus*), XP_001271891.1 (GID:121707620, *A. clavatus*),
15 XP_751811.1 (GID:70993928, *A. fumigatus*), XP_659621.1 (GID:67523121, *A. nidulans*), XP_001266999.1 (GID:119500484, *N. fischeri*) and XP_001258585.1 (GID:119473371, *N. fischeri*), which are all incorporated herein by reference.

Alternatively, a transglucosidase herein may have an amino acid sequence that is at least 90% or 95% identical with the amino acid sequence of any of the
20 foregoing disclosed transglucosidase sequences, and have hydrolytic activity toward alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in saccharides. All of the foregoing transglucosidases, when used in a hydrolysis reaction herein, are preferably in a mature form lacking an N-terminal signal peptide.

A transglucosidase enzyme in certain embodiments herein may comprise
25 the amino acid sequence of SEQ ID NO:1 (TG L-2000), which is an *A. niger* transglucosidase (U.S. Patent Appl. Publ. No. 2008/0229514). Alternatively, a transglucosidase may comprise an amino acid sequence that is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:1 and have hydrolytic activity toward alpha-1,3 and/or alpha-1,6 glucosyl-
30 glucose linkages in saccharides. Any of SEQ ID NO:1 or variants thereof can be produced following the disclosure of U.S. Patent Appl. Publ. No. 2008/0229514,

for example, which is incorporated herein by reference. SEQ ID NO:1 is a mature transglucosidase that lacks an N-terminal signal peptide. Since SEQ ID NO:1 does not begin with a methionine residue, it would be understood that an N-terminal start-methionine would typically be added to SEQ ID NO:1 (directly or via an intervening heterologous amino acid sequence such as an epitope) if expressing it without using a signal peptide (such as with an expression system where the enzyme is expressed intracellularly and obtained from a cell lysate).

A glucoamylase (EC 3.2.1.3; alpha-1,4-glucan glucohydrolase) can be used in certain embodiments herein as an alpha-glucosidase. For example, a glucoamylase can be included with a transglucosidase in each of the hydrolysis reaction settings/conditions disclosed herein. In this context, a glucoamylase can be used to hydrolyze (i) an alpha-1,5 glucosyl-fructose linkage, and/or (ii) an alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkage present in a saccharide containing any of these linkage types. This class of enzymes has previously been recognized as exo-acting enzymes that catalyze hydrolysis of both alpha-1,4 and alpha-1,6 glycosidic linkages from non-reducing ends of glucose-containing di-, oligo- and poly-saccharides. Glucoamylases as now disclosed herein also have hydrolytic activity toward alpha-1,5 glucosyl-fructose linkages. In certain embodiments, an alpha-glucosidase is not a glucoamylase.

A glucoamylase enzyme herein may be derived from any microbial source, such as a bacteria or fungus. Examples of bacterial glucoamylases include, but are not limited to, those of *Bacillus* species (e.g., *B. alkalophilus*, *B. amyloliquefaciens*, *B. lentus*, *B. licheniformis*, *B. stearothermophilus*, *B. subtilis*, *B. thuringiensis*) and *Streptomyces* species (e.g., *S. lividans*). Examples of fungal glucoamylases include, but are not limited to, those of *Trichoderma* species (e.g., *T. reesei*, *T. longibrachiatum*, *T. strictipilis*, *T. asperellum*, *T. konilangbra*, *T. hazianum*), *Aspergillus* species (e.g., *A. niger*, *A. oryzae*, *A. terreus*, *A. clavatus*, *A. nidulans*, *A. kawachi*, *A. awamori*), *Rhizopus* species (e.g., *R. oryzae*, *R. niveus*), *Talaromyces* species (e.g., *T. emersonii*, *T. thermophilus*, *T. duponti*), *Mucor* species, *Hypocrea* species (e.g., *H. gelatinosa*, *H. orientalis*, *H. vinosa*, *H. citrina*), *Fusarium* species (e.g., *F. oxysporum*, *F.*

roseum, *F. venenatum*), *Neurospora* species (e.g., *N. crassa*), *Humicola* species (e.g., *H. grisea*, *H. insolens*, *H. lanuginosa*), *Penicillium* species (e.g., *P. notatum*, *P. chrysogenum*) and *Saccharomycopsis* species (e.g., *S. fibuligera*).

Examples of these bacterial and fungal glucoamylases for use herein are disclosed in U.S. Pat. Appl. Publ. No. 2013/0102035, which is incorporated herein by reference. Other examples of glucoamylase enzymes useful herein are described in Svensson et al. (1983, *Carlsberg Res. Commun.* 48:529-544), Boel et al. (1984, *EMBO J.* 3:1097-1102); Hayashida et al. (1989, *Agric. Biol. Chem.* 53:923-929); U.S. Pat. No. 5024941, U.S. Pat. No. 4794175, U.S. Pat. No. 4247637, U.S. Pat. No. 6255084, U.S. Pat. No. 6620924, Ashikari et al. (1986, *Agric. Biol. Chem.* 50:957-964), Ashikari et al. (1989, *Appl. Microbiol. Biotechnol.* 32:129-133), U.S. Pat. No. 4863864; U.S. Pat. No. 4618579, Houghton-Larsen et al. (2003, *Appl. Microbiol. Biotechnol.* 62:210-217) and U.S. Pat. No. 7413887, all of which are incorporated herein by reference.

Alternatively, a glucoamylase herein may have an amino acid sequence that is at least 90% or 95% identical with the amino acid sequence of any of the foregoing disclosed glucoamylase sequences, and have hydrolytic activity toward (i) alpha-1,5 glucosyl-fructose linkages and/or (ii) alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages. All of the foregoing glucoamylases, when used in a hydrolysis reaction herein, are preferably in a mature form lacking an N-terminal signal peptide. Commercially available glucoamylases useful herein include OPTIDEX L-400, GC 147, GC 321, G ZYME G990 4X, OPTIMAX 7525, DEXTROZYME, DISTILLASE and GLUCZYME, for example.

A glucoamylase enzyme in certain embodiments herein may comprise the amino acid sequence of SEQ ID NO:2 (GC 321), which is a *T. reesei* glucoamylase. Alternatively, a glucoamylase may comprise an amino acid sequence that is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:2 and have hydrolytic activity toward (i) alpha-1,5 glucosyl-fructose linkages and/or (ii) alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages. Any of SEQ ID NO:2 or variants thereof can be produced following the disclosures of U.S. Pat. No. 7413887 or U.S. Pat. Appl.

Publ. No. 2013/0102035, for example, which are incorporated herein by reference. SEQ ID NO:2 is a mature glucoamylase that lacks an N-terminal signal peptide. Since SEQ ID NO:2 does not begin with a methionine residue, it would be understood that an N-terminal start-methionine would typically be added to SEQ ID NO:2 (directly or via an intervening heterologous amino acid sequence such as an epitope) if expressing it without using a signal peptide (such as with an expression system where the enzyme is expressed intracellularly and obtained from a cell lysate).

An alpha-glucosidase enzyme herein such as a transglucosidase or glucoamylase may be from a commercial source (e.g., DuPont Industrial Biosciences / Genencor, USA; Megazyme International, Ireland; Amano Enzyme Inc., Japan). Alternatively, such an enzyme may be produced by any means known in the art, such as described in U.S. Pat. Appl. Publ. No. 2008/0229514, U.S. Pat. No. 7413887 or U.S. Pat. Appl. Publ. No. 2013/0102035, which are incorporated herein by reference. For example, an alpha-glucosidase may be produced recombinantly in a heterologous expression system, such as a microbial or fungal heterologous expression system. Examples of heterologous expression systems include bacterial (e.g., *E. coli*, *Bacillus* sp.) and eukaryotic systems. Eukaryotic systems can employ yeast (e.g., *Pichia* sp., *Saccharomyces* sp.) or fungal (e.g., *Trichoderma* sp. such as *T. reesei*; *Aspergillus* species such as *A. niger*) expression systems, for example. The transglucosidase of SEQ ID NO:1 and glucoamylase of SEQ ID NO:2, and variants thereof, can be expressed in a *T. reesei* host, for example.

An alpha-glucosidase enzyme when used in a hydrolysis reaction herein is preferably in a mature form lacking an N-terminal signal peptide. An expression system for producing a mature alpha-glucosidase enzyme herein may employ an enzyme-encoding polynucleotide that further comprises sequence encoding an N-terminal signal peptide to direct extra-cellular secretion. The signal peptide in such embodiments is cleaved from the enzyme during the secretion process. The signal peptide may either be native or heterologous to the transglucosidase or glucoamylase. Alternatively, an alpha-glucosidase enzyme in a mature form

can be provided by expressing it without using a signal peptide, such as with an expression system where the enzyme is expressed intracellularly and obtained from a cell lysate. In either scenario (secretion or intracellularly expressed), a heterologous amino acid sequence such as an epitope can optionally be included at the N-terminus of the alpha-glucosidase.

An alpha-glucosidase enzyme in certain embodiments may be provided in a hydrolysis reaction herein by direct use of a cell that expresses the enzyme(s). In other words, an alpha-glucosidase that is contacted with a saccharide can be present by virtue of its expression from a cell placed in the suitable conditions for hydrolysis. Such a cell could thus be used in place of adding an isolated alpha-glucosidase preparation to the hydrolysis reaction. A cell for this purpose can be a bacterial, yeast, or fungal cell, for example. Examples of yeast include those from the genera *Saccharomyces* (e.g., *S. cerevisiae*), *Kluyveromyces*, *Candida*, *Pichia*, *Schizosaccharomyces*, *Hansenula*, *Kloeckera*, and *Schwanniomyces*.

Other expression systems useful herein are disclosed in U.S. Patent. Appl. Publ. No. 2013/0323822, which is incorporated herein by reference.

A saccharide herein comprises at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage. Thus, depending on the length of the saccharide, it may contain 1, 2, 3, 4, 5, 6, 7, or 8 alpha-1,5 glucosyl-glucose linkages, for example. A saccharide preferably contains 1, 2, or 3 linkages of this type. A saccharide in other preferred embodiments only has alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages. In other embodiments, a saccharide can have one or more alpha-1,5 glucosyl-fructose linkages.

Since a saccharide herein comprises at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, the saccharide comprises at least two glucose units. In certain embodiments, a saccharide herein comprises only glucose units, or both glucose and fructose units. Such a composition may characterize the disaccharide and oligosaccharide byproducts of a glucan synthesis reaction. Alternatively, a saccharide herein may contain other monosaccharides in addition to glucose and fructose, such as galactose, ribose and xylose.

A saccharide hydrolyzed in certain embodiments of the disclosed invention can be an oligosaccharide. An oligosaccharide herein can have, for example, 2, 3, 4, 5, 6, 7, 8, or 9 monosaccharide units. As would be understood in the art, an oligosaccharide herein can be referenced with respect to its degree of polymerization (DP) number, which specifies the number of monomeric units in the oligosaccharide. A DP3 oligosaccharide has 3 monomeric units, for example. Thus, the oligosaccharide can be a DP3, DP4, DP5, DP6, DP7, DP8, or DP9 oligosaccharide, for example. The DP of a saccharide in certain embodiments is 3 to 7 (i.e., DP 3-7).

An oligosaccharide herein with 3 or more monosaccharide units, for example, can comprise other linkages in addition to at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage. For example, there may also be one or more alpha-1,5 glucosyl-fructose linkages in the oligosaccharide, which are also susceptible to hydrolysis by alpha-glucosidases as shown herein.

An oligosaccharide in certain embodiments comprises only glucose monomers linked by alpha-1,3 and/or alpha-1,6 glycosidic linkages. Thus, such oligosaccharides comprise only alpha-1,3 glucosyl-glucose and/or alpha-1,6 glucosyl-glucose linkages. Examples of such an oligosaccharide contain only alpha-1,3 linkages or alpha-1,6 linkages. An oligosaccharide can comprise at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% glucosyl-glucose linkages in certain embodiments. In other embodiments, there can be about 75-85% alpha-1,3 glucosyl-glucose linkages and about 15-25% alpha-1,6 glucosyl-glucose linkages in oligosaccharides herein. Alternatively, oligosaccharides herein can comprise any percentage (any integer value between 1% and 99%) of alpha-1,3 glucosyl-glucose linkages and any percentage (any integer value between 1% and 99%) of alpha-1,6 glucosyl-glucose linkages, just so long that the total of these percentages is not greater than 100%. Any of these oligosaccharides can be in a fraction from a glucan synthesis reaction that produces (i) an insoluble alpha-glucan (e.g., poly alpha-1,3-glucan), or (ii) a soluble alpha-glucan product, for example. This linkage content can characterize (i) each oligosaccharide individually, or (ii) a group of

oligosaccharides (i.e., average linkage content). Oligosaccharides comprising only glucose monomers linked by alpha-1,3 and/or alpha-1,6 glycosidic linkages can be DP2-DP7, or DP3-DP7, for example. It should be understood that the exact distribution of linkages in oligosaccharides can vary depending on the conditions of the glucan synthesis reaction (e.g., gtf enzyme) producing oligosaccharide byproducts. It should further be understood that the exact linkage distribution is not critical to the presently disclosed methods.

The Examples herein demonstrate that alpha-glucosidases (e.g., transglucosidase and glucoamylase enzymes) can hydrolyze both (i) leucrose, which comprises an alpha-1,5 glucosyl-fructose linkage, and (ii) oligosaccharides comprising only alpha-1,3 glucosyl-glucose and/or alpha-1,6 glucosyl-glucose linkages. Therefore, an alpha-glucosidase can be used, for example, in a reaction for hydrolyzing alpha-1,5 glucosyl-fructose linkages, alpha-1,3 glucosyl-glucose linkages and/or alpha-1,6 glucosyl-glucose linkages.

At least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide herein can be hydrolyzed by an alpha-glucosidase herein. Alternatively, it is believed that 2, 3, 4, 5, or more of such linkages in a saccharide can be hydrolyzed by an alpha-glucosidase, for example. Hydrolysis of at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage can occur at the non-reducing-end of a saccharide in certain embodiments.

The amount of a saccharide is reduced in the disclosed hydrolysis method compared to the amount of the saccharide that was present prior to the contacting step. This reduction results from hydrolytic cleavage of at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in the saccharide. The amount (e.g., concentration) of a saccharide after the contacting step in a hydrolysis method herein can be less than about 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (or any integer value between 1% and 90%) of the amount of the saccharide that was present prior to the contacting step (prior to contacting alpha-glucosidase with a saccharide under suitable conditions).

The amount of a saccharide is reduced in the disclosed hydrolysis method compared to the amount of the saccharide that was present prior to the contacting step. It would be understood that such a comparison can be made in any number of ways. For example, the saccharide concentration can be measured both before and after performing the hydrolysis method. Alternatively, the comparison can be made with respect to a control reaction having the same conditions, except that an alpha-glucosidase as presently disclosed is not added to the control reaction.

An alpha-glucosidase in certain embodiments herein may be immobilized. The enzyme may be immobilized using any method and/or means known in the art, such as those disclosed in U.S. Pat. Nos. 5541097 and 4713333, both of which are incorporated herein by reference. For example, one or more enzymes can be immobilized by contacting the enzyme(s) with a solution of an amine-reactive material (e.g., glutaraldehyde) to form an adduct (e.g., enzyme-glutaraldehyde adduct), after which the adduct is bonded to a solid carrier that has been treated with a polyamine (e.g., a polyethylenimine such as EPOMIN P-1050).

A solid carrier (solid support) to which an alpha-glucosidase enzyme can be immobilized in certain embodiments can be an inorganic or organic material. Such materials include, for example, gamma-alumina, titania, activated granular carbon, granular diatomaceous earth, glass beads, porous glass, pumice-stone, silica gel, metal oxide and aluminum oxide.

A polyamine can be used to treat a solid carrier such that subsequent exposure of the solid carrier to an adduct comprising an enzyme and amine-reactive material leads to attachment of the enzyme to the solid carrier. Examples of polyamines useful herein include polyethylenediamine, a polyethylenimine (e.g., polydiethylenetriamine, polytriethylenetetramine, polypentaethylenehexamine, polyhexamethylenediamine), polymethylenedicyclohexylamine, polymethylenedianiline, polytetraethylenepentamine, polyphenylenediamine and blends of two or more of

these polyamine compounds. Preferred polyamines are water-soluble and/or have a molecular weight of about from 500 to 100,000 Daltons. A polyethylenimine such as EPOMIN P-1050 can be used in certain embodiments.

5 An amine-reactive material useful for preparing an adduct comprising an enzyme herein can be, for example, an aldehyde, organic halide, anhydride, azo compound, isothiocyanate, and/or isocyanate. Examples of these amine-reactive materials include glutaraldehyde, succindialdehyde, terephthaldehyde, bis-diazobenzidine-2,2'-disulfonic acid, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone, diphenyl-4,4'-dithiocyanate-2,2'-disulfonic acid, 3-methoxydiphenylmethane-4,4'-
10 diisocyanate, toluene-2-isocyanate-4-isothiocyanate, toluene-2,-4-diisothiocyanate, diazobenzidine, diazobenzidine-3,3'-dianisidine, N,N'-hexamethylene bisiodoacetamide, hexamethylene diisocyanate, cyanuric chloride, and/or 1,5-difluoro-2,4-dinitrobenzene. Preferably, the amine-reactive material is an aldehyde such as glutaraldehyde.

15 An alpha-glucosidase enzyme adducted with an amine-reactive compound can be contacted with a polyamine-treated solid carrier, thereby immobilizing the enzyme onto the solid carrier. An immobilized enzyme herein can be employed in various reactor systems, such as in a column (e.g., packed column) or stirred tank reactor, for performing hydrolysis reaction as disclosed herein.

20 Suitable conditions for contacting a saccharide herein with an alpha-glucosidase (e.g., transglucosidase) are those conditions that support the hydrolysis of one or more alpha-1,3 or alpha-1,6 glucosyl-glucose linkages in the saccharide by the alpha-glucosidase. Examples of suitable conditions are
25 disclosed in the below Examples. Conditions (e.g., temperature, pH, time) for contacting an alpha-glucosidase with a sugar substrate are also disclosed in U.S. Pat. Appl. Publ. No. 2008/0229514, U.S. Pat. No. 7413887 and U.S. Pat. Appl. Publ. No. 2013/0102035 (all of which are incorporated herein by reference), and may also be applicable to the disclosed hydrolysis method.

30 The disaccharides and oligosaccharides in the disclosed hydrolysis method are typically soluble in water or an aqueous solution. Thus, contacting a

saccharide herein with an alpha-glucosidase is preferably performed under suitable conditions that are aqueous, in which the saccharide is dissolved.

Aqueous conditions can characterize a solution or mixture comprising at least about 20 wt% water. Alternatively, aqueous conditions herein are at least about 20, 30, 40, 50, 60, 70, 80, 85, 90, or 95 wt% water (or any integer value between 20 and 95 wt%), for example. Aqueous conditions can further comprise a buffer, for example, such as an acidic, neutral, or alkaline buffer, at a suitable concentration and selected based on the pH range provided by the buffer. Examples of buffers/buffering agents include citrate, acetate (e.g., sodium acetate), KH_2PO_4 , MOPS, CHES, borate, sodium carbonate, and sodium bicarbonate.

The pH of a hydrolysis reaction herein can be about 3.0 to 9.0, for example. Hydrolysis reaction pH can be, for example, about 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0. Alternatively, the pH can be about 4-5.

Techniques for setting pH include the use of buffers, alkalis, and/or acids, for example, and are well known in the art.

The temperature of a hydrolysis reaction herein can be about 20 °C to about 80 °C, for example. Hydrolysis reaction temperature can be, for example, about 20, 30, 40, 50, 60, 70, or 80 °C (or any integer value between 20 and 80 °C). A hydrolysis temperature of about 60 °C, 65 °C, or 60-65 °C is preferred in certain embodiments.

A hydrolysis reaction herein can be performed for a period of at least about 10 minutes to about 90 hours, for example. The time of a hydrolysis reaction can be, for example, at least about 0.5, 1, 2, 3, 4, 8, 12, 16, 20, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, or 90 hours (or any integer value between 0.5 and 72 hours). In certain embodiments, a hydrolysis reaction can be performed in less than 4 hours (e.g., 0.5-4 hours) for example. The time period required to achieve a desired level of hydrolysis will vary on the exact conditions used, and would be understood by one skilled in the art. For example, increasing the amount of enzyme added to a reaction or immobilized on a solid support used in a reaction will reduce the contact time.

One or more of alpha-glucosidase enzymes herein may be used in a hydrolysis reaction in certain embodiments. Both a transglucosidase and glucoamylase can be used in a reaction, for example. The amount of an alpha-glucosidase in a hydrolysis reaction herein can be plus/minus 10% to 20% (or 5% to 10%) from any of the amounts used in the Examples below (e.g., Example 2), for example. Alternatively, about 0.1-0.5 vol% or 0.1-1.0 vol% of alpha-glucosidase can be used in a hydrolysis reaction. Alternatively still, an alpha-glucosidase herein can be used at about, or at least about, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 ppm in a hydrolysis reaction. A transglucosidase unit (TGU) can be defined as the amount of a transglucosidase enzyme that will produce one micromole of panose per minute under the conditions of the following assay, for example. Transglucosidase activity can be assayed as follows, for example: a transglucosidase is brought up in 100 mM sodium acetate buffer, pH 4.5, containing 4 mM para-nitrophenyl-alpha-glucoside and 1 mg/ml bovine serum albumin (BSA). After 30 min incubation at 30 °C, the reaction is terminated by the addition of an equal volume 1 M sodium carbonate and OD₄₀₅ is recorded. A glucoamylase unit (GAU) can be defined, for example, as the amount of glucoamylase enzyme that will produce 1 g of reducing sugar calculated as glucose per hour from a soluble starch substrate (4% DS [degree of substitution]) at pH 4.2 and 60 °C.

The initial concentration of a saccharide in a hydrolysis reaction in certain embodiments of the disclosed invention can be about 1 wt% to 50 wt%, for example. For example, the concentration of leucrose can be about 5, 10, 15, 20, 25, 30, 35, or 40 wt% (or any integer value between 5 and 40 wt%). As another example, the concentration of one or more oligosaccharides (e.g., DP2, DP3, DP4, DP2-DP7, DP3-DP7) in a hydrolysis reaction herein can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 wt%. Those skilled in the art would recognize that the concentration of total sugars (which includes disaccharides and oligosaccharides) can have an impact on the activity of alpha-glucosidase enzymes; preferred concentrations of total sugars in a hydrolysis reaction to

maximize enzyme activity can be less than 50 wt% dry solids (DS), with a most preferred concentration of 20-35 wt% DS in some aspects.

Suitable conditions in certain embodiments for contacting a saccharide
5 with an alpha-glucosidase herein can comprise (i) a glucan synthesis reaction, or
(ii) a fraction obtained from a glucan synthesis reaction, where the saccharide is
a byproduct of the glucan synthesis reaction. In other words, a hydrolysis
reaction herein may be conducted in the context of a glucan synthesis reaction or
a fraction of a glucan synthesis reaction, though it is typically conducted in the
10 latter. A glucan synthesis reaction herein can produce one or more insoluble
and/or soluble alpha-glucan products, for example. Thus, a glucan synthesis
reaction can be characterized in some embodiments herein as an "alpha-glucan
synthesis reaction".

A glucan synthesis reaction generally refers to a solution comprising at
15 least sucrose, water and one active glucosyltransferase enzyme, and optionally
other components. Other components that can be in a glucan synthesis reaction
include fructose, glucose, leucrose, soluble oligosaccharides (e.g., DP2-DP7),
and soluble glucan product(s). Also, one or more alpha-glucanohydrolase
enzymes can be comprised in a glucan synthesis reaction in some aspects. It
20 would be understood that certain glucan products, such as poly alpha-1,3-glucan
with a DP of at least 8 or 9, may be water-insoluble and thus are not dissolved in
a glucan synthesis reaction, but rather may be present out of solution. Thus, a
glucan produced by glucan synthesis reaction herein can be insoluble. An alpha-
glucosidase enzyme herein can be added to a glucan synthesis reaction at any
25 stage thereof, such as during initial preparation of the reaction or when the
reaction is near (e.g., 80 to 90% complete) or at completion, where the latter two
time points are preferred.

