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CA 2938128 A1 2015/10/01

(21) 2 938 128

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) **Date de dépôt PCT/PCT Filing Date:** 2015/03/24
(87) **Date publication PCT/PCT Publication Date:** 2015/10/01
(85) **Entrée phase nationale/National Entry:** 2016/07/28
(86) **N° demande PCT/PCT Application No.:** US 2015/022135
(87) **N° publication PCT/PCT Publication No.:** 2015/148440
(30) **Priorité/Priority:** 2014/03/25 (US61/969,958)

(51) **Cl.Int./Int.Cl.** ***C12P 19/18*** (2006.01)

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(54) **Titre : PRODUCTION DE POLYMERES DE GLUCANE A PARTIR DE SOURCES ALTERNATIVES DE SACCHAROSE**
(54) **Title: PRODUCTION OF GLUCAN POLYMERS FROM ALTERNATE SUCROSE SOURCES**

(57) **Abrégé/Abstract:**
Reaction solutions are disclosed herein comprising water, incompletely refined sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DPw) of at least 100. The yield of poly alpha-1,3-glucan by a reaction solution herein is at least 7% of the weight of sucrose that was converted in the reaction solution. Further disclosed are methods of producing poly alpha-1,3-glucan using incompletely refined sucrose, and poly alpha-1,3-glucan produced by these methods.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 October 2015 (01.10.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/148440 A1

(51) **International Patent Classification**:
C12P 19/18 (2006.01)

(21) **International Application Number**:
PCT/US2015/022135

(22) **International Filing Date**:
24 March 2015 (24.03.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/969,958 25 March 2014 (25.03.2014) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

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

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

WO 2015/148440 A1

(54) **Title**: PRODUCTION OF GLUCAN POLYMERS FROM ALTERNATE SUCROSE SOURCES

(57) **Abstract**: Reaction solutions are disclosed herein comprising water, incompletely refined sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100. The yield of poly alpha-1,3-glucan by a reaction solution herein is at least 7% of the weight of sucrose that was converted in the reaction solution. Further disclosed are methods of producing poly alpha-1,3-glucan using incompletely refined sucrose, and poly alpha-1,3-glucan produced by these methods.

TITLE

PRODUCTION OF GLUCAN POLYMERS FROM ALTERNATE SUCROSE SOURCES

This application claims the benefit of U.S. Provisional Application No. 61/969,958 (filed March 25, 2014), which is incorporated herein by reference in its entirety.

FIELD OF INVENTION

The invention is in the field of polysaccharide synthesis. For example, this invention pertains to producing insoluble poly alpha-1,3-glucan using sucrose that is not completely refined.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 20150324_CL6221USNP_SequenceListing created on March 16, 2015, and having a size of 569 kilobytes and is filed concurrently with the specification. The sequence listing contained in this ASCII-formatted document is part of the specification and is herein incorporated by reference in its entirety.

BACKGROUND

Driven by a desire to find new structural polysaccharides using enzymatic syntheses or genetic engineering of microorganisms, researchers have discovered polysaccharides that are biodegradable and can be made economically from renewably sourced feedstocks. One such polysaccharide is poly alpha-1,3-glucan, a glucan polymer characterized by having alpha-1,3-glycosidic linkages.

Poly alpha-1,3-glucan has been isolated by contacting an aqueous solution of sucrose with a glucosyltransferase (gtf) enzyme isolated from *Streptococcus salivarius* (Simpson et al., *Microbiology* 141:1451-1460, 1995). U.S. Patent 7,000,000 disclosed the preparation of a polysaccharide fiber using an *S. salivarius* gtfJ enzyme. At least 50% of the hexose units within the polymer of this fiber were linked via alpha-1,3-glycosidic linkages. The disclosed polymer formed a liquid crystalline solution when it was dissolved above a critical

concentration in a solvent or in a mixture comprising a solvent. From this solution continuous, strong, cotton-like fibers, highly suitable for use in textiles, were spun and used.

Enzymatic synthesis of poly alpha-1,3-glucan has previously been performed using white, refined sucrose. Since this form of sucrose is relatively expensive, it is desirable to develop new enzymatic processes for poly alpha-1,3-glucan synthesis using sucrose that is unrefined or otherwise incompletely refined.

SUMMARY OF INVENTION

In one embodiment, the disclosure concerns a reaction solution comprising water, sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100, wherein the sucrose is unrefined or partially refined. The yield of poly alpha-1,3-glucan by the reaction solution is at least 7% of the weight of sucrose that was converted to products in the reaction solution.

In another embodiment of the reaction solution, the sucrose is from sugar beet and has not been crystallized.

In another embodiment of the reaction solution, the sucrose is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.

In another embodiment of the reaction solution, the sucrose has an ICUMSA value greater than 150.

In another embodiment of the reaction solution, the relative reaction rate of the reaction solution is at least 0.8 with respect to the reaction rate of a reaction solution comprising water, white refined sucrose and the glucosyltransferase enzyme.

In another embodiment of the reaction solution, the poly alpha-1,3-glucan produced by the reaction solution has an *L** value less than 93.

In another embodiment, the glucosyltransferase enzyme comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:4, SEQ ID

NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

In another embodiment, the disclosure concerns a method for producing poly alpha-1,3-glucan comprising the step of contacting water, sucrose, and a glucosyltransferase enzyme, wherein the sucrose is unrefined or partially refined. The poly alpha-1,3-glucan produced in the contacting step has at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100. The yield of poly alpha-1,3-glucan produced in the method is at least 7% of the weight of the sucrose that was converted to products in the contacting step. The method optionally comprises isolating the poly alpha-1,3-glucan produced in the contacting step.

In another embodiment, the sucrose used in the method is from sugar beet and has not been crystallized.

In another embodiment, the sucrose used in the method is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.

In another embodiment, the sucrose used in the method has an ICUMSA value greater than 150.

In another embodiment, the relative reaction rate of producing poly alpha-1,3-glucan in the contacting step of the method is at least 0.8 with respect to the reaction rate of the contacting step if white refined sucrose is used instead of unrefined or partially refined sucrose.

In another embodiment, the poly alpha-1,3-glucan optionally isolated in the method has an *L** value less than 93.

In another embodiment, the glucosyltransferase enzyme used in the method comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

In another embodiment, the disclosure concerns isolated poly alpha-1,3-glucan produced by the above method, wherein the poly alpha-1,3-glucan has an *L** value less than 93.

BRIEF DESCRIPTION OF THE SEQUENCES

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

Description	Nucleic acid SEQ ID NO.	Protein SEQ ID NO.
"0874 gtf", Streptococcus sobrinus. DNA codon-optimized for expression in E. coli. The first 156 amino acids of the protein are deleted compared to GENBANK Identification No. 450874; a start methionine is included.	1	2 (1435 aa)
"6855 gtf", Streptococcus salivarius SK126. DNA codon-optimized for expression in E. coli. The first 178 amino acids of the protein are deleted compared to GENBANK Identification No. 228476855; a start methionine is included.	3	4 (1341 aa)
"2379 gtf", Streptococcus salivarius. DNA codon-optimized for expression in E. coli. The first 203 amino acids of the protein are deleted compared to GENBANK Identification No. 662379; a start methionine is included.	5	6 (1247 aa)
"7527" or "gtfJ", Streptococcus salivarius. DNA codon-optimized for expression in E. coli. The first 42 amino acids of the protein are deleted compared to GENBANK Identification No. 47527; a start methionine is included.	7	8 (1477 aa)
"1724 gtf", Streptococcus downei. DNA codon-optimized for expression in E. coli. The first 162 amino acids of the protein are deleted compared to GENBANK Identification No. 121724; a start methionine is included.	9	10 (1436 aa)
"0544 gtf", Streptococcus mutans. DNA codon-optimized for expression in E. coli. The first 164 amino acids of the protein are deleted compared to GENBANK Identification No. 290580544; a start methionine is included.	11	12 (1313 aa)
"5926 gtf", Streptococcus dentirousetti. DNA codon-optimized for expression in E. coli. The first 144 amino acids of the protein are deleted compared to GENBANK Identification No. 167735926; a start methionine is included.	13	14 (1323 aa)
"4297 gtf", Streptococcus oralis. DNA codon-optimized for expression in E. coli. The first 228 amino acids of the protein are deleted compared to GENBANK Identification No. 7684297; a start methionine is included.	15	16 (1348 aa)
"5618 gtf", Streptococcus sanguinis. DNA codon-	17	18

