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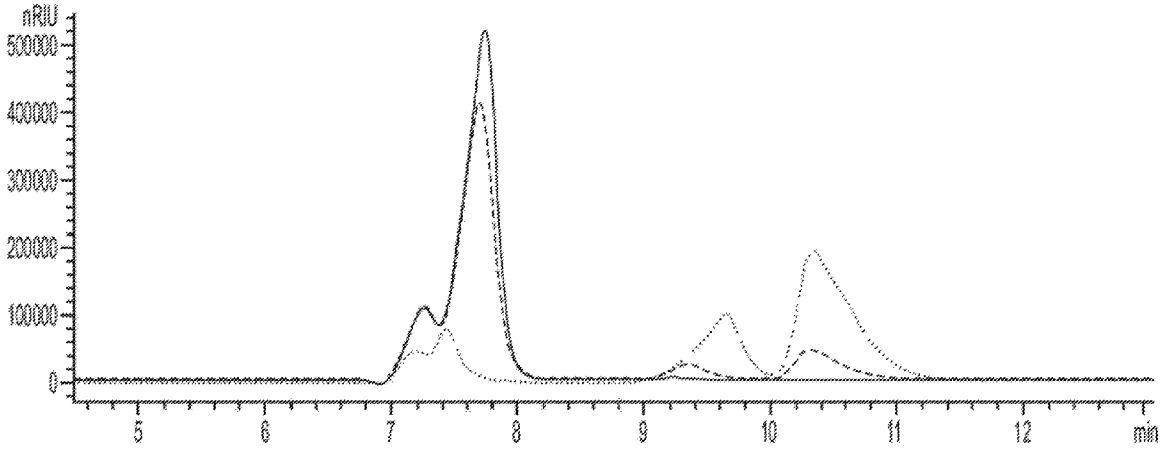
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(54) Title: ENZYMATIC PRODUCTION OF TAGATOSE

FIG. 7



(57) Abrégé/Abstract:
Disclosed herein are improved processes for making tagatose including the steps of converting F6P to T6P, catalyzed by a F6PE; and converting the T6P to tagatose, catalyzed by a T6PP, using enzymes with higher activities compared to F6PEs and T6PPs previously used in a process to produce tagatose.

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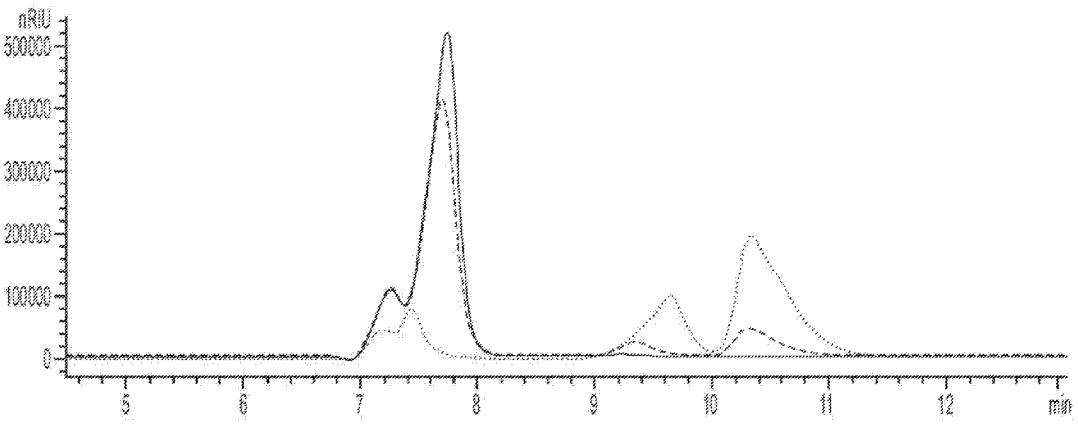


FIG. 7

(57) Abstract: Disclosed herein are improved processes for making tagatose including the steps of converting F6P to T6P, catalyzed by a F6PE; and converting the T6P to tagatose, catalyzed by a T6PP, using enzymes with higher activities compared to F6PEs and T6PPs previously used in a process to produce tagatose.

WO 2020/081959 A1

ENZYMATIC PRODUCTION OF TAGATOSE

Cross Reference to Related Applications

[001] This application claims priority to U.S. Application No. 62/747,877, filed on October 19, 2018, and to U.S. Application No. 62/790,788, filed on January 10, 2019, each herein incorporated by reference.

Field of the Invention

[002] The invention relates to the field of biotechnology pertaining to D-tagatose production. More specifically, the invention provides improved D-tagatose preparation methods capable of enzymatically converting saccharides (e.g., polysaccharides, oligosaccharides, disaccharides, sucrose, D-glucose, and D-fructose) into D-tagatose.

Background

[003] D-tagatose (tagatose hereafter) is a low-calorie, natural sweetener that has 92% the sweetness of sucrose, but only 38% of the calories. Due to its high selling prices, its use as a sweetener has been limited. Tagatose boasts a myriad of health benefits: it is non-cariogenic; it is low-calorie; it has a very low glycemic index of 3; it attenuates the glycemic index of glucose by 20%; it can lower average blood glucose levels; it helps prevent cardiovascular disease, strokes, and other vascular diseases by promoting high-density lipoprotein (HDL) cholesterol; and it is a verified prebiotic and antioxidant. Lu et al., Tagatose, a New Antidiabetic and Obesity Control Drug, *Diabetes Obes. Metab.* 10(2): 109-34 (2008). As such, tagatose clearly has a variety of applications in consumable products and in a variety of industries, such as the pharmaceutical, biotechnological, academic, food, beverage, dietary supplement, and grocery industries.

[004] Tagatose is produced predominantly through the hydrolysis of lactose by lactase to form D-glucose and D-galactose (*see* WO 2011/150556, CN 103025894, US 5,002,612, US 6,057,135, and US 8,802,843). The D-galactose is then isomerized to D-tagatose either chemically by calcium hydroxide under alkaline conditions or enzymatically by L-arabinose isomerase under pH neutral conditions. The final product is isolated by a combination of filtration and ion exchange chromatography. This method suffers because of the costly separation of D-glucose and D-galactose, and low product yields. Several methods via microbial cell fermentation are being developed, but none have been proven to be a

practical alternative due to their dependence on costly feedstock (e.g., galactitol and D-psicose), low product yields, and costly separation. Other processes for preparing tagatose have also been reported. *See e.g.*, Lee et al., Scientific Reports | 7: *1934* | DOI:10.1038/s41598-017-02211-3, pp. 1-8; U.S. Patent Publication No. 2018/0023073; International Patent Application Publication Nos. WO 2014/196811, WO 2018/004310, WO 2018/021894, WO 2018/182344, WO 2018/182345, WO 2018/182354, WO 2018/182355, and WO 2016/064146.

[005] International Patent Application Publication No. WO 2017/059278 recently described the enzymatic synthesis of tagatose, in a process that involves a steps of converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P), catalyzed by an epimerase, fructose 6-phosphate epimerase, and a step of converting the T6P to tagatose, catalyzed by a phosphatase, tagatose 6-phosphate phosphatase. However, despite improvements in enzymatic tagatose production, there is still a desire and need for providing further improved processes of producing tagatose that can, e.g., provide a higher yield with lower amounts of enzymes. There is a strong industrial and commercial interest in decreasing the cost of tagatose production, and this decrease involves the use of a reduced amount of enzymes and use of combinations of enzymes that are more effective than previously used enzymes.