A glucan synthesis reaction herein may be one that, in addition to
producing a glucan product, produces byproducts such as leucrose and/or
30 soluble oligosaccharides. A glucan in some aspects is a poly alpha-glucan.
Thus, a glucan synthesis reaction herein can be for producing poly alpha-1,3-

glucan or mutan, for example, which are typically co-produced with at least leucrose and/or oligosaccharide byproducts in a glucan synthesis reaction.

A glucan synthesis reaction in certain embodiments comprises a glucosyltransferase enzyme that produces a poly alpha-glucan such as poly
5 alpha-1,3-glucan. Examples of such glucosyltransferase enzymes useful herein are disclosed in U.S. Pat. No. 7000000, and U.S. Pat. Appl. Publ. Nos. 2013/0244288, 2013/0244287 and 2014/0087431 (all of which are incorporated herein by reference).

A glucosyltransferase enzyme herein may be derived from any microbial
10 source, such as a bacteria or fungus. Examples of bacterial glucosyltransferase enzymes are those derived from a *Streptococcus* species, *Leuconostoc* species or *Lactobacillus* species. Examples of *Streptococcus* species include *S. salivarius*, *S. sobrinus*, *S. dentirosetti*, *S. downei*, *S. mutans*, *S. oralis*, *S. gallolyticus* and *S. sanguinis*. Examples of *Leuconostoc* species include *L.*
15 *mesenteroides*, *L. amelibiosum*, *L. argentinum*, *L. carnosum*, *L. citreum*, *L. cremoris*, *L. dextranicum* and *L. fructosum*. Examples of *Lactobacillus* species include *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*, *L. casei*, *L. curvatus*, *L. plantarum*, *L. sakei*, *L. brevis*, *L. buchneri*, *L. fermentum* and *L. reuteri*.

A glucosyltransferase enzyme herein can be primer-independent or
20 primer-dependent. Primer-independent glucosyltransferase enzymes do not require the presence of a primer to perform glucan synthesis. A primer-dependent glucosyltransferase enzyme requires the presence of an initiating molecule in the reaction solution to act as a primer for the enzyme during glucan
25 polymer synthesis. The term "primer" as used herein refers to any molecule that can act as the initiator for a glucosyltransferase enzyme. Primers that can be used in certain embodiments include dextran and other carbohydrate-based primers, such as hydrolyzed glucan, for example. U.S. Appl. Publ. No. 2013/0244287, which is incorporated herein by reference, discloses preparation
30 of hydrolyzed glucan using poly alpha-1,3-glucan as the starting material.

Dextran for use as a primer can be dextran T10 (i.e., dextran having a molecular weight of 10 kD), for example.

A glucosyltransferase enzyme for a glucan synthesis reaction herein may be produced by any means known in the art. For example, a glucosyltransferase enzyme may be produced recombinantly in a heterologous expression system, such as a microbial heterologous expression system. Examples of heterologous expression systems include bacterial (e.g., *E. coli* such as TOP10 or MG1655; *Bacillus* sp.) and eukaryotic (e.g., yeasts such as *Pichia* sp. and *Saccharomyces* sp.) expression systems.

A glucosyltransferase enzyme described herein may be used in any purification state (e.g., pure or non-pure). For example, a glucosyltransferase enzyme may be purified and/or isolated prior to its use. Examples of glucosyltransferase enzymes that are non-pure include those in the form of a cell lysate. A cell lysate or extract may be prepared from a bacteria (e.g., *E. coli*) used to heterologously express the enzyme. For example, the bacteria may be subjected to disruption using a French pressure cell. In alternative embodiments, bacteria may be homogenized with a homogenizer (e.g., APV, Rannie, Gaulin). A glucosyltransferase enzyme is typically soluble in these types of preparations. A bacterial cell lysate, extract, or homogenate herein may be used at about 0.15-0.3% (v/v), for example, in a reaction solution for producing a poly alpha-glucan such as poly alpha-1,3-glucan from sucrose.

The temperature of a glucan synthesis reaction herein can be controlled, if desired. In certain embodiments, the temperature of the reaction is between about 5 °C to about 50 °C. The temperature in certain other embodiments is between about 20 °C to about 40 °C.

The initial concentration of sucrose in a glucan synthesis reaction herein can be about 20 g/L to about 400 g/L, for example. Alternatively, the initial concentration of sucrose can be about 75 g/L to about 175 g/L, or from about 50 g/L to about 150 g/L. Alternatively still, the initial concentration of sucrose can be about 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, or 160 g/L (or any integer value between 40 and 160 g/L), for example. "Initial concentration of

sucrose" refers to the sucrose concentration in a gtf reaction solution just after all the reaction solution components have been added (at least water, sucrose, gtf enzyme).

Sucrose used in a glucan synthesis reaction herein can be highly pure ($\geq$ 99.5%) or be of any other purity or grade. For example, sucrose can have a purity of at least 99.0%, or can be reagent grade sucrose. As another example, incompletely refined sucrose can be used. Incompletely refined sucrose herein refers to sucrose that has not been processed to white refined sucrose. Thus, incompletely refined sucrose can be completely unrefined or partially refined.

Examples of unrefined sucrose are "raw sucrose" ("raw sugar") and solutions thereof. Examples of partially refined sucrose have not gone through one, two, three, or more crystallization steps. The ICUMSA (International Commission for Uniform Methods of Sugar Analysis) of incompletely refined sucrose herein can be greater than 150, for example. Sucrose herein may be derived from any renewable sugar source such as sugar cane, sugar beets, cassava, sweet sorghum, or corn. Suitable forms of sucrose useful herein are crystalline form or non-crystalline form (e.g., syrup, cane juice, beet juice), for example. Additional suitable forms of incompletely refined sucrose are disclosed in U.S. Appl. No. 61/969,958.

Methods of determining ICUMSA values for sucrose are well known in the art and disclosed by the International Commission for Uniform Methods of Sugar Analysis in ICUMSA Methods of Sugar Analysis: Official and Tentative Methods Recommended by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) (Ed. H.C.S. de Whalley, Elsevier Pub. Co., 1964), for example, which is incorporated herein by reference. ICUMSA can be measured, for example, by ICUMSA Method GS1/3-7 as described by R.J. McCowage, R.M. Urquhart and M.L. Burge (Determination of the Solution Colour of Raw Sugars, Brown Sugars and Coloured Syrups at pH 7.0 – Official, Verlag Dr Albert Bartens, 2011 revision), which is incorporated herein by reference.

The pH of a glucan synthesis reaction in certain embodiments can be between about 4.0 to about 8.0. Alternatively, the pH can be about 4.0, 4.5, 5.0,

5.5, 6.0, 6.5, 7.0, 7.5, or 8.0. The pH can be adjusted or controlled by the addition or incorporation of a suitable buffer, including but not limited to: phosphate, tris, citrate, or a combination thereof. Buffer concentration in a glucan synthesis reaction can be from 0 mM to about 100 mM, or about 10, 20, or 50 mM, for example.

Poly alpha-1,3-glucan produced in a glucan synthesis reaction herein may have at least about 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% (or any integer value between 50% and 100%) glycosidic linkages that are alpha-1,3. In such embodiments, accordingly, the poly alpha-1,3-glucan has less than about 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, or 0% (or any integer value between 0% and 50%) of glycosidic linkages that are not alpha-1,3.

Poly alpha-1,3-glucan herein preferably has a backbone that is linear/unbranched. In certain embodiments, the poly alpha-1,3-glucan has no branch points or less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% branch points as a percent of the glycosidic linkages in the polymer. Examples of branch points include alpha-1,6 branch points.

The molecular weight of poly alpha-1,3-glucan produced in a glucan synthesis reaction herein can be measured as number-average molecular weight (M_n) or weight-average molecular weight (M_w). Alternatively, molecular weight can be measured in Daltons or grams/mole. It may also be useful to refer to the DP_w (weight average degree of polymerization) or DP_n (number average degree of polymerization) of the poly alpha-1,3-glucan polymer.

The M_n or M_w of poly alpha-1,3-glucan herein may be at least about 1000. Alternatively, the M_n or M_w can be at least about 1000 to about 600000 (or any integer value between 1000 and 600000), for example. Alternatively still, poly alpha-1,3-glucan in can have a molecular weight in DP_n or DP_w of at least about 100, or of at least about 100 to 1000 (or any integer value between 100 and 1000).

A fraction of a glucan synthesis reaction may constitute suitable conditions for contacting a saccharide with an alpha-glucosidase as presently disclosed. A fraction can be a portion of, or all of, the liquid solution from a glucan synthesis reaction. Typically, a fraction has been separated from soluble or insoluble glucan product(s) synthesized in the reaction. For example, a fraction can be separated from one or more glucan products that are insoluble in water (e.g., poly alpha-1,3-glucan) which fall out of solution during their synthesis. A fraction in certain preferred embodiments of the present disclosure is from a poly alpha-1,3-glucan synthesis reaction.

The volume of a fraction (before optionally diluting or concentrating the fraction, see below) in certain embodiments can be at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% (or any integer value between 10% and 90%) of the volume of the glucan synthesis reaction from which it is obtained. Typically, in glucan synthesis reactions producing an insoluble glucan (e.g., poly alpha-1,3-glucan), the fraction will be a portion of (not all of) the liquid solution component of the reaction. A fraction can be obtained at any stage of a glucan synthesis reaction, but is preferably obtained near (e.g., greater than 80 or 90% complete) or after completion of the reaction.

Examples of a fraction of a glucan synthesis reaction in certain embodiments include filtrates and supernatants. Thus, in those embodiments in which an insoluble glucan product is synthesized, a fraction herein can be obtained (separated) from a glucan synthesis reaction using a funnel, filter (e.g., press filter), centrifuge, or any other method or equipment known in the art that allows removal of some or all liquids from solids. Filtration can be by gravity, vacuum, or press filtration, for example. Filtration preferably removes all or most of an insoluble glucan; any filter material (e.g., filter paper) with an average pore size (e.g., ~40-50 micron) sufficient to remove solids from liquids can be used. A fraction typically retains all or most of its dissolved components, such as byproducts of the glucan synthesis reaction.

A fraction herein can optionally be diluted or concentrated, if desired. Concentration of a fraction can be performed using any other method or

equipment known in the art suitable for concentrating a solution. For example, a fraction can be concentrated by evaporation, such as with a rotary evaporator (e.g., set at a temperature of about 40-50 °C). A fraction in some aspects herein can be concentrated down to a volume that is about 75%, 80%, 85%, 90%, or 95% of the original fraction volume. A concentrated fraction (e.g., concentrated filtrate) can optionally be referred to as a syrup.

A fraction in some aspects can comprise water that replaces the water that was present in the composition from which the fraction was obtained. For example, saccharide byproduct(s) from a glucan synthesis reaction can be separated in certain chromatographic methods in which the original solvent is replaced with another solvent (e.g., saccharide byproducts that are bound to a column [thus removed from the original solvent] can be eluted into a new solvent).

A fraction in some aspects may be treated in a manner to have any of the suitable conditions (e.g., temperature, pH and time) disclosed above for contacting a saccharide with an alpha-glucosidase. For example, a fraction can be modified to have a pH of about 4 to 5 before an alpha-glucosidase is added to the fraction. As another example, the temperature of a hydrolysis reaction with a fraction can be about 55-65 °C (e.g., about 60 °C). A fraction that has been concentrated down to a syrup can be used in a hydrolysis reaction in yet another example.

A fraction in certain preferred embodiments herein is from a poly alpha-1,3-glucan synthesis reaction; such a fraction is preferably a filtrate. A fraction of a poly alpha-1,3-glucan synthesis reaction herein comprises at least water, fructose and one or more types of saccharide (leucrose and/or oligosaccharides such as DP2-DP7). Other components that may be in this type of fraction include sucrose (i.e., residual sucrose not consumed in the gtf reaction), one or more gtf enzymes, glucose, buffer, salts, FermaSure[®], borates, sodium hydroxide, hydrochloric acid, cell lysate components, proteins and/or nucleic acids, for example. Minimally, the components of a fraction from a poly alpha-1,3-glucan synthesis reaction include water, fructose, glucose, one or more types

of saccharide (leucrose and/or oligosaccharides such as DP2-DP7), and optionally sucrose, for example. It would be understood that the composition of a fraction depends, in part, on the conditions of the glucan synthesis reaction from which the fraction is obtained. In those fractions containing one or more gtf
5 enzymes, it is preferable that such one or more gtf enzymes are deactivated (e.g., heat-deactivated) before using the fraction in a hydrolysis reaction herein.

It should be understood that the exact distribution of sugar byproducts produced via polymerization of sucrose in a glucan synthesis reaction can vary based on the reaction conditions and gtf enzyme used, especially on temperature
10 and sucrose concentration. It should also be understood that the exact composition of sugars in a fraction of a glucan synthesis reaction is not critical to the disclosed hydrolysis process. Generally, as the amount of sucrose is increased, the selectivity of the reaction towards both leucrose and oligosaccharides will increase. Conversely, as the temperature increases, the
15 selectivity of the reaction towards leucrose tends to decrease, while the selectivity towards oligosaccharides is largely unaffected. It should also be understood that the ratio of sugars to water, i.e., wt% dry solids (DS), which is calculated by dividing the mass of sugar to total solution weight, can be adjusted either by evaporating water, preferably at temperatures below 50 °C under
20 vacuum, or addition of water, without significant impact to the relative distribution of sugars in a fraction of a glucan synthesis reaction. It is also possible to increase the percentage of sucrose in a fraction by stopping the gtf reaction before complete conversion (to glucan) is achieved, either by reducing the pH below the active range for the gtf enzyme or by thermal deactivation of the gtf
25 enzyme.

In certain embodiments, a glucan synthesis reaction herein can produce one or more soluble alpha-glucan products. A soluble alpha-glucan product ("soluble fiber", alternatively) can be (i) a direct product of a glucosyltransferase,
30 or (ii) a product of the concerted action of both a glucosyltransferase and an

alpha-glucanohydrolase capable of hydrolyzing glucan polymers having one or more alpha-1,3-glycosidic linkages or one or more alpha-1,6-glycosidic linkages.

A soluble alpha-glucan herein can comprise, for example:

- a) at least 75% alpha-1,3-glycosidic linkages;
- 5 b) less than 25% alpha-1,6-glycosidic linkages;
- c) less than 10% alpha-1,3,6-glycosidic linkages;
- d) an M_w of less than 5000 Daltons;
- e) a viscosity of less than 0.25 Pascal second (Pa·s) at 12 wt% in water at 20 °C;
- 10 f) a dextrose equivalence (DE) in the range of 4 to 40;
- g) a digestibility of less than 10% as measured by the Association of Analytical Communities (AOAC) method 2009.01;
- h) a solubility of at least 20% (w/w) in pH 7 water at 25 °C; and
- i) a polydispersity index (PDI) of less than 5.

15 Such a soluble alpha-glucan can be produced as disclosed in U.S. Appl. No. 62/004,290.

As an example, a soluble alpha-glucan fiber composition can comprise at least 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% alpha-(1,3) glycosidic
20 linkages.

As another example, in addition to the alpha-(1,3) glycosidic linkage embodiments described above, a soluble alpha-glucan fiber composition can further comprise less than 25%, preferably less than 10%, more preferably 5% or less, and even more preferably less than 1% alpha-(1,6) glycosidic linkages.

25 As another example, in addition to the alpha-(1,3) and alpha-(1,6) glycosidic linkage content embodiments described above, a soluble alpha-glucan fiber composition can further comprise less than 10%, preferably less than 5%, and most preferably less than 2.5% alpha-(1,3,6) glycosidic linkages.

As another example, a soluble alpha-glucan fiber composition can
30 comprise 93 to 97% alpha-(1,3) glycosidic linkages and less than 3% alpha-(1,6) glycosidic linkages and has a weight-average molecular weight corresponding to

a DP of 3 to 7 mixture. In a further embodiment, a soluble alpha-glucan fiber composition can comprise about 95% alpha-(1,3) glycosidic linkages and about 1% alpha-(1,6) glycosidic linkages and has a weight-average molecular weight corresponding to a DP of 3 to 7 mixture. In a further aspect of the above
5 embodiment, a soluble alpha-glucan fiber composition can further comprise 1 to 3% alpha-(1,3,6) linkages or preferably about 2 % alpha-(1,3,6) linkages.

As another example, in addition to the above-mentioned glycosidic linkage content embodiments, a soluble alpha-glucan fiber composition can further comprise less than 5%, preferably less than 1 %, and most preferably less than
10 0.5 % alpha-(1,4) glycosidic linkages.

As another example, in addition the above-mentioned glycosidic linkage content embodiments, a soluble alpha-glucan fiber composition can comprise a weight average molecular weight (M_w) of less than 5000 Daltons, preferably less than 2500 Daltons, more preferably between 500 and 2500 Daltons, and most
15 preferably about 500 to about 2000 Daltons.

As another example, in addition to any of the above features, a soluble alpha-glucan fiber composition can comprise a viscosity of less than 250 centipoise (0.25 Pa·s), preferably less than 10 cP (0.01 Pa·s), preferably less than 7 cP (0.007 Pa·s), more preferably less than 5 cP (0.005 Pa·s), more
20 preferably less than 4 cP (0.004 Pa·s), and most preferably less than 3 cP (0.003 Pa·s) at 12 wt% in water at 20 °C.

A soluble alpha-glucan fiber composition can have, in certain embodiments, a digestibility of less than 10%, or preferably less than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% digestibility as measured by the Association of
25 Analytical Communities (AOAC) method 2009.01. In another aspect, the relative level of digestibility may alternatively be determined using AOAC 2011.25 (Integrated Total Dietary Fiber Assay) (McCleary et al., 2012, *J. AOAC Int.*, 95 (3), 824-844).

In addition to any of the above embodiments, a soluble alpha-glucan fiber
30 composition can have a solubility of at least 20% (w/w), preferably at least 30%, 40%, 50%, 60%, or 70% in pH 7 water at 25 °C.

In one embodiment, a soluble alpha-glucan fiber composition can comprise a reducing sugar content of less than 10 wt%, preferably less than 5 wt%, and most preferably 1 wt% or less.

In one embodiment, a soluble alpha-glucan fiber composition can
5 comprise a caloric content of less than 4 kcal/g, preferably less than 3 kcal/g, more preferably less than 2.5 kcal/g, and most preferably about 2 kcal/g or less.

As another example, a soluble alpha-glucan herein can comprise:

- a) 10% to 30% alpha-1,3-glycosidic linkages;
- b) 65% to 87% alpha-1,6-glycosidic linkages;
- 10 c) less than 5% alpha-1,3,6-glycosidic linkages;
- d) a weight average molecular weight (Mw) of less than 5000 Daltons;
- e) a viscosity of less than 0.25 Pascal second (Pa·s) at 12 wt% in water at 20 °C;
- f) a dextrose equivalence (DE) in the range of 4 to 40, preferably 10
15 to 40;
- g) a digestibility of less than 10% as measured by the Association of Analytical Communities (AOAC) method 2009.01;
- h) a solubility of at least 20% (w/w) in pH 7 water at 25 °C; and
- i) a polydispersity index (PDI) of less than 5.

20 Such a soluble alpha-glucan can be produced as disclosed in U.S. Appl. No. 62/004,308.

As another example, a soluble alpha-glucan herein can comprise:

- a) 25-35 alpha-1,3-glycosidic linkages;
- b) 55-75% alpha-1,6-glycosidic linkages;
- 25 c) 5-15% alpha-1,3,6-glycosidic linkages;
- d) a weight average molecular weight of less than 5000 Daltons ;
- e) a viscosity of less than 0.25 Pascal second (Pa·s) at 12 wt% in water at 20 °C;
- f) a dextrose equivalence (DE) in the range of 4 to 40;
- 30 g) a digestibility of less than 10% as measured by the Association of Analytical Communities (AOAC) method 2009.01;

- h) a solubility of at least 20% (w/w) in water at 25 °C; and
- i) a polydispersity index of less than 5.

Such a soluble alpha-glucan can be produced as disclosed in U.S. Appl. No. 62/004,312.

5 As another example, a soluble alpha-glucan herein can comprise:

- a) at least 95% alpha-1,6-glycosidic linkages;
- b) 1% or less alpha-1,3-glycosidic linkages;
- c) less than 2% alpha-1,3,6-glycosidic linkages;
- d) less than 1.5% alpha-1,4-glycosidic linkages;

10 e) a weight average molecular weight of less than 20000 Daltons;

f) a viscosity of less than 0.25 Pascal second (Pa•s) at 12 wt% in water at 20 °C;

g) a dextrose equivalence (DE) in the range of 1 to 30;

h) a digestibility of less than 10% as measured by the Association of

15 Analytical Communities (AOAC) method 2009.01;

i) a solubility of at least 20% (w/w) in pH 7 water at 25 °C; and

j) a polydispersity index of less than 5.

Such a soluble alpha-glucan can be produced as disclosed in U.S. Appl. No. 62/004,314.

20 As another example, a soluble alpha-glucan herein can comprise:

a) a range of:

i) 1% to 50% of alpha-1,3-glycosidic linkages; or

ii) greater than 10% but less than 40 % alpha-1,4-glycosidic linkages; or

25 iii) any combination of i) and ii);

b) 1 to 50% alpha-1,2-glycosidic linkages;

c) 0-25% alpha-1,3,6-glycosidic linkages;

d) less than 98% alpha-1,6-glycosidic linkages;

e) a weight average molecular weight of less than 300 kDa;

30 f) a viscosity of less than 0.25 Pascal second (Pa•s) at 12 wt% in water at 20 °C;

g) a digestibility of less than 20% as measured by the Association of Analytical Communities (AOAC) method 2009.01;

h) a solubility of at least 20% (w/w) in pH 7 water at 25 °C; and

i) a polydispersity index of less than 26, preferably less than 5.

5 Such a soluble alpha-glucan can be produced as disclosed in U.S. Appl. No. 62/004,305.

In certain embodiments, a soluble alpha-glucan is a direct product of a glucosyltransferase. Such a glucosyltransferase, and conditions for use thereof in a suitable glucan synthesis reaction, can be as disclosed herein, or as
10 disclosed in any of U.S. Patent Appl. Nos. 62/004,290, 62/004,308, 62/004,312, 62/004,314, and/or 62/004,305, for example.

A soluble alpha-glucan can alternatively be a product, for example, of the concerted action of both a glucosyltransferase and an alpha-glucanohydrolase that is capable of hydrolyzing glucan polymers having one or more alpha-1,3-
15 glycosidic linkages or one or more alpha-1,6-glycosidic linkages. In some aspects, a glucan synthesis reaction for producing a soluble alpha-glucan product can comprise both at least one glucosyltransferase and at least one alpha-glucanohydrolase. In other aspects, a glucan synthesis reaction can initially comprise one or more glucosyltransferases as the only enzyme
20 component(s). Such a reaction produces a first alpha-glucan product that has not yet been subject to modification by an alpha-glucanohydrolase. Then, at least one alpha-glucanohydrolase is added to the reaction for a suitable period of time to allow modification of the first product to a soluble alpha-glucan product. Thus, there are different ways by which to synthesize a soluble alpha-glucan
25 product through the concerted action of both a glucosyltransferase and an alpha-glucanohydrolase. Conditions for performing a glucan synthesis reaction in which one or more alpha-glucanohydrolase enzymes are included during glucan synthesis reaction and/or after glucan synthesis can be as disclosed herein, or as disclosed in any of U.S. Patent Appl. Nos. 62/004,290, 62/004,308, 62/004,312,
30 62/004,314, and/or 62/004,305, for example.

An alpha-glucanohydrolase herein can be, for example, a dextranase (capable of hydrolyzing alpha-1,6-linked glycosidic bonds; E.C. 3.2.1.11), a mutanase (capable of hydrolyzing alpha-1,3-linked glycosidic bonds; E.C. 3.2.1.59), a mycodextranase (capable of endohydrolysis of (1-4)-alpha-D-glucosidic linkages in alpha-D-glucans containing both (1-3)- and (1-4)-bonds; EC 3.2.1.61), a glucan 1,6-alpha-glucosidase (EC 3.2.1.70), and an alternanase (capable of endohydrolytically cleaving alternan; E.C. 3.2.1.-; see U.S. Patent No. 5786196).