optimized for expression in E. coli. The first 223 amino acids of the protein are deleted compared to GENBANK Identification No. 328945618; a start methionine is included.		(1348 aa)
"2765 gtf", unknown Streptococcus sp. C150. DNA codon-optimized for expression in E. coli. The first 193 amino acids of the protein are deleted compared to GENBANK Identification No. 322372765; a start methionine is included.	19	20 (1340 aa)
"4700 gtf", Leuconostoc mesenteroides. DNA codon-optimized for expression in E. coli. The first 36 amino acids of the protein are deleted compared to GENBANK Identification No. 21654700; a start methionine is included.	21	22 (1492 aa)
"1366 gtf", Streptococcus criceti. DNA codon-optimized for expression in E. coli. The first 139 amino acids of the protein are deleted compared to GENBANK Identification No. 146741366; a start methionine is included.	23	24 (1323 aa)
"0427 gtf", Streptococcus sobrinus. DNA codon-optimized for expression in E. coli. The first 156 amino acids of the protein are deleted compared to GENBANK Identification No. 940427; a start methionine is included.	25	26 (1435 aa)
"2919 gtf", Streptococcus salivarius PS4. DNA codon-optimized for expression in E. coli. The first 92 amino acids of the protein are deleted compared to GENBANK Identification No. 383282919; a start methionine is included.	27	28 (1340 aa)
"2678 gtf", Streptococcus salivarius K12. DNA codon-optimized for expression in E. coli. The first 188 amino acids of the protein are deleted compared to GENBANK Identification No. 400182678; a start methionine is included.	29	30 (1341 aa)
"2381 gtf", Streptococcus salivarius. DNA codon-optimized for expression in E. coli. The first 273 amino acids of the protein are deleted compared to GENBANK Identification No. 662381; a start methionine is included.	31	32 (1305 aa)
"3929 gtf", Streptococcus salivarius JIM8777. DNA codon-optimized for expression in E. coli. The first 178 amino acids of the protein are deleted compared to GENBANK Identification No. 387783929; a start methionine is included.	33	34 (1341 aa)
"6907 gtf", Streptococcus salivarius SK126. DNA codon-optimized for expression in E. coli. The first	35	36 (1331 aa)

161 amino acids of the protein are deleted compared to GENBANK Identification No. 228476907; a start methionine is included.		
“6661 gtf”, Streptococcus salivarius SK126. DNA codon-optimized for expression in E. coli. The first 265 amino acids of the protein are deleted compared to GENBANK Identification No. 228476661; a start methionine is included.	37	38 (1305 aa)
“0339 gtf”, Streptococcus gallolyticus ATCC 43143. DNA codon-optimized for expression in E. coli. The first 213 amino acids of the protein are deleted compared to GENBANK Identification No. 334280339; a start methionine is included.	39	40 (1310 aa)
“0088 gtf”, Streptococcus mutans. DNA codon-optimized for expression in E. coli. The first 189 amino acids of the protein are deleted compared to GENBANK Identification No. 3130088; a start methionine is included.	41	42 (1267 aa)
“9358 gtf”, Streptococcus mutans UA159. DNA codon-optimized for expression in E. coli. The first 176 amino acids of the protein are deleted compared to GENBANK Identification No. 24379358; a start methionine is included.	43	44 (1287 aa)
“8242 gtf”, Streptococcus gallolyticus ATCC BAA-2069. DNA codon-optimized for expression in E. coli. The first 191 amino acids of the protein are deleted compared to GENBANK Identification No. 325978242; a start methionine is included.	45	46 (1355 aa)
“3442 gtf”, Streptococcus sanguinis SK405. DNA codon-optimized for expression in E. coli. The first 228 amino acids of the protein are deleted compared to GENBANK Identification No. 324993442; a start methionine is included.	47	48 (1348 aa)
“7528 gtf”, Streptococcus salivarius. DNA codon-optimized for expression in E. coli. The first 173 amino acids of the protein are deleted compared to GENBANK Identification No. 47528; a start methionine is included.	49	50 (1427 aa)
“3279 gtf”, Streptococcus sp. C150. DNA codon-optimized for expression in E. coli. The first 178 amino acids of the protein are deleted compared to GENBANK Identification No. 322373279; a start methionine is included.	51	52 (1393 aa)
“6491 gtf”, Leuconostoc citreum KM20. DNA codon-optimized for expression in E. coli. The first 244 amino acids of the protein are deleted compared to	53	54 (1262 aa)

GENBANK Identification No. 170016491; a start methionine is included.		
"6889 gtf", Streptococcus salivarius SK126. DNA codon-optimized for expression in E. coli. The first 173 amino acids of the protein are deleted compared to GENBANK Identification No. 228476889; a start methionine is included.	55	56 (1427 aa)
"4154 gtf", Lactobacillus reuteri. DNA codon-optimized for expression in E. coli. The first 38 amino acids of the protein are deleted compared to GENBANK Identification No. 51574154; a start methionine is included.	57	58 (1735 aa)
"3298 gtf", Streptococcus sp. C150. The first 209 amino acids of the protein are deleted compared to GENBANK Identification No. 322373298; a start methionine is included.		59 (1242 aa)
"Wild type gtfJ", Streptococcus salivarius. GENBANK Identification No. 47527.		60 (1518 aa)
Wild type gtf corresponding to 2678 gtf, Streptococcus salivarius K12. GENBANK Identification No. 400182678.		61 (1528 aa)
Wild type gtf corresponding to 6855 gtf, Streptococcus salivarius SK126. GENBANK Identification No. 228476855.		62 (1518 aa)
Wild type gtf corresponding to 2919 gtf, Streptococcus salivarius PS4. GENBANK Identification No. 383282919.		63 (1431 aa)
Wild type gtf corresponding to 2765 gtf, Streptococcus sp. C150. GENBANK Identification No. 322372765.		64 (1532 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 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 "poly alpha-1,3-glucan", "alpha-1,3-glucan polymer" and the like are used interchangeably herein. Poly alpha-1,3-glucan is a polymer comprising glucose monomeric units linked together by glycosidic linkages (i.e., glucosidic linkages), wherein at least about 50% of the glycosidic linkages are alpha-1,3-

glycosidic linkages. Poly alpha-1,3-glucan is a type of polysaccharide. 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 terms "glycosidic linkage" and "glycosidic bond" are used interchangeably herein and refer to the type of covalent bond that joins a carbohydrate (sugar) molecule to another group such as another carbohydrate. 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.

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

The term "sucrose" herein refers to a non-reducing disaccharide 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.

"White refined" sucrose herein refers to sucrose comprising at least 99.0 wt% sucrose. Additionally, or alternatively, white refined sucrose can refer to sucrose having an ICUMSA value of 150 or less (e.g., 45 or less), a minimum polarization of 99.70%, and/or an *L** value of at least 87.0.

An "ICUMSA" (International Commission for Uniform Methods of Sugar Analysis) value, or "standard ICUMSA" value, is an international unit for expressing the purity of a sucrose sample in solution, and is directly related to the color of the sucrose. The greater the ICUMSA value of a sucrose sample, the darker the sucrose sample is. Methods of determining ICUMSA values for sucrose samples are well known in the art and are 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. ICUMSA values can be expressed in "reference base units" (RBU).

ICUMSA values herein can be measured by a method very similar to ICUMSA Method GS1/3-7, but differing by using a cellulose acetate filter instead of a cellulose nitrate filter. Thus, ICUMSA values disclosed herein can alternatively be referred to as "modified ICUMSA" values. Given how ICUMSA is measured, it would be understood that ICUMSA values provided herein for a solid sugar samples (e.g., raw cane sugar) were obtained using an aqueous solution of the sugar sample (about 200 g/L).

The "polarization" ("pol") of a sucrose sample herein refers to the apparent sucrose content in the sample expressed as a mass percent measured by the optical rotation of polarized light passing through a solution comprising the sucrose sample at 20 °C. The greater the polarization of a sucrose sample, the more pure the sucrose in the sample is.

Sucrose that is "not completely refined" ("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 of incompletely refined sucrose herein is greater than 150.

The terms sucrose "crystallization" "crystallization step", "fractional crystallization" and the like are used interchangeably herein and refer to a process of crystallizing sucrose from a solution comprising incompletely refined sucrose and separating the sucrose crystals from the supernatant (mother liquor). The crystals resulting from this process typically represents sucrose that is more pure compared to the sucrose as it existed before the crystallization step. It is important to note, however, that incompletely refined sucrose having gone through one, two, three, or more crystallization steps may still constitute incompletely refined sucrose (i.e., the crystallized sucrose may not have the

purity of white refined sucrose). Cane sugar typically requires three or more crystallization steps to prepare white refined sugar, whereas beet juice in certain embodiments may only need one crystallization step to reach such purity. Various means are known in the art for crystallizing sucrose, such as evaporation, boiling, and/or vacuum-drying processes.

The term "*L** value" as used herein refers to the lightness component of the CIE 1976 (*L**, *a**, *b**) ("CIELAB") three-dimensional color space specified by the International Commission on Illumination (CIE, Vienna, Austria). The three coordinates of the *L*a*b** color space represent, respectively, lightness of the color of a solid (*L** = 0 indicates black and *L** = 100 indicates diffuse white), the color of the object along a scale between red/magenta and green (*a**, negative values indicate green while positive values indicate magenta), and the color of the object along a scale between yellow and blue (*b**, negative values indicate blue and positive values indicate yellow). The asterisks (*) used in referring to a *L*a*b** color space of an object are pronounced as "star" (e.g., *L** is "L-star") and serve to distinguish this color space from Hunter's *L*, *a*, *b* color system. The *L**, *a**, and *b** components of a CIELAB color space of an object can be calculated using the formulae disclosed by J. Schwiegerling (Field Guide to Visual and Ophthalmic Optics, SPIE Press, Bellingham, WA, 2004), which is incorporated herein by reference. *L**, *a**, *b** values herein are with respect to solid material such as dry sucrose or dry poly alpha-1,3-glucan.