Summary of the Invention

[006] The invention provides improved tagatose preparation methods capable of enzymatically converting saccharides (e.g., polysaccharides, oligosaccharides, disaccharides, sucrose, D-glucose, and D-fructose) into tagatose. In one aspect, an improved process of the invention for the production of tagatose from a saccharide includes a step of converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) wherein the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7. In another aspect, an improved process according to the invention for the production of tagatose from a saccharide includes a step of converting T6P to tagatose using tagatose-6-phoshpate phosphatase (T6PP), wherein the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. In some embodiments of the invention, the improved process includes a step of converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) wherein the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and a step of converting T6P to tagatose using a tagatose-6-phoshpate phosphatase (T6PP), wherein the T6PP

comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

[007] In some improved processes of the invention, the process for preparing tagatose also involves the step of converting glucose 6-phosphate (G6P) to the F6P, where the step is catalyzed by phosphoglucose isomerase (PGI). Some processes according to the invention further include the step of converting glucose 1-phosphate (G1P) to the G6P catalyzed by phosphoglucomutase (PGM). Some process of the invention further include the steps of converting a saccharide to G1P, catalyzed by at least one enzyme.

[008] The saccharides used in any of the processes can be selected from the group consisting of a starch or its derivative, cellulose or its derivative, and sucrose. The starch or its derivative can be amylose, amylopectin, soluble starch, amylodextrin, maltodextrin, maltose, maltotriose, or glucose. In some improved processes of the invention, the process for preparing tagatose involves converting starch to a starch derivative by enzymatic hydrolysis or by acid hydrolysis of starch. In other processes, a starch derivative is prepared by enzymatic hydrolysis of starch catalyzed by isoamylase, pullulanase, alpha-amylase, or a combination of two or more of these enzymes. Some processes of the invention can additionally involve adding 4-glucan transferase (4GT).

[009] Other processes of the invention for preparing tagatose further include a step of converting fructose to F6P, catalyzed by at least one enzyme. Other processes of the invention further include a step of converting sucrose to the fructose, catalyzed by at least one enzyme. G6P to be used in some processes for preparing tagatose can also be generated by converting glucose to the G6P, catalyzed by at least one enzyme. Glucose can in turn be produced by converting sucrose to glucose, catalyzed by at least one enzyme.

[010] Process of the invention are conducted under reaction conditions including at a temperature ranging from about 37˚C to about 85˚C, at a pH ranging from about 5.0 to about 9.0, and/or for about 1 hour to about 48 hours, or as continuous reactions. In some embodiments, the steps of a process for preparing tagatose are conducted under those reaction conditions in one bioreactor. In other embodiments, the steps are conducted under those reaction conditions in a plurality of bioreactors arranged in series.

[011] In some processes of the invention, the steps for preparing tagatose are conducted ATP-free, NAD(H)-free, at a phosphate concentration from about 0.1 mM to about 150 mM, the phosphate is recycled, and/or the step of converting T6P to tagatose involves an energetically favorable chemical

reaction.

Brief Description of the Figures

[012] FIG. 1 is a schematic diagram illustrating an enzymatic pathway converting starch or its derived products to tagatose. The following abbreviations are used: αGP, alpha-glucan phosphorylase or starch phosphorylase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase; IA, isoamylase; PA, pullulanase; MP, maltose phosphorylase; PPGK, polyphosphate glucokinase.

[013] FIG. 2 shows an enzymatic pathway converting cellulose or its derived products to tagatose. CDP, cellodextrin phosphorylase; CBP, cellobiose phosphorylase; PPGK, polyphosphate glucokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase.

[014] FIG. 3 is a schematic diagram illustrating an enzymatic pathway converting fructose to tagatose. PPFK, polyphosphate fructokinase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase.

[015] FIG. 4 is a schematic diagram illustrating an enzymatic pathway converting glucose to tagatose. PPGK, polyphosphate glucokinase; PGI, phosphoglucoisomerase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase.

[016] FIG. 5 shows an enzymatic pathway converting sucrose or its derived products to tagatose. SP, sucrose phosphorylase; PPFK, polyphosphate fructokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase.

[017] FIG. 6 shows the Reaction Gibbs Energy between intermediates based on formation Gibbs energy for the conversion of glucose 1-phosphate to tagatose.

[018] FIG. 7 shows the conversion of F6P to tagatose as described in Example 3 in an HPLC chromatogram. (solid line) 0 hour chromatogram; (dashed line) 2 hour with F6PE (Uniprot ID B5YBD7) and T6PP (Uniprot ID O29805); and (dotted line) 2 hour with the improved process with F6PE (Uniprot ID A0A0P6XN50) and T6PP (Uniprot ID D6YBK5); peaks shown are (1) Void, (2) F6P and T6P, (3) free phosphate, (4) tagatose, and (5) fructose.

Detailed Description

[019] The invention generally relates to improved enzymatic processes for the conversion of saccharides to tagatose. For example, the invention relates to improved processes for the conversion of

saccharides such as starch, cellulose, sucrose, glucose, and fructose and their derived products to tagatose using cell-free enzyme cocktails. In contrast to cell-based manufacturing methods, the invention involves a cell-free preparation of tagatose, has relatively high reaction rates due to the elimination of the cell membrane, which often slows down the transport of substrate/product into and out of the cell. The processes of the invention also result in a final product free of nutrient-rich fermentation media/cellular metabolites.

[020] In one aspect, the invention relates to improved processes for making tagatose including the steps of converting F6P to T6P, catalyzed by a F6PE; and converting the T6P to tagatose, catalyzed by a T6PP, using enzymes with improved activities compared to F6PEs and/or T6PPs previously used in a process to produce tagatose. *See e.g.*, International Patent Application Publication WO2017/059278, disclosing F6PEs and T6PPs: F6PE from *Anaerolinea thermophila* UNI-1 (Uniprot ID E8N0N6); F6PE from *Caldicellulosiruptor kronotskyensis* (Uniprot ID E4SEH3); F6PE from *Caldilinea aerophila* (Uniprot ID I0I507); F6PE from *Caldithrix abyssi* (Uniprot ID H1XRG1); and F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7); T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805); T6PP from *Archaeoglobus profundus* (Uniprot ID D2RHV2_ARCPA); and T6PP from *Archaeoglobus veneficus* (Uniprot ID F2KMK2_ARCVS). Using enzymes with higher activities allows for using lower amounts of enzymes, thereby reducing the cost of the overall process.

[021] In the improved processes of the invention, F6PEs have a higher activity compared to that of the previously disclosed Thermophilic F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7). *See* International Patent Application Publication WO2017/059278. Preferably, F6PEs used in the processes of the invention have an enzymatic activity improved by at least 10%, at least 30%, at least 80%, at least 100%, at least 150%, at least 180% or at least 200%, relative to the activity of Thermophilic F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7). For instance, as shown in Example 1, Thermophilic F6PE from *Thermanaerothrix daxensis* (Uniprot A0A0P6XN50) has enzymatic activity improved by approximately 150% relative to Thermophilic F6PE from *Dictyoglomus thermophilum* (Uniprot ID: B5YBD7), Thermophilic F6PE from *Candidatus Thermofonsia Clade 3* (Uniprot ID A0A2M8QBR9) has enzymatic activity improved by approximately 120% relative to Thermophilic F6PE from *Dictyoglomus thermophilum* (Uniprot ID: B5YBD7), and Thermophilic F6PE from *Thermoanaerobacterium thermosaccharolyticum* (Uniprot A0A223HVJ3) has enzymatic activity improved by approximately 178% relative to Thermophilic F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7). The examples below provide protocols to those skilled in the art for determining activity of F6PEs, which involve incubating the enzyme with its substrate, and then measuring the amounts of reactants and products

via HPLC. Measurements of relative activities any two enzymes are performed under identical reaction conditions such as buffer, pH, temperature, etc.