A mutanase comprising SEQ ID NO:47 can be used in certain aspects. Alternatively, a mutanase can comprise an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:47 and have mutanase activity, for example.

A glucan synthesis reaction as presently disclosed for producing one or more soluble alpha-glucan products can serve directly as suitable conditions in which to perform a hydrolysis reaction herein in which an alpha-glucosidase is used to hydrolyze an alpha-1,5 glucosyl-fructose linkage. Such hydrolysis can be performed following any of the conditions disclosed above regarding hydrolytic treatment of a glucan synthesis reaction that produces poly alpha-1,3-glucan, for example. Alternatively, a fraction (e.g., chromatographic fraction) of a glucan synthesis reaction for producing one or more soluble alpha-glucan products can be used as suitable conditions in which to perform alpha-glucosidase-mediated hydrolysis of alpha-1,5 glucosyl-fructose linkages.

A fraction in certain embodiments herein can be a chromatographic fraction of a glucan synthesis reaction. For example, a fraction can be a chromatographic fraction of a glucan synthesis reaction that produces one or more soluble alpha-glucan products as disclosed herein. Such a reaction can optionally include one or more alpha-glucanohydrolases during glucan synthesis, and/or after completion of glucan synthesis. A fraction in any of these types of embodiments typically has been obtained for the purpose of separating all of, or most of (e.g., at least about 60%, 70%, 80%, 90%, 95%), a soluble alpha-glucan product from a reaction composition from which it was produced. Once

separated from all or most of a soluble alpha-glucan product, a fraction can be subjected to any of the alpha-1,5 glucosyl-fructose hydrolysis processes disclosed herein using one or more alpha-glucanases.

A chromatographic fraction herein can typically be obtained using a suitable type of liquid chromatography. Liquid chromatography can be performed using size-exclusion chromatography (SEC), column chromatography, high-performance liquid chromatography (HPLC), ion-exchange chromatography, affinity chromatography, ultrafiltration, microfiltration, or dialysis, for example.

The disclosed invention also concerns a composition produced by contacting a saccharide with an alpha-glucosidase (e.g., transglucosidase), wherein (i) the saccharide is a disaccharide or oligosaccharide comprising at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, and (ii) the alpha-glucosidase hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide. The composition produced in this manner comprises a reduced amount of the saccharide compared to the amount of the saccharide that was present prior to the contacting. Examples of the composition include any of those disclosed herein, such as a hydrolyzed filtrate from a glucan synthesis reaction, or a hydrolyzed fraction of a glucan synthesis reaction used to produce soluble alpha-glucan. Any of the features disclosed above and in the Examples regarding a hydrolysis method and products thereof can characterize the composition. The following features of the composition are examples.

An alpha-glucosidase enzyme in certain embodiments of the composition can comprise an amino acid sequence that is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, or that of DIAZYME RDF ULTRA (DuPont Industrial Biosciences). A transglucosidase in certain embodiments of the composition can comprise an amino acid sequence that is at least 90% identical to SEQ ID NO:1. Alternatively, any of the alpha-glucosidases disclosed herein can be used to produce the disclosed composition.

A saccharide in certain embodiments of the composition has a degree of polymerization before hydrolysis of 3 to 7.

A composition produced by a hydrolysis method herein can have, for example, a concentration of a saccharide that is less than 50% of the concentration of the saccharide that was present prior to contacting the
5 saccharide with an alpha-glucosidase.

A composition produced by a hydrolysis method in certain embodiments herein can be a glucan synthesis reaction, or a fraction thereof, in which a saccharide byproduct of the glucan synthesis reaction is contacted with an alpha-
10 glucosidase. A fraction in this embodiment can be a filtrate of the glucan synthesis reaction, or a fraction of a glucan synthesis reaction used to produce soluble alpha-glucan, for example. The saccharide in this embodiment can have a degree of polymerization of 3 to 7 before hydrolysis, for example.

15 It would be understood by a skilled artisan that the presently disclosed embodiments are useful, in part, for saccharifying disaccharides and oligosaccharides that can otherwise be difficult to breakdown. This feature can be taken advantage of to perform enhanced methods of (i) fructose enrichment and (ii) fermentation, for example.

20 Example 6 below demonstrates that fructose enrichment by chromatography is enhanced when using a glucan filtrate hydrolyzed by an alpha-glucosidase (transglucosidase), as compared to using a filtrate that was not hydrolyzed.

Thus, the disclosed invention further concerns a method of enriching
25 fructose that is present in a fraction of a glucan synthesis reaction. This method comprises (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase (e.g., transglucosidase) under suitable conditions, wherein the enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the
30 fraction; and (b) separating fructose from the hydrolyzed fraction of step (a) to

obtain a composition having a higher concentration of fructose compared to the fructose concentration of the fraction of step (a).

The features of the disclosed fructose enrichment method regarding alpha-glucosidase (e.g., transglucosidase) enzymes, and fractions of a glucan
5 synthesis reaction, for example, can be according to any of the disclosures provided herein concerning each of these features.

Step (b) of separating fructose can be performed by any means known in the art. For example, chromatography can be employed as disclosed in the below Examples, or by following the disclosure of European Patent Publ. No.
10 EP2292803B1, which is incorporated herein by reference.

A composition (e.g., fructose solution or fructose syrup) having a higher concentration of fructose resulting from the disclosed enrichment method can have at least about 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 wt% fructose.

A fructose enrichment method herein can perform better than one which
15 utilizes a filtrate that has not been hydrolyzed with an alpha-glucosidase as presently disclosed. Such increased performance can be measured in terms of a percent fructose recovery of at least 40%, 45%, or 50%.

The present disclosure further concerns a fermentation method
20 comprising (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme (e.g., transglucosidase or glucoamylase) under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,5 glucosyl-fructose linkage of a disaccharide or oligosaccharide comprised within the fraction; (b) fermenting the fraction of step (a) with a
25 microbe to yield a product; and (c) optionally, isolating the product of (b). The fermenting step of (b) can be performed after step (a) or simultaneously with step (a). Significantly, this method can be used to produce ethanol, for example, by fermenting a hydrolyzed filtrate of a glucan synthesis reaction. The ethanol yield from such a process is higher than the ethanol yield obtained when fermenting a
30 glucan filtrate that has not been hydrolyzed.

The features of the disclosed fermentation method regarding alpha-glucosidase (e.g., transglucosidase or glucoamylase) enzymes, disaccharides and oligosaccharides, fractions of a glucan synthesis reaction, and suitable contacting conditions, for example, can be according to any of the disclosures
5 provided herein concerning each of these features.

A microbe for use in a fermentation method herein can be a bacteria, yeast, or fungus, for example. Examples of bacteria useful herein include *Lactobacillus* species, *Streptococcus* species, *Bifidobacterium* species, *Leuconostoc* species, *Escherichia* species (e.g., *E. coli*) and *Bacillus* species.
10 Examples of yeast useful herein include *Saccharomyces* species such as *S. cerevisiae* and *S. bayanus*.

A fermentation method herein can yield a product such as ethanol or an acid (e.g., lactic acid). It is believed, however, that other products can be produced if desired. It would be understood by one of skill in the art that
15 production of certain products using a fermentation method as disclosed would depend on various conditions such as the microbe(s) used in the fermentation. Conditions for fermentation herein can be as disclosed in the below Examples, or as disclosed in El-Mansi et al. (2006, Fermentation Microbiology and Biotechnology, Second Edition, CRC Press) and Stanbury et al. (1999, Principles of Fermentation Technology, Second Edition, Butterworth-Heinemann), for
20 example, which are both incorporated herein by reference.

The yield of a product in certain embodiments of a fermentation method herein is higher than the product yield obtained when fermenting a glucan filtrate that has not been hydrolyzed with an alpha-glucosidase herein. This comparison
25 can be with respect to a control fermentation, for example, which used a non-hydrolyzed fraction of a glucan synthesis reaction. Product yield of a fermentation herein can be increased by at least about 10%, 20%, 40%, 60%, 80%, or 100% (or any integer value between 10% and 100%), for example. In addition, the rate of product formation by a fermentation herein can be increased.

30

Example 7 below demonstrates that leucrose can be fermented to ethanol by yeast provided a feed comprising glucan filtrate that had not been hydrolyzed. Thus, further disclosed herein is a method of using a microbe to ferment leucrose to a product (e.g., ethanol). Such a method can comprise fermenting a glucan filtrate that (i) has, or (ii) has not been, hydrolyzed with an alpha-glucosidase as disclosed herein. Regardless of whether the leucrose is provided in a glucan filtrate or another form (e.g., semi-purified or enriched form), a method for fermenting leucrose can comprise adapting a microbe (e.g., yeast such as *S. cerevisiae*) for utilizing leucrose. Such adaptation can comprise growing a microbe in the presence of leucrose, and optionally other sugars, over at least 2 or 3 growth cycles, for example, afterwhich the microbe utilizes more leucrose for fermenting a product. In certain embodiments, a microbe can be (i) grown in a first feed comprising leucrose (1 cycle complete), (ii) removed from the first feed, (iii) grown in a second feed comprising leucrose (two cycles complete), (iv) optionally removed from the second feed, and (v) optionally grown in a third feed (three cycles complete). A microbe adapted in this manner can have an increased capacity to ferment leucrose in certain embodiments.

Example 9 below demonstrates that almost all (e.g., >98% or >99%) the leucrose present in a glucan filtrate can be used for fermentation by yeast when the glucan filtrate is hydrolyzed with a transglucosidase while at the same time fermented with yeast. Thus, an enhanced leucrose fermentation method herein can comprise hydrolysis of leucrose with an alpha-glucosidase (e.g., transglucosidase or glucoamylase) while simultaneously fermenting the leucrose with a microbe.

Non-limiting examples of compositions and methods disclosed herein include:

1. A method of hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide comprising at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein the saccharide is a disaccharide or oligosaccharide, and wherein the method comprises:

contacting the saccharide with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide,

5 and wherein the amount of the saccharide is reduced compared to the amount of the saccharide that was present prior to the contacting.

2. The method of embodiment 1, wherein the alpha-glucosidase enzyme is immobilized.

3. The method of embodiment 1 or 2, wherein the degree of polymerization
10 of the saccharide before hydrolysis is 3 to 7.

4. The method of embodiment 1, 2, or 3, wherein the concentration of the saccharide after the contacting step is less than 50% of the concentration of the saccharide that was present prior to the contacting.

5. The method of embodiment 1, 2, 3, or 4, wherein the suitable conditions
15 comprise (i) a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction;
wherein the saccharide is a byproduct of the glucan synthesis reaction.

6. The method of embodiment 5, wherein the glucan synthesis reaction produces at least one insoluble alpha-glucan product.

20 7. The method of embodiment 6, wherein the fraction is a filtrate of the glucan synthesis reaction.

8. The method of embodiment 5, wherein the glucan synthesis reaction produces at least one soluble alpha-glucan product that is
(i) a product of a glucosyltransferase, or

25 (ii) a product of the concerted action of both a glucosyltransferase and an alpha-glucanohydrolase capable of hydrolyzing glucan polymers having one or more alpha-1,3-glycosidic linkages or one or more alpha-1,6-glycosidic linkages.

9. The method of embodiment 8, wherein the fraction is a chromatographic
30 fraction of the glucan synthesis reaction.

10. The method of any one of embodiments 1-9, wherein the alpha-glucosidase enzyme is a transglucosidase.
11. A composition produced by contacting a saccharide with an alpha-glucosidase enzyme,
5 wherein the saccharide is a disaccharide or oligosaccharide and comprises at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein the enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide, and wherein the composition comprises a reduced amount of the
10 saccharide compared to the amount of the saccharide that was present prior to the contacting.
12. The composition of embodiment 11, wherein the degree of polymerization of the saccharide before hydrolysis is 3 to 7.
13. The composition of embodiment 11 or 12, wherein the saccharide is in (i)
15 a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction;
wherein the saccharide is a byproduct of the glucan synthesis reaction.
14. A method of enriching fructose present in a fraction of a glucan synthesis reaction, comprising:
20 (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the fraction; and
25 (b) separating fructose from the hydrolyzed fraction of step (a) to obtain a composition having a higher concentration of fructose compared to the fructose concentration of the fraction of step (a).
15. A fermentation method comprising:
30 (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme under suitable conditions, wherein the alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or

alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the fraction;

(b) fermenting the fraction of step (a) with a microbe to yield a product, wherein the fermenting is performed after step (a) or simultaneously with step (a); and

(c) optionally, isolating the product of (b); wherein the yield of the product of (b) is increased compared to the product yield of fermenting a fraction of the glucan synthesis reaction that has not been contacted with the alpha-glucosidase enzyme.

EXAMPLES

The disclosed invention is further defined in the following Examples. It should be understood that these Examples, while indicating certain preferred aspects of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

Abbreviations

The meaning of some of the abbreviations used herein is as follows: "g" means gram(s), "h" means hour(s), "mL" means milliliter(s), "psi" means pound(s) per square inch, "wt%" means weight percentage, "μm" means micrometer(s), "%" means percent, "°C" means degrees Celsius, "mg" means milligram(s), "mm" means millimeter(s), "mL/min" means milliliters per minute, "m" means meter(s), "μL" means microliter(s), "mmol" means millimole(s), "min" means minute(s), "mol%" means mole percent, "M" means molar, "mg/g" means milligram per gram, "rpm" means revolutions per minute, "MPa" means megaPascals.

GENERAL METHODS

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Sucrose was obtained from VWR (Radnor, PA).

Preparation of Crude Extracts of Glucosyltransferase (gtf) Enzymes

The *Streptococcus salivarius* gtfJ enzyme (SEQ ID NO:3) was expressed in *E. coli* strain DH10B using an isopropyl beta-D-1-thiogalactopyranoside (IPTG)-induced expression system. SEQ ID NO:3 has an N-terminal 42-residue deletion compared to the *S. salivarius* gtfJ amino acid sequence in GENBANK Identification No. 47527, but includes a start methionine. Briefly, *E. coli* DH10B cells were transformed to express SEQ ID NO:3 from a DNA sequence codon-optimized to express the gtfJ enzyme in *E. coli*. This DNA sequence was contained in the expression vector, pJexpress404[®] (DNA 2.0, Menlo Park CA).

The transformed cells were inoculated to an initial optical density (OD at 600_{nm}) of 0.025 in LB medium (10 g/L Tryptone; 5 g/L yeast extract, 10 g/L NaCl) and allowed to grow at 37 °C in an incubator while shaking at 250 rpm. The cultures were induced by addition of 1 mM IPTG when they reached an OD₆₀₀ of 0.8-1.0. Induced cultures were left on the shaker and harvested 3 hours post induction.

GtfJ enzyme (SEQ ID NO:3) was harvested by centrifuging cultured cells (25 °C, 16000 rpm) in an Eppendorf[®] centrifuge, re-suspending the cells in 5.0 mM phosphate buffer (pH 7.0) and cooling to 4 °C on ice. The cells were broken using a bead beater with 0.1-mm silica beads, and then centrifuged at 16000 rpm at 4 °C to pellet the unbroken cells and cell debris. The crude extract (containing soluble GtfJ enzyme, SEQ ID NO:3) was separated from the pellet and analyzed by Bradford protein assay to determine protein concentration (mg/mL).

The *Streptococcus* sp. C150 gtf-S enzyme (SEQ ID NO:40) was prepared as follows. SG1184 is a *Bacillus subtilis* expression strain that expresses a truncated version of the glycosyltransferase Gtf-S ("GTF0459") from *Streptococcus* sp.C150 (GENBANK[®] GI:321278321). The gene (SEQ ID NO:41) encoding an N-terminal truncated protein GTF0459 (SEQ ID NO:42) from *E. coli* expression plasmid pMP79 was cloned into the NheI and HindIII sites of the *Bacillus subtilis* integrative expression plasmid p4JH under the aprE promoter and fused with the *B. subtilis* AprE signal peptide on the vector. The construct was first transformed into *E. coli* DH10B and selected on LB with ampicillin (100 µg/mL) plates. The confirmed construct pDCQ984 expressing GTF0459 was

then transformed into *B. subtilis* BG6006 containing nine protease deletions (*amyE::xylRPxylAcomK-ermC*, *degUHy32*, *oppA*, Δ *spolIE3501*, Δ *aprE*, Δ *nprE*, Δ *epr*, Δ *ispA*, Δ *bpr*, Δ *vpr*, Δ *wprA*, Δ *mpr-ybfJ*, Δ *nprB*) and selected on LB plates with chloramphenicol (5 μ g/mL). The colonies grown on LB plates with 5 μ g/mL chloramphenicol were streaked several times onto LB plates with 25 μ g/mL chloramphenicol. The resulting *B. subtilis* expression strain, SG1184, was first grown in LB medium with 25 μ g/mL chloramphenicol and then subcultured into GrantsII medium containing 25 μ g/mL chloramphenicol grown at 30 °C for 2-3 days. The cultures were spun at 15,000 g for 30 min at 4 °C and the supernatant was filtered through 0.22- μ m filters. The filtered supernatant was aliquoted and frozen at -80 °C.

B. subtilis SG1184 strain, expressing GTF0459 (SEQ ID NO:42), was grown under an aerobic submerged condition by conventional fed-batch fermentation. A nutrient medium was used containing 0-0.25% corn steep solids (Roquette), 5-25 g/L sodium and potassium phosphate, a solution of 0.3-0.6 M ferrous sulfate, manganese chloride and calcium chloride, 0.5-4 g/L magnesium sulfate, and a solution of 0.01-3.7 g/L zinc sulfate, cuprous sulfate, boric acid and citric acid. An antifoam agent, FOAMBLAST 882, at 2-4 mL/L was added to control foaming. A 10-L fermentation was fed with 50% (w/w) glucose feed when initial glucose in batch was non-detectable. The glucose feed rate was ramped over several hours. The fermentation was controlled at 30 °C and 20% DO, and at initial agitation of 750 rpm. The pH was controlled at 7.2 using 50% (v/v) ammonium hydroxide. Fermentation parameters such as pH, temperature, airflow, and DO% were monitored throughout the entire 2-day fermentation run. The culture broth was harvested at the end of the run and centrifuged to obtain supernatant. The supernatant containing GTF0459 (SEQ ID NO:42) was then stored frozen at -80 °C.

The *S. mutans* MT-4239 gtf-C enzyme (SEQ ID NO:43) was prepared as follows. A gene encoding a truncated version of a glucosyltransferase (gtf) enzyme identified in GENBANK® as GI:3130088 (SEQ ID NO:43; gtf-C from *S. mutans* MT-4239) was synthesized using codons optimized for expression in

Bacillus subtilis and synthesized by GenScript. The gene (SEQ ID NO:44) encoding GTF0088BsT1 with an N-terminal truncation and a C-terminal T1 truncation (SEQ ID NO:45) was amplified from the GENSCRIPT plasmid and cloned into the NheI and HindIII sites of the *Bacillus subtilis* integrative expression plasmid p4JH under the aprE promoter and fused with the *B. subtilis* AprE signal peptide on the vector. The construct was first transformed into *E. coli* DH10B and selected on LB with ampicillin (100 µg/mL) plates. The confirmed construct pDCQ1021 expressing GTF0088BsT1 was then transformed into *B. subtilis* BG6006 containing nine protease deletions (amyE::xylRPxylAcomK-ermC, degUHy32, oppA, ΔspolIE3501, ΔaprE, ΔnprE, Δepr, ΔispA, Δbpr, Δvpr, ΔwprA, Δmpr-ybfJ, ΔnprB) and selected on the LB plates with chloramphenicol (5 µg/mL). The colonies grown on LB plates with 5 µg/mL chloramphenicol were streaked several times onto LB plates with 25 µg/mL chloramphenicol. The resulting *B. subtilis* expression strain SG1221 was first grown in LB medium with 25 µg/mL chloramphenicol and then subcultured into GrantsII medium containing 25 µg/mL chloramphenicol grown at 30 °C for 2-3 days. The cultures were spun at 15,000 g for 30 min at 4 °C and the supernatant was filtered through 0.22-µm filters. The filtered supernatant was aliquoted and frozen at -80 °C.

B. subtilis SG1221 strain, expressing GTF0088BsT1 (SEQ ID NO:45), was grown under an aerobic submerged condition by conventional fed-batch fermentation. A nutrient medium was used containing 0-0.25% corn steep solids (Roquette), 5-25 g/L sodium and potassium phosphate, a solution of 0.3-0.6 M ferrous sulfate, manganese chloride and calcium chloride, 0.5-4 g/L magnesium sulfate, and a solution of 0.01-3.7 g/L zinc sulfate, cuprous sulfate, boric acid and citric acid. An antifoam agent, FOAMBLAST 882, at 2-4 mL/L was added to control foaming. A 2-L fermentation was fed with 50% (w/w) glucose feed when initial glucose in batch was non-detectable. The glucose feed rate was ramped over several hours. The fermentation was controlled at 30 °C and 20% DO, and at an initial agitation of 400 rpm. The pH was controlled at 7.2 using 50% (v/v) ammonium hydroxide. Fermentation parameters such as pH, temperature,

airflow, and DO% were monitored throughout the entire 2-day fermentation run. The culture broth was harvested at the end of run and centrifuged to obtain supernatant. The supernatant containing GTF088BsT1 (SEQ ID NO:45) was then stored frozen at -80 °C.

5 Determination of the Glucosyltransferase GTF0459 and GTF0088BsT1 Activity

Glucosyltransferase activity assay was performed by incubating 1-10% (v/v) crude protein extract containing GTF enzyme with 200 g/L sucrose in 25 mM or 50 mM sodium acetate buffer at pH 5.5 in the presence or absence of 25 g/L dextran (MW ~1500, Sigma-Aldrich, Cat.#31394) at 37 °C and 125 rpm
10 orbital shaking. One aliquot of reaction mixture was withdrawn at 1 h, 2 h and 3 h and heated at 90 °C for 5 min to inactivate the GTF. The insoluble material was removed by centrifugation at 13,000xg for 5 min, followed by filtration through 0.2-µm RC (regenerated cellulose) membrane. The resulting filtrate was analyzed by HPLC using two AMINEX HPX-87C columns series at 85 °C (Bio-
15 Rad, Hercules, CA) to quantify sucrose concentration. The sucrose concentration at each time point was plotted against the reaction time and the initial reaction rate was determined from the slope of the linear plot. One unit of GTF activity was defined as the amount of enzyme needed to consume one micromole of sucrose in one minute under the assay conditions.

20 Preparation of a Crude Extract of Alpha-(1,3)-Glucanohydrolase (mutanase)

A gene encoding the *Penicillium marneffei* ATCC® 18224™ mutanase identified in GENBANK® as GI:212533325 was synthesized by GenScript (Piscataway, NJ). The nucleotide sequence (SEQ ID NO:46) encoding protein sequence (MUT3325; SEQ ID NO:47) was subcloned into plasmid pTrex3 at
25 SacII and AscI restriction sites, a vector designed to express the gene of interest in *Trichoderma reesei*, under control of *CBHI* promoter and terminator, with *Aspergillus niger* acetamidase for selection. The resulting plasmid was transformed into *T. reesei* by biolistic injection. The detailed method of biolistic transformation is described in International PCT Patent Application Publication
30 WO2009/126773 A1, which is incorporated herein by reference. A 1-cm² agar plug with spores from a stable clone, TRM05-3, was used to inoculate the

production media (described below). The culture was grown in shake flasks for 4-5 days at 28 °C and 220 rpm. To harvest the secreted proteins, the cell mass was first removed by centrifugation at 4000g for 10 min and the supernatant was filtered through 0.2-µm sterile filters. The expression of mutanase MUT3325

5 (SEQ ID NO:47) was confirmed by SDS-PAGE.

The production media component is listed below.