"Dry" sucrose as used herein can characterize sucrose that comprises no more than 2.0, 1.5, 1.0, 0.5, 0.25, 0.10, 0.05, or 0.01 wt% water.

The "molecular weight" of poly alpha-1,3-glucan herein can be represented as number-average molecular weight (M_n) or as weight-average molecular weight (M_w). 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 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 the substrate sucrose to make the products poly alpha-1,3-glucan and fructose. Other products (byproducts) of a gtf 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). Leucrose is a disaccharide composed of glucose and fructose linked by an alpha-1,5 linkage. Wild type forms of glucosyltransferase enzymes generally contain (in the N-terminal to C-terminal direction) a signal peptide, a variable domain, a catalytic domain, and a glucan-binding domain. A gtf 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).

A “reaction solution” as used herein generally refers to a solution comprising sucrose, water, at least one active glucosyltransferase enzyme, and optionally other components. A reaction solution can alternatively be referred to herein as a “glucan synthesis reaction”, “glucan reaction”, or “gtf reaction”, for example. Other components that can be in a glucan synthesis reaction include fructose, glucose, leucrose, and soluble oligosaccharides (e.g., DP2-DP7). 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. It is in the reaction solution where the step of contacting water, sucrose and a glucosyltransferase enzyme is performed. The term “under suitable reaction conditions” as used herein, refers to reaction conditions that support conversion of sucrose to poly alpha-1,3-glucan via glucosyltransferase enzyme activity.

A “control” reaction solution as used herein can refer to a reaction solution comprising white refined sucrose instead of incompletely refined sucrose. All the

other features (e.g., sucrose concentration, temperature, pH, type of gtf) of a control reaction solution can be the same as the reaction solution to which it is being compared.

The "percent dry solids" of a glucan synthesis reaction refers to the wt% of all the sugars in the 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.

The "yield" of poly alpha-1,3-glucan by a reaction solution herein represents the weight of poly alpha-1,3-glucan product expressed as a percentage of the weight of sucrose substrate that is converted in the reaction. For example, if 100 g of sucrose in a reaction solution is converted to products, and 10 g of the products is poly alpha-1,3-glucan, the yield of the poly alpha-1,3-glucan would be 10%. This yield calculation can be considered as a measure of selectivity of the reaction toward poly alpha-1,3-glucan.

The term "relative reaction rate" as used herein refers to the rate of a particular glucan synthesis reaction as compared to another glucan synthesis reaction. For example, if reaction A has a rate of *x*, and reaction B has a rate of *y*, then the relative reaction rate of reaction A with respect to the reaction rate of reaction B can be expressed as *x*/*y* (*x* divided by *y*). The terms "reaction rate" and "rate of reaction" are used interchangeably herein to refer to the change in concentration/amount of reactant(s) or the change in concentration/amount of product(s) per unit time per unit of enzyme. Preferred reactant and product herein of a glucan synthesis reaction are, respectively, sucrose and poly alpha-1,3-glucan.

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 solid glucan product synthesized in the reaction. A fraction can alternatively be referred to as a "mother liquor." 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.

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.

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: [(volume of solute)/(volume of solution)] x 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.

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).

Various polynucleotide and polypeptide 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%, 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 nucleotide or amino acid sequence 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.

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, an isolated polynucleotide or polypeptide molecule can be comprised within a cell or organism in a heterologous manner. Other examples are isolated glucosyltransferase and isolated poly alpha-1,3-glucan. It is believed that the glucosyltransferase reaction processes disclosed herein are synthetic, non-naturally occurring processes.

Enzymatic synthesis of poly alpha-1,3-glucan has previously been performed using white, refined sucrose. Since this form of sucrose is relatively expensive, it is desirable to develop new enzymatic processes for poly alpha-1,3-glucan synthesis using sucrose that is unrefined or otherwise incompletely refined.

Embodiments of the present disclosure concern a reaction solution comprising at least water, sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100, wherein the sucrose is unrefined or partially refined (i.e., not completely refined). The reaction solution produces insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a DP_w of at least 100. The yield of poly alpha-1,3-glucan by the reaction solution is at least 7% of the weight of sucrose that was converted to products in the reaction solution.

Significantly, embodiments of this reaction solution produce poly alpha-1,3-glucan with yields and molecular weights comparable to the glucan yields and molecular weights produced by reaction solutions using white refined sucrose instead of incompletely refined sucrose. These results indicate that the

contaminants present in incompletely refined sucrose generally do not impede its use by glucosyltransferase in polymerizing poly alpha-1,3-glucan.

Sucrose that is not completely refined can be used in a reaction solution as presently disclosed. Such sucrose has not been processed to white refined sucrose. Examples include unrefined sucrose compositions comprising raw sucrose (raw sugar). Other forms of incompletely refined sucrose useful herein include forms of sucrose derived from sugarcane (*Saccharum* spp. such as *S. officenarum*), sugar beet (*Beta* spp. such as *B. vulgaris*) (can alternatively be referred to herein as "beet"), date palm (*Phoenix dactylifera*), sorghum (*Sorghum* spp. such as *S. vulgare* and *S. bicolor*), sugar maple (*Acer saccharum*), cassava (*Manihot esculenta*), or corn, for example. Incompletely refined forms of sugarcane and/or sugar beet sucrose can be used in preferred embodiments herein. Sugarcane contains about 20% sucrose in its juice, whereas sugar beet contains about 10 to 15% sucrose in its juice.

Incompletely refined sucrose in certain embodiments may be from a plant that produces sucrose and, optionally, is grown for sucrose production. Such a plant, such as those listed above, can be from any region of the world where the plant is typically grown. For example, sucrose herein may be from a plant grown in South America (e.g., Brazil, Colombia, Argentina, Guyana), North America (e.g., U.S.A., Mexico, West Indies, Central America [e.g., Belize]), Australia, Asia (e.g., India, China, Russia, Turkey, Thailand, Pakistan, Philippines, Indonesia), Africa (e.g., Egypt, Mozambique, Zimbabwe) and Europe (e.g., France, Germany, Ukraine, Russia, Turkey).

Incompletely refined sucrose may be provided as a composition obtained at any stage of a process of sucrose purification from a juice of a plant (e.g., sugarcane or sugar beet) containing sucrose. Such processes are disclosed in Handbook of Sugar Refining: A Manual for the Design and Operation of Sugar Refining Facilities (Ed. C.C. Chou, John Wiley & Sons, Inc., 2000), Chen and Chou (Cane Sugar Handbook: A Manual for Cane Sugar Manufacturers and Their Chemists, 12th Edition, John Wiley & Sons, Inc., 1993), and Asadi (Beet-

Sugar Handbook, 1st Edition, Wiley-Interscience, 2006), for example, which are all incorporated herein by reference. Some preferred compositions and processes from these references are discussed as follows.

Incompletely refined sucrose herein may be provided as a composition resulting from any step of a sucrose-purification process, such as (i) initial extraction (e.g., hot water extraction) of "raw juice" from plant material; (ii) juice purification by carbonatation (i.e., using lime and carbon dioxide to form a calcium carbonate precipitate that co-precipitates impurities), yielding "thin juice"; (iii) evaporation of water from thin juice, yielding "thick juice", and/or (iv) boiling or vacuum-concentrating thick juice to crystallize sucrose (such once-crystallized sucrose typically is not white refined sugar) (the crystals can be removed from the supernatant by centrifugation, for example). The products of steps (i) and (ii) can be filtered in certain embodiments before further processing, for example. The supernatant of crystallization (iv) can be recycled and mixed with other supernatant and/or thick juice, which is then subject to a crystallization (yielding crystals as in step [iv] that can also be used herein). Recycling of supernatant eventually results in "molasses". Thus, incompletely refined sucrose can be provided as raw juice, thin juice, thick juice, molasses, and/or sucrose crystals that have gone through no more than one crystallization, for example. These forms of incompletely refined sucrose, and respective process steps used to obtain them, preferably characterize examples of incompletely refined sucrose obtained from sugar beet, but may also characterize sucrose obtained from other sources such as sugarcane.

Examples of incompletely refined sucrose from sugar beets useful herein include beet raw juice, beet thin juice (comprises about 10-20 wt% sucrose), beet thick juice (comprises about 60-90 wt% sucrose) and sugar beet molasses (about 50-60 wt% sucrose). Beet thin and/or thick juice are used in certain embodiments. ICUMSA values herein can be at least 1000 (e.g., ~1000-1300) for beet thin juice, at least 1300 (e.g., ~1300-1800) for beet thick juice, and/or at least 50000 for beet molasses (e.g., ~50000-60000), for example.