[022] F6PEs used in processes of the invention are specific for F6P and T6P; the epimerization catalyzed by F6PE is a reversible reaction. Specific means having a higher activity for F6P/T6P over other phosphorylated monosaccharides present in the reaction. For instance, F6PE has a higher epimerization activity on F6P/T6P than on, for example, G6P.

[023] Examples of F6PEs for use in the improved process of the invention include but are not limited to the following proteins: Thermophilic F6PE from *Thermanaerothrix daxensis* (Uniprot ID A0A0P6XN50) with the amino acid sequence as listed in SEQ ID NO: 1; Thermophilic F6PE from *Thermoanaerobacterium thermosaccharolyticum* (Uniprot ID A0A223HVJ3) with the amino acid sequence as listed in SEQ ID NO: 2; Thermophilic F6PE from *Candidatus Thermofonsia Clade 3* (Uniprot ID A0A2M8QBR9), with the amino acid sequence as listed in SEQ ID NO: 7;and F6PEs having at least 90%, at least 95%, at least 97%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7. An improved process for the production of tagatose from a saccharide according to the invention includes the step of converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a F6PE, where the F6PE comprises an amino acid sequence having at least 90%, amino acid sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7.

[024] F6PEs used in processes of the invention are epimerases which can convert F6P to T6P. F6PEs utilize a divalent metal cofactor, such as magnesium, manganese, cobalt, or zinc, preferably magnesium F6PEs for use in processes of the invention comprises an amino acid sequence having at least 90% at least 95%, at least 97%, at least 99%, or 100%, amino acid sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and preferably contain an aldolase-type TIM barrel. *See* Wichelecki et al.,(2015) J. Biol. Chem., v290, pp. 28963-76.

[025] In the improved processes of the invention, T6PPs have a higher activity compared to that of the previously disclosed T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805). *See* International Patent Application Publication WO2017/059278. Preferably, T6PPs used in the processes of the invention have an enzymatic activity improved by at least 10%, at least 30%, at least 80%, at least 100%, at least 150%, at least 300%, at least 500%, at least 600%, at least 900%, at least 1200%, at least 1500%, at least 1800%, or at least 2100% relative to the activity of T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805). For instance, as shown in Example 2, T6PP from *Methanosarcina thermophila CHTI-55* (Uniprot ID A0A0E3NCH4) has enzymatic activity improved by approximately 614% relative to T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805), T6PP from *Thermobispora bispora* strain ATCC 19993

(Uniprot D6YBK5) has enzymatic activity improved by approximately 1328% relative to T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805), T6PP from *Spirochaeta thermophila ATCC 49972* (Uniprot ID E0RT70) has enzymatic activity improved by approximately 2075% relative to T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805), and T6PP from *Sphaerobacter thermophilus DSM 20745* (Uniprot ID D1C7G9) has enzymatic activity improved by approximately 814% relative to T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805). The examples below provide protocols to those skilled in the art for determining activity of T6PPs, which involve incubating the enzyme with its substrate, and then measuring the amounts of reactants and products via HPLC. Measurements of relative activities any two enzymes are performed under identical reaction conditions such as buffer, pH, temperature, etc.

[026] T6PPs used in processes of the invention are specific for T6P. For T6PP, specific means having a higher dephosphorylation activity on T6P over other phosphorylated monosaccharides in the process. For instance, T6PP has a higher dephosphorylation activity on T6P than on, for example G1P, G6P, and F6P.

[027] Examples of T6PPs for use in the processes of the invention include but are not limited to the following proteins: a T6PP from *Methanosarcina thermophila CHTI-55* (Uniprot ID A0A0E3NCH4) with the amino acid sequence as listed in SEQ ID NO: 3; a T6PP from *Thermobispora bispora strain ATCC 19993* (Uniprot ID D6YBK5) with the amino acid sequence as listed in SEQ ID NO: 4; a T6PP from *Spirochaeta thermophila strain ATCC 49972* (Uniprot ID E0RT70) with the amino acid sequence as listed in SEQ ID NO: 5; a T6PP from *Sphaerobacter thermophilus strain DSM 20745* (Uniprot ID D1C7G9) with the amino acid sequence as listed in SEQ ID NO: 6; and T6PPs having at least 90%, at least 95%, at least 97%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. A process according to the invention for the production of tagatose from a saccharide includes converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) and converting the T6P produced to tagatose using a tagatose-6-phoshpate phosphatase (T6PP), where the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

[028] In processes of the invention, T6PPs are phosphatases which convert T6P to tagatose. T6PPs utilize a divalent metal cofactor, such as zinc, manganese, cobalt, or magnesium, preferably magnesium. In processes of the invention, T6PPs have at least 90%, at least 95%, at least 97%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6; and preferably contain a Rossmanoid fold domain for catalysis and additionally, a C1 capping domain for

substrate specificity; a DxD signature in the 1st β-strand of the Rossmanoid fold for coordinating magnesium, where the second Asp is a general acid/base catalyst; a Thr or Ser at the end of the 2nd β-strand of the Rossmanoid fold that helps stability of reaction intermediates; a Lys at the N-terminus of the α-helix C-terminal to the 3rd β-strand of the Rossmanoid fold that helps stability of reaction intermediates; and a E(D/N) signature at the end of the 4th β-strand of the Rossmanoid fold for coordinating divalent metal cations, such as magnesium. *See e.g.*, Burroughs *et al*., Evolutionary Genomics of the HAD Superfamily: Understanding the Structural Adaptations and Catalytic Diversity in a Superfamily of Phosphoesterases and Allied Enzymes. J. Mol. Biol. 2006; 361; 1003–1034.

[029] A preferred enzymatic process according to the invention includes a step of converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) where the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and a step of converting T6P to tagatose using a tagatose-6-phoshpate phosphatase (T6PP), where the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. More preferably, the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 2, and the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 5. Most preferably, the F6PE has the amino acid sequence as listed in SEQ ID NO:2, and the T6PP has the amino acid sequence as listed in SEQ ID NO: 5.

[030] A process for preparing tagatose from a saccharide according to the invention also includes the step of enzymatically converting glucose 6-phosphate (G6P) to the F6P, where the step is catalyzed by phosphoglucose isomerase (PGI). Exemplary PGIs which may be used include those disclosed in International Patent Application Publication WO2017/059278: PGI from *Clostridium thermocellum* (Uniprot ID A3DBX9) and PGI from *Thermus thermophilus* (Uniprot ID Q5SLL6).

[031] A process for preparing tagatose according to the invention additionally includes the step of converting glucose 1-phosphate (G1P) to the G6P, where the step is catalyzed by phosphoglucomutase (PGM). An example of a PGM is PGM from *Thermococcus kodakaraensis (*Uniprot ID Q68BJ6), disclosed in International Patent Application Publication WO2017/059278.

[032] Additionally, the processes according to the invention may further comprise the step of converting a saccharide to the G1P, where the step is catalyzed by at least one enzyme, and the saccharide is selected from the group consisting of a starch or derivative thereof (FIG. 1), cellulose or a derivative thereof (FIG. 2), fructose (FIG. 3), glucose (FIG. 4), and sucrose (FIG. 5). The enzyme or enzymes used in the step of converting a saccharide to the G1P in the processes according to the

invention can be alpha-glucan phosphorylase (αGP), maltose phosphorylase, sucrose phosphorylase, cellodextrin phosphorylase, cellobiose phosphorylase, and/or cellulose phosphorylase, and mixtures thereof. The choice of the enzyme or enzyme combination to arrive at F6P depends on the saccharide used in the process.