NREL-Trich Lactose Defined

Formula	Amount	Units
ammonium sulfate	5	g
PIPPS	33	g
BD BACTO casamino acid	9	g
KH ₂ PO ₄	4.5	g
CaCl ₂ .2H ₂ O	1.32	g
MgSO ₄ .7H ₂ O	1	g
<i>T. reesei</i> trace elements	2.5	mL
NaOH pellet	4.25	g
Adjust pH to 5.5 with 50% NaOH		
Bring volume to	920	mL
Add to each aliquot: FOAMBLAST	5	drops
Autoclave, then add 20 % lactose filter sterilized	80	mL

10 *T. reesei* trace elements

Formula	Amount	Units
citric acid.H ₂ O	191.41	g
FeSO ₄ .7H ₂ O	200	g
ZnSO ₄ .7H ₂ O	16	g
CuSO ₄ .5H ₂ O	3.2	g

MnSO ₄ .H ₂ O	1.4	g
H ₃ BO ₃ (boric acid)	0.8	g
Bring volume to	1	L

Fermentation seed culture was prepared by inoculating 0.5 L of minimal medium in a 2-L baffled flask with 1.0 mL frozen spore suspension of the MUT3325 expression strain TRM05-3 (The minimal medium was composed of 5 g/L ammonium sulfate, 4.5 g/L potassium phosphate monobasic, 1.0 g/L magnesium sulfate heptahydrate, 14.4 g/L citric acid anhydrous, 1 g/L calcium chloride dihydrate, 25 g/L glucose and trace elements including 0.4375 g/L citric acid, 0.5 g/L ferrous sulfate heptahydrate, 0.04 g/L zinc sulfate heptahydrate, 0.008 g/L cupric sulfate pentahydrate, 0.0035 g/L manganese sulfate monohydrate and 0.002 g/L boric acid. The pH was 5.5.). The culture was grown at 32 °C and 170 rpm for 48 hours before being transferred to 8 L of the production medium in a 14-L fermenter. The production medium was composed of 75 g/L glucose, 4.5 g/L potassium phosphate monobasic, 0.6 g/L calcium chloride dehydrate, 1.0 g/L magnesium sulfate heptahydrate, 7.0 g/L ammonium sulfate, 0.5 g/L citric acid anhydrous, 0.5 g/L ferrous sulfate heptahydrate, 0.04 g/L zinc sulfate heptahydrate, 0.00175 g/L cupric sulfate pentahydrate, 0.0035g/L manganese sulfate monohydrate, 0.002 g/L boric acid and 0.3 mL/L FOAMBLAST 882.

The fermentation was first run with batch growth on glucose at 34 °C, 500 rpm for 24 h. At the end of 24 h, the temperature was lowered to 28 °C and the agitation speed was increased to 1000 rpm. The fermenter was then fed with a mixture of glucose and sophorose (62% w/w) at a specific feed rate of 0.030 g glucose-sophorose solids / g biomass / hr. At the end of run, the biomass was removed by centrifugation and the supernatant containing the MUT3325 mutanase (SEQ ID NO:47) was concentrated about 10-fold by ultrafiltration using 10-kD Molecular Weight Cut-Off ultrafiltration cartridge (UFP-10-E-35; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The concentrated protein was stored at -80 °C.

Determination of Alpha-Glucanohydrolase (Mutanase) Activity

Insoluble mutan polymers required for determining mutanase activity were prepared using secreted enzymes produced by *Streptococcus sobrinus* ATCC[®] 33478[™]. Specifically, one loop of glycerol stock of *S. sobrinus* ATCC[®] 33478[™] was streaked on a BHI agar plate (Brain Heart Infusion agar, Teknova, Hollister, CA), and the plate was incubated at 37 °C for 2 days. A few colonies were picked using a loop to inoculate 2X 100 mL BHI liquid medium in the original medium bottle from Teknova, and the culture was incubated at 37 °C, held static for 24 h. The resulting cells were removed by centrifugation and the resulting supernatant was filtered through a 0.2-µm sterile filter; 2X 101 mL of filtrate was collected. To the filtrate was added 2X 11.2 mL of 200 g/L sucrose (final sucrose 20 g/L). The reaction was incubated at 37 °C with no agitation for 67 h. The resulting polysaccharide polymers were collected by centrifugation at 5000xg for 10 min. The supernatant was carefully decanted. The insoluble polymers were washed 4 times with 40 mL of sterile water. The resulting mutan polymers were lyophilized for 48 h. Mutan polymer (390 mg) was suspended in 39 mL of sterile water to make a 10 mg/mL suspension. The mutan suspension was homogenized by sonication (40% amplitude until large lumps disappear, ~10 min in total). The homogenized suspension was aliquoted and stored at 4 °C.

A mutanase assay was initiated by incubating an appropriate amount of enzyme with 0.5 mg/mL mutan polymer (prepared as described above) in 25 mM KOAc buffer at pH 5.5 and 37 °C. At various time points, an aliquot of reaction mixture was withdrawn and quenched with equal volume of 100 mM glycine buffer (pH 10). The insoluble material in each quenched sample was removed by centrifugation at 14,000xg for 5 min. The reducing ends of oligosaccharide and polysaccharide polymer produced at each time point were quantified by the *p*-hydroxybenzoic acid hydrazide solution (PAHBAH) assay (Lever M., *Anal. Biochem.*, (1972) 47:273-279) and the initial rate was determined from the slope of the linear plot of the first three or four time points of the time course. The PAHBAH assay was performed by adding 10 µL of reaction sample supernatant to 100 µL of PAHBAH working solution and heated at 95 °C for 5 min. The

working solution was prepared by mixing one part of reagent A (0.05 g/mL p-hydroxy benzoic acid hydrazide and 5% by volume of concentrated hydrochloric acid) and four parts of reagent B (0.05 g/mL NaOH, 0.2 g/mL sodium potassium tartrate). The absorption at 410 nm was recorded and the concentration of the reducing ends was calculated by subtracting appropriate background absorption and using a standard curve generated with various concentrations of glucose as standards.

Analysis of Reaction Profiles by HPLC

Periodic samples from reactions were taken and analyzed using an Agilent® 1260 HPLC equipped with a refractive index detector. An Aminex® HP-87C column (BioRad, Hercules, CA) having deionized water at a flow rate of 0.6 mL/min and 85 °C was used to quantitate the level of sucrose, glucose, leucrose and fructose in gtf reactions. An Aminex® HP-42A column (BioRad) having deionized water at a flow rate of 0.6 mL/min and 85 °C was used to quantitate soluble oligosaccharide byproducts (DP2-DP7) in gtf reactions.

A Dionex® UltiMate™ 3000 HPLC (Thermo Scientific) equipped with a refractive index detector was used for samples involving immobilized enzymes (Example 4). A Phenomenex® Rezex™ calcium monosaccharide column having deionized water at a flow rate of 0.3 mL/min and 85 °C was used to analyze the sugars.

Analysis of oligosaccharide linkage by NMR

NMR data were acquired on an Agilent DD2 spectrometer operating at 500 MHz for ¹H using a 5-mm cryogenic triple-resonance pulsed-field gradient (PFG) probe. Water suppression was obtained by carefully placing the observe transmitter frequency on resonance for the residual water signal in a "presat" experiment, and then using the first slice of a NOESY experiment with a full phase cycle (multiple of 32) and a mix time of 10 ms. One-dimensional ¹H spectra were acquired with a spectral width of 6410 Hz, acquisition time of 5.1 s, 65536 data points, 4 s presaturation and a 90-degree pulse of 5.85 μs. Sample temperature was maintained at 25 °C. Samples were prepared by adding 50 μL to a 5-mm NMR tube along with 450 μL of D₂O and 60 μL of D₂O containing 12.4

mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt) internal reference with the methyl resonance set to 0 ppm. Chemical shift assignments for different anomeric linkages were taken from: Goffin et al. (2009, *Bull Korean Chem. Soc.* 30:2535-2541. Peak assignments were 5.35 ppm for alpha(1,3) linkages, 5.1 ppm for leucrose, and 4.95 for alpha(1,6) linkages. Reducing ends (RE) were assigned to be 5.2 for alpha RE and 4.65 for beta RE.

EXAMPLE 1

Production of Sugar Syrup by Polymerization of Sucrose

This example discloses the general manner in which a mixture of soluble sugars was produced by polymerization of sucrose with a gtf enzyme in a glucan synthesis reaction. Specifically, a filtrate of a glucan synthesis reaction was prepared, which was then concentrated to a syrup.

Sucrose (3000 g) was added to a clean 5-gallon polyethylene bucket. Water (18.1 L) and Fermasure™ (10 mL) were added to the bucket, and the pH was adjusted to 7.0 by addition of 5 vol% NaOH and 5 vol% H₂SO₄. The final volume was ~20 L and the initial concentration of sucrose as measured by HPLC was 152.5 g/L. The glucan polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:3) extract prepared as described in the General Methods section. This extract contained about 2.9 mg/mL of protein. Agitation to the reaction solution was provided using an overhead mechanical motor equipped with a glass shaft and PTFE blade.

After 48 hours, HPLC analysis revealed that 96% of the sucrose had been consumed and the reaction was deemed to be complete. The insoluble poly-alpha-1,3-glucan product of the reaction was removed by filtration with a Buchner filter funnel using 325-mesh steel screen and 40-micron filter paper. The mother liquor (filtrate) was then concentrated using a rotary evaporator (bath temp of 40-50 °C) to a total sugar concentration of about 320 g/L sugars. The composition of the concentrated filtrate is provided in Table 2.

Table 2Composition of a Concentrated Filtrate of a Glucan Synthesis Reaction

	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total
g/L	13.5	130.6	25.5	103.8	18.3	28.3	320.1
wt%	4.2	40.8	8	32.4	5.7	8.9	100

Table 2 indicates that the concentrated filtrate of the glucan synthesis
 5 reaction contains sucrose, fructose, glucose, leucrose and oligosaccharides of
 DP2-DP7.

EXAMPLE 2Effect of Enzymes on Hydrolysis of Sugars in a Filtrate of a Glucan Synthesis
 Reaction

10 This example measures the activity of various glucoamylase (EC 3.2.1.3),
 transglucosidase (EC 2.4.1.24), beta-glucosidase (EC 3.2.1.21), alpha-amylase
 (EC 3.2.1.1) and glucosidase (EC 3.2.1) enzymes for the purpose of reducing the
 concentration of leucrose and/or oligosaccharide byproducts in a concentrated
 filtrate of a glucan synthesis reaction. Certain enzymes such as DIAZYME RDF
 15 ULTRA, transglucosidase (EC 2.4.1.24) and glucoamylase (EC 3.2.1.3), which
 are all alpha-glucosidase, were found to be particularly effective at reducing the
 amount of these byproducts, resulting in a corresponding increase in
 monosaccharides (glucose and fructose) in the treated filtrate.

20 A filtrate of a glucan synthesis reaction was first prepared and
 concentrated to a syrup according to the procedure outline in Example 1. The
 composition of this concentrated filtrate is provided in Table 3. NMR analysis
 revealed that the ratio of alpha(1,3) to alpha (1,6) linkages present in the syrup
 was 78:22.

Table 3Composition of a Concentrated Filtrate of a Glucan Synthesis Reaction

	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total
g/L	161	210	93	302	33	61	860
wt%	18.7	24.4	10.8	35.1	3.8	7.1	100.0

The syrup of Table 3 was used to test the hydrolytic activity of various enzymes against leucrose and oligosaccharide byproducts of the glucan synthesis reaction. It was not obvious at the outset of these experiments what enzyme could be used to hydrolyze both these byproducts, given that leucrose contains an unusual linkage [$\alpha(1,5)$ -glucosyl fructose] and that the oligosaccharides comprise primarily $\alpha(1,3)$ and $\alpha(1,6)$ glucosyl-glucose linkages. Enzymes with various activities were selected for this analysis (Table 4).

Table 4

Enzymes Evaluated for Leucrose and Oligosaccharide Hydrolysis

Enzyme	Source	Function	Activity or protein concentration
DIAZYME RDF ULTRA	DuPont IB ^a	1,4- α -glucosidase	710 U/g
Oligo-1,6-glucosidase	Megazyme	1,6- α -glucosidase	320 U/mg
SPEZYME FRED	DuPont IB	α -amylase	1-5%
SPEZYME RSL	DuPont IB	α -amylase	1-5%
OPTIMAX L-1000	DuPont IB	Pullulanase	1-5%
TRANSGLUCOSIDASE L-2000	DuPont IB	Transglucosidase	>1700 TGU/g
purified TRANSGLUCOSIDASE L-2000	DuPont IB	Transglucosidase	22.7 mg/mL
ACCELLERASE BG	DuPont IB	Beta-glucosidase	3000 U/g
NOVO 188	Sigma-Aldrich	Beta-glucosidase	> 250 U/g
SUMIZYME BFS-L	Shin Nihon Chemical	Beta-glucosidase	100 U/g
SUMIZYME BGA	Shin Nihon Chemical	Beta-glucosidase	2000 U/g
ACCELERASE TRIO	DuPont IB	Cellulase	5-10%
ACCELERASE 1500	DuPont IB	Cellulase/Beta-glucosidase	5-10%/0.5-4%
OPTIDEX L-400	DuPont IB	Glucoamylase	> 350 GAU/g
GC 147	DuPont IB	Glucoamylase	400 GAU/g
GC 321	DuPont IB	Glucoamylase	> 350 GAU/g

^a DuPont Industrial Biosciences

Conditions for treating the syrup of Table 3 with each of the above enzymes are provided in Table 5 (enzyme loading, time, temperature, pH, sugar concentration). The syrup was diluted with water to reach the sugar

concentration used in each hydrolysis reaction. Table 5 further provides the percent hydrolysis of the leucrose and DP3+ (at least DP3-DP7) oligosaccharides by each enzyme. Percent DP3+ hydrolysis was calculated as $(1 - (\text{wt\% DP3+ oligosaccharides in the final syrup}) / (\text{wt\% DP3+ oligosaccharides in the initial syrup}))$. Similarly, percent leucrose hydrolysis was calculated as $(1 - (\text{wt\% leucrose in the final syrup}) / (\text{wt\% leucrose in the initial syrup}))$.

Table 5

Hydrolysis of Leucrose and Oligosaccharides in a Concentrated Filtrate by Various Enzymes

Example	Enzyme	Enzyme loading (vol %)	Temp (°C)	Time (hr)	pH	Sugar concentration (g/L) ^a	DP3+ ^b hydrolysis (%)	Leucrose hydrolysis (%)
2.1	DIAZYME RDF ULTRA	0.5	60	88	4.0	300	36	13
2.2	Oligo-1,6-glucosidase	5	40	72	5.5	400	43	<2
2.3	SPEZYME FRED	0.5	60	66	4.0	280	<2	<2
2.4	SPEZYME RSL	0.5	60	48	4	290	<2	<2
2.5	OPTIMAX L-1000	0.5	60	48	4	290	<2	<2
2.6	TG L-2000	0.25	60	70	4.0	260	54	>98
2.7	TG L-2000	2	60	48	4.5	300	96	>98
2.8	PURIFIED TG L-2000	0.5	60	48	4.5	260	56	>98
2.9	ACCELERASE BG	0.5	60	70	4	300	11	<2
2.10	NOVO 188	0.25	60	70	4	300	49	36
2.11	NOVO 188	5	60	40	5.5	340	93	29
2.12	SUMIZYME BFS-L	0.5	60	48	4.5	260	55	46
2.13	SUMIZYME BGA	0.1 wt%	60	48	4.5	260	26	77
2.14	ACCELERASE TRIO	0.5	60	48	4	290	28	6.6
2.15	ACCELERASE 1500	0.5	60	66	4	280	26	4
2.16	GC 147	0.5	60	40	4	300	55	12
2.17	GC 321	5	60	72	5.5	400	74	64
2.18	OPTIDEX L-400	0.5	60	70	4	300	27	25

^a Sugar concentration (total concentration of sucrose, glucose, fructose, leucrose and oligosaccharides) measured by HPLC; reported values are rounded to nearest 10 g/L increment.

5 ^b DP3+ contains DP3-DP7, but may also contain larger soluble oligosaccharides that have a high ratio of alpha-1,6 linkages to alpha-1,3 linkages, when produced using certain gtf enzymes.

Table 5 indicates that 1,4-alpha-glucosidase and 1,6-alpha-glucosidase showed some (Example 2.1) or very little (Example 2.2) hydrolysis of leucrose, but did release some glucose from the oligosaccharides. Use of alpha-amylase (Example 2.3 and Example 2.4) showed very little activity against the compounds of interest. Similarly, use of a pullulanase (Example 2.5) showed very little activity.

Cellulases (Examples 2.14 and 2.15) were largely ineffective at hydrolyzing leucrose, but did hydrolyze some of the oligosaccharides.

Although the oligosaccharides did not contain beta linkages, surprisingly, beta-glucosidase enzymes also showed a range of hydrolytic conversion from very low (ACCELERASE BG, Example 2.9) to very high (NOVO 188, Examples 2.10 and 2.11). The relative efficacy of these enzymes varied quite dramatically. In some cases, the amount of oligosaccharide that was hydrolyzed greatly exceeded (Example 2.11), or was close to (Example 2.12), the percentage of leucrose that was hydrolyzed. In other cases, leucrose was highly hydrolyzed by beta-glucosidase while the oligosaccharides were moderately hydrolyzed (Example 2.13). The high disparity amongst the results observed with beta-glucosidase suggests that the presence of other enzymes in the tested beta-glucosidase formulations, such as glucoamylase or another type of alpha-glucosidase, could be responsible for the observed activity.

Conversely, the results in Table 5 indicate that transglucosidase (TG L-2000, Example 2.6) showed very high activity at hydrolyzing both the oligosaccharides and leucrose. Leucrose hydrolysis by transglucosidase appeared quantitative under certain circumstances, and greater than 95% of the DP3+ material was hydrolyzed to glucose and DP2 at high enzyme loadings (Example 2.7). Use of a purified version of transglucosidase revealed similar

activity (Example 2.8), indicating that the observed hydrolysis is due to the transglucosidase enzyme and not background activity.

Glucoamylases (Examples 2.16-2.18) showed a range of activity against leucrose and the oligosaccharides. Only one tested glucoamylase (Example
5 2.18) gave less than 30% hydrolysis of both the leucrose and oligosaccharides.

The results in Table 5 indicate that alpha-glucosidases such as DIAZYME RDF ULTRA, glucoamylase and transglucosidase can hydrolyze leucrose byproduct present in a glucan reaction filtrate. The ability of alpha-glucosidases to hydrolyze leucrose indicates that these enzymes can hydrolyze alpha-1,5
10 glucosyl-fructose linkages. While this activity was shown above using leucrose as a substrate, it is believed that this activity can also be extended to oligosaccharides comprising alpha-1,5 glucosyl-fructose linkages.

The results in Table 5 further indicate that alpha-glucosidases such as glucoamylase and transglucosidase can hydrolyze oligosaccharide byproducts present in a glucan reaction filtrate. Since these oligosaccharides are mostly
15 comprised of glucose monomer units linked by alpha-1,3 and/or alpha-1,6 linkages (Example 3), the data in Table 5 indicate that alpha-glucosidase enzymes can hydrolyze alpha-1,3 glucosyl-glucose and/or alpha-1,6 glucosyl-glucose linkages.

20 Since alpha-glucosidase enzymes were generally effective at hydrolyzing the leucrose and/or oligosaccharide byproducts of a glucan synthesis reaction, these enzymes can be used alone or in combination to reduce the processing time necessary to generate a high purity syrup from a glucan reaction filtrate containing an increased amount of monosaccharides and reduced amount of
25 sugar byproducts. An example of an effective enzyme combination could be a transglucosidase such as TG L-2000, for leucrose hydrolysis, and a glucoamylase (e.g., GC 321) enzyme that efficiently hydrolyzes oligosaccharide byproducts.

Thus, alpha-glucosidase enzymes can individually hydrolyze (i) alpha-1,5
30 glucosyl-fructose linkages and (ii) alpha-1,3 and alpha-1,6 glucosyl-glucose linkages in certain saccharides.

EXAMPLE 3

Comparison of Linkage Distributions of Glucan Reaction Filtrate Components before and after Enzyme Hydrolysis

This example measures the hydrolytic activity of transglucosidase (EC
5 2.4.1.24) and beta-glucosidase (EC 3.2.1.21) enzymes against leucrose and
oligosaccharide byproducts present in a concentrated filtrate of a glucan
synthesis reaction. Transglucosidase was found to reduce the amount of these
byproducts, resulting in a corresponding increase in monosaccharides (glucose
and fructose) in the treated filtrate.

10 The oligosaccharide byproducts present in the filtrate of the above glucan
synthesis reaction comprise >90% glucose-glucose linkages, as determined by
NMR (General Methods). Of the glucose-glucose linkages, ~78% represent
alpha-1,3 linkages and ~22% represent alpha-1,6 linkages.

NMR was used to determine the linkage profile of material generated in
15 Example 2.11 above after hydrolysis. As shown in Figure 1, the peak
corresponding to alpha-1,3 linkages was reduced by 86%, the peak
corresponding to alpha-1,6 linkages was reduced by only 2.3%, and the peak
corresponding to leucrose peaks was reduced by 21%. While sucrose was very
nearly quantitatively hydrolyzed by this enzyme, Novo 188 does not appear to be
20 capable of hydrolyzing alpha-1,6 linkages.

NMR was similarly used to determine the linkage profile of material
generated using TG L-2000 (SEQ ID NO:1) transglucosidase (Figure 2). 210 µL
of concentrated filtrate from the material in Table 3, 300 µL of D₂O, and 90 µL of
D₂O containing 12.4 mM DSS as internal reference were mixed in an NMR tube
25 to give a total sugar concentration of 300 g/L and heated to 60 °C. A time zero
spectrum (starting material in Figure 2) was acquired after thermal equilibration
at 60 °C, and then 0.5 vol% of enzyme was added. The sample was re-
equilibrated in the probe at 60 °C and shimmed, and measurements were taken
within a few minutes of analysis. After 10 hours of treatment with TG L-2000
30 enzyme (treated material in Figure 2), the peaks corresponding to alpha-1,3
linkages were reduced by 41%, the peaks corresponding to alpha-1,6 linkages

5 These results demonstrate that a transglucosidase can convert oligosaccharides containing alpha-1,3 and alpha-1,6 linkages into glucose and can convert leucrose into fructose and glucose. Thus, transglucosidase can hydrolyze (i) alpha-1,5 glucosyl-fructose linkages and (ii) alpha-1,3 and alpha-1,6 glucosyl-glucose linkages in certain saccharides.

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(PEI, EPOMIN P-1050). 3500 mL of the PEI solution was then pumped upflow and effluent was recycled through the beds for 2 hours. The granular diatomaceous earth beds were then washed upflow with distilled water for 2 hours to remove free PEI at room temperature. In this manner, granular
5 diatomaceous earth-PEI carriers were obtained.

In the meantime, 3.5 mL of glucoamylase GC-147 having activity defined in Table 4 was added to 315 ml of 0.02 M acetate buffer (pH 4.5). 1.575 g of 50% w/w glutaraldehyde (Protectol® GA-50) was then slowly added to the aqueous solution of glucoamylase with gentle mixing, and the glutaraldehyde
10 was allowed to react with the aqueous glucoamylase solution for 4 hours at a temperature of 20-25 °C with gentle agitation, which resulted in formation of a treated enzyme-glutaraldehyde adduct containing treated glucoamylase.

Separately, these steps were repeated using the transglucosidase TG L-2000 having activity defined in Table 4 instead of the glucoamylase, thereby resulting
15 in the formation of a treated enzyme-glutaraldehyde adduct containing treated transglucosidase.

Each of the treated enzyme-glutaraldehyde adducts was then circulated for 4 hours (20-25 °C) in its own column prepared as above containing granular diatomaceous earth-PEI carrier. Excess treated adduct was then washed out of
20 the carriers with water. Columns with immobilized glucoamylase or transglucosidase were thus prepared.

A glucan filtrate having the composition defined in Table 3 was diluted to 180 g/L, adjusted to pH 4.5, and passed through a column containing an immobilized enzyme. Column temperature was controlled to 60 °C. After 16
25 hours of column equilibration, samples were taken periodically at different flow rates. Sugar compositions of hydrolysis reaction products were determined by HPLC (Table 6). Every time the flow rate setting was changed, the column was allowed to re-equilibrate for at least 1-2 bed volumes before sampling. The degree of hydrolysis of leucrose and oligosaccharides was calculated using the
30 manner described in Example 2. Three column configurations were tested: 1)

immobilized glucoamylase, 2) immobilized transglucosidase, and 3) immobilized glucoamylase followed by immobilized transglucosidase.