Alternatively, incompletely refined sucrose herein may be "raw sucrose" ("raw sugar"), which is provided by removing all the water from raw juice (i.e., raw sucrose is solid). Alternatively still, incompletely refined sucrose herein may be "VHP sucrose" ("VHP", "VHP sugar", "very high polarization" sucrose), which is provided by first carbonatating and filtering raw juice, followed by evaporating raw juice to crystallize a portion of the sucrose therein; the crystallized sucrose removed from the supernatant is VHP sucrose. VHP sucrose has thus gone through one crystallization. Alternatively still, incompletely refined sucrose herein may be "VVHP sucrose" ("VVHP", "VVHP sugar", "very very high polarization" sucrose), which is provided by dissolving VHP sucrose in water and re-crystallizing the sucrose. VVHP sucrose has thus been through two crystallizations. These forms of incompletely refined sucrose (raw sucrose, VHP, VVHP), and respective process steps used to obtain them, preferably characterize examples of incompletely refined sucrose obtained from sugarcane, but may also characterize sucrose obtained from other sources such as sugar beets.

Raw sucrose (e.g., "raw cane sugar") herein can have a polarization value of 94% to 97%, an ICUMSA value of about 600 to 1200, and/or an L^* value below 87.0 (e.g., less than 85, 80, 75, 70, 65, 60, 55, or 50). It should be understood that raw sucrose is not "brown sugar", which is a product of mixing a molasses syrup with white refined sugar followed by drying. VHP sucrose herein can have a polarization value of at least 99.30%, and/or an ICUMSA value of about 300 to 1000, for example. VHP sucrose can optionally have any of the following characteristics: 0.15% maximum moisture content, 0.15% maximum ash content, 97% solubility in water, and/or golden brown color. VVHP sucrose herein can have a polarization value of at least 99.50%, and/or an ICUMSA value of over 150 to about 400, for example.

Incompletely refined sucrose herein is not white refined sucrose. White refined sucrose herein refers to sucrose comprising at least 99.0 wt% sucrose (e.g., at least 99.5 wt% or 99.9 wt%). Additionally, or alternatively, white refined sucrose can refer to sucrose having an ICUMSA value of 150 or less (e.g., 45 or

less), a minimum polarization of 99.70% (e.g., at least 99.80%), and/or an *L** value of at least 87.0 (e.g., at least 87.5, 88.0, or 88.5). White refined sucrose in certain embodiments can also have any of the following characteristics: 0.04% maximum moisture content, 0.04% maximum ash content, 100% solubility in water, sparkling white color, and/or fine granulation.

In certain embodiments, incompletely refined sucrose has not been crystallized. There can be mentioned incompletely refined sucrose obtained sugar beet, including, for example, beet raw juice, beet thin juice and beet thick juice. Alternatively, incompletely refined sucrose herein has had no more than one, two, three, or more crystallization steps. Incompletely refined sucrose from sugar cane that has had no more than two or three crystallizations can be used, for example. Alternatively still, incompletely refined sucrose can be used if it has been through one, two, three, or more crystallizations, but has an ICUMSA greater than 150. A crystallization step can comprise, for example, boiling and/or vacuum-drying an aqueous solution comprising sucrose at least to the point that dissolved sucrose begins to fall out of the solution as crystals.

The ICUMSA value of incompletely refined sucrose herein can be greater than 150, for example. Alternatively, incompletely refined sucrose can have an ICUMSA value of at least about 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 20000, 30000, 40000, 50000, or 60000 (or any integer value between 151 and 60000), for example. The ICUMSA in certain embodiments can range from about 1000-1300, such as when the incompletely refined sucrose is beet thin juice. The ICUMSA in other embodiments herein can range from about 1300-1800, such as when the incompletely refined sucrose is beet thick juice, or about 50000 to 60000, such as when the incompletely refined sucrose is beet molasses. Still in other embodiments, the ICUMSA can range from about 600-1200, such as when the incompletely refined sucrose is raw sucrose; about 300-1000, such as when the incompletely refined sucrose is VHP sucrose; or over 150 to about 400, such as when the incompletely refined sucrose is VVHP sucrose.

It is believed that ICUMSA values of sucrose compositions herein ("modified ICUMSA") are the same as, or very similar to, the ICUMSA values that would be measured for the compositions using other ICUMSA methods.

A reaction solution herein refers to a solution comprising at least incompletely refined sucrose, water and an 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), for example. It 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. A reaction solution herein may be one that, in addition to producing insoluble glucan product, produces byproducts such as leucrose and/or soluble oligosaccharides.

A reaction solution as disclosed herein comprises a glucosyltransferase enzyme that produces poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a DP_w of at least 100. Examples of such glucosyltransferase enzymes useful herein are disclosed in U.S. Pat. No. 7000000, and U.S. Pat. Appl. Publ. Nos. 2013/0244288 and 2013/0244287 (all of which are incorporated herein by reference). Still other examples of glucosyltransferases that can be used in a reaction solution herein for producing poly alpha-1,3-glucan are disclosed in U.S. Pat. Appl. Publ. No. 2014/0087431 (U.S. Pat. Appl. No. 14/036,049), which is incorporated herein by reference. For example, a glucosyltransferase enzyme herein can (i) comprise, or consist of, an amino acid sequence that is 100% identical to, or at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to, SEQ ID NO:4, 8, 10, 12, 14, 20, 26, 28, 30, or 34, and (ii) have glucosyltransferase activity.

A reaction solution in certain other embodiments comprises a glucosyltransferase enzyme that (i) comprises, or consists of, an amino acid sequence that is 100% identical to, or at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to, SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18,

20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 59, 60, 61, 62, 63, or 64, and (ii) has glucosyltransferase activity.

A glucosyltransferase enzyme herein may be derived from any microbial 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. dentirousetti*, *S. downei*, *S. mutans*, *S. oralis*, *S. gallolyticus* and *S. sanguinis*. Examples of *Leuconostoc* species include *L. 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 in some aspects herein produces poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages in a glucan synthesis reaction in which incompletely refined sucrose is used. It is believed that a glucosyltransferase enzyme in certain embodiments synthesizes poly alpha-1,3-glucan in which at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% (or any integer between 60% and 100%) of the constituent glycosidic linkages are alpha-1,3 linkages. Accordingly, the glucosyltransferase enzyme in the foregoing embodiments synthesizes poly alpha-1,3-glucan in which there is 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.

In other aspects herein, a glucosyltransferase enzyme can synthesize poly alpha-1,3-glucan with 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, such as those that are present in mutan polymer.

A glucosyltransferase enzyme in some aspects herein can synthesize poly alpha-1,3-glucan having a molecular weight in DP_n or DP_w of at least about 100.

Alternatively, the glucosyltransferase enzyme can synthesize poly alpha-1,3-glucan having a molecular weight in DP_n or DP_w of at least about 400. Alternatively still, the glucosyltransferase enzyme can synthesize poly alpha-1,3-glucan having a molecular weight in DP_n or DP_w of at least about 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 (or any integer between 100 and 1000).

One or more different glucosyltransferase enzymes may be used in certain aspects. The glucosyltransferase enzyme in certain embodiments does not have, or has very little (less than 1%), dextransucrase, reuteransucrase, or alternansucrase activity. A reaction solution herein may contain one, two, or more glucosyltransferase enzymes, for example.

A glucosyltransferase enzyme herein can be primer-independent or 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 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. Dextran for use as a primer can be dextran T10 (i.e., dextran having a molecular weight of 10 kD), for example.

Examples of glucosyltransferase enzymes herein can be any of the amino acid sequences disclosed herein and that further include 1-300 (or any integer there between [e.g., 10, 15, 20, 25, 30, 35, 40, 45, or 50]) residues on the N-terminus and/or C-terminus. Such additional residues may be from a corresponding wild type sequence from which the glucosyltransferase enzyme is derived, or may be a heterologous sequence such as an epitope tag (at either N- or C-terminus) or a heterologous signal peptide (at N-terminus), for example.

A glucosyltransferase enzyme herein typically lacks an N-terminal signal peptide. An expression system for producing a glucosyltransferase enzyme

herein may employ an enzyme-encoding polynucleotide that further comprises sequence encoding an N-terminal signal peptide to direct extra-cellular secretion, if desired. 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 glucosyltransferase. An example of a signal peptide useful herein is one from a bacterial (e.g., a *Bacillus* species such as *B. subtilis*) or fungal species. An example of a bacterial signal peptide is an aprE signal peptide, such as one from *Bacillus* (e.g., *B. subtilis*, see Vogtentanz et al., *Protein Expr. Purif.* 55:40-52, which is incorporated herein by reference).