[033] Cellulose is the most abundant bio resource and is the primary component of plant cell walls. Non-food lignocellulosic biomass contains cellulose, hemicellulose, and lignin as well as other minor components. Pure cellulose, including Avicel (microcrystalline cellulose), regenerated amorphous cellulose, bacterial cellulose, filter paper, and so on, can be prepared via a series of treatments. The partially hydrolyzed cellulosic substrates include water-insoluble cellodextrins whose degree of polymerization is more than 7, water-soluble cellodextrins with degree of polymerization of 3-6, cellobiose, glucose, and fructose.

[034] In certain processes according to the invention, cellulose and its derived products can be converted to tagatose through a series of steps. See FIG. 2. For example, a process according to the invention provides a pathway that involves the following steps: generating G1P from cellodextrin and cellobiose and free phosphate catalyzed by cellodextrin phosphorylase (CDP) and cellobiose phosphorylase (CBP), respectively; converting G1P to G6P catalyzed by PGM; converting G6P to F6P catalyzed by PGI; converting F6P to tagatose as described above, and the phosphate ions can be recycled by the step of converting cellodextrin and cellobiose to G1P.

[035] Several enzymes may be used to hydrolyze solid cellulose to water-soluble cellodextrins and cellobiose. Such enzymes include endoglucanase and cellobiohydrolase, but not including beta-glucosidase (cellobiase). Prior to cellulose hydrolysis and G1P generation, cellulose and biomass can be pretreated to increase their reactivity and decrease the degree of polymerization of cellulose chains. Cellulose and biomass pretreatment methods include dilute acid pretreatment, cellulose solvent-based lignocellulose fractionation, ammonia fiber expansion, ammonia aqueous soaking, ionic liquid treatment, and partially hydrolyzed by using concentrated acids, including hydrochloric acid, sulfuric acid, phosphoric acid and their combinations.

[036] When the saccharides include cellobiose, and the enzymes contain cellobiose phosphorylase, G1P is generated from cellobiose by cellobiose phosphorylase. When the saccharides contain cellodextrins and the enzymes include cellodextrin phosphorylase, G1P is generated from cellodextrins by cellodextrin phosphorylase. When the saccharides include cellulose, and enzymes contain cellulose phosphorylase, the G1P is generated from cellulose by cellulose phosphorylase.

[037] When the saccharides include maltose and the enzymes contain maltose phosphorylase, the G1P is generated from maltose by maltose phosphorylase. If the saccharides include sucrose, and enzymes contain sucrose phosphorylase, the G1P is generated from sucrose by sucrose phosphorylase.

[038] When the saccharide is starch or a starch derivative, the derivative may be selected from the group consisting of amylose, amylopectin, soluble starch, amylodextrin, maltodextrin, maltose, maltotriose, and glucose, and mixtures thereof. In certain processes of the invention, the enzymes used to convert a saccharide to G1P contain αGP. In this step, when the saccharides include starch, the G1P is generated from starch by αGP; when the saccharides contain soluble starch, amylodextrin, or maltodextrin, the G1P is produced from soluble starch, amylodextrin, or maltodextrin by αGP. An example of αGP is αGP from *Thermotoga maritima (*Uniprot *ID* G4FEH8), disclosed in International Patent Application Publication WO2017/059278.

[039] Some processes according to the invention may further comprise the step of converting starch to a starch derivative, where the starch derivative is prepared by enzymatic hydrolysis of starch or by acid hydrolysis of starch. In certain processes of the invention, maltose phosphorylase (MP) can be used to increase tagatose yields by phosphorolytically cleaving the degradation product maltose into G1P and glucose. Alternatively, 4-glucan transferase (4GT) can be used to increase tagatose yields by recycling the degradation products glucose, maltose, and maltotriose into longer maltooligosaccharides; which can be phosphorolytically cleaved by αGP to yield G1P. An example of 4GT is 4GT from *Thermococcus litoralis* (Uniprot ID O32462), disclosed in International Patent Application Publication WO2017/059278. In some processes of the invention, polyphosphate and polyphosphate glucokinase (PPGK) can be added to the process, thus increasing yields of tagatose by phosphorylating the degradation product glucose to G6P.

[040] Starch is the most widely used energy storage compound in nature and is mostly stored in plant seeds. Natural starch contains linear amylose and branched amylopectin. Examples of starch derivatives include amylose, amylopectin, soluble starch, amylodextrin, maltodextrin, maltose, fructose, and glucose. Examples of cellulose derivatives include pretreated biomass, regenerated amorphous cellulose, cellodextrin, cellobiose, fructose, and glucose. Sucrose derivatives include fructose and glucose.

[041] Where the processes use a starch derivative, the starch derivative can be prepared by enzymatic hydrolysis of starch catalyzed by isoamylase, pullulanase, α-amylase, or their combination. Corn starch contains many branches that impede αGP action. Isoamylase can be used to de-branch starch, yielding linear amylodextrin. Isoamylase-pretreated starch can result in a higher F6P concentration in the final

product. Isoamylase and pullulanase cleave alpha-1,6-glycosidic bonds, which allows for more complete degradation of starch by alpha-glucan phosphorylase. Alpha-amylase cleaves alpha-1,4-glycosidic bonds, therefore alpha-amylase is used to degrade starch into fragments for quicker conversion to tagatose.

[042] Tagatose can also be produced from fructose. *See* FIG. 3. Processes according to the inventions can also comprise the step of converting fructose to F6P, wherein the step is catalyzed by at least one enzyme and, optionally, the step of converting sucrose to the fructose, wherein the step is catalyzed by at least one enzyme. For example, the process involves generating F6P from fructose and polyphosphate catalyzed by polyphosphate fructokinase (PPFK). The conversion of F6P to tagatose is described above. The fructose can be produced, for example, by an enzymatic conversion of sucrose. The phosphate ions generated when T6P is converted to tagatose can then be recycled in the steps of converting sucrose to G1P.

[043] Tagatose can also be produced from glucose. *See* FIG. 4. Processes according to the inventions can also comprise the step of converting glucose to G6P, catalyzed by at least one enzyme, and, optionally, the step of converting sucrose to the fructose, wherein the step is catalyzed by at least one enzyme. For example, the process involves generating G6P from glucose and polyphosphate catalyzed by polyphosphate glucokinase (PPGK). The glucose can be produced, for example, by an enzymatic conversion of sucrose. *See* FIG. 5.

[044] In some methods of the invention, the phosphate ions generated when T6P is converted to tagatose are recycled in the step of converting starch derivatives to G1P (*See, e.g.*, FIG. 1), cellulose derivatives to G1P (*See e.g.*, FIG. 2), or sucrose to G1P (*See* FIG. 5), especially if the process is conducted in a single reaction vessel. Additionally, PPFK and polyphosphate can be used to increase tagatose yields by producing F6P from fructose generated by the phosphorolytic cleavage of sucrose by SP.

[045] Processes for preparing tagatose from a saccharide, for example, include the following steps: (i) converting a saccharide to glucose 1-phosphate (G1P) using one or more enzymes; (ii) converting G1P to G6P using phosphoglucomutase (PGM, EC 5.4.2.2); (iii) converting G6P to F6P using phosphoglucoisomerase (PGI, EC 5.3.1.9); (iv) converting F6P to T6P via fructose 6-phosphate epimerase (F6PE), and (v) converting T6P to tagatose via tagatose 6-phosphate phosphatase (T6PP). In improved processes of the invention, the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and/or the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. In such processes, for example, the enzyme in step (i) is αGP. Typically, the ratios of enzyme units used in the process are 1:1:1:1:1 (αGP:PGM:PGI:F6PE:T6PP). An enzyme unit is the amount of

enzyme needed to convert 1 umol of substrate to product in 1 minute. Accordingly, an enzyme with a higher activity will have a lower amount of enzyme, in terms of mg of enzyme per one enzyme unit, compared to an enzyme with a lower activity which catalyzes the same reaction. To optimize product yields, these ratios can be adjusted in any number of combinations. For example, a particular enzyme may be present in an amount about 2x, 3x, 4x, 5x, etc. relative to the amount of other enzymes.