Table 6

Application of Immobilized Glucoamylase and Transglucosidase Enzymes to
Hydrolyze Oligosaccharides and Leucrose

Immobilized Enzyme	Mean contact time (hr)	DP3+ hydrolysis (%)	Leucrose hydrolysis (%)
GC 147	0.7	16	17
GC 147	1.0	20	22
GC 147	1.3	25	29
GC 147	3.0	39	47
TG L-2000	0.7	28	>95
TG L-2000	1.0	32	>95
TG L-2000	1.3	37	>95
TG L-2000	3.0	47	>95
GC 147 + TG L-2000	6.0	55	>95

5

Table 6 indicates that, as the mean contact time (defined as the nominal column volume divided by the mean flow rate) was increased, the degree of hydrolysis of leucrose and oligosaccharides generally increased. Use of the immobilized transglucosidase to hydrolyze leucrose was particularly effective, as no significant difference was observed even using the fastest flow rate that was tested. While each column individually showed reasonable conversion, the combination of the glucoamylase and transglucosidase gave the highest hydrolysis of oligosaccharides.

10

Thus, use of an immobilized glucoamylase or transglucosidase, or both types of immobilized enzymes, represents an effective technique to hydrolyze oligosaccharides containing alpha-1,3 and alpha-1,6 glucosyl-glucose linkages, as well as leucrose. These results are consistent with those of Example 2. Immobilization of other alpha-glucosidase enzymes should give similar results.

15

EXAMPLE 5

20 Enrichment of Fructose from a Glucan Reaction Filtrate by Chromatography

This example discloses how fructose in a glucan reaction filtrate can be further enriched through chromatography.

Generally, when separating sugar molecules by chromatography, components elute inversely to molecular size so that the largest molecules elute first. Thus, with respect to a filtrate of a glucan synthesis reaction, oligosaccharides elute first, followed by disaccharides, and then monosaccharides. Separations using a sodium cation resin did not separate fructose and glucose well, and all of leucrose, sucrose, and DP2 co-eluted. Use of ion exchange resins where the cation is calcium are preferred to separate glucose and fructose.

A filtrate of a glucan synthesis reaction was first prepared and concentrated to a syrup according to the procedure outline in Example 1. The composition of this concentrated filtrate is provided in Table 7.

Table 7

Composition of a Concentrated Filtrate of a Glucan Synthesis Reaction

	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total
g/L	126	202	93	295	40	65	821
wt%	15.4	24.6	11.3	36.0	4.8	7.9	100

The syrup of Table 7 was filtered and diluted to 25 g dry solids/100 g solution with ion-exchanged water, and fed to a column containing a crosslinked strong acid ion exchange resin in the calcium form. The physical parameters of this column appear in Table 8. Diluted syrup (15.8 L) was fed to the column, which was maintained at 65 °C, after which the column was eluted using water at a flow rate of 30 L/hr.

Table 8

Physical Parameters of the Column

Resin Type	FINEX CS11GC
Ion form	Ca ²⁺
Crosslinking, % divinyl benzene	5.5
Particle size (mm)	0.34
Bed length (m)	5.0
Column diameter (m)	0.225

In this separation, leucrose remained in the column longer than sucrose, perhaps due to complexation of leucrose with the calcium cation, and in fact, co-

eluted with glucose. Two fractions containing fructose were isolated. Fraction 5.1 eluted between 47 and 120 minutes, and fraction 5.2 eluted between 120 and 172 minutes. Of the fructose fed to the chromatographic separation, 95.7% of the fructose was isolated in >90% purity. The product distribution in each fraction (5.1 and 5.2) as measured by HPLC appears in Table 9.

Table 9

Product Distribution of Chromatographic Fractions Containing Significant Amounts of Fructose

Fraction	Sucrose	Leucrose	Glucose	Fructose	DP3+	Others	Total	% Fructose recovered
5.1	31.9	34.8	20.8	3.9	5.4	4.8	100	3.9
5.2	0.0	1.0	0.8	97.7	0.0	0.6	100	95.7

As the feed composition for this separation comprised 36.0% fructose, a total of 34.5% of the total stream was recovered as a fructose syrup with >90 wt% DS fructose. If the sucrose in the feed is neglected, 40.7% of the sugars were recovered as a fructose syrup with >90 wt% DS fructose.

Thus, fructose in a glucan reaction filtrate can be further enriched through chromatography. Example 6 below demonstrates that this process can be enhanced using glucan filtrate hydrolyzed with a transglucosidase.

EXAMPLE 6

Enrichment of Fructose from a Hydrolyzed Glucan Reaction Filtrate by Chromatography

This example demonstrates that fructose isolation from a glucan filtrate in which the oligosaccharides and leucrose have been hydrolyzed results in an increased yield of high purity fructose syrup compared to when isolating fructose from a non-hydrolyzed glucan filtrate.

A syrup was prepared by concentrating (vacuum at 50 °C) a glucan filtrate that had been treated with 1 vol% of transglucosidase TG L-2000 (SEQ ID NO:1) for 24 hr at 60 °C and pH 4.5. Some oligosaccharide formation was observed during the concentration process, which was expected since transglucosidase enzymes are known to create oligosaccharides at high concentrations of

monosaccharides. The syrup had the final product distribution described in Table A.

Table A

Composition of a Concentrated Glucan Filtrate that Was Hydrolyzed before
Concentration

	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total
g/L	3	<10	294	409	73	81	~870
wt%	0.3	1.1	33.7	47.0	8.4	9.3	100

The syrup described in Table A was filtered and diluted to 25.4 g DS/100 g solution with ion-exchanged water and was fed to a column containing a crosslinked strong acid cation exchange resin in the calcium form. The physical parameters of the column appear in Table B. Diluted syrup (169 g) was then fed to the column, which was maintained at 65 °C, afterwhich the column was eluted using water at a flow rate of 50 mL/min.

Table B

Physical Parameters of the Column

Resin Type	FINEX CS11GC
Ion form	Ca ²⁺
Crosslinking, % divinyl benzene	5.5
Particle size (mm)	0.34
Bed length (m)	1.69
Column diameter (m)	0.093

Two fractions containing fructose were isolated. Fraction 6.1 eluted between 73 and 103 minutes, and fraction 6.2 eluted between 103 and 120 minutes. Of the fructose fed to the chromatographic separation, 93.0% of the fructose fed to the column was isolated in fraction 6.2 in >90% purity. The product distribution in each fraction (6.1 and 6.2) as measured by HPLC appears in Table C.

Table CProduct Distribution of Chromatographic Fractions Containing Fructose from a Hydrolyzed Glucan Filtrate

Fraction	Sucrose	Leucrose	Glucose	Fructose	DP3+	Others	Total	% Fructose recovered
6.1	7.7	13.9	63.9	7.3	1.5	5.7	100	5.9
6.2	0.0	0.6	3.0	91.8	0.0	4.6	100	93.0

5 The reduced separation efficiency in this example compared to Example 5 can be attributed to differences in the scale of the column and the higher glucose fraction of the sample. Even so, chromatographic purification of this material resulted in an increased yield of high purity fructose syrup compared to that achieved in Example 5, in which syrup was chromatographically prepared from a glucan filtrate that had not been hydrolyzed by a transglucosidase. As the feed composition for this separation comprised 47% fructose (Table A), 43.7% of the total stream was recovered as a fructose syrup with >90 wt% DS fructose. This 43.7% recovery is significantly better than the 34.5% recovery in Example 5.

15 Thus, fructose isolation from a glucan filtrate that has been hydrolyzed with transglucosidase results in an increased yield of fructose compared to when isolating fructose from a non-hydrolyzed glucan filtrate.

EXAMPLE 7Production of Ethanol by Fermenting a Filtrate of a Glucan Synthesis Reaction

This example discloses yeast fermentation of glucan filtrate to ethanol.

20 Yeast (*S. cerevisiae*) cream (Tonon mill, Brazil) was washed by suspending the cream in tap water (2.4 L, optical density of 65 at 600 nm) and then centrifuging the yeast cream for 5 minutes using a LEGEND XTR centrifuge (Thermo Scientific) at 4500 g. After decanting the supernatant, the yeast cells were resuspended and concentrated by centrifugation two additional times. After 25 the third wash, the pH was adjusted to 2 by addition of 5 wt% sulfuric acid. The optical density was measured using a GENESYS 20 4001 spectrophotometer (Thermo Scientific) and adjusted to 100 at 600 nm by addition of tap water. The adjusted yeast cream (1.5 L) was added to a 7.5-L BIOFLO310 fermenter vessel

(New Brunswick). The fermenter was set to maintain temperature at 30 °C and agitation at 100 rpm. Although pH was measured during fermentation, it was not controlled by the addition of acid or base solutions.

A feed solution containing yeast extract (10 g/L), peptone (20 g/L), and 200 g/L of sugars from a glucan filtrate was prepared and sterilized using a PHOENIX AV-250 PLUS autoclave at 121 °C for 15 minutes. The feed solution was allowed to cool to 25 °C (room temperature) before the fermentation began. The sterilized feed solution (3.5 L) was added to the fermenter over approximately 5 hours at a rate of 684 mL/hr, and the fermentation was allowed to proceed for 22 hours.

Periodic samples were taken during the fermentation and analyzed for optical density using a GENESYS 20 4001 spectrophotometer, Brix using a PAL-3 refractometer (Atago), and sugar and ethanol concentrations by HPLC (General Methods). These results are summarized in Table 10.

15

Table 10

Feed and Time Course Fermentation Profiles for the First Ethanol Fermentation

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	9	70	19	76	7	19	200	0
0	0.2	0.0	0.0	0.1	-	-	-	7
1	<1	18	0.3	<1	-	-	-	15
2	<1	30	0.4	<1	-	-	-	21
3	<1	40	0.0	<1	-	-	-	25
4	<1	46	0.0	<1	-	-	-	28
5	<1	49	0.0	<1	-	-	-	29
6	<1	53	0.0	<1	-	-	-	32
8	<1	53	0.0	<1	-	-	-	33
22	<1	53	0.0	<1	5	18	76	33

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-22 hours) are listed.

20

When the fermentation was over, the yeast cells were separated by centrifugation using a LEGEND XTR centrifuge at 4500 g for 5 minutes. After decanting the supernatant, the yeast were resuspended and concentrated by centrifugation two additional times. After the third wash, the pH was adjusted to

2 by addition of 5 wt% sulfuric acid. The optical density was measured using a GENESYS 20 4001 spectrophotometer and adjusted to 100 at 600 nm by addition of tap water. Two additional fermentation cycles, each using fresh feed, were performed using recycled yeast cells from the previous fermentation following the same conditions described above. The fermentation results obtained using first-time and second-time recycled yeast are provided in Tables 11 and 12, respectively.

Table 11

Feed and Time Course Fermentation Profiles Using the First Recycle of Yeast

Cells

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	13	69	21	77	7	18	206	0
0	0	4	0	0	-	-	-	5
1	<1	19	0	<1	-	-	-	18
4	<1	35	0	<1	-	-	-	23
4	<1	40	0	<1	-	-	-	26
5	<1	45	0	<1	-	-	-	29
6	<1	53	0	<1	-	-	-	32
7	<1	50	0	<1	-	-	-	32
7	<1	51	0	<1	-	-	-	33
21	<1	42	0	<1	6	18	65	37

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-21 hours) are listed.

Table 12

Feed and Time Course Fermentation Profiles Using the Second Recycle of Yeast

Cells

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	10	70	19	76	6	19	201	0
0	<1	0	0	<1	-	-	-	11
1	<1	32	0	<1	-	-	-	24
4	<1	40	0	<1	-	-	-	29
4	<1	45	0	<1	-	-	-	31
5	<1	46	0	<1	-	-	-	33
6	<1	45	0	<1	-	-	-	34

6	<1	16	0	<1	-	-	-	48
7	<1	7	0	<1	-	-	-	52
21	<1	5	0	<1	5	16	27	54

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-21 hours) are listed.

Very little leucrose was consumed in the first fermentation, although the yeast cells started to acclimate and consume leucrose by the second recycle. Ethanol fermentation titers increased from 33 g/L (Table 10, 22 hours) to 54 g/L (Table 12, 21 hours) after three fermentation cycles with recycled yeast, although significant amounts of leucrose were present in the medium, even after the last cycle.

Thus, glucan filtrate can be used in a fermentation process to produce ethanol.

EXAMPLE 8

Production of Ethanol by Fermenting Hydrolyzed Glucan Filtrate

This example demonstrates that fermenting a glucan filtrate in which the leucrose and oligosaccharide byproduct components have previously been saccharified results in increased ethanol yields.

Fermentations were performed following the procedure outlined in Example 7, but using a glucan filtrate that was previously treated with a transglucosidase (TG L-2000, SEQ ID NO:1). Hydrolyzed glucan filtrate was prepared as follows. Glucan filtrate was adjusted to 300 g sugars/L and then the pH was adjusted to 4.0 using 1.0 M sodium hydroxide and 5 wt% sulfuric acid. The final volume of this preparation was 6.75 L. The filtrate solution was then sterilized using a PHOENIX AV-250 PLUS autoclave at 121 °C for 15 minutes, and then the temperature was adjusted to 60 °C. TG L-2000 enzyme extract as described in Table 4 (135 mL) was mixed with the sterilized filtrate and the solution was incubated in an incubator-shaker (IKA KS4000) at 60 °C and 100 rpm for 72 hours. Hydrolyzed glucan filtrate was thus prepared.

Yeast (*S. cerevisiae*) cream (Bom Retiro mill, Brazil) was washed by suspending the cream in tap water (2.4 L, optical density of 65 at 600 nm) and then centrifuging the yeast cream for 5 minutes using a LEGEND XTR centrifuge

at 4500 g. After decanting the supernatant, the yeast were resuspended and concentrated by centrifugation two additional times. After the third wash, the pH was adjusted to 4.5 by addition of 5 wt% sulfuric acid and the optical density was measured using a GENESYS 20 4001 spectrophotometer and adjusted to 100 at 5 600 nm by addition of tap water. The adjusted yeast cream (1.5 L) was added to a 7.5-L BIOFLO310 fermenter vessel. The fermenter was set to maintain temperature at 30 °C, agitation at 100 rpm, and pH at 4.5 using 4 M aqueous ammonium hydroxide or 5 wt% aqueous sulfuric acid.

A feed solution containing yeast extract (10 g/L), peptone (20 g/L), and 10 200 g/L of sugars from the hydrolyzed filtrate was prepared and sterilized using a PHOENIX AV-250 Plus autoclave at 121 °C for 15 minutes. The feed solution was allowed to cool to 25 °C (room temperature) before the fermentation began. The sterilized feed solution (3.5 L) was added to the fermenter over approximately 5 hours at a rate of 684 mL/hr, and the fermentation was allowed 15 to proceed for 22 hours.

Periodic samples were taken during the fermentation and analyzed for optical density using a GENESYS 20 4001 spectrophotometer, Brix using a PAL-3 refractometer, and sugar and ethanol concentrations by HPLC (General Methods). These results are summarized in Table 13.

20 Table 13
Feed and Time Course Fermentation Profiles for the First Ethanol Fermentation
Using Hydrolyzed Glucan Filtrate

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	7	4	65	97	11	3	186	0
0	0	0	0	0	-	-	-	7
1	<1	1	<1	<1	-	-	-	20
2	<1	3	<1	<1	-	-	-	32
3	<1	4	<1	<1	-	-	-	40
4	<1	4	<1	<1	-	-	-	49
5	<1	4	<1	<1	-	-	-	53
6	<1	5	<1	<1	-	-	-	55
8	<1	5	<1	<1	-	-	-	57
22	<1	5	<1	<1	8	3	16	54

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-22 hours) are listed.

When the fermentation was over, the yeast cells were separated by centrifugation using a LEGEND XTR centrifuge at 4500 g for 5 minutes. After decanting the supernatant, the yeast cells were resuspended and concentrated by centrifugation two additional times. After the third wash, the pH was adjusted to 2 by addition of 5 wt% sulfuric acid. The optical density was measured using a GENESYS 20 4001 spectrophotometer and adjusted to 100 at 600 nm by addition of tap water. Two additional fermentation cycles, each using fresh feed, were performed using recycled yeast cells from the previous fermentation following the same conditions described above. The fermentation results obtained using first-time and second-time recycled yeast cells are provided in Tables 14 and 15, respectively.

Table 14

Feed and Time Course Fermentation Profiles Using the First Recycle of Yeast

Cells with Hydrolyzed Glucan Filtrate

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	7	4	69	104	7	4	194	0
0	<1	0	<1	<1	-	-	-	10
1	<1	7	<1	<1	-	-	-	25
4	<1	5	<1	<1	-	-	-	39
4	<1	4	<1	<1	-	-	-	45
5	<1	5	<1	<1	-	-	-	51
6	<1	5	<1	<1	-	-	-	57
6.2	<1	5	<1	<1	-	-	-	60
7	<1	5	<1	<1	-	-	-	59
21	<1	5	<1	<1	9	5	19	58

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-21 hours) are listed.

Table 15

Feed and Time Course Fermentation Profiles Using the Second Recycle of YeastCells with Hydrolyzed Glucan Filtrate

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	7	4	70	105	7	5	197	0
0	0	0	0	0	-	-	-	10
1	<1	7	<1	<1	-	-	-	25
3.5	<1	5	<1	<1	-	-	-	39
4	<1	4	<1	<1	-	-	-	45
5	<1	5	<1	<1	-	-	-	51
6	<1	5	<1	<1	-	-	-	57
6.2	<1	5	<1	<1	-	-	-	60
7	<1	5	<1	<1	-	-	-	59
21	<1	5	<1	<1	9	5	19	58

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-21 hours) are listed.

All of the fermentations were essentially complete within about six hours of initiating fermentation, and resulted in ethanol titers of 57-60.0 g/L. Comparing these fermentations with those in Example 7 demonstrates that hydrolyzing a glucan filtrate before subjecting it to fermentation results in faster and greater ethanol yields than those obtained from fermentations using non-hydrolyzed glucan filtrate.

Thus, fermenting a glucan filtrate in which the leucrose and oligosaccharide byproduct components have been saccharified results in increased ethanol yields at faster rates. This saccharification can be done using a transglucosidase, for example.

EXAMPLE 9Simultaneous Saccharification and Fermentation of a Glucan Filtrate Solution

This example discloses that simultaneous saccharification and fermentation of a feed containing glucan filtrate can result in enhanced fermentation properties.

Yeast (*S. cerevisiae*) cream (Bom Retiro mill, Brazil) was washed by suspending the cream in tap water (2.4 L, optical density of 65 at 600 nm) and

then centrifuging the yeast cream for 5 minutes using a LEGEND XTR centrifuge at 4500 g. After decanting the supernatant, the yeast cells were resuspended and concentrated by centrifugation two additional times. After the third wash, the pH was adjusted to 4.5 by addition of 5 wt% sulfuric acid and the optical density was measured using a GENESYS 20 4001 spectrophotometer and adjusted to 100 at 600 nm by addition of tap water. The adjusted yeast cream (1.5 L) was added to a 7.5-L BIOFLO310 fermenter vessel. The fermenter was set to maintain temperature at 30 °C, agitation at 100 rpm, and pH at 4.5 using 4 M aqueous ammonium hydroxide or 5 wt% aqueous sulfuric acid.

A feed solution containing yeast extract (10 g/L), peptone (20 g/L), and 200 g/L of sugars from a glucan filtrate was prepared and sterilized using a PHOENIX AV-250 PLUS autoclave at 121 °C for 15 minutes. The feed solution was allowed to cool to 25 °C (room temperature) before the fermentation began. TG L-2000 transglucosidase enzyme extract as described in Table 4 (1% v/v) was added to the sterilized feed solution immediately before adding the solution to the fermenter. The feed solution (3.5 L) containing TG L-2000 enzyme was added to the fermenter over approximately 5 hours at 684 mL/hr, and the fermentation was allowed to proceed for 48 hours.

Periodic samples were taken during the fermentation and analyzed for optical density using a GENESYS 20 4001 spectrophotometer, Brix using a PAL-3 refractometer (Atago), and sugar and ethanol concentrations by HPLC (General Methods). These results are summarized in Table 16.

Table 16

Feed and Time Course Fermentation Profiles for Simultaneous Saccharification and Ethanol Fermentation of Glucan Filtrate

Time (hr)	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3+	Total Sugar	EtOH
Feed	7	82	12	79	6	20	206	0
0	0	0	0	0	0	0	0	2
1	<1	13	<1	3	-	-	-	11
2	<1	21	<1	4	-	-	-	30
3	<1	21	<1	3	-	-	-	38
4	<1	20	<1	3	11	16	50	43

5	<1	14	<1	2	-	-	-	45
6	<1	<1	<1	2	-	-	-	59
22	<1	<1	<1	1	-	-	-	62
25	<1	<1	<1	<1	-	-	-	63
27	<1	<1	<1	<1	-	-	-	63
31	<1	<1	<1	<1	-	-	-	57
46	<1	<1	<1	<1	-	-	-	57
48	<1	<1	<1	<1	1	11	12	62

Concentrations (g/L) of ethanol (EtOH) and sugar compounds in the feed and at various fermentation time points (0-48 hours) are listed.

The fermentation was nominally complete in 6 hours, similar to the fermentations where the filtrate was hydrolyzed prior to the fermentation step (Example 8), and gave a slightly superior titer of ethanol (62 g/L) compared to using unhydrolyzed filtrate (Example 7). In addition, almost all of the leucrose was consumed by 6 hours (compare Table 16 with Tables 13-15). In addition to adding a saccharifying enzyme, such as TG L-2000, to a feed containing glucan filtrate just prior to fermentation, similar results should be obtained if the saccharifying enzyme is added to the fermentation directly.

Thus, simultaneous saccharification and fermentation of a feed containing glucan filtrate can result in enhanced fermentation properties such as increased (i) consumption of glucan filtrate components (e.g., leucrose) and (ii) ethanol yield and rate of production.

EXAMPLE 10

Preparation of Various Alpha-Glucosidases

This example discloses preparing various alpha-glucosidases in addition to those alpha-glucosidases (transglucosidase, glucoamylase, DIAZYME RDF ULTRA) used in some of the foregoing Examples. These additional alpha-glucosidases were tested for hydrolytic activity against oligosaccharides comprising alpha-1,5 glucosyl-fructose linkages or alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in Examples 11, 12, 15 and 16 provided below.

Discovery of an *Aspergillus clavatus* alpha-glucosidase (*Acglu1*)

A strain of *Aspergillus clavatus* was selected as a potential source of other enzymes that may be useful in various industrial applications. One of the genes identified in *Aspergillus clavatus* encodes an alpha-glucosidase and the sequence of this gene, called "*Acglu1*", is provided in SEQ ID NO:4. The corresponding protein encoded by SEQ ID NO:4 is provided in SEQ ID NO:5. *Acglu1* belongs to Glycosyl hydrolase family 31 based on a PFAM search (pfam.sanger.ac.uk web link). At the N-terminus, the protein (SEQ ID NO:5) has a signal peptide with a length of 19 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.*, 2011, *Nature Methods*, 8:785-786). The presence of a signal sequence suggests that *Acglu1* is a secreted enzyme. The amino acid sequence of the predicted mature form of *Acglu1* is set forth as SEQ ID NO:6.

Expression of *Aspergillus clavatus* alpha-glucosidase *Acglu1*

A synthetic *Acglu1* gene was cloned into pTrex3gM expression vector (described in U.S. Patent Appl. Publ. No. 2011/0136197, incorporated herein by reference) and the resulting plasmid was designated as pJG294. The sequence of the *Acglu1* gene was confirmed by DNA sequencing.

Plasmid pJG294 was transformed into a quad deleted *Trichoderma reesei* strain (described in WO05/001036) using a biolistic method (Te'o VS *et al.*, J Microbiol Methods, 51:393-9, 2002). The protein, which was predicted to comprise SEQ ID NO:6, was secreted into the extracellular medium and filtered culture medium was used to perform SDS-PAGE and alpha-glucosidase activity assays to confirm enzyme expression.