Several glucosyltransferase enzyme sequences disclosed herein lack an N-terminal signal peptide (as well as a variable domain) (refer to Table 1). An N-terminal start-methionine (amino acid position 1) has been added to each sequence for intracellular expression purposes (expressed enzyme can be obtained in a cell lysate, for example). One of skill in the art would understand that an intervening heterologous amino acid sequence such as an epitope and/or signal peptide could optionally be added between the start methionine and glucosyltransferase sequence. Thus, for example, a glucosyltransferase enzyme herein may comprise, or consist of, an amino acid sequence that (i) is 100% identical to, or at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to, the amino acid sequence beginning at position 2 of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 59, 60, 61, 62, 63, or 64, and (ii) has glucosyltransferase activity.

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.

In certain embodiments, a heterologous gene expression system may be one that is designed for protein secretion. The glucosyltransferase enzyme comprises a signal peptide (signal sequence) in such embodiments. The signal peptide may be either its native signal peptide or a heterologous signal peptide.

A glucosyltransferase enzyme described herein may be used in any purification state (e.g., pure or non-pure). For example, the 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) in a reaction solution for producing poly alpha-1,3-glucan from sucrose.

The activity of a glucosyltransferase enzyme herein can be determined using any method known in the art. For example, glucosyltransferase enzyme activity can be determined by measuring the production of reducing sugars (fructose and glucose) in a reaction solution containing sucrose (50 g/L), dextran T10 (1 mg/mL) and potassium phosphate buffer (pH 6.5, 50 mM), where the solution is held at 22-25 °C for 24-30 hours. The reducing sugars can be measured by adding 0.01 mL of the reaction solution to a mixture containing 1 N NaOH and 0.1% triphenyltetrazolium chloride and then monitoring the increase in absorbance at OD_{480nm} for five minutes.

The temperature of a reaction solution 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, or about 20 °C to about 30 °C (e.g., about 25 °C).

The initial concentration of sucrose in a reaction solution 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, incompletely refined sucrose, gtf enzyme). All of, or a portion of, the sucrose in a reaction solution can be from incompletely refined sucrose added to the solution. Though it is preferable that all the sucrose be incompletely refined, white refined sucrose may additionally be used in a reaction solution.

It would be understood that, with certain incompletely refined sucrose compositions that are in liquid form (e.g., beet thin juice, beet thick juice, molasses), such compositions would be added accordingly to a reaction solution to achieve a particular initial concentration of sucrose in a particular reaction volume. For example, an incompletely refined sucrose composition from sugar beets (e.g., beet thin juice, beet thick juice, molasses) could be diluted into a reaction solution such that the initial sucrose concentration of the reaction is about 70-90 g/L or 80-85 g/L.

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. A suitable amount of DTT (dithiothreitol, e.g., about 1.0 mM) can optionally be added to a reaction solution.

A reaction solution herein may be contained within any vessel suitable for applying one or more of the reaction conditions disclosed herein. For example, a reaction solution herein may be in a stainless steel, plastic, or glass vessel or

container of a size suitable to contain a particular reaction. Such a vessel can optionally be equipped with a stirring device.

Examples of other conditions and components suitable for carrying out a reaction solution herein are disclosed in U.S. Patent No. 7000000, and U.S. Pat. Appl. Publ. Nos. 2013/0244288, 2013/0244287, 2013/0196384, 2013/0157316, and 2014/0087431, all of which are incorporated herein by reference.

The yield of poly alpha-1,3-glucan by a reaction solution of the present disclosure is at least 7% of the weight of sucrose that was converted to products in the reaction solution. Alternatively, the yield of poly alpha-1,3-glucan can be at least about 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, or 47%. In certain embodiments, the yield of poly alpha-1,3-glucan in a reaction solution herein is about the same as the yield of poly alpha-1,3-glucan by a control reaction solution in which white refined sucrose is used instead of incompletely refined sucrose. All the foregoing yields can be obtained using a reaction solution maintained at a temperature of about 20-30 °C (e.g., 25 °C) and/or using a gtf comprising SEQ ID NO:8, for example. Certain of these embodiments may use thick beet juice or thin beet juice as incompletely refined sucrose.

In certain embodiments, the relative reaction rate of a reaction solution is at least about 0.8 with respect to the reaction rate of a reaction solution comprising water, white refined sucrose and a glucosyltransferase enzyme. For example, the relative reaction rate of a reaction solution is at least about 0.8 with respect to a control reaction (i.e., the reaction rate of a reaction solution herein is at least 80% of the rate of a control reaction). The relative reaction rate herein can alternatively be at least about 0.82, 0.84, 0.86, 0.88, 0.90, 0.92, 0.94, 0.96, 0.98, 1.00, 1.02, or 1.04, for example. The reaction rate of a reaction solution can be expressed in terms the change in concentration/amount of reactant(s) (e.g., sucrose) and/or the change in concentration/amount of product(s) (e.g.,

poly alpha-1,3-glucan) per unit time per unit concentration of active glucosyltransferase enzyme.

A reaction solution herein can produce poly alpha-1,3-glucan having an *L** value less than 93, for example. Alternatively, the *L** value of poly alpha-1,3-glucan produced by a reaction solution herein can be less than 92, 90, 88, 86, 84, 82, 80, 78, 76, 74, 72, 70, 68, 66, 64, 62, or 60. Examples of ranges of *L** values of poly alpha-1,3-glucan products herein can be about 82-87 (e.g., when beet thin juice or beet thick juice is used in a reaction solution) or 80-82 (e.g., when VHP sucrose is used in a reaction solution). *L** values can be determined, for example, for poly alpha-1,3-glucan that has been removed from a reaction solution, washed with at least one half reaction volume of water in two displacement washes (e.g., wash with at least one 1-L of water if the reaction volume was 2 L), and then dried, ground and sieved through 60-mesh sieve. Drying should be performed at a temperature that does not discolor the poly alpha-1,3-glucan. Thus, any color in the poly alpha-1,3-glucan should be derived from the incompletely refined sucrose.

A reaction solution herein produces poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages. It is believed that in certain embodiments poly alpha-1,3-glucan is produced in which at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% (or any integer between 60% and 100%) of the constituent glycosidic linkages are alpha-1,3 linkages. Accordingly, the poly alpha-1,3-glucan produced in the foregoing embodiments has less than about 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, or 0% (or any integer value between 0% and 50%) glycosidic linkages that are not alpha-1,3.

The glycosidic linkage profile of a poly alpha-1,3-glucan product herein can be determined using any method known in the art. For example, linkage profile can be determined using methods that use nuclear magnetic resonance (NMR) spectroscopy (e.g., ^{13}C NMR or ^{1}H NMR). These and other methods that can be used are disclosed in Food Carbohydrates: Chemistry, Physical Properties, and Applications (S. W. Cui, Ed., Chapter 3, S. W. Cui, Structural

Analysis of Polysaccharides, Taylor & Francis Group LLC, Boca Raton, FL, 2005), which is incorporated herein by reference.

A reaction solution herein produces poly alpha-1,3-glucan having a molecular weight in DP_n or DP_w of at least about 100. Alternatively, poly alpha-1,3-glucan produced in a reaction solution herein can have a molecular weight in DP_n or DP_w of at least about 400. Alternatively still, the poly alpha-1,3-glucan can have a molecular weight in DP_n or DP_w of at least about 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 (or any integer between 100 and 1000).

The molecular weight of poly alpha-1,3-glucan herein can be measured using any of several means known in the art. For example, glucan polymer molecular weight can be measured using high-pressure liquid chromatography (HPLC), size exclusion chromatography (SEC), or gel permeation chromatography (GPC).

The present disclosure also concerns a method for producing poly alpha-1,3-glucan comprising the step of contacting at least water, sucrose, and a glucosyltransferase enzyme, wherein the sucrose is unrefined or partially refined (i.e., not completely refined). The poly alpha-1,3-glucan produced in this method, which can optionally be isolated, has at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100. Further, the yield of poly alpha-1,3-glucan produced in the method is at least 7% of the weight of the sucrose that was converted to products by being contacted with the water and glucosyltransferase enzyme. Any of the features of a reaction solution herein as disclosed above and in the Examples can characterize this method. The following features of the method are examples.

Incompletely refined sucrose in certain embodiments of the method can be from sugar beet (e.g., beet thin juice or beet thick juice) and has not been crystallized. In another example, incompletely refined sucrose is from sugar cane (e.g., raw sucrose, VHP, or VVHP sucrose) and has had no more than two or three crystallization steps.

Incompletely refined sucrose used in the disclosed method can have an ICUMSA value greater than 150, for example.

Poly alpha-1,3-glucan produced in certain embodiments of the method has an *L** value less than 93. *L** values herein can be determined, for example, with respect to poly alpha-1,3-glucan that has been removed from a reaction solution; optionally washed one, two, or more times with water; and dried.

The relative reaction rate of producing poly alpha-1,3-glucan in the contacting step of the method is at least 0.8 with respect to the reaction rate if white refined sucrose is used instead of incompletely refined sucrose.