[046] A process for preparing tagatose according to the invention may include the following additional steps: generating glucose from polysaccharides and oligosaccharides by enzymatic hydrolysis or acid hydrolysis, converting glucose to G6P catalyzed by at least one enzyme, generating fructose from polysaccharides and oligosaccharides by enzymatic hydrolysis or acid hydrolysis, and converting fructose to G6P catalyzed by at least one enzyme. Examples of the polysaccharides and oligosaccharides are enumerated above.

[047] Processes to prepare tagatose according the invention can be conducted in a single bioreactor or reaction vessel. Alternatively, the steps can also be conducted in a plurality of bioreactors, or reaction vessels, that are arranged in series. In a preferred process, the enzymatic production of tagatose is conducted in a single reaction vessel.

[048] The enzymes used in the invention may take the form of soluble, immobilized, assembled, or aggregated proteins. These enzymes could be adsorbed on insoluble organic or inorganic supports commonly used to improve functionality, as known in the art. These include polymeric supports such as agarose, methacrylate, polystyrene, or dextran, as well as inorganic supports such as glass, metal, or carbon-based materials. These materials are often produced with large surface-to-volume ratios and specialized surfaces that promote attachment and activity of immobilized enzymes. The enzymes might be affixed to these solid supports through covalent, ionic, or hydrophobic interactions. The enzymes could also be affixed through genetically engineered interactions such as covalent fusion to another protein or peptide sequence with affinity to the solid support, most often a poly-histidine sequence. The enzymes might be affixed either directly to the surface or surface coating, or they might be affixed to other proteins already present on the surface or surface coating. The enzymes can be immobilized all on one carrier, on individual carriers, or a combination of the two (e.g., two enzyme per carrier then mix those carriers). These variations can be mixed evenly or in defined layers to optimize turnover in a continuous reactor. For example, the beginning of the reactor may have a layer of aGP to ensure a high initial G1P increase. Enzymes may be immobilized all on one carrier, on individual carriers, or in groups. These enzymes may be mixed evenly or in defined layers or zones to optimize turnover.

[049] Any suitable biological buffer known in the art can be used in a process of the invention, such as HEPES, PBS, BIS-TRIS, MOPS, DIPSO, Trizma, etc. The reaction buffer for all embodiments can have a pH ranging from 5.0-9.0. More preferably, the reaction buffer pH can range from about 6.0 to about 7.3. For example, the reaction buffer pH can be 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, or 7.3.

[050] In some improved processes of the invention the reaction buffer contains divalent metal cations. Examples include Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+}, and the like, preferably Mg^{2+} The concentration of divalent metal cations can range from about 0 mM to about 150 mM, from about 0 mM to about 100 mM, from about 1 mM to about 50 mM, preferably from about 5 mM to about 50 mM, or more preferably from about 10 mM to about 50 mM. For instance, the divalent metal cation concentration can be about 0.1 mM, about 0.5 mM, about 1 mM, about 1.5 mM, about 2 mM, about 2.5 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, or about 55 mM.

[051] The reaction temperature at which the process steps are conducted can range from 37-85 ˚C. More preferably, the steps can be conducted at a temperature ranging from about 37˚C to about 85˚C. The temperature can be, for example, about 40˚C, about 45˚C, about 50˚C, about 55˚C, or about 60˚C. Preferably, the reaction temperature is about 50˚C. In some processes of the invention, the reaction temperature is constant, and is not changed during the process.

[052] The reaction time of the disclosed processes can be adjusted as necessary and can range from about 1 hour to about 48 hours. For example, the reaction time can be about 16 hours, about 18 hours, about 20 hours, about 22 hours, about 24 hours, about 26 hours, about 28 hours, about 30 hours, about 32 hours, about 34 hours, about 36 hours, about 38 hours, about 40 hours, about 42 hours, about 44 hours, about 46 hours, or about 48 hours. More preferably, the reaction time is about 24 hours.

[053] The reaction can be run in batch or in a continuous process using a packed bed reactor or similar device. In the continuous process, a solution maltodextrin would be pumped through a bed of immobilized enzyme at such a rate that conversion to tagatose would be complete when the solution leaves the column for downstream processing. For example, 200 g/L of maltodextrin can be pumped through a column packed with immobilized enzymes (maintained at, for example, 50˚C) such that when the maltodextrin leaves the column maximum tagatose yield is achieved. This methodology offers greater volumetric productivity over batch methods. This limits the time our product is in contact with the column and reaction conditions, which decreases chances of product degradation (e.g., potential hydroxymethylfurfural formation). Whether in batch or continuous mode the various steps of processes

of the invention may be conducted using the same reaction conditions as the other steps. For example, in a particular process of the invention using a single bioreactor or reaction vessel, the reaction conditions such as pH and temperature, and reaction buffer are kept constant for all steps of the process.

[054] Phosphate ions produced by T6PP dephosphorylation of T6P can then be recycled in the process step of converting a saccharide to G1P, particularly when all process steps are conducted in a single bioreactor or reaction vessel. The ability to recycle phosphate in the disclosed processes allows for non-stoichiometric amounts of phosphate to be used, which keeps reaction phosphate concentrations low. This affects the overall pathway and the overall rate of the processes, but does not limit the activity of the individual enzymes and allows for overall efficiency of the tagatose making processes.

[055] For example, reaction phosphate concentrations can range from about 0 mM to about 300 mM, from about 0 mM to about 150 mM, from about 1 mM to about 50 mM, preferably from about 5 mM to about 50 mM, or more preferably from about 10 mM to about 50 mM. For instance, the reaction phosphate concentration can be about 0.1 mM, about 0.5 mM, about 1 mM, about 1.5 mM, about 2 mM, about 2.5 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, or about 55 mM.

[056] Therefore, low phosphate concentrations result in decreased production costs due to low total phosphate and thus lowered cost of phosphate removal. It also prevents inhibition of T6PP by high concentrations of free phosphate and decreases the potential for phosphate pollution.

[057] Furthermore, the processes disclosed herein can be conducted without added ATP as a source of phosphate, i.e., ATP-free. The processes can also be conducted without having to add NAD(H), i.e., NAD(H)-free. Other advantages also include the fact that at least one step of the disclosed processes for making tagatose involves an energetically favorable chemical reaction. FIG. 6. While the use of enzymes with higher activities will not affect the overall energetics, the ability to use lower amounts of enzymes in the improved processes is advantageous. The advantage is the reduction of the overall cost of enzyme in the total production cost of the product.

[058] The processes according to the invention can achieve high yields due to the very favorable equilibrium constant for the overall reaction. Theoretically, up to 99% yields can be achieved if the starting material is completely converted to an intermediate. Also, the step of converting T6P to tagatose according to the invention is an irreversible phosphatase reaction, regardless of the feedstock. Therefore, tagatose is produced with a very high yield.

[059] Processes of the invention use low-cost starting materials and reduce production costs by decreasing costs associated with the feedstock and product separation. Starch, cellulose, sucrose and their derivatives are less expensive feedstocks than, for example, lactose. When tagatose is produced from lactose, glucose and galactose and tagatose are separated via chromatography, which leads to higher production costs.

[060] Processes according to the invention allow for easy recovery of tagatose, and separation costs are minimized. Preferably, in processes of the invention, the recovery of tagatose is not via chromatographic separation. Following production of tagatose in a continuous reaction, the product is instead passed through microfiltration, ion exchange (cation then anion, not mixed bed), concentration, crystallization, crystal isolation, and drying. Due to high yields of tagatose, the crystallization step is all that is needed to purify tagatose. To further purify tagatose prior to crystallization, one can employ nanofiltration to eliminate the risk of enzyme being present in the crystallization process and to remove any unconverted dextrins that may co-crystallize with tagatose or limit the recyclability of the mother liquor (maltodextrin, maltotetraose, maltotriose, maltose, etc.).