Discovery of *Neosartorya fischeri* alpha-glucosidase *Nfiglu1*

A strain of *Neosartorya fischeri* was selected as a potential source of other enzymes that may be useful in various industrial applications. One of the genes identified in *Neosartorya fischeri* encodes an alpha-glucosidase and the sequence of this gene, called "*Nfiglu1*", is provided in SEQ ID NO:7. The corresponding protein encoded by SEQ ID NO:7 is provided in SEQ ID NO:8.

Nfiglu1 belongs to Glycosyl hydrolase family 31 based on a PFAM search (pfam.sanger.ac.uk web link). At the N-terminus, the protein (SEQ ID NO:8) has a signal peptide with a length of 19 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.*, 2011, *Nature Methods*, 8:785-786). The presence of a signal sequence suggests that Nfiglu1 is a secreted enzyme. The amino acid sequence of the predicted mature form of Nfiglu1 is set forth as SEQ ID NO: 9.

Expression of *Neosartorya fischeri* alpha-glucosidase Nfiglu1

A synthetic *Nfiglu1* gene was cloned into pTrex3gM expression vector (described in U.S. Patent Appl. Publ. No. 2011/0136197) and the resulting plasmid was designated as pJG295. The sequence of the *Nfiglu1* gene was confirmed by DNA sequencing.

Plasmid pJG295 was transformed into a quad deleted *Trichoderma reesei* strain (described in WO05/001036) using a biolistic method (Te'o VS *et al.*, J Microbiol Methods, 51:393-9, 2002). The protein, which was predicted to comprise SEQ ID NO:9, was secreted into the extracellular medium and filtered culture medium was used to perform SDS-PAGE and alpha-glucosidase activity assays to confirm enzyme expression.

Discovery of *Neurospora crassa* alpha-glucosidase Ncrglu1

A strain of *Neurospora crassa* was selected as a potential source of other enzymes that may be useful in various industrial applications. One of the genes identified in *Neurospora crassa* encodes an alpha-glucosidase and the sequence of this gene, called "*Ncrglu1*", is provided in SEQ ID NO:10. The corresponding protein encoded by SEQ ID NO:10 is provided in SEQ ID NO:11. Ncrglu1 belongs to Glycosyl hydrolase family 31 based on a PFAM search (pfam.sanger.ac.uk web link). At the N-terminus, the protein (SEQ ID NO:11) has a signal peptide with a length of 22 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.*, 2011, *Nature Methods*, 8:785-786). The presence of a signal sequence suggests that Ncrglu1 is a secreted enzyme. The

amino acid sequence of the predicted mature form of Ncrglu1 is set forth as SEQ ID NO:12.

Expression of *Neurospora crassa* alpha-glucosidase Ncrglu1

A synthetic *Ncrglu1* gene was cloned into pTrex3gM expression vector (described in U.S. Patent Appl. Publ. No. 2011/0136197) and the resulting plasmid was designated as pJG296. The sequence of the *Ncrglu1* gene was confirmed by DNA sequencing.

Plasmid pJG296 was transformed into a quad deleted *Trichoderma reesei* strain (described in WO05/001036) using a biolistic method (Te'o VS et al., J Microbiol Methods, 51:393-399, 2002). The protein, which was predicted to comprise SEQ ID NO:12, was secreted into the extracellular medium and filtered culture medium was used to perform SDS-PAGE and alpha-glucosidase activity assays to confirm enzyme expression.

Discovery of *Rasamsonia composticola* alpha-glucosidase TauSec098

A strain of *Rasamsonia composticola* was selected as a potential source of other enzymes that may be useful in various industrial applications. One of the genes identified in *Rasamsonia composticola* encodes an alpha-glucosidase and the sequence of this gene, called "TauSec098", is provided in SEQ ID NO:13. The corresponding protein encoded by SEQ ID NO:13 is provided in SEQ ID NO:14. TauSec098 belongs to Glycosyl hydrolase family 31 and contains an N-terminal CBM 20 domain based on a PFAM search (pfam.sanger.ac.uk web link). At the N-terminus, the protein (SEQ ID NO:14) has a signal peptide with a length of 22 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen et al., 2011, *Nature Methods*, 8:785-786). The presence of a signal sequence suggests that TauSec098 is a secreted enzyme. The amino acid sequence of the predicted mature form of TauSec098 is set forth as SEQ ID NO:15.

Expression of *Rasamsonia composticola* alpha-glucosidase TauSec098

A synthetic *TauSec098* gene was cloned into the *Trichoderma reesei* expression vector pGXT (a pTTT-derived plasmid) by Generay Biotech Co.

(Shanghai, China) and the resulting plasmid was designated as pGX256-TauSec098. The sequence of the *TauSec098* gene was confirmed by DNA sequencing.

Plasmid pGX256-TauSec098 was transformed into a quad-deleted
 5 *Trichoderma reesei* strain (described in WO05/001036) using protoplast transformation (Te'o et al., J. Microbiol. Methods 51:393-399, 2002). Transformants were selected on a medium containing acetamide as a sole source of nitrogen (acetamide 0.6 g/L; cesium chloride 1.68 g/L; glucose 20 g/L; potassium dihydrogen phosphate 15 g/L; magnesium sulfate heptahydrate 0.6
 10 g/L; calcium chloride dihydrate 0.6 g/L; iron (II) sulfate 5 mg/L; zinc sulfate 1.4 mg/L; cobalt (II) chloride 1 mg/L; manganese (II) sulfate 1.6 mg/L; agar 20 g/L; pH 4.25). Transformed colonies (about 50-100) appeared in about 1 week. After growth on acetamide plates, the spores of transformants were collected and transferred into new acetamide agar plates. After 5 days of growth on acetamide
 15 plates, 1×10^8 spores were inoculated into 30 ml Glucose/Sophorose defined media in a 250-mL shake flask. The shake flask was shook at 28 °C for 5 days. Supernatants from these cultures were used to confirm expression (SDS PAGE) and activity of mature TauSec098 enzyme (SEQ ID NO:15).

20 Discovery of *Rasamsonia composticola* alpha-glucosidase *TauSec099*

A strain of *Rasamsonia composticola* was selected as a potential source of other enzymes that may be useful in various industrial applications. One of the genes identified in *Rasamsonia composticola* encodes an alpha-glucosidase and the sequence of this gene, called "*TauSec099*", is provided in SEQ ID
 25 NO:16. The corresponding protein encoded by SEQ ID NO:16 is provided in SEQ ID NO:17. TauSec099 belongs to Glycosyl hydrolase family 31 based on a PFAM search (pfam.sanger.ac.uk web link). At the N-terminus, the protein (SEQ ID NO:17) has a signal peptide with a length of 17 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen et al., 2011, *Nature Methods*, 8:785-786).
 30 The presence of a signal sequence suggests that TauSec099 is a secreted

enzyme. The amino acid sequence of the predicted mature form of TauSec099 is set forth as SEQ ID NO:18.

Expression of *Rasamsonia composticola* alpha-glucosidase TauSec099

A synthetic *TauSec099* gene was cloned into the *Trichoderma reesei* expression vector pGXT (a pTTT-derived plasmid) by Generay Biotech Co. (Shanghai, China) and the resulting plasmid was designated as pGX256-TauSec099. The sequence of the *TauSec099* gene was confirmed by DNA sequencing.

Plasmid pGX256-TauSec099 was transformed into a quad-deleted *Trichoderma reesei* strain (described in WO05/001036) using protoplast transformation (Te'o et al., J. Microbiol. Methods 51:393-399, 2002). Transformants were selected on a medium containing acetamide as a sole source of nitrogen (acetamide 0.6 g/L; cesium chloride 1.68 g/L; glucose 20 g/L; potassium dihydrogen phosphate 15 g/L; magnesium sulfate heptahydrate 0.6 g/L; calcium chloride dihydrate 0.6 g/L; iron (II) sulfate 5 mg/L; zinc sulfate 1.4 mg/L; cobalt (II) chloride 1 mg/L; manganese (II) sulfate 1.6 mg/L; agar 20 g/L; pH 4.25). Transformed colonies (about 50-100) appeared in about 1 week. After growth on acetamide plates, the spores of transformants were collected and transferred into new acetamide agar plates. After 5 days of growth on acetamide plates, 1×10^8 spores were inoculated into 30 ml Glucose/Sophorose defined media in a 250-mL shake flask. The shake flask was shook at 28 °C for 5 days. Supernatants from these cultures were used to confirm expression (SDS PAGE) and activity of mature TauSec099 enzyme (SEQ ID NO:18).

Sequences of *Bifidobacterium longum* alpha-glucosidase BloGlu1

An alpha-glucosidase gene, "*BloGlu1*", was identified from *Bifidobacterium longum* subsp. *longum* JDM301. The nucleic acid sequence for the *BloGlu1* gene (SEQ ID NO:19, GENBANK Acc. No. NC014169.1, complement sequence from positions 140600 to 142414) and the amino acid sequence of the hypothetical protein (SEQ ID NO:20) encoded by SEQ ID NO:19 were found in GENBANK Acc. No. YP_003660432.1.

Expression of *Bifidobacterium longum* alpha-glucosidase BloGlu1

The DNA sequence encoding the entire BloGlu1 protein (SEQ ID NO:20) was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:21) and inserted into the p3JM plasmid by Generay Biotech Co. (Shanghai, China), resulting in p3JM-BloGlu1. The p3JM-BloGlu1 plasmid contains an aprE promoter to drive expression of the optimized *BloGlu1* sequence (SEQ ID NO:21).

Plasmid p3JM-BloGlu1 was used to transform *B. subtilis* cells (degUHy32, Δ nprB, Δ vpr, Δ epr, Δ scoC, Δ wprA, Δ mpr, Δ ispA, Δ bpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BloGlu1 protein (SEQ ID NO:20).

Sequences of *Bifidobacterium longum* alpha-glucosidase BloGlu2

An alpha-glucosidase, BloGlu2, was identified from *Bifidobacterium longum*. The amino acid sequence (SEQ ID NO:22) of BloGlu2 was found in the NCBI database (GENBANK Acc. No. WP_007054665.1).

Expression of *Bifidobacterium longum* alpha-glucosidase BloGlu2

A DNA sequence encoding BloGlu2 protein was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:23) and inserted into the p3JM plasmid by Generay Biotech Co., resulting in p3JM-BloGlu2. SEQ ID NO:23 encodes the amino acid sequence of SEQ ID NO:24. The p3JM-BloGlu2 plasmid contains an aprE promoter to drive expression of the optimized *BloGlu2* sequence (SEQ ID NO:23).

Plasmid p3JM-BloGlu2 was used to transform *B. subtilis* cells (degUHy32, Δ nprB, Δ vpr, Δ epr, Δ scoC, Δ wprA, Δ mpr, Δ ispA, Δ bpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD

medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BloGlu2 protein (SEQ ID NO:24).

Sequences of *Bifidobacterium longum* alpha-glucosidase BloGlu3

5 An alpha-glucosidase gene, "*BloGlu3*", was identified from *Bifidobacterium longum* subsp. *longum* F8. The nucleic acid sequence for the *BloGlu3* gene (SEQ ID NO:25, GENBANK Acc. No. NC_021008.1, positions 2130627 to 2132441), and the amino acid sequence of the hypothetical protein (SEQ ID NO:26) encoded by SEQ ID NO:25 were found in GENBANK Acc. No.
10 YP_007768249.1.

Expression of *Bifidobacterium longum* alpha-glucosidase BloGlu3

The DNA sequence encoding the entire BloGlu3 protein (SEQ ID NO:26) was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:27) and inserted into the p3JM plasmid by Generay Biotech Co., resulting in
15 p3JM-BloGlu3. The p3JM-BloGlu3 plasmid contains an aprE promoter to drive expression of the optimized *BloGlu3* sequence (SEQ ID NO:27).

Plasmid p3JM-BloGlu3 was used to transform *B. subtilis* cells (degUHy32, ΔnprB , Δvpr , Δepr , ΔscoC , ΔwprA , Δmpr , ΔispA , Δbpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A
20 colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BloGlu3 protein (SEQ ID NO:26).

Sequences of *Bifidobacterium pseudolongum* alpha-glucosidase BpsGlu1

An alpha-glucosidase, BpsGlu1, was identified from *Bifidobacterium pseudolongum*. The amino acid sequence (SEQ ID NO:28) of BpsGlu1 was found in the NCBI database (GENBANK Acc. No. WP_022858408.1).

Expression of *Bifidobacterium pseudolongum* alpha-glucosidase BpsGlu1

30 A DNA sequence encoding BpsGlu1 protein was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:29) and inserted into the

p3JM plasmid by Generay Biotech Co., resulting in p3JM-BpsGlu1. SEQ ID NO:29 encodes the amino acid sequence of SEQ ID NO:30. The p3JM-BpsGlu1 plasmid contains an aprE promoter to drive expression of the optimized *BpsGlu1* sequence (SEQ ID NO:29)

5 Plasmid p3JM-BpsGlu1 was used to transform *B. subtilis* cells (degUHy32, Δ nprB, Δ vpr, Δ epr, Δ scoC, Δ wprA, Δ mpr, Δ ispA, Δ bpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask
10 with MBD medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BpsGlu1 protein (SEQ ID NO:30).

Sequences of *Bifidobacterium thermophilum* alpha-glucosidase BthGlu1

An alpha-glucosidase gene, "*BthGlu1*", was identified from *Bifidobacterium*
15 *thermophilum* RBL67. The nucleic acid sequence of the *BthGlu1* gene (SEQ ID NO:31, GENBANK Acc. No. NC_020546.1, positions 150690 to 152495), and the amino acid sequence of the hypothetical protein (SEQ ID NO:32) encoded by SEQ ID NO:31 were found in GENBANK Acc. No. YP_007592840.1.

Expression of *Bifidobacterium thermophilum* alpha-glucosidase BthGlu1

20 The DNA sequence encoding the entire BthGlu1 protein (SEQ ID NO:32) was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:33) and inserted into the p3JM plasmid by Generay Biotech Co., resulting in p3JM-BthGlu1. The p3JM-BthGlu1 plasmid contains an aprE promoter to drive expression of the optimized *BthGlu1* sequence (SEQ ID NO:33).

25 Plasmid p3JM-BthGlu1 was used to transform *B. subtilis* cells (degUHy32, Δ nprB, Δ vpr, Δ epr, Δ scoC, Δ wprA, Δ mpr, Δ ispA, Δ bpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD
30 medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BthGlu1 protein (SEQ ID NO:32).

Sequences of *Bifidobacterium breve* alpha-glucosidase BbrGlu2

An alpha-glucosidase, BbrGlu2, was identified from *Bifidobacterium breve*. The amino acid sequence (SEQ ID NO:34) of BbrGlu2 was found in the NCBI
 5 database (GENBANK Acc. No. WP_003827971.1).

Expression of *Bifidobacterium breve* alpha-glucosidase BbrGlu2

A DNA sequence encoding BbrGlu2 protein was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:35) and inserted into the p3JM plasmid by Generay Biotech Co., resulting in p3JM-BbrGlu2. SEQ ID
 10 NO:35 encodes the amino acid sequence of SEQ ID NO:36. The p3JM-BbrGlu2 plasmid contains an aprE promoter to drive expression of the optimized *BbrGlu2* sequence (SEQ ID NO:35)

Plasmid p3JM-BbrGlu2 was used to transform *B. subtilis* cells (degUHy32, ΔnprB, Δvpr, Δepr, ΔscoC, ΔwprA, Δmpr, ΔispA, Δbpr), and the transformed cells
 15 were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl₂) to express SEQ ID NO:36.

Sequences of *Bifidobacterium breve* alpha-glucosidase BbrGlu5

An alpha-glucosidase gene, "*BbrGlu5*", was identified from *Bifidobacterium breve* ACS-071-V-Sch8b. The nucleic acid sequence of the *BbrGlu5* gene (SEQ ID NO:37, GENBANK Acc. No. NC_017218.1, complement
 25 of sequence from positions 2241075 to 2242895), and the amino acid sequence of the hypothetical protein (SEQ ID NO:38) encoded by SEQ ID NO:37 were found in GENBANK Acc. No. YP_005583701.1.

Expression of *Bifidobacterium breve* alpha-glucosidase BbrGlu5

The DNA sequence encoding the entire BbrGlu5 protein (SEQ ID NO:38)
 30 was optimized for expression in *B. subtilis*, then synthesized (yielding SEQ ID NO:39) and inserted into the p3JM plasmid by Generay Biotech Co., resulting in

p3JM-BbrGlu5. The p3JM-BbrGlu5 plasmid contains an aprE promoter to drive expression of the optimized *BbrGlu5* sequence (SEQ ID NO:39).

Plasmid p3JM-BbrGlu5 was used to transform *B. subtilis* cells (degUHy32, Δ nprB, Δ vpr, Δ epr, Δ scoC, Δ wprA, Δ mpr, Δ ispA, Δ bpr), and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol. A colony with correct insertion, as confirmed by PCR and sequencing, was selected and subjected to fermentation in a 250-mL shake flask with MBD medium (a MOPS-based defined medium supplemented with an additional 5 mM CaCl_2) to express BbrGlu5 protein (SEQ ID NO:38).

Purification of alpha-glucosidases from expression cultures

AclGlu1 and NcrGlu1

Both AclGlu1 (SEQ ID NO:6) and NcrGlu1 (SEQ ID NO:12) alpha-glucosidases were purified using two chromatography steps. For each purification, the crude broth from the shake flask was concentrated, after which ammonium sulfate was added to a final concentration of 2 M. The solution was loaded onto a 50-mL phenyl HP column pre-equilibrated with 20 mM Tris pH 8.0, 2 M ammonium sulfate. The target protein (SEQ ID NO:6 or SEQ ID NO:12) was eluted from the column with 1 M ammonium sulfate, 20 mM Tris pH 8.0.

Respective fractions were pooled, concentrated and buffer-exchanged into 20 mM Tris pH 8.0 (buffer A), using a VIVAFLOW 200 ultrafiltration device (Sartorius Stedim). The resulting solution was applied to a 40-mL Q HP column pre-equilibrated with buffer A. The target protein was eluted from the column with 0.3 M NaCl in buffer A. The fractions containing target protein were then pooled and concentrated using 10K AMICON ULTRA-15 devices, and stored in 40% glycerol at -20 °C until usage.

NfiGlu1

NfiGlu1 alpha-glucosidase (SEQ ID NO:9) was purified using two hydrophobic interaction chromatography steps. The crude broth from the shake flask was concentrated, after which ammonium sulfate was added to a final concentration of 1 M. The solution was loaded onto a 50-mL phenyl HP column

pre-equilibrated with 20 mM Tris pH 8.0, 1 M ammonium sulfate. The target protein (SEQ ID NO:9) flowed through the column. Flow-through fractions were pooled, after which ammonium sulfate was added to a final concentration of 2 M. The solution was loaded onto the same phenyl HP column pre-equilibrated with 20 mM Tris pH 8.0, 2 M ammonium sulfate. The target protein was eluted from the column with 1 M ammonium sulfate, 20 mM Tris pH 8.0. The fractions containing target protein were then pooled and concentrated using 10K AMICON ULTRA-15 devices, and stored in 40% glycerol at -20 °C until usage.

TauSec098 and TauSec099

Both TauSec098 (SEQ ID NO:15) and TauSec099 (SEQ ID NO:18) alpha-glucosidases were purified via hydrophobic interaction chromatography. For each purification, ammonium sulphate was added to about 180 mL of concentrated crude broth from a 7-L fermenter to a final concentration of 1 M. This solution was then loaded onto a 50-mL HIPREP phenyl-FF Sepharose column (GE Healthcare) pre-equilibrated with 20 mM sodium acetate pH 5.0, 1 M ammonium sulphate (buffer A). After washing with the same buffer with three column volumes (CVs), the column was eluted stepwise with 75%, 50% and 0% buffer A using three CVs each, followed by two CVs of MILLIQ H₂O. All fractions were analyzed by SDS-PAGE. The target protein (SEQ ID NO:15 or SEQ ID NO:18) was mainly present in the flow-through fraction, which was concentrated and buffer-exchanged to remove excess ammonium sulfate using 10 KDa AMICON ULTRA-15 devices. The final product, which was greater than 90% pure, was stored in 40% glycerol at -80 °C until usage.

BloGlu1, BloGlu2 and BloGlu3

BloGlu1 (SEQ ID NO:20), BloGlu2 (SEQ ID NO:24) and BloGlu3 (SEQ ID NO:26) alpha-glucosidases were all purified in three steps. For each purification, the crude broth from a 1-L DASGIP fermenter was concentrated, after which ammonium sulfate was added to 60% saturation. The solution was stirred at 4 °C for 1 hr, and then centrifuged at 8000 X g for 30 min. The resulting pellet was re-suspended in 20 mM Tris pH 8.0 (buffer A). Ammonium sulfate was added to the resulting solution to a final concentration of 1 M; this preparation was then

loaded onto a 40-mL HiPrep™ Phenyl FF column pre-equilibrated with 20 mM Tris pH 8.0, 1 M ammonium sulfate (buffer B). After washing, the column was eluted stepwise with 75%, 50%, and 0% buffer B and H₂O in three column volumes each. All fractions were analyzed using SDS-PAGE and activity assays.

- 5 The fractions containing target protein (SEQ ID NO:20, SEQ ID NO:24, or SEQ ID NO:26) were pooled, concentrated and subsequently loaded onto a HiLoad™ 26/60 Superdex™ 75 column pre-equilibrated with 20 mM sodium phosphate pH 7.0, 0.15 M NaCl. Flow-through fractions containing the target protein were then pooled and concentrated using 10K AMICON ULTRA-15 devices, and stored in
10 40% glycerol at -20 °C until usage.

BpsGlu1 and BthGlu1

- Both BpsGlu1 (SEQ ID NO:30) and BthGlu1 (SEQ ID NO:32) alpha-glucosidases were purified in two steps. For each purification, the crude broth from a 1-L DASGIP fermenter was concentrated, after which ammonium sulfate
15 was added to 60% saturation. The solution was stirred at 4 °C for 1 hr, and then centrifuged at 8000 X g for 30 min. The resulting pellet was re-suspended in 20 mM Tris pH 8.0 (buffer A). Ammonium sulfate was added to the resulting solution to a final concentration of 1 M; this preparation was then loaded onto a 40-mL HiPrep™ Phenyl FF column pre-equilibrated with 20 mM Tris pH 8.0, 1 M
20 ammonium sulfate (buffer B). After washing, the column was eluted stepwise with 75%, 50%, and 0% buffer B and H₂O in three column volumes each. All fractions were analyzed using SDS-PAGE and activity assays. The target protein (SEQ ID NO:30 or SEQ ID NO:32) was present in the eluate from the 0% buffer B elution step; this eluate was pooled and concentrated using 10K
25 AMICON ULTRA-15 devices. The final product, which was greater than 95% pure, was stored in 40% glycerol at -20 °C until usage.

BbrGlu2 and BbrGlu5

- Both BbrGlu2 (SEQ ID NO:36) and BbrGlu5 (SEQ ID NO:38) alpha-glucosidases were purified in four steps. For each purification, the crude broth
30 from a 1-L DASGIP fermenter was concentrated, after which ammonium sulfate was added to 60% saturation. The solution was stirred at 4 °C for 1 hr, and then

centrifuged at 8000 X g for 30 min. The resulting pellet was re-suspended in 20 mM HEPES pH 7.0 (buffer A). Ammonium sulfate was added to the resulting solution to a final concentration of 1 M; this preparation was then loaded onto a HiPrep™ Phenyl FF column pre-equilibrated with 20 mM HEPES pH 7.0, 1 M ammonium sulfate. The target protein (SEQ ID NO:36 or SEQ ID NO:38) was eluted from the column with 0.5 M ammonium sulfate. Respective fractions were pooled, concentrated and buffer-exchanged into buffer A using a VIVAFLOW 200 ultrafiltration device (Sartorius Stedim). The resulting solution was applied to a HiPrep™ Q FF 16/10 column pre-equilibrated with buffer A. Target protein was eluted from the column with a linear gradient of 0-0.5 M NaCl in buffer A. Fractions containing target protein were pooled, concentrated and subsequently loaded onto a HiLoad™ 26/60 Superdex™ 75 column pre-equilibrated with 20 mM HEPES pH 7.0, 0.15 M NaCl. The fractions containing target protein were then pooled and concentrated using 10K AMICON ULTRA-15 devices, and stored in 40% glycerol at -20 °C until usage.