The disclosed method comprises contacting at least water, incompletely refined sucrose, and a glucosyltransferase enzyme. This contacting step can comprise providing a reaction solution comprising water, incompletely refined sucrose and a glucosyltransferase enzyme. It will be understood that, as the glucosyltransferase enzyme synthesizes poly alpha-1,3-glucan, the reaction solution becomes a reaction mixture given that insoluble poly alpha-1,3-glucan falls out of solution as indicated by clouding of the reaction. The contacting step of the disclosed method can be performed in any number of ways. For example, the desired amount of incompletely refined sucrose can first be dissolved or mixed in water (optionally, other components may also be added at this stage of preparation, such as buffer components), followed by addition of a glucosyltransferase enzyme. The solution may be kept still, or agitated via stirring or orbital shaking, for example. The reaction can be, and typically is, cell-free.

Completion of a reaction in certain embodiments can be determined visually (no more accumulation of insoluble poly alpha-1,3-glucan) and/or by measuring the amount of sucrose left in the solution (residual sucrose), where a percent sucrose consumption of over about 90% can indicate reaction completion. Typically, a reaction of the disclosed process will take about 12, 24, 36, 48, 60, 72, 84, or 96 hours to complete, depending on certain parameters such as the amount of sucrose and glucosyltransferase enzyme used in the reaction.

The percent sucrose consumption of a reaction in certain embodiments of the disclosed method is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the sucrose initially contacted with water and a glucosyltransferase enzyme. Alternatively, the percent sucrose consumption may be >90% or >95%.

The yield of poly alpha-1,3-glucan produced in some aspects of a glucan synthesis method herein can be at least about 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, or 47% based on the weight of sucrose converted in the reaction.

Poly alpha-1,3-glucan produced in the disclosed method may optionally be isolated. For example, insoluble poly alpha-1,3-glucan may be separated by centrifugation or filtration. In doing so, poly alpha-1,3-glucan is separated from most of the reaction solution, which may comprise water, fructose and certain byproducts (e.g., leucrose, soluble oligosaccharides DP2-DP7). This solution may also comprise residual sucrose and glucose monomer. Isolation can optionally further comprise washing the poly alpha-1,3-glucan one, two, or more times with water, and/or drying the poly alpha-1,3-glucan.

The present disclosure also concerns poly alpha-1,3-glucan produced by a reaction solution or method disclosed herein. This poly alpha-1,3-glucan has an *L** value that is less than 93. Any of the features of poly alpha-1,3-glucan as disclosed above and in the Examples can characterize poly alpha-1,3-glucan of this embodiment. The following features are examples.

A poly alpha-1,3-glucan product herein may be isolated, and can additionally be provided in a dry form, for example. In certain embodiments, a poly alpha-1,3-glucan product is provided in an isolated, dry amount of at least 1 gram (e.g., at least 100 g or 500 g). “Dry” poly alpha-1,3-glucan comprises no more than 2.0, 1.5, 1.0, 0.5, 0.25, 0.10, 0.05, or 0.01 wt% water, for example.

It is believed that a poly alpha-1,3-glucan product in certain embodiments

may contain one or more of the following compounds: caramels, melanoidins, hexose alkaline degradation products (HADPs) (polymeric C6 sugar condensation products formed under alkaline conditions), polyphenol-iron complexes (e.g., iron catechol complexes), melanins. One or more of these compounds are further believed to provide darker coloration to the poly alpha-1,3-glucan product, compared to the coloration, if any, of a poly alpha-1,3-glucan product rendered by a reaction solution in which only white refined sucrose is used. Such coloration differences can be determined using *L** values, for example.

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

1. A reaction solution comprising water, sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100, wherein the sucrose is unrefined or partially refined;
wherein the yield of poly alpha-1,3-glucan by the reaction solution is at least 7% of the weight of sucrose that was converted to products in the reaction solution.
2. The reaction solution of embodiment 1, wherein the sucrose is from sugar beet and has not been crystallized.
3. The reaction solution of embodiment 1, wherein the sucrose is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.
4. The reaction solution of embodiment 1, 3, or 3, wherein the sucrose has an ICUMSA value greater than 150.
5. The reaction solution of embodiment 1, 2, 3, or 4, wherein the relative reaction rate of the reaction solution is at least 0.8 with respect to the reaction rate of a reaction solution comprising water, white refined sucrose and the glucosyltransferase enzyme.

6. The reaction solution of embodiment 1, 2, 3, 4, or 5, wherein the poly alpha-1,3-glucan produced by the reaction solution has an *L** value less than 93.

7. The reaction solution of embodiment 1, 2, 3, 4, 5, or 6, wherein the glucosyltransferase enzyme comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

8. A method for producing insoluble poly alpha-1,3-glucan comprising:

(a) contacting at least water, sucrose, and a glucosyltransferase enzyme, wherein the sucrose is unrefined or partially refined, whereby poly alpha-1,3-glucan is produced having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100; and

b) optionally, isolating the poly alpha-1,3-glucan produced in step (a);

wherein the yield of poly alpha-1,3-glucan is at least 7% of the weight of sucrose converted to products in step (a).

9. The method of embodiment 8, wherein the sucrose is from sugar beet and has not been crystallized.

10. The method of embodiment 8, wherein the sucrose is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.

11. The method of embodiment 8, 9, or 10, wherein the sucrose has an ICUMSA value greater than 150.

12. The method of embodiment 8, 9, 10, or 11, wherein the relative reaction rate of producing poly alpha-1,3-glucan in step (a) is at least 0.8 with respect to the reaction rate of step (a) if white refined sucrose is used instead of the unrefined or partially refined sucrose.

13. The method of embodiment 8, 9, 10, 11, or 12, wherein the poly alpha-1,3-glucan isolated in step (b) has an *L** value less than 93.

14. The method of embodiment 8, 9, 10, 11, 12, or 13, wherein the glucosyltransferase enzyme comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

15. Isolated poly alpha-1,3-glucan produced by the method of claim 8, 9, 10, 11, 12, 13, or 14, wherein the poly alpha-1,3-glucan has an *L** value less than 93.

EXAMPLES

The present disclosure is further exemplified in Examples 2-12 provided below. It should be understood that these Examples, while indicating certain preferred aspects herein, 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 the disclosed embodiments, and without departing from the spirit and scope thereof, can make various changes and modifications to adapt the disclosed embodiments to various uses and conditions.

GENERAL METHODS

Sucrose ICUMSA Measurement

ICUMSA measurements were made closely following ICUMSA Method GS1/3-7 (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 essential steps of ICUMSA color measurement of a sucrose sample was as follows. Sucrose was added to deionized water (dissolved if in solid form such as cane sugar, diluted if in liquid form such as beet juice) to a specified concentration based on expected ICUMSA range, as specified in ICUMSA Method GS1/3-7. The sucrose solution was filtered to remove any undissolved impurities and the absorbance of the filtered sucrose solution was measured. The ICUMSA color of the solution was then calculated according to Equation 1:

$$Color\,\mathrm{ICUMSA}(UI) = \frac{Abs}{bxc} x1000$$

where Abs = sample solution absorbance reading; b = Optical cell path (cm); c = sucrose concentration in g/mL.

The above ICUMSA method follows ICUMSA Method GS1/3-7, but with the following modifications:

1. Cellulose acetate 0.45-micron filters (square 50 mm) were used instead of cellulose nitrate 0.45-micron filters.

2. Instead of calculating RDS (“refractometric dry substance”) and density, sucrose concentration of a sample was determined by the following steps: Effective ppt salinity was measured using a refractometer and converted to %Brix using a linear relationship obtained from published data (%Brix = 0.1258 ppt salinity + 0.0152). The refractive index measurements were performed on additionally diluted samples and the

Brix values converted back to standard concentrations used in the UV measurement.

Deionized water was used for sucrose dissolution. Sucrose solution samples were not de-aerated as no foam or bubbles were observed in the solutions. Cellulose acetate filters were pre-housed in a plastic funnel for sterifilter applications.

*L*a*b** Color Measurement of Poly Alpha-1,3-Glucan

Poly alpha-1,3-glucan color was measured using the CIE *L*a*b** measurement system. Two sample preparation methods were used depending on the amount of poly alpha-1,3-glucan available. The two methods gave equivalent *L*a*b** measurements.

In the first method, dried poly alpha-1,3-glucan was ground in a coffee grinder to a fine powder. 0.77 g of powder was transferred to an evacuable 13-mm KBr Pellet Die and the poly alpha-1,3-glucan was formed into a pellet at 7000 pounds. The color of the pellet was measured using a Konica Minolta 2600D spectrophotometer.

In the second method, dried poly alpha-1,3-glucan was ground in a coffee grinder to a fine powder. Ground poly alpha-1,3-glucan was sieved through a 60-mesh screen and filled into a 1-cm cuvette. The color of the ground poly alpha-1,3-glucan was measured using a HUNTERLAB COLORQUEST XE spectrophotometer.