[061] An improved process for preparing tagatose according to the invention includes the following steps: (i) converting a saccharide to glucose 1-phosphate (G1P) using one or more enzymes; (ii) converting G1P to G6P using phosphoglucomutase (PGM, EC 5.4.2.2); (iii) converting G6P to F6P using phosphoglucoisomerase (PGI, EC 5.3.1.9); (iv) converting F6P to T6P via fructose 6-phosphate epimerase (F6PE), and (v) converting T6P to tagatose via tagatose 6-phosphate phosphatase (T6PP), where the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and/or where the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6. This process is preferably conducted in a single bioreactor or reaction vessel.

[062] Preferably, an improved process for preparing tagatose according to the invention includes the following steps: (i) converting a saccharide to glucose 1-phosphate (G1P) using αGP, where the saccharide is selected from the group consisting of starch, one or more derivatives of starch, or a combination thereof; (ii) converting G1P to G6P using phosphoglucomutase (PGM, EC 5.4.2.2); (iii) converting G6P to F6P using phosphoglucoisomerase (PGI, EC 5.3.1.9); (iv) converting F6P to T6P via fructose 6-phosphate epimerase (F6PE), and (v) converting T6P to tagatose via tagatose 6-phosphate phosphatase (T6PP), where the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7, and/or where the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID

NO: 6. The process is preferably conducted in a single reactor vessel and may incorporate one or more of the various process conditions discussed above.

[063] Examples

[064] Materials and Methods

[065] All chemicals, including glucose 1-phosphate, magnesium chloride, sodium phosphate (mono and dibasic), are reagent grade or higher and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. E. coli BL21 (DE3) (Sigma-Aldrich, St. Louis, MO, USA) was used as a host cell for recombinant protein expression. ZYM-5052 media including 50 mg L-1 kanamycin was used for E. coli cell growth and recombinant protein expression.

[066] Production and purification of recombinant enzymes

[067] The *E. coli* BL21 (DE3) strain harboring a protein expression plasmid (pET28a) was incubated in a 1-L Erlenmeyer flask with 100 mL of ZYM-5052 media containing 50 mg L^{-1} kanamycin. Cells were grown at 37°C with rotary shaking at 220 rpm for 16-24 hours. The cells were harvested by centrifugation at 12°C and washed once with either 20 mM HEPES (pH 7.5) containing 50 mM NaCl and 5 mM $MgCl_2$ (heat precipitation) or 20 mM HEPES (pH 7.5) containing 300 mM NaCl and 5 mM imidazole (Ni purification). The cell pellets were re-suspended in the same buffer and lysed by sonication. After centrifugation, the target proteins in the supernatants were purified. His-tagged proteins were purified by the Profinity IMAC Ni-Charged Resin (Bio-Rad, Hercules, CA, USA). Heat precipitation at 50-80°C for 5-30 min was used to purify thermostable enzymes. The purity of the recombinant proteins was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

[068] Example 1: F6PEs with higher activity

[069] The relative activities of different F6PEs were measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 38.5 mM G1P, 0.05 g/L PGM, 0.05 g/L PGI, 0.05 g/L F6PE, and 0.075 g/L T6PP from *Methanosarcina thermophila* CHTI-55 (Uniprot ID A0A0E3NCH4) at 50°C for 1 hour. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, tagatose, was evaluated using an Agilent Hi-Plex H-column and refractive index detector. The sample was run in 5 mM H_2SO_4 at 0.6 mL/min for 15 min at 65°C. Table 1 shows that F6PEs used in the improved processes of the invention have higher activity relative to the previously disclosed F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7). *See* International Patent Application Publication WO2017/059278.

Table 1: Relative activities of F6PEs

F6PE (Uniprot ID)	Relative activity (%)
B5YBD7 (Comparative)	100
A0A0P6XN50 (SEQ ID NO: 1)	250
A0A223HVJ3 (SEQ ID NO: 2)	278
A0A2M8QBR9 (SEQ ID NO: 7)	220

[070] Example 2: T6PPs with higher activity

[071] The relative activities of T6PPs were measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl2, 0.5 mM $MnCl_2$, 38.5 mM G1P, 0.05 g/L PGM, 0.05 g/L PGI, 0.25 g/L F6PE from *Thermanaerothrix daxensis* (Uniprot ID A0A0P6XN50), and 0.05 g/L T6PP at 50°C for 1 hour. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, tagatose, was evaluated using an Agilent Hi-Plex H-column and refractive index detector. The sample was run in 5 mM H_2SO_4 at 0.6 mL/min for 15 min at 65°C. Table 2 shows that T6PPs used in the improved processes of the invention have higher activity relative to the previously disclosed T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805). *See* International Patent Application Publication WO 2017/059278.

Table 2: Relative activities of T6PP

T6PP Uniprot ID	Relative activity (%)
O29805 (Comparative)	100
A0A0E3NCH4 (SEQ ID NO 3)	714
D6YBK5 (SEQ ID NO 4)	1428
E0RT70 (SEQ ID NO 5)	2175
D1C7G9 (SEQ ID NO 6)	914

[072] Example 3: Improved tagatose production from F6P

[073] The conversion of F6P to tagatose using previously disclosed enzymes, F6PE (Uniprot ID B5YBD7) and T6PP (Uniprot ID O29805), was compared with the conversion of F6P to tagatose with enzymes useful in the improved processes of the invention, F6PE (Uniprot ID A0A0P6XN50) and T6PP (Uniprot ID

D6YBK5). A 0.20 mL reaction mixture containing 38.5 mM F6P, 50 mM HEPES pH 7.2, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 g/L F6PE, and 0.033 g/L T6PP was incubated at 50°C for 2 hours.

[074] The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO) and analyzed via HPLC (Agilent 1100 series) using an Agilent Hi-Plex H-column and refractive index detector. The sample was run in 5 mM H_2SO_4 at 0.6 mL/min for 15.5 minutes at 65 °C. Results show a 4.4-fold improvement in tagatose production with the enzymes of the improved process over the previously disclosed enzymes (FIG. 7). A slight amount of fructose (see FIG. 7, shoulder,5, on peak 4) is present in the reaction with the enzymes of the improved process. This may be an artifact due to the T6PP initially seeing a large abundance of F6P compared to T6P for the reaction here and is not an issue in the full pathway.

Sequence Listing

Alpha-glucan phosphorylase

Thermotoga maritima (Uniprot ID G4FEH8) - SEQ ID NO: 8

MLEKLPENLKELESLAYNLWWSWSRPAQRLWRMIDSEKWEEHRNPVKILREVSKERLEELSKDEDFIALYELTLERFTDY
MEREDTWFNVNYPEWDEKIVYMCMEYGLTKALPIYSGGLGILAGDHLKSASDLGLPLIAVGLLYKHGYFTQQIDSDGR
QIEIFPEYDIEELPMKPLRDEDGNQVIVEVPIDNDTVKARVFEVQVGRVKLYLLDTDFEENEDRFRKICDYLYNPEPDVRV
SQEILLGIGGMKLLKTLKIKPGVIHLNEGHPAFSSLERIKSYMEEGYSFTEALEIVRQTTVFTTHTPVPAGHDRFPFDFVEKK
LTKFFEGFESKELLMNLGKDEDGNFNMTYLALRTSSFINGVSKLHADVSRRMFKNVWKGVPVEEIPIEGITNGVHMGT
WINREMRKLFDRYLGRVWREHTDLEGIWYGVDRIPDEELWEAHLNAKKRFIDYIRESIKRRNERLGINEPLPEISENVLII
GFARRFATYKRAVLLFSDLERLKRIVNNSERPVYIVYAGKAHPRDEGGKEFLRRIYEVSQMPDFKNKIIVLENYDIGMARL
MVSGVDVWLNNPRRPMEASGTSGMKAAANGVLNASVYDGWWVEGYNGRNGWVIGDESVLPETEADDPKDAEAL
YELLENEIIPTYYENREKWIFMMKESIKSVAPKFSTTRMLKEYTEKFYIKGLVNREWLERRENVEKIGAWKERILKNWENV
SIERIVLEDSKSVEVTVKLGDLTPNDVIVELVAGRGEGMEDLEVWKVIHIRRYRKENDLFVYTYTNGVLGHLGSPGWFYA
VRVIPYHPRLPIKFLPEVPVVWKKVL