Thus, various additional alpha-glucosidases were expressed and purified. These alpha-glucosidases were tested for hydrolytic activity against alpha-1,5 glucosyl-fructose linkages and alpha-1,3 and/or alpha-1,6 glucosyl-glucose linkages in Examples 11, 12, 15 and 16 provided below.

EXAMPLE 11

Testing Alpha-Glucosidases for Hydrolytic Activity Against Various Glycosidic Linkages

This example discloses testing whether alpha-glucosidases have hydrolytic activity beyond that previously associated with this class of enzymes (EC 3.2.1.20). Alpha-glucosidases from Example 10 were shown to have hydrolytic activity against alpha-1,5 glucosyl-fructose linkages and alpha-1,3 and alpha-1,6 glucosyl-glucose linkages.

Substrate Specificity of Alpha-Glucosidases

The substrate specificity of each alpha-glucosidase disclosed in Example 10 was assayed based on the release of glucose from a particular substrate (isomaltose, maltose, panose, leucrose, or nigerose) when the substrate was

incubated with alpha-glucosidase. The rate of glucose release was measured using a coupled glucose oxidase/peroxidase (GOX/HRP) method (1980, *Anal. Biochem.* 105:389-397). Glucose release was quantified as the rate of oxidation of 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) by peroxide that was generated from coupled GOX/HRP enzymes reacted with glucose.

Individual substrate solutions were prepared by mixing a 9 mL solution of substrate (1% in water, w/v) with 1 mL of 0.5 M pH 5.0 sodium acetate buffer and 40 μ L of 0.5 M calcium chloride in a 15-mL conical tube. Coupled enzyme (GOX/HRP) solution with ABTS was prepared in 50 mM sodium acetate buffer (pH 5.0), with the final concentrations of 2.74 mg/mL ABTS, 0.1 U/mL HRP, and 1 U/mL GOX. Serial dilutions of individual alpha-glucosidase samples and glucose standard were prepared in MILLIQ water. For nigerose, alpha-glucosidase samples were tested with only one dosage at 10 ppm due to a limited stock of substrate solutions. Each alpha-glucosidase sample (10 μ L) was transferred into a new microtiter plate (Corning 3641) containing 90 μ L of substrate solution pre-incubated at 50 °C for 5 min at 600 rpm. Reactions were carried out at 50 °C for 10 min (for isomaltose, maltose, panose, and nigerose substrates), or for 60 min (for leucrose substrate) with shaking (600 rpm) in a THERMOMIXER (Eppendorf). 10 μ L of each reaction mix, as well as 10 μ L of serial dilutions of glucose standard, were then quickly transferred to new microtiter plates (Corning 3641), respectively, to which 90 μ L of ABTS/GOX/HRP solution was then added accordingly. The microtiter plates containing reaction mixes were immediately measured at 405 nm at 11 second intervals for 5 min using a SOFTMAX PRO plate reader (Molecular Devices). The output was the reaction rate, V_o , for each enzyme concentration. Linear regression was used to determine the slope of the plot V_o vs. enzyme dose. The specific activity of each alpha-glucosidase was calculated based on the glucose standard curve using Equation 1:

$$\text{Specific Activity (Unit/mg)} = \text{Slope (enzyme)} / \text{slope (std)} \times 1000 \quad (1),$$

where 1 Unit = 1 μ mol glucose / min.

For nigerose, the value of the reaction rate with enzyme dosage at 10 ppm was directly used to indicate enzyme activity.

Using the foregoing method, the specificity of each alpha-glucosidase was determined against each substrate. The activities of an oligo-1,6-glucosidase (purchased from Megazyme, see Table 4) and a transglucosidase (TG L-2000, see Table 4) against each substrate were also measured. The results of this analysis are provided in Table 17.

Table 17

Activity of Various Alpha-Glucosidases Against Different Substrates

Enzyme	SEQ ID NO.	Enzyme Activity (U/mg) as Measured on:				
		Isomaltose	Maltose	Panose	Leucrose	Nigerose ^a
Oligo-1,6-glucosidase		118.2	0.0	54.3	1.3	19.6
TG L-2000	1	194.0	235.6	127.7	68.9	254.0
AcIGlu1	6	255.7	401.9	180.9	113.7	315.1
NfiGlu1	9	521.2	360.0	126.9	89.4	264.3
NcrGlu1	12	282.7	34.9	15.9	61.6	200.4
TauSec098	15	54.9	123.8	23.8	1.8	305.6
TauSec099	18	244.0	97.7	50.8	70.6	184.8
BloGlu1	20	71.1	66.9	23.1	2.5	165.0
BloGlu2	24	65.9	86.7	19.9	3.5	217.9
BloGlu3	26	120.1	175.5	31.4	9.0	272.6
BspGlu1	30	64.2	247.9	60.8	27.3	254.6
BthGlu1	32	108.3	93.3	21.1	68.4	128.5
BbrGlu2	36	106.6	167.5	26.9	6.1	258.8
BbrGlu5	38	925.8	0.0	279.7	2.8	22.1

^a Each enzyme was used at one dosage (10 ppm) against nigerose.

Interestingly, it was found that alpha-glucosidases, besides exhibiting hydrolytic activity against alpha-1,4 glucosyl-glucose linkage (maltose), also exhibit hydrolytic activity against alpha-1,6 glucosyl-glucose linkage (isomaltose),

alpha-1,3 glucosyl-glucose linkage (nigerose), and alpha-1,5 glucosyl-fructose linkage (leucrose) (Table 17).

Thus, alpha-glucosidases have hydrolytic activity beyond that previously associated with EC 3.2.1.20 enzymes. Specifically, alpha-glucosidases have hydrolytic activity against alpha-1,5 glucosyl-fructose linkages and alpha-1,3 and alpha-1,6 glucosyl-glucose linkages.

EXAMPLE 12

Hydrolysis of Leucrose and Oligosaccharides in Glucan Reaction Filtrate Using Alpha-Glucosidase

This Example describes using alpha-glucosidase to hydrolyze leucrose and other oligosaccharides present in filtrate obtained from a glucan synthesis reaction. Specifically, the effect of alpha-glucosidases disclosed in Example 10 on the hydrolysis of leucrose and oligosaccharides DP2, DP3 and HS (higher sugars, DP4+) in a filtrate of an insoluble glucan (poly alpha-1,3-glucan) synthesis reaction was studied.

Isolation and Analysis of Oligosaccharides for Testing Against Alpha-Glucosidase Activity

First, a concentrated filtrate of a glucan synthesis reaction was prepared as per Example 1.

Briefly, oligosaccharides were isolated from the concentrated filtrate by chromatographic separation, and analyzed for glycosidic linkage profile. Chromatographic separation employing a strong acid cation-exchange resin was used to isolate the oligosaccharide fraction of the concentrated filtrate. The physical parameters of the column used for this separation were as follows: FINEX CS11GC, #227 resin; Na⁺ ion form; 5% divinyl benzene (crosslinking); 0.34 mm particle size; 1.64 m bed length; 0.093 m column diameter.

In more detail, the concentrated sugar solution (i.e., concentrated filtrate) described in Table 3 was filtered and diluted to 25 g dry solids/100 g solution using tap water. Prior to addition of this sugar solution to the column resin, the resin was washed with six bed volumes (BV) of sodium chloride solution (three BV at 10 wt% sodium chloride followed by three BV at 5 wt% sodium chloride) to

convert the resin to the sodium form. The sugar solution (0.6 L) was then fed to the column, after which the column was eluted using water at a flow rate of 50 mL/min. The run conditions of the chromatographic separation are summarized as follows: 0.6 L feed size, 25 g dry solids/100 g solution, 65 °C column temperature, 50 mL/min flow rate. An oligosaccharide solution was eluted between 11 and 21 minutes. A small amount of salts – indicated by an increase in conductivity – was eluted at the same time. The oligosaccharide fraction thus prepared was analyzed by HPLC to determine its product distribution. In total, the fraction contained >89% of oligosaccharides containing three or more hexose units and less than 1.5% of identifiable mono- and di-saccharides. This fraction was concentrated to a total dry weight of 317 g/L using a thin film evaporator (LCI Corporation, Charlotte, NC) followed by rotary evaporation with a ROTAVAPOR (R-151; Buchi, New Castle, DE). The product distribution of the concentrated fraction as measured by HPLC appears in Table 18.

Table 18

Product Distribution of Concentrated Oligosaccharide Fraction

	Sucrose	Leucrose	Glucose	Fructose	DP2	DP3	DP4	DP5	DP6	DP7	Total
g/L	0.0	2.5	0.0	0.7	31.5	75.9	101.8	62.1	26.9	15.3	316.7
wt%	0.0	0.8	0.0	0.2	9.9	23.9	32.1	19.6	8.5	4.8	100

Primary Screening of Alpha-Glucosidases on Glucan Oligomer Hydrolysis

The activities of eleven different alpha-glucosidases (Example 10), as well as the activities of two benchmark enzymes, oligo-1,6-glucosidase (purchased from Megazyme) and transglucosidase (TG L-2000), were individually evaluated against the purified oligosaccharide fraction prepared above (Table 18). Each alpha-glucosidase (dosed at 1 mg/mL) was incubated in a solution containing oligosaccharide substrates (2.9% dry solids) and 2 mM calcium chloride at pH 5.0 at 60 °C. Each reaction was quenched after 24 hours of incubation by adding 50 µL of 0.5 M NaOH.

The oligosaccharide/monosaccharide contents of the quenched reactions were determined as follows. A sample of each reaction was diluted 5-fold in water for HPLC analysis. HPLC separation was done using an Agilent 1200

series HPLC system with an AMINEX HPX-42A column (300 mm x 7.8 mm) at 85 °C. The sample (10 µL) was applied to the HPLC column and separated with an isocratic gradient of MILLI-Q water as the mobile phase at a flow rate of 0.6 mL/min. Oligosaccharide products were detected using a refractive index detector. The numbers provided in Table 19 below reflect the average of peak area percentages (from duplication of each sample) of each DP_n as a fraction of the total from DP1 to DP7.

Table 19

Analysis Glucan Filtrate Oligosaccharides Following Treatment with Alpha-Glucosidase

Enzyme	SEQ ID NO	DP7%	DP6%	DP5%	DP4%	DP3%	DP2%	DP1%
Oligo-1,6-glucosidase		0.0	0.6	8.7	27.9	21.9	12.2	28.7
TG L-2000	1	0.0	0.0	0.0	0.0	0.0	3.0	97.0
Nfiglu1	9	0.0	0.0	0.0	0.0	0.0	1.9	98.1
Ncrglu1	12	0.0	0.0	0.0	2.8	1.6	7.0	88.6
TauSec098	15	0.0	0.0	0.0	15.3	0.0	4.5	80.2
TauSec099	18	0.0	0.0	0.0	15.7	0.0	0.5	83.7
BloGlu1	20	0.0	0.0	6.7	36.3	35.6	0.0	21.3
BloGlu2	24	0.0	0.7	8.6	31.2	34.9	14.0	10.7
BloGlu3	26	0.0	0.6	8.0	28.4	33.5	13.9	15.6
BspGlu1	30	0.0	0.5	5.2	15.4	16.2	8.2	54.5
BthGlu1	32	0.0	0.0	13.0	12.6	2.0	1.9	70.5
BbrGlu2	36	0.0	0.0	21.5	30.7	24.6	0.0	23.3
BbrGlu5	38	0.0	0.0	8.1	23.8	12.1	15.5	40.5
Blank		0.0	0.0	16.2	38.6	30.4	0.0	14.8

As indicated with shading in Table 19, the oligosaccharide content of the reactions generally shifted toward smaller sized sugars, in comparison with the control reaction ("Blank") in which there was no enzyme. These results indicate that alpha-glucosidase can be used to hydrolyze oligosaccharides comprised within a glucan synthesis reaction and a fraction thereof. Also, given the linkage profile of the oligosaccharides (Examples 3 and 4), and the activity of alpha-glucosidase against various glycosidic linkages in addition to alpha-1,4 linkages (Example 11), it is apparent that alpha-glucosidase can be used to break down

oligosaccharides with alpha-1,5 glucosyl-fructose linkages and/or alpha-1,3 and alpha-1,6 glucosyl-glucose linkages. The results provided in Table 19 also suggest that fungal alpha-glucosidases have better hydrolytic activity towards soluble oligosaccharides compared with the bacterial alpha-glucosidases.

5 Confirmation of Alpha-Glucosidase Hydrolytic Activity Toward Oligosaccharide Products of Glucan Synthesis Reactions

Reactions were prepared comprising one or two alpha-glucosidases and a concentrated filtrate obtained from a poly alpha-1,3-glucan synthesis reaction (Table 3). Alpha-glucosidase reactions were dosed with enzyme at 4 ppm, or for
10 blends, each enzyme was used at a 1:1 ratio with a final dosage of 4 ppm. The concentrated filtrate was loaded in each reaction at 10% dry solids. Each reaction further comprised 2 mM calcium chloride at pH 5.0, and was carried out at 60 °C or 65 °C. The reactions were quenched by adding 50 µL of 0.5 M NaOH after a 23-hour incubation.

15 The oligosaccharide/monosaccharide contents of the quenched reactions were determined as follows. A sample of each reaction diluted 25-fold in water for HPLC analysis. HPLC separation was done using an Agilent 1200 series HPLC system with an AMINEX HPX-42A column (300 mm x 7.8 mm) at 85 °C. The sample (10 µL) was applied to the HPLC column and separated with an
20 isocratic gradient of MILLI-Q water as the mobile phase at a flow rate of 0.6 mL/min. Oligosaccharide products were detected using a refractive index detector. The numbers provided in Table 20 below reflect the average of peak area percentages (from duplication of each sample) of each DP_n as a fraction of the total. The results provided in Table 20 generally confirm the activity of certain
25 alpha-glucosidases as discussed above regarding the results provided in Table 19.

Table 20
Analysis Glucan Filtrate Sugars Following Treatment with Alpha-Glucosidase

Temp.	Enzyme	Seq ID	DP7+%	DP7%	DP6%	DP5%	DP4%	DP3%	DP2%	Leucrose%	Glucose%	Fructose%
60 °C	TGL-2000	1	6.8	0.2	0.4	1.2	1.8	2.0	6.2	0.0	32.0	30.4
	TauSec098	15	5.4	0.0	0.1	0.2	0.3	1.9	3.4	25.4	26.2	31.1
	TauSec099	18	6.0	0.2	0.5	1.2	1.8	2.3	6.8	0.0	30.4	30.8
	TauSec098+	15,										
	TauSec099	18	6.4	0.1	0.1	0.2	0.5	2.2	8.1	0.0	32.4	31.0
	TauSec098+	15,										
	TGL-2000	1	6.3	0.1	0.1	0.2	0.4	1.8	8.1	0.0	32.9	30.1
	Blank		5.4	0.2	0.4	1.0	1.6	2.4	2.9	25.1	22.7	35.4
	TGL-2000	1	6.3	0.1	0.4	1.1	1.7	2.2	6.7	0.0	31.6	30.8
	TauSec098	15	5.8	0.1	0.2	0.2	0.3	1.6	3.3	24.5	27.2	36.1
65 °C	TauSec099	18	6.3	0.2	0.5	1.1	1.7	2.1	6.1	0.0	32.3	30.1
	TauSec098+	15,										
	TauSec099	18	6.7	0.0	0.1	0.2	0.4	1.7	7.8	0.0	34.0	30.2
	TGL-2000	1	6.8	0.1	0.2	0.2	0.4	1.8	7.6	0.0	33.9	30.1
	Blank		6.0	0.1	0.3	0.9	1.4	2.1	3.0	24.5	23.8	31.1
	TauSec098+	15,										
	TauSec099	18										

Thus, alpha-glucosidase can be used to hydrolyze leucrose and other oligosaccharides present in a fraction (e.g., filtrate) obtained from a glucan synthesis reaction, such as a poly alpha-1,3-glucan synthesis reaction.

EXAMPLE 13

5 Isolation of Oligomer/Leucrose Fraction from gtf-S/MUT3325 Reaction

Sucrose (4.50 kg) was dissolved in distilled deionized water to a final total volume of 9.5 L and the resulting solution was heated with stirring at 80 °C for 5 minutes and then cooled to 47 °C. With stirring, 500 grams of a crude extract containing 0.6 g/L of gtf-S enzyme (GTF0459, SEQ ID NO:42) and 15.0 mL of a
 10 crude extract containing 10 g/L of mutanase (MUT3325, SEQ ID NO:47) was added with stirring (see General Methods for enzyme preparations). The pH of the resulting mixture was immediately adjusted to between pH 5.5 to pH 6.0 by slowly adding a 1:10 (v/v) dilution of 37 wt% HCl with stirring. The reaction temperature and pH were maintained at 47 °C and pH 5.5-6.0, respectively, until
 15 sucrose conversion was >95% per HPLC analysis, after which the reaction mixture was immediately adjusted to pH 7.0 to 7.5 and heated to 90 °C for 20 min, then cooled to 25 °C for immediate filtration to remove particulates and precipitate. The resulting filtrate was held at 5 °C prior to IEX/SEC column chromatography using the following resin and conditions: FINEX CS 11 GC SAC
 20 in Ca²⁺ form, column i.d = 9.3 cm, resin bed height 1.58 m, T= 70 °C, flow rate = 51 mL/min, linear flow rate = 0.44 m/h, feed size = 0.6 L = 171 g, feed RI-DS = 25.1 g/100 g, sample interval = 3 min. The column fractions collected between 30 min and 67 min were combined, concentrated by evaporation to 66% dissolved solids and analyzed by HPLC as described in the General Methods.
 25 Table 21 indicates the oligosaccharide and monosaccharide components of the isolated fraction thus prepared.

Table 21

Analysis of Oligomer/Leucrose Fraction from gtf-S/MUT3325 Reaction

DP7+ (%DS)	DP6 (%DS)	DP5 (%DS)	DP4 (%DS)	DP3 (%DS)	DP2 (%DS)	sucrose (%DS)	leucrose (%DS)	glucose (%DS)	fructose (%DS)
0	1.1	2.9	7.2	16.2	12.7	13.5	30.7	7.9	7.9

In this Example, a glucan synthesis reaction was used to produce at least one soluble alpha-glucan product. This soluble product resulted from the concerted action of both a glucosyltransferase (GTF0459, SEQ ID NO:42) and an alpha-glucanohydrolase (MUT3325, SEQ ID NO:47) that were both present in the glucosyltransferase reaction. This Example also demonstrated the preparation of a chromatographic fraction from the glucan synthesis reaction. This fraction was used in Examples 15 and 16 below to test the activity of alpha-glucosidases thereupon.

EXAMPLE 14

Isolation of Oligomer/Leucrose Fraction from Gtf-C Reaction

Sucrose (4.50 kg) was dissolved in distilled deionized water to a final total volume of 9.5 L and the resulting solution was heated with stirring at 80 °C for 5 minutes and then cooled to 47 °C . With stirring, 500 grams of a crude extract containing 0.41 g/L of gtf-C enzyme (GTF0088BsT1, SEQ ID NO:45) was added with stirring (see General Methods for enzyme preparation). The pH of the resulting mixture was immediately adjusted to between pH 5.5 to pH 6.0 by slowly adding a 1:10 (v/v) dilution of 37 wt% HCl with stirring. The reaction temperature and pH were maintained at 47 °C and pH 5.5-6.0, respectively, until sucrose conversion was >95% per HPLC analysis, after which the reaction mixture was immediately adjusted to pH 7.0 to 7.5 and heated to 90 °C for 20 min, then cooled to 25 °C for immediate filtration to remove particulates and precipitate. The resulting filtrate held at 5 °C prior to IEX/SEC column chromatography using the following resin and conditions: FINEX CS 11 GC SAC in Ca²⁺ form, column i.d = 9.3 cm, resin bed height 1.58 m, T= 70 °C, flow rate = 50 mL/min, linear flow rate = 0.44 m/h, feed size = 0.6 L = 171 g, feed RI-DS = 25.8 g/100 g, sample interval = 3 min. The column fractions collected between 34 min and 72 min were combined, concentrated by evaporation to 67% dissolved solids and analyzed by HPLC as described in the General Methods. Table 22 indicates the oligosaccharide and monosaccharide components of the isolated fraction thus prepared.

Table 22

Analysis of Oligomer/Leucrose Fraction from Gtf-C Reaction

DP7+ (%DS)	DP6 (%DS)	DP5 (%DS)	DP4 (%DS)	DP3 (%DS)	DP2 (%DS)	sucrose (%DS)	leucrose (%DS)	glucose (%DS)	fructose (%DS)
1.2	0.9	1.5	2.6	5.4	13.2	2.5	59.9	6.9	5.7

In this Example, a glucan synthesis reaction was used to produce at least one soluble alpha-glucan product. This Example also demonstrated the preparation of a chromatographic fraction from a glucan synthesis reaction that produced a soluble alpha-glucan product. This fraction was used in Examples 15 and 16 below to test the activity of alpha-glucosidases thereupon.

EXAMPLE 15

Primary Screening of Alpha-Glucosidases Using Oligomer/Leucrose Fractions from Gtf-S/MUT3325 and Gtf-C Reactions

This Example describes using alpha-glucosidase to hydrolyze leucrose and other oligosaccharides present in chromatographic fractions obtained from glucan synthesis reactions that produced soluble alpha-glucan product.

Specifically, study was made on the effect of alpha-glucosidases disclosed in Example 10 on the hydrolysis of leucrose and oligosaccharides in the fractions prepared in Examples 13 and 14.

A total of twelve alpha-glucosidases and two benchmark enzymes (oligo-1,6-glucosidase and TG L-2000 transglucosidase) were screened using oligomer/leucrose fractions from gtf-S/MUT3325 (Example 13) and gtf-C (Example 14) reactions as substrate material. All the enzymes (alpha-glucosidases and benchmark enzymes) were dosed at equal protein concentrations. Each alpha-glucosidase (dosed at 100 ppm) was incubated in a solution containing oligomer/leucrose substrates (10% dry solids) and 2 mM calcium chloride at pH 5.5 at 47 °C. Each reaction was quenched after 21 hours of incubation by adding 50 µL of 0.5 M NaOH.

The oligosaccharide/monosaccharide contents of the quenched reactions were determined as follows. A sample from each reaction was centrifuged and supernatant therefrom was diluted 25-fold in water for HPLC analysis (General

Methods). The percentages reported in Table 23 reflect the average of peak area percentages (from duplicate analyses of each sample) of each DP_n as a fraction of the total. The results indicate that the fungal alpha-glucosidases had better hydrolytic activity towards glucan oligomers when compared to the

5 bacterial alpha-glucosidases.