Preparation of Crude Extracts of Glucosyltransferase (gtf) Enzyme

Gtf enzymes (e.g., SEQ ID NO:8)) were prepared as follows. *E. coli* TOP10® cells (Invitrogen, Carlsbad California) were transformed with a pJexpress404®-based construct containing a particular gtf-encoding DNA sequence. Each sequence was codon-optimized to express the gtf enzyme in *E. coli.* Individual *E. coli* strains expressing a particular gtf enzyme were grown in LB (Luria broth) medium (Becton, Dickinson and Company, Franklin Lakes, NJ) with ampicillin (100 μg/mL) at 37 °C with shaking to OD_{600} = 0.4-0.5, at which time IPTG (isopropyl beta-D-1-thiogalactopyranoside, Cat. No. I6758, Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 0.5 mM. The

cultures were incubated for 2-4 hours at 37 °C following IPTG induction. Cells were harvested by centrifugation at 5,000 x *g* for 15 minutes and resuspended (20% w/v) in 50 mM phosphate buffer pH 7.0 supplemented with dithiothreitol (DTT, 1.0 mM). Resuspended cells were passed through a French Pressure Cell (SLM Instruments, Rochester, NY) twice to ensure >95% cell lysis. Lysed cells were centrifuged for 30 minutes at 12,000 x *g* at 4 °C. The resulting supernatant was analyzed by the BCA (bicinchoninic acid) protein assay (Sigma-Aldrich) and SDS-PAGE to confirm expression of the gtf enzyme, and the supernatant was stored at -20 °C.

Relative Reaction Rate of Gtf

The enzymatic reaction of sucrose to poly alpha-1,3-glucan by glucosyltransferase follows Michaelis-Menten kinetics. At high sucrose concentrations the reaction rate is zeroth order in sucrose. The concentration of sucrose was measured by HPLC periodically throughout the reaction. The reaction rate was calculated as the rate of sucrose consumption during the zeroth order reaction. That is, the reaction rate was calculated as the negative of the slope of the linear region of a sucrose concentration vs. time graph. The reaction rate was then divided by the enzyme activity loaded to the reactor to give a normalized reaction rate, which eliminated reaction rate differences due to variations in enzyme concentration. Finally the normalized reaction rate was divided by the normalized reaction rate for white refined sugar to give Relative Reaction Rate.

Determination of Gtf Enzymatic Activity

Gtf enzyme (e.g., SEQ ID NO:8) activity was confirmed following a protocol such as the following, which measures the production of reducing sugars (fructose and glucose) in a gtf reaction solution. A reaction solution is prepared by adding a crude gtf extract to a mixture containing sucrose (50 or 150 g/L), potassium phosphate buffer (pH 6.5, 50 mM), and optionally dextran (1 mg/mL, dextran T10, Cat. No. D9260, Sigma-Aldrich); the gtf extract is added to 2.5%-5% by volume. The reaction solution is then incubated at 22-25 °C for 24-30 hours, after which it is centrifuged. Supernatant (0.01 mL) is added to a

mixture containing 1 N NaOH and 0.1% triphenyltetrazolium chloride (Sigma-Aldrich). The mixture is incubated for five minutes after which its OD_{480nm} is determined using an ULTROSPEC spectrophotometer (Pharmacia LKB, New York, NY) to gauge the presence of the reducing sugars fructose and glucose.

Determination of Weight Average Degree of Polymerization (DP_W)

The DP_W of a glucan product synthesized by a gtf enzyme (e.g., SEQ ID NO:8) was determined by size-exclusion chromatography (SEC). An example SEC protocol is as follows. Dry poly alpha-1,3-glucan polymer is dissolved at 5 mg/mL in N,N-dimethyl-acetamide (DMAc) and 5% LiCl with overnight shaking at 100 °C. The SEC system is an Alliance™ 2695 separation module from Waters Corporation (Milford, MA) coupled with three on-line detectors: a differential refractometer 2410 from Waters, a multiangle light scattering photometer Heleos™ 8+ from Wyatt Technologies (Santa Barbara, CA), and a differential capillary viscometer ViscoStar™ from Wyatt. The columns used for SEC are four styrene-divinyl benzene columns from Shodex (Japan) and two linear KD-806M, KD-802 and KD-801 columns to improve resolution at the low molecular weight region of a polymer distribution. The mobile phase is DMAc with 0.11% LiCl. The chromatographic conditions used are 50 °C in the column and detector compartments, 40 °C in the sample and injector compartment, a flow rate of 0.5 mL/min, and an injection volume of 100 μL. The software packages used for data reduction are Empower™ version 3 from Waters (calibration with broad glucan polymer standard) and Astra® version 6 from Wyatt (triple detection method with column calibration).

Determination of Glycosidic Linkages

Glycosidic linkages in a glucan product synthesized by a gtf enzyme (e.g., SEQ ID NO:8) can be determined by following a ^{13}C NMR (nuclear magnetic resonance) such as the following. Dry glucan polymer (25-30 mg) is dissolved in 1 mL of deuterated dimethyl sulfoxide (DMSO) containing 3% by weight of LiCl with stirring at 50 °C. Using a glass pipet, 0.8 mL of the solution is transferred into a 5-mm NMR tube. A quantitative ^{13}C NMR spectrum is acquired using a Bruker Avance 500-MHz NMR spectrometer (Billerica, MA) equipped with a

CPDUL cryoprobe at a spectral frequency of 125.76 MHz, using a spectral window of 26041.7 Hz. An inverse gated decoupling pulse sequence using waltz decoupling is used with an acquisition time of 0.629 second, an inter-pulse delay of 5 seconds, and 6000 pulses. The time domain data is transformed using an exponential multiplication of 2.0 Hz.

EXAMPLE 1 (Comparative)

Preparation of Poly Alpha-1,3-Glucan Using White Refined Sucrose

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using white refined sucrose.

An 80 g/L sucrose solution was prepared as follows. 1500 g of deionized water was charged to a jacketed, agitated 2-L glass reactor controlled at 23 °C. 2.7 g of KH_2PO_4 buffer was added to the reactor. Next, 160 g of white refined sucrose (ICUMSA 47; United Sugars Corporation, Bloomington, MN) was added to the reactor, afterwhich the volume in the reactor was adjusted to 2 L with more deionized water. FermaSure® (DuPont) was then added (1 mL/L reaction), and the pH was adjusted to 5.5 using 5 wt% aqueous sodium hydroxide or 5 wt% aqueous sulfuric acid. The glucan polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:8) extract (General Methods), and maintained at 23 °C.

After the reaction was determined to be complete by either complete consumption of sucrose or no change in sucrose concentration between measurements, 200 mL of the reaction slurry was filtered using a FILTRATEST (Bokela GmbH Karlsruhe, Germany). This filtration separated mother liquor (filtrate) from poly alpha-1,3-glucan wet cake. Residual sugars in the wet cake were washed out with two displacement washes (200-L each) of deionized water. The wet cake was then dried in a convection oven at 80 °C for approximately 24 hours. The polymerization yield was calculated using the final weight of dried polymer divided by the amount of sucrose reacted.

The molecular weight of the poly alpha-1,3-glucan product was measured by SEC (General Methods) and is presented as DP_W (Table 2), which can be calculated as the average polymer molecular weight divided by the monomer

molecular weight. The color of the dried glucan polymer product was measured according to the General Methods and is presented as *L** in Table 2.

EXAMPLE 2

Preparation of Poly Alpha-1,3-Glucan Using VHP Sucrose

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using VHP, which is a type of incompletely refined sucrose.

The polymerization procedure of Example 1 was followed except that VHP sucrose (ICUMSA 501, Iracema Mill, Brazil) was used instead of white refined sucrose.