Phosphoglucomutase

Thermococcus kodakaraensis (Uniprot ID Q68BJ6) - SEQ ID NO: 9

MGKLFGTFGVRGIANEEITPEFALKIGMAFGTLLKREGRERPLVVVGRDTRVSGEMLKDALISGLLSTGCDVIDVGIAPTP
AIQWATNHFNADGGAVITASHNPPEYNGIKLLEPNGMGLKKEREAIVEELFFSEDFHRAKWNEIGELRKEDIIKPYIEAIK
NRVDVEAIKKRRPFVVVDTSNGAGSLTLPYLLRELGCKVVSVNAHPDGHFPARNPEPNEENLKGFMEIVKALGADFGV
AQDGDADRAVFIDENGRFIQGDKTFALVADAVLRENGGGLLVTTIATSNLLDDIAKRNGAKVMRTKVGDLIVARALLEN
NGTIGGEENGGVIFPDFVLGRDGAMTTAKIVEIFAKSGKKFSELIDELPKYYQFKTKRHVEGDRKAIVAKVAELAEKKGYKI
DTTDGTKIIFDDGWVLVRASGTEPIIRIFSEAKSEEKAREYLELGIKLLEEALKG

Fructose 6-phosphate 4-epimerases

(Comparative) Dictyoglomus thermophilum (Uniprot ID B5YBD7) - SEQ ID NO: 10

MWLSKDYLRKKGVYSICSSNPYVIEASVEFAKEKNDYILIEATPHQINQFGGYSGMTPEDFKNFVMGIIKEKGIEEDRVIL
GGDHLGPLPWQDEPSSSAMKKAKDLIRAFVESGYKKIHLDCSMSLSDDPVVLSPEKIAERERELLEVAEETARKYNFQPV

YVVGTDVPVAGGGEEEGITSVEDFRVAISSLKKYFEDVPRIWDRIIGFVIMLGIGFNYEKVFEYDRIKVRKILEEVKKENLFV
EGHSTDYQTKRALRDMVEDGVRILKVGPALTASFRRGVFLLSSIEDELISEDKRSNIKKVVLETMLKDDKYWRKYYKDSER
LELDIWYNLLDRIRYYWEYKEIKIALNRLFENFSEGVDIRYIYQYFYDSYFKVREGKIRNDPRELIKNEIKKVLEDYHYAVNL

Thermanaerothrix daxensis (Uniprot ID A0A0P6XN50) - SEQ ID NO: 1

MVTYLDFVVLSHRFRRPLGITSVCSAHPYVIEAALRNGMMTHTPVLIEATCNQVNQYGGYTGMTPADFVRYVENIAAR
VGSPRENLLLGGDHLGPLVWAHEPAESAMEKARALVKAYVEAGFRKIHLDCSMPCADDRDFSPKVIAERAAELAQVAE
STCDVMGLPLPNYVIGTEVPPAGGAKAEAETLRVTRPEDAAETIALTRAAFFKRGLESAWERVVALVVQPGVEFGDHQI
HVYRREEAQALSRFIESQPGLVYEAHSTDYQPRDALRALVEDHFAILKVGPALTFAFREAVFALASIEDWVCDSPSRILEV
LETTMLANPVYWQKYYLGDERARRIARGYSFSDRIRYYWSAPAVEQAFERLRANLNRVSIPLVLLSQYLPDQYRKVRDG
RLPNQFDALILDKIQAVLEDYNVACGVRIGE

Thermoanaerobacterium thermosaccharolyticum (Uniprot ID A0A223HVJ3) - SEQ ID NO: 2

MAKEHPLKELVNKQKSGISEGIVSICSSNEFVIEASMERALTNGDYVLIESTANQVNQYGGYIGMTPIEFKKFVFSIAKKV
DFPLDKLILGGDHLGPLIWKNESSNLALAKASELIKEYVLAGYTKIHIDTSMRLKDDTDFNTEIIAQRSAVLLKAAENAYME
LNKNNKNVLHPVYVIGSEVPIPGGSQGSDESLQITDAKDFENTVEIFKDVFSKYGLINEWENIVAFVVQPGVEFGNDFVH
EYKRDEAKELTDALKNYKTFVFEGHSTDYQTRESLKQMVEDGIAILKVGPALTFALREALIALNNIENELLNNVDSIKLSNF
TNVLVSEMINNPEHWKNHYFGDDARKKFLCKYSYSDRCRYYLPTRNVKNSLNLLIRNLENVKIPMTLISQFMPLQYDNIR
RGLIKNEPISLIKNAIMNRLNDYYYAIKP

Candidatus Thermofonsia Clade 3 bacterium (Uniprot ID A0A2M8QBR9) - SEQ ID NO: 7

MTYLDYLTASHHSGKPIGLSSICSAHPWVLRTALQGERPVLIESTCNQVNQFGGYSGMKPADFVRFVHSLAAENGFPTE
KILLGGDHLGPSPWQNEPAEQAMAKAIEMVRAYVQAGYTKIHLDCSMPLGGERQLPVEVIAQRTVQLAEAAEQAAHE
SRLTSHTLRYVIGSEVPPPGGAIGPHEGLRVTPVEEARQTLEVMQAAFHQAGLEAAWERVRALVVQPGVEFGDDFVH
DYDPAAAAGLARFIETVPNLVYEAHSTDYQTPESLAALVRDHFAILKVGPALTFALREAVFALAMIENELFPAEERSHLVE
RLEAAMLRQPGHWQRHYHGEERQQALARKYSFSDRIRYYWGDPDVQAAFRQLLANLERVAPLPLTLLSQYLPEMFGE
IRRGLLLNHPAAFLERKIQAVLDTYRAACGEED

Tagatose 6-phosphate phosphatases

(Comparative) Archaeoglobus fugidis (Uniprot ID O29805) - SEQ ID NO: 11

MFKPKAIAVDIDGTLTDRKRALNCRAVEALRKVKIPVILATGNISCFARAAAKLIGVSDVVICENGGVVRFEYDGEDIVLG
DKEKCVEAVRVLEKHYEVELLDFEYRKSEVCMRRSFDINEARKLIEGMGVKLVDSGFAYHIMDADVSKGKALKFVAERL
GISSAEFAVIGDSENDIDMFRVAGFGIAVANADERLKEYADLVTPSPDGEGVVEALQFLGLLR

Methanosarcina thermophila CHTI-55 (Uniprot ID A0A0E3NCH4) - SEQ ID NO: 3

MLKALIFDMDGVLVDSMPFHAAAWKKAFFEMGMEIQDSDIFAIEGSNPRNGLPLLIRKARKEPEAFDFEAITSIYRQEFK
RVFEPKAFEGMKECLEVLKKRFLLSVVSGSDHVIVHSIINRLFPGIFDIVVTGDDIINSKPHPDPFLKAVELLNVRREECVVIE
NAILGVEAAKNARIYCIGVPTYVEPSHLDKADLVVEDHRQLMQHLLSLEPANGFRQ