Table 23

Sugar Composition Analysis of Primary Screening of Alpha-Glucosidases Using Oligomer/Leucrose Fractions from

Gtf-S/MUT3325 and Gtf-C reactions

Substrate	Enzyme	SEQ ID NO	DP6+%	DP6%	DP5%	DP4%	DP3%	DP2%	Leucrose%	Glucose%	Fructose%
oligomer/ leucrose fraction from Gtf- C reaction	Oligo-1,6-glucosidase		8	0.1	0.2	0.4	1.1	6.8	30.7	19.6	13.1
	TG L-2000	1	0.7	0.1	0.2	0.5	1.1	5.7	3.8	48.6	39.3
	Adglu1	6	0.8	0.1	0.2	0.4	1	4.2	0	51.8	41.6
	Nfiglu1	9	0.7	0.1	0.2	0.4	1	3.6	0	52.2	41.7
	Norglu1	12	0.7	0	0.2	0.4	1	3.6	4	49.9	40.2
	BioGlu1	20	0.1	0.3	0.4	0.7	0.6	2.9	20.6	40.7	33.6
	BioGlu2	24	0.8	0.2	0.6	1	0.9	4	30.9	34	27.5
	BioGlu3	26	1.1	0.3	0.5	1	1	4	28.8	35.4	28.1
	BspGlu1	30	0.9	0.1	0.1	0.1	0.3	2.5	27.9	39.5	28.7
	BthGlu1	32	0.8	0.2	0.5	0.7	0.7	2.9	28.6	37.1	28.4
	BbrGlu2	36	1.1	0.1	0.6	1.1	1.2	4.7	36.8	30.7	23.7
	BbrGlu5	38	1.4	0.1	0.2	0.5	0.9	4	44.6	29.1	19.4
	TauSec098	15	3.1	0.2	0.4	0.7	1.7	4.9	59.3	17.9	11.6
	TauSec099	18	0.1	0	0.1	0.4	1	3.3	0	61.2	43.6
	blank		1.1	0.3	0.6	1.5	2.9	7.9	65.4	11.7	8.5
	Oligo-1,6-glucosidase		10.8	0.8	2.8	7.5	15.7	0	27.8	18.9	15.6
	TG L-2000	1	1.4	0.8	2.7	7.3	13.2	2.3	0	43.8	28.6
	Adglu1	6	1.8	0.8	2.5	6.9	12.3	1.2	0	45.8	29
	Nfiglu1	9	1.8	0.8	2.6	7	13.6	0.9	0	44.4	29.1
oligomer/ leucrose fraction from Gtf- S/											

MUT3325 reaction	NcrGlu1	12	2.1	0.7	2.2	6.6	13.9	0	0	45.2	29.3
	BloGlu1	20	3.5	0.7	2.4	6.5	11.7	2.1	14.8	35.6	22.6
	BloGlu2	24	1.5	0.8	2.6	7	14.6	2.8	24.9	28	17.8
	BloGlu3	26	2	0.8	2.5	6.9	14.1	2.9	22.4	29.9	18.6
	BspGlu1	30	1.7	0.6	1.5	3	3.4	2	18.8	42.3	20.8
	BthGlu1	32	1.4	0.7	2.5	6.2	12.2	2.1	16.3	36.7	21.9
	BbrGlu2	36	1.5	0.8	2.6	7	14.7	3	25	28	17.6
	BbrGlu5	38	2.7	0.7	2.5	5.9	17.2	0	23.9	26.6	20.5
	TauSec098	15	2.9	0	0.1	0.3	1.1	3.6	37.1	41.8	13.1
	TauSec099	18	1.4	0.8	2.8	7.6	16.2	0	0	40.8	30.4
	blank		1.4	0.9	3.1	8.7	19.4	0	37.1	15.6	13.8

As indicated with shading in Table 23, the oligosaccharide content of the reactions generally shifted toward smaller sized sugars, in comparison with the control reactions ("Blank") in which there was no enzyme. These results indicate that alpha-glucosidase can be used to hydrolyze oligosaccharides comprised within a glucan synthesis reaction and a fraction thereof, particularly a chromatographic fraction of a glucan synthesis reaction that produced soluble alpha-glucan product. Also, given the linkage profile of the oligosaccharides (Examples 13 and 14), and the activity of alpha-glucosidase against various glycosidic linkages in addition to alpha-1,4 linkages (Example 11), it is apparent that alpha-glucosidase can be used to break down oligosaccharides with alpha-1,5 glucosyl-fructose linkages and also likely alpha-1,3 and alpha-1,6 glucosyl-glucose linkages. The results provided in Table 23 also suggest that fungal alpha-glucosidases have better hydrolytic activity towards soluble oligosaccharides compared with the bacterial alpha-glucosidases.

Thus, alpha-glucosidase can be used to hydrolyze leucrose and other oligosaccharides present in a fraction (e.g., chromatographic fraction) obtained from a glucan synthesis reaction, such as one that synthesizes a soluble alpha-glucan product.

EXAMPLE 16

Select Screening of Alpha-Glucosidases using Oligomer/Leucrose Fractions from Gtf-S/MUT3325 and Gtf-C Reactions

This Example is further to Example 15, describing the use of alpha-glucosidase to hydrolyze leucrose and other oligosaccharides present in chromatographic fractions obtained from glucan synthesis reactions that produced soluble alpha-glucan product.

Evaluation of alpha-glucosidases that were most active for hydrolysis of oligomer/leucrose fractions from gtf-S/MUT3325 and gtf-C reactions (Example 15) was performed by analyzing sugar compositions resulting in reactions containing enzymes dosed at equal protein concentrations. Incubations of alpha-glucosidases (dosed at 4 ppm; for blends, the ratio of the two enzymes was 1:1 and total dosage was 4 ppm) and oligomer/leucrose substrate (10% ds) were

performed at pH 5.5 in the presence of 2 mM calcium chloride at 60 °C and 65 °C, respectively. The reactions were quenched by adding 50 µL of 0.5 M NaOH after 23 hours of incubation.

5 The oligosaccharide/monosaccharide contents of the quenched reactions were determined as follows. A sample from each reaction was centrifuged and supernatant therefrom was diluted 25-fold in water for HPLC analysis (General Methods). The percentages reported in Table 24 (below) reflect the average of peak area percentages (from duplicate analyses of each sample) of each DP_n as a fraction of the total. The results indicate that TauSec098 was efficacious for
10 hydrolysis of DP2 to DP7 oligomers and TauSec099 outperformed TG L-2000 for leucrose hydrolysis when the incubation was performed at 65 °C. The blends of TauSec098 with TauSec099 (or TG L-2000) were effective for hydrolysis of oligomers and leucrose for DP1 production.

Thus, alpha-glucosidase can be used to hydrolyze leucrose and other
15 oligosaccharides present in a fraction (e.g., chromatographic fraction) obtained from a glucan synthesis reaction, such as one that synthesizes a soluble alpha-glucan product.

Table 24
Sugar Composition Analysis of Select Screening of Alpha-Glucosidases Using Oligomer/Leucrose Fractions from
Gtf-S/MUT3325 and Gtf-C reactions

Temp	Substrate	Enzyme	SEQ ID NO	DP7+ (%)	DP7 (%)	DP6 (%)	DP5 (%)	DP4 (%)	DP3 (%)	DP2 (%)	Leucrose (%)	Glucose (%)	Fructose (%)
60 °C	oligomer/leucrose fraction from Gtf-C reaction	TG L-2000	1	2.5	0.4	0.5	0.9	1.9	5.3	22.9	18.4	21.4	25.8
		TauSec098	15	5.4	0.3	0.5	1.0	1.4	2.6	6.3	71.8	0.0	10.7
		TauSec099	18	2.9	0.3	0.5	1.0	1.9	4.7	23.3	21.5	19.7	24.1
		TauSec098+ TauSec099	15, 18	2.7	0.3	0.5	0.9	1.4	3.3	19.9	34.9	18.2	17.8
		TauSec098+ TG L-2000	15, 1	2.7	0.3	0.5	0.8	1.4	4.5	23.7	27.1	18.6	20.3
		Blank		5.2	0.4	0.6	1.2	1.8	3.2	8.3	70.0	0.0	9.2
		TG L-2000	1	4.1	0.3	1.2	3.2	8.1	17.2	13.2	0.0	27.9	24.8
		TauSec098	15	3.4	0.2	0.0	0.4	1.1	3.5	12.3	32.3	35.3	11.5
		TauSec099	18	4.2	0.0	1.2	3.2	8.2	17.4	15.3	0.0	25.4	25.1
		TauSec098+ TauSec099	15, 18	3.5	0.2	0.4	0.9	2.3	6.2	16.8	21.2	31.7	16.9
65 °C	oligomer/leucrose fraction from Gtf-S/MUT3325 reaction	TauSec098+ TG L-2000	15, 1	3.2	0.1	0.3	0.7	2.0	6.0	26.0	17.0	29.1	15.6
		Blank		4.6	0.4	1.2	3.2	7.9	17.5	15.1	36.6	0.0	13.5
		TG L-2000	1	2.5	0.3	0.5	1.0	1.8	4.9	24.9	26.0	17.4	20.8
		TauSec098	15	2.8	0.4	0.5	1.0	1.4	2.6	6.6	73.6	0.0	11.1
		TauSec099	18	2.2	0.3	0.4	1.0	2.0	4.9	23.2	17.4	21.6	27.0
		TauSec098+ TauSec099	15, 18	4.5	0.3	0.5	0.9	1.4	3.4	20.3	28.6	20.1	20.1
		TauSec098+ TG L-2000	15, 1	5.1	0.3	0.5	0.9	1.2	2.9	21.1	34.4	18.0	15.7
		Blank		7.0	0.4	0.7	1.3	1.8	3.2	7.9	68.4	0.0	9.4

WO 2015/130881													
oligomer/ leucrose fraction from Gtf-S/ MUT3325 reaction	TG L-2000	1	2.9	0.2	1.1	3.1	8.1	18.0	11.7	16.5	19.3	19.0	
	TauSec098	15	2.6	0.0	0.1	0.3	0.9	3.3	12.0	33.1	36.6	11.1	
	TauSec099	18	4.4	0.0	1.2	3.1	7.8	16.1	14.4	0.0	27.4	25.5	
	TauSec098+ TauSec099	15, 18	3.9	0.2	0.4	0.8	2.1	5.7	16.2	19.4	33.7	17.6	
	TauSec098+ TG L-2000	15, 1	3.7	0.2	0.3	0.7	1.8	5.0	24.9	20.5	29.4	13.6	
	Blank		3.1	0.6	1.1	2.5	6.3	13.6	13.0	31.1	17.0	11.8	

CLAIMSWhat is claimed is:

1. A method of hydrolyzing an alpha-1,3 or alpha-1,6 glucosyl-glucose linkage in a saccharide comprising at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein the saccharide is a disaccharide or oligosaccharide, and wherein the method comprises:
contacting the saccharide with an alpha-glucosidase enzyme under suitable conditions, wherein said alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide,
and wherein the amount of the saccharide is reduced compared to the amount of the saccharide that was present prior to said contacting.
2. The method of claim 1, wherein the alpha-glucosidase enzyme is immobilized.
3. The method of claim 1, wherein the degree of polymerization of the saccharide before hydrolysis is 3 to 7.
4. The method of claim 1, wherein the concentration of the saccharide after the contacting step is less than 50% of the concentration of the saccharide that was present prior to said contacting.
5. The method of claim 1, wherein the suitable conditions comprise (i) a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction;
wherein the saccharide is a byproduct of the glucan synthesis reaction.
6. The method of claim 5, wherein the glucan synthesis reaction produces at least one insoluble alpha-glucan product.

7. The method of claim 6, wherein the fraction is a filtrate of the glucan synthesis reaction.
8. The method of claim 5, wherein the glucan synthesis reaction produces at least one soluble alpha-glucan product that is
- 5 (i) a product of a glucosyltransferase, or
- (ii) a product of the concerted action of both a glucosyltransferase and an alpha-glucanohydrolase capable of hydrolyzing glucan polymers having one or more alpha-1,3-glycosidic linkages or one or more alpha-1,6-
- 10 glycosidic linkages.
9. The method of claim 8, wherein the fraction is a chromatographic fraction of the glucan synthesis reaction.
10. The method of claim 1, wherein the alpha-glucosidase enzyme is a transglucosidase.
11. A composition produced by contacting a saccharide with an alpha-glucosidase enzyme,
- 20 wherein said saccharide is a disaccharide or oligosaccharide and comprises at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage, wherein said enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of the saccharide, and wherein said composition comprises a reduced amount of the
- 25 saccharide compared to the amount of the saccharide that was present prior to said contacting.
12. The composition of claim 11, wherein the degree of polymerization of the saccharide before hydrolysis is 3 to 7.

13. The composition of claim 11, wherein the saccharide is in (i) a glucan synthesis reaction, or (ii) a fraction obtained from the glucan synthesis reaction;

wherein the saccharide is a byproduct of the glucan synthesis reaction.

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14. A method of enriching fructose present in a fraction of a glucan synthesis reaction, comprising:

- (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme under suitable conditions, wherein said alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within the fraction; and
- (b) separating fructose from the hydrolyzed fraction of step (a) to obtain a composition having a higher concentration of fructose compared to the fructose concentration of the fraction of step (a).

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15. A fermentation method comprising:

- (a) contacting a fraction obtained from a glucan synthesis reaction with an alpha-glucosidase enzyme under suitable conditions, wherein said alpha-glucosidase enzyme hydrolyzes at least one alpha-1,3 or alpha-1,6 glucosyl-glucose linkage of a disaccharide or oligosaccharide comprised within said fraction;
- (b) fermenting the fraction of step (a) with a microbe to yield a product, wherein said fermenting is performed after step (a) or simultaneously with step (a); and
- (c) optionally, isolating the product of (b);

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wherein the yield of the product of (b) is increased compared to the product yield of fermenting a fraction of the glucan synthesis reaction that has not been contacted with the alpha-glucosidase enzyme.

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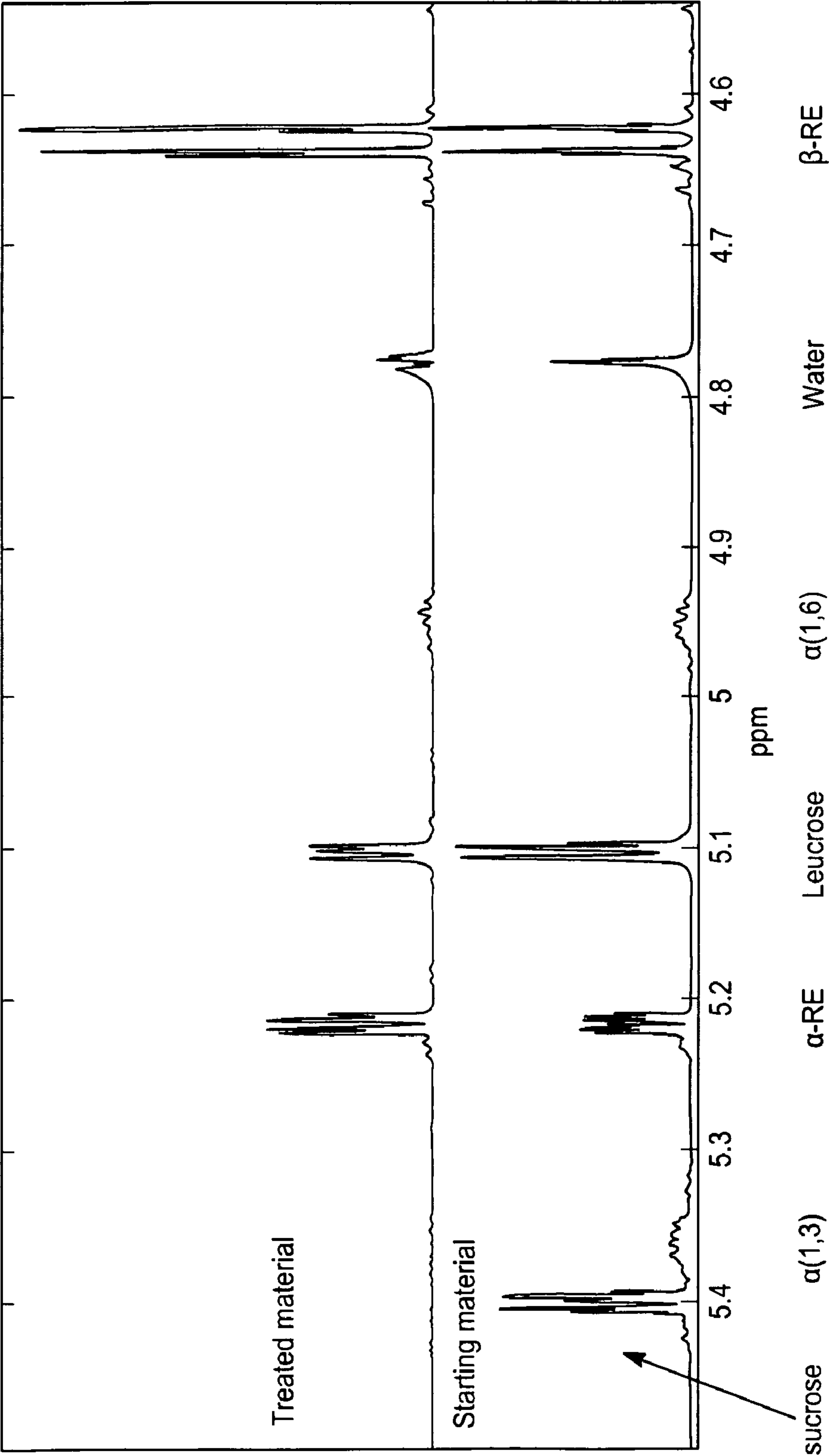


FIG. 1

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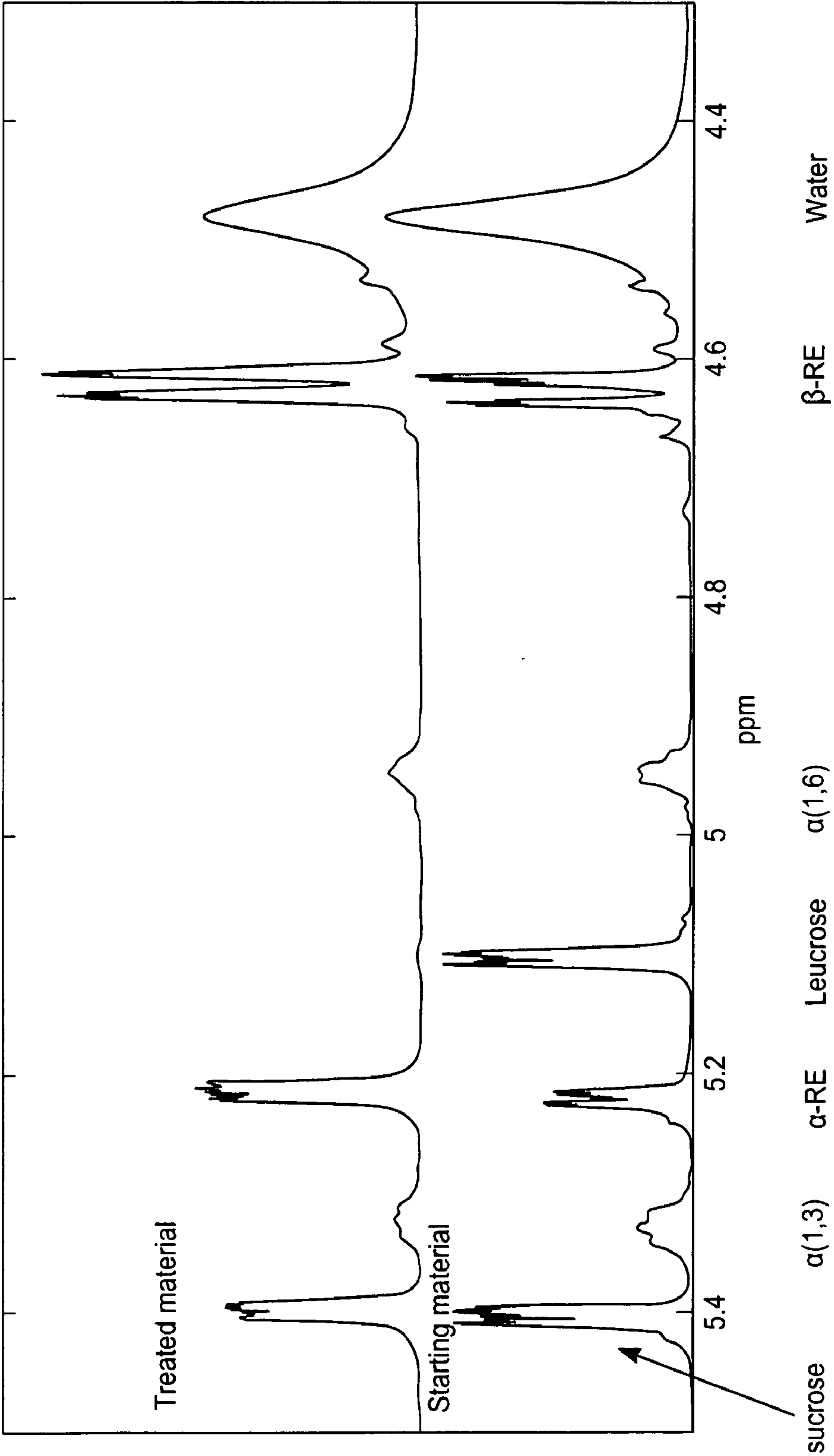


FIG. 2

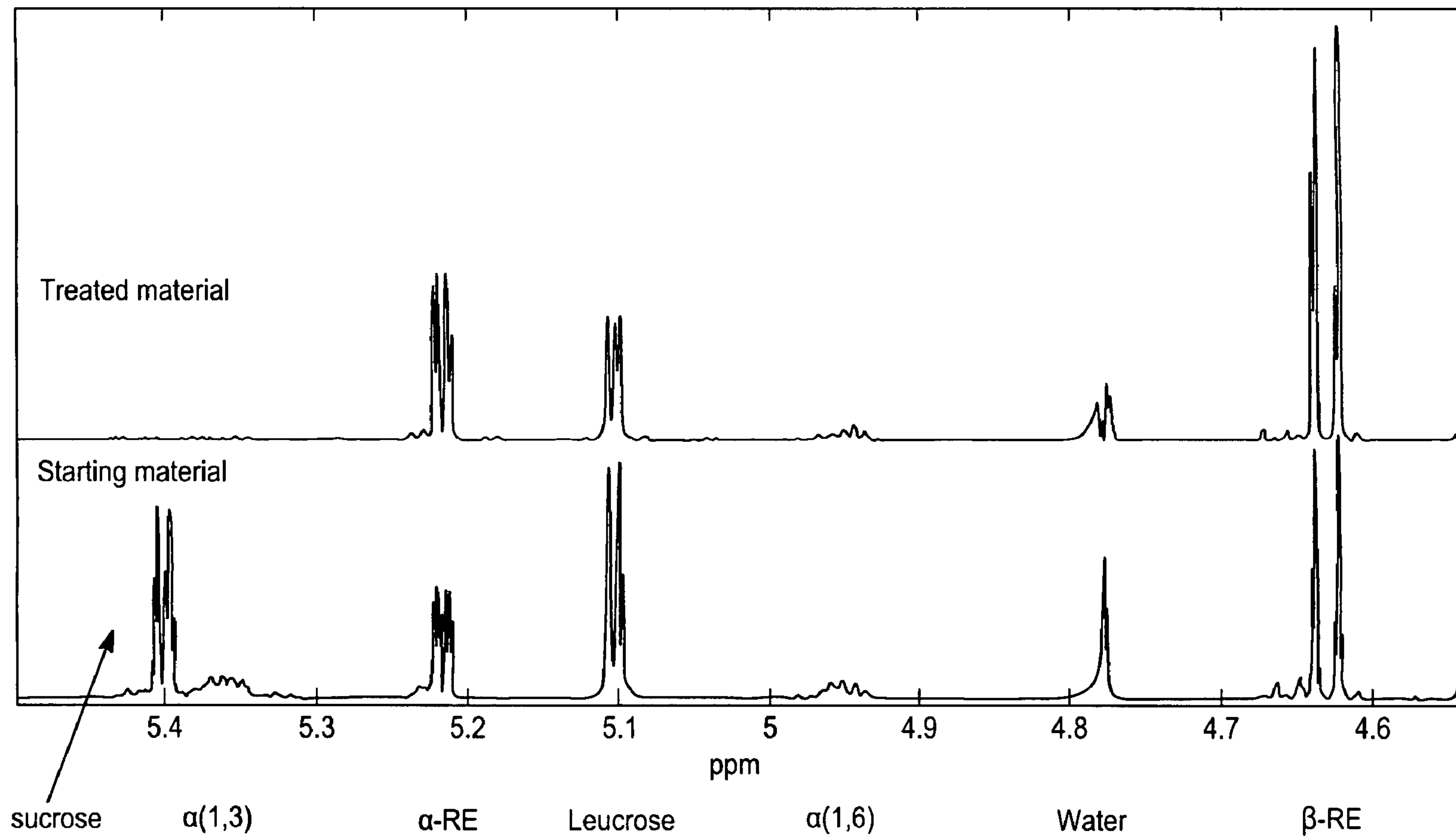


FIG. 1