After the reaction was determined to be complete by either complete consumption of sucrose or no change in sucrose concentration between measurements, the reaction slurry was filtered using a Buchner funnel and vacuum flask. This filtration separated mother liquor (filtrate) from poly alpha-1,3-glucan wet cake. Residual sugars in the wet cake were washed out with two displacement washes (1-L each) of deionized water. The wet cake was then dried in a vacuum oven at 40 °C and 360 mm Hg for approximately 48 hours. The polymerization yield was calculated using the final weight of dried polymer divided by the amount of sucrose reacted. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 3

Preparation of Poly Alpha-1,3-Glucan Using VVHP Sucrose

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using VVHP, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 2 were followed except that VVHP sucrose (ICUMSA 421, Ferrari Mill, Brazil) was used instead of VHP sucrose. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 4

Preparation of Poly Alpha-1,3-Glucan Using Beet Thick Juice

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using beet thick juice, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 2 were generally followed except that beet thick juice sucrose (ICUMSA 1414, Southern Minnesota Beet Sugar Cooperative) was used instead of VHP sucrose. The polymerization procedure was conducted as follows. 235 g of beet thick juice was added to the reactor and diluted with deionized water until the sucrose concentration was 80 g/L (approximately 1765 mL of water was added). 2.72 g of KH_2PO_4 buffer was added and the pH was adjusted to 5.5 with 5 wt% sodium hydroxide. The glucan polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:8) extract (General Methods), and maintained at 23 °C. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 5

Preparation of Poly Alpha-1,3-Glucan Using Beet Thin Juice

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using beet thin juice, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 2 were generally followed except that beet thin juice sucrose (ICUMSA 1158, Southern Minnesota Beet Sugar Cooperative) was used instead of VHP sucrose and a one 1-L wash followed by a 500-mL water wash was used instead of two 1-L washes. 1229 mL of beet thin juice was added to 771 mL of deionized water to prepare a starting sucrose concentration of 80 g/L. 2.72 g of KH_2PO_4 buffer was added and the pH was adjusted to 5.5 using 5 wt% sulfuric acid. The glucan polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:8) extract (General Methods), and maintained at 23 °C. The molecular weight, yield

and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 6

Preparation of Poly Alpha-1,3-Glucan Using Beet Molasses

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using beet molasses, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 2 were generally followed except that beet molasses (ICUMSA 57781, Southern Minnesota Beet Sugar Cooperative) was used instead of VHP sucrose and three 1-L water washes were used instead of two 1-L washes. 291 mL of beet molasses was added to 1709 mL of deionized water to prepare a starting sucrose concentration of 83.4 g/L. 2.72 g of KH_2PO_4 buffer was added and the pH was adjusted to 5.5 using 5 wt% sodium hydroxide. The glucan polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:8) extract (General Methods), and maintained at 23 °C. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 7

Preparation of Poly Alpha-1,3-Glucan Using Brazil Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using Brazil raw cane sugar, which is a type of incompletely refined sucrose.

In a 1-liter Erlenmeyer flask, 75 g of Brazil raw cane sugar (ICUMSA 2655) was dissolved in approximately 500 mL of deionized water. 1.02 g KH_2PO_4 and 0.15 mL of FermaSure® were added, afterwhich water was added to a volume of 750 mL. The pH was adjusted to 5.5 using 5 wt% sodium hydroxide. The flask was placed in an incubation stirrer oven at 25 °C. The glucan

polymerization reaction was initiated by adding 0.3 vol% of crude gtf enzyme (SEQ ID NO:8) extract (General Methods), and maintained at 25 °C. The reaction was completed in 30 hours. After reaction completion, the reaction was filtered with a Buchner funnel and vacuum flask. The resulting cake was displacement-washed with two 800-mL water washes and one 200-mL water wash. The poly alpha-1,3-glucan was dried in a vacuum oven at 40 °C. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 8

Preparation of Poly Alpha-1,3-Glucan Using New Orleans Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using New Orleans raw cane sugar, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 7 were followed except that New Orleans raw cane sugar (ICUMSA 2850) was used as the sucrose component. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 9

Preparation of Poly Alpha-1,3-Glucan Using Mozambique Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using Mozambique raw cane sugar, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 7 were followed except that Mozambique raw cane sugar (ICUMSA 3022) was used as the sucrose component. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 10

Preparation of Poly Alpha-1,3-Glucan Using Zimbabwe Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using Zimbabwe raw cane sugar, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 7 were followed except that Zimbabwe raw cane sugar (ICUMSA 4183) was used as the sucrose component. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 11

Preparation of Poly Alpha-1,3-Glucan Using Belize Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using Belize raw cane sugar, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 7 were followed except that Belize raw cane sugar (ICUMSA 5150) was used as the sucrose component. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

EXAMPLE 12

Preparation of Poly Alpha-1,3-Glucan Using Guyana Raw Cane Sugar

This Example describes producing alpha-1,3-glucan in a gtf-catalyzed reaction using Guyana raw cane sugar, which is a type of incompletely refined sucrose.

The polymerization and polymer isolation procedures of Example 7 were followed except that Guyana raw cane sugar (ICUMSA 8153) was used as the

sucrose component. The molecular weight, yield and color of poly alpha-1,3-glucan prepared in this procedure are presented in Table 2.

Thus, poly alpha-1,3-glucan can be synthesized in a gtf reaction solution comprising incompletely refined sucrose.

Table 2

Poly Alpha-1,3-Glucan Produced Using Various Types of Incompletely Refined Sucrose

Example	Sucrose Used In Reaction			Relative Reaction Rate[a]	Poly Alpha-1,3-Glucan Product		
	Source	Type	ICUMSA		DP_W	*L**	Yield
1	United Sugars Corporation	White Refined	47	1.00	708	93.9[b]	18%
2	Iracema Mill, Brazil	VHP	501	0.88	765	78	21%
3	Ferrari Mill, Brazil	VVHP	421	0.88	876	88	17%
4	Southern Minnesota Beet Sugar Cooperative	Beet Thick Juice	1414	0.97	760	86	21%
5	Southern Minnesota Beet Sugar Cooperative	Beet Thin Juice	1158	1.01	781	83	13%
6	Southern Minnesota Beet Sugar Cooperative	Beet Molasses	57781	0.92	756	65	8%
7	Brazil	Raw Cane Sugar	2655	1.00	565	78	16%
8	New Orleans	Raw Cane Sugar	2850	0.92	573	86	17%
9	Mozambique	Raw Cane Sugar	3022	1.01	532	82	16%
10	Zimbabwe	Raw Cane Sugar	4183	0.88	553	71	16%
11	Belize	Raw Cane Sugar	5150	0.74	721	62	11%
12	Guyana	Raw Cane Sugar	8153	0.95	577	62	16%

[a] Relative rates of the reactions in Examples 2-12 were calculated with respect to the rate of the reaction in Example 1.

[b] This *L** was determined for polymer produced following the Example 1 procedure, except that a 500-mL reaction was used instead of 2-L and the product was dried in an oven at 40 °C and 360 mm Hg for approximately 48 hours.

The data in Table 2 generally indicate that gtf reaction solutions comprising incompletely refined sucrose (Examples 2-12) can perform at the same level of, or even better than, gtf reaction solutions comprising white refined sucrose (Example 1). For the most part, reactions containing incompletely refined sucrose had reaction rates that were nearly or completely equivalent with the rate of a reaction containing white refined sucrose. Also, several reactions containing incompletely refine sucrose produced glucan with DP_W and/or yield greater than what was observed using white refined sucrose (e.g., Examples 2-6).

Thus, multiple different types of incompletely refined sucrose can be used to enzymatically produce poly alpha-1,3-glucan.

CLAIMS

What is claimed is:

1. A reaction solution comprising water, sucrose, and a glucosyltransferase enzyme that synthesizes insoluble poly alpha-1,3-glucan having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100, wherein the sucrose is unrefined or partially refined;
wherein the yield of poly alpha-1,3-glucan by the reaction solution is at least 7% of the weight of sucrose that was converted to products in the reaction solution.

2. The reaction solution of claim 1, wherein the sucrose is from sugar beet and has not been crystallized.

3. The reaction solution of claim 1, wherein the sucrose is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.

4. The reaction solution of claim 1, wherein the sucrose has an ICUMSA value greater than 150.

5. The reaction solution of claim 1, wherein the relative reaction rate of said reaction solution is at least 0.8 with respect to the reaction rate of a reaction solution comprising water, white refined sucrose and said glucosyltransferase enzyme.

6. The reaction solution of claim 1, wherein the poly alpha-1,3-glucan produced by the reaction solution has an *L** value less than 93.

7. The reaction solution of claim 1, wherein the glucosyltransferase enzyme comprises an amino acid sequence that is at least 90% identical to SEQ

ID NO:4, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

8. A method for producing insoluble poly alpha-1,3-glucan comprising:
   (a) contacting at least water, sucrose, and a glucosyltransferase enzyme, wherein the sucrose is unrefined or partially refined, whereby poly alpha-1,3-glucan is produced having at least 50% alpha-1,3 glycosidic linkages and a weight average degree of polymerization (DP_w) of at least 100; and
   b) optionally, isolating the poly alpha-1,3-glucan produced in step (a);
wherein the yield of poly alpha-1,3-glucan is at least 7% of the weight of sucrose converted to products in step (a).

9. The method of claim 8, wherein the sucrose is from sugar beet and has not been crystallized.

10. The method of claim 8, wherein the sucrose is from sugar cane and (i) has not been crystallized, or (ii) has been crystallized using no more than three crystallization steps.

11. The method of claim 8, wherein the sucrose has an ICUMSA value greater than 150.

12. The method of claim 8, wherein the relative reaction rate of producing poly alpha-1,3-glucan in step (a) is at least 0.8 with respect to the reaction rate of step (a) if white refined sucrose is used instead of said unrefined or partially refined sucrose.

13. The method of claim 8, wherein the poly alpha-1,3-glucan isolated in step (b) has an *L** value less than 93.

14. The method of claim 8, wherein the glucosyltransferase enzyme comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:20, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, or SEQ ID NO:34.

15. Isolated poly alpha-1,3-glucan produced by the method of claim 8, wherein the poly alpha-1,3-glucan has an *L** value less than 93.