Thermobispora bispora strain ATCC 19993 (Uniprot ID D6YBK5) - SEQ ID NO: 4

MDVVLFDMDGLLVDTERLWFAVETEVVERLGGSWGPEHQRQLVGGSLKRAVAYMLEHTGADVDPDVVAGWLIEG
MERRLTESVDPMPGAMELLTALRDEGIPTGLVTSSRRPLADAVLKHIGREHFDVVVTADDVSHAKPHPEPYLTALAMLS
ADPARSVALEDSPNGVASAVAAGCRVVAVPSLLPIPEQPGVTVLPALTHADVGLLRSLVG

Spirochaeta thermophila ATCC 49972 (Uniprot ID E0RT70) - SEQ ID NO: 5

MRKRRECAPPGIRAAIFDMDGTLVNSEDVYWDADCAFLDRYGIPHDDALREYMIGRGTKGFIEWMRTQKEIPRSDEEL
AREKMEVFLAHARGRVQVFPEMRRLLGLLEEAGMPCALASGSPRGIIEVLLEETGLAGFFRVVVSADEVARPKPAPDVF
LEAAGRLGVEPGGCVVFEDSEPGVRAGLDAGMVCVAIPTLVKDRYPEVFYQADVLFEGGMGEFSAERVWEWLGCGV
GVGR

Sphaerobacter thermophilus DSM 20745 (Uniprot ID D1C7G9) - SEQ ID NO: 6

MSQGVRGVVFDLDGLLVESEEYWEQARREFVSRYGGTWGDDAQQAVMGANTRQWSRYIREAFDIPLTEEEIAAAVI
ARMQELYHDHLPLLPGAIPAVRALADRYPLAVASSSPPVLIRFVLAEMGVAECFQSVTSSDEVAHGKPAPDVYHLACER
LGVAPEQAVAFEDSTAGIAAALAAGLRVIAVPNRSYPPDPDVLRRADLTLPSLEEFDPAVLEQW

<u>4-glucan transferase</u>

Thermococcus litoralis (Uniprot ID O32462) - SEQ ID NO: 12

MERINFIFGIHNHQPLGNFGWVFEEAYNRSYRPFMEILEEFPEMKVNVHFSGPLLEWIEENKPDYLDLLRSLIKRGQLEIV
VAGFYEPVLAAIPKEDRLVQIEMLKDYARKLGYDAKGVWLTERVWQPELVKSLREAGIEYVVVDDYHFMSAGLSKEELF

WPYYTEDGGEVITVFPIDEKLRYLIPFRPVKKTIEYLESLTSDDPSKVAVFHDDGEKFGVWPGTYEWVYEKGWLREFFDAI
TSNEKINLMTYSEYLSKFTPRGLVYLPIASYFEMSEWSLPAKQAKLFVEFVEQLKEEGKFEKYRVFVRGGIWKNFFFKYPE
SNFMHKRMLMVSKAVRDNPEARKYILKAQCNDAYWHGVFGGIYLPHLRRTVWENIIKAQRYLKPENKILDVDFDGRA
EIMVENDGFIATIKPHYGGSIFELSSKRKAVNYNDVLPRRWEHYHEVPEATKPEKESEEGIASIHELGKQIPEEIRRELAYD
WQLRAILQDHFIKPEETLDNYRLVKYHELGDFVNQPYEYEMIENGVKLWREGGVYAEEKIPARVEKKIELTEDGFIAKYR
VLLEKPYKALFGVEINLAVHSVMEKPEEFEAKEFEVNDPYGIGKVRIELDKAAKVWKFPIKTLSQSEAGWDFIQQGVSYT
MLFPIEKELEFTVRFREL

What is claimed is:

1. An improved process for the production of tagatose from a saccharide, the improvement comprising converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) wherein the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7.

2. An improved process for the production of tagatose from a saccharide, the improvement comprising converting T6P to tagatose using a tagatose-6-phoshpate phosphatase (T6PP), wherein the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6.

3. The process of claim 2, further comprising a step of converting fructose-6-phosphate (F6P) to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE) wherein the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7.

4. The process of any one of claims 1-3, further comprising a step of converting glucose 6-phosphate (G6P) to the F6P, wherein the step is catalyzed by a phosphoglucose isomerase (PGI).

5. The process of claim 4, further comprising the step of converting glucose 1-phosphate (G1P) to the G6P, wherein the step is catalyzed by a phosphoglucomutase (PGM).

6. The process of claim 5, further comprising the step of converting a saccharide to the G1P, wherein the step is catalyzed by at least one enzyme, wherein the saccharide is selected from the group consisting of a starch or derivative thereof, cellulose or a derivative thereof and sucrose.

7. The process of claim 6, wherein the at least one enzyme is selected from the group consisting of alpha-glucan phosphorylase (αGP), maltose phosphorylase, sucrose phosphorylase, cellodextrin phosphorylase, cellobiose phosphorylase, and cellulose phosphorylase.

8. The process of claim 6, wherein the saccharide is starch or a derivative thereof selected from the group consisting of amylose, amylopectin, soluble starch, amylodextrin, maltodextrin, maltose, maltotriose, and glucose.

9. The process of claim 8, further comprising the step of converting starch to a starch derivative wherein the starch derivative is prepared by enzymatic hydrolysis of starch or by acid hydrolysis of starch.

10. The process of claim 9, wherein a 4-glucan transferase (4GT) is added to the process.

11. The process of claim 9, wherein the starch derivative is prepared by enzymatic hydrolysis of starch catalyzed by an isoamylase, a pullulanase, an alpha-amylase, or a combination thereof.

12. The process of any one of claims 1-3, further comprising:
a step of converting fructose to F6P catalyzed by at least one enzyme; and
optionally, a step of converting sucrose to fructose catalyzed by at least one enzyme.

13. The process of claim 4, further comprising:
a step of converting glucose to G6P catalyzed by at least one enzyme, and
optionally, a step of converting sucrose to glucose catalyzed by at least one enzyme.

14. The process of claim 2, wherein the process is an enzymatic process for the production of tagatose comprising the steps of:

(i) converting a saccharide to glucose 1-phosphate (G1P) using one or more enzymes, wherein the saccharide is selected from the group consisting of starch, one or more derivatives of starch, or a combination thereof;

(ii) converting G1P to glucose 6-phosphate (G6P) using a phosphoglucomutase (PGM);

(iii) converting G6P to fructose 6-phosphate (F6P) using a phosphoglucoisomerase (PGI);

(iv) converting the F6P to tagatose 6-phopsphate (T6P) using a fructose 6-phosphate epimerase (F6PE), and

(v) converting the T6P to tagatose using a tagatose 6-phosphate phosphatase (T6PP), wherein the T6PP comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NOs 3 or 4;

wherein process steps (i)-(v) are conducted in a single reaction vessel.

15. The process of claim 14, wherein the F6PE comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 7.

16. The process of claim 14 or 15, wherein the process steps are conducted under at least one of the following process conditions:

at a temperature ranging from about 37˚C to about 85˚C,

at a pH ranging from about 5.0 to about 9.0, or

for about 1 hour to about 48 hours.

17. The process of any one of claim 14-16, wherein the process steps are conducted under at least one of the following process conditions:

without adenosine triphosphate (ATP) as a source of phosphate,

without nicotinamide adenosine dinucleotide,

at a phosphate concentration from about 0.1 mM to about 150 mM,

at a Mg^{2+} concentration from about 0.1 mM to 50 mM,

wherein phosphate is recycled, and

wherein at least one step of the process involves an energetically favorable chemical reaction.

18. The process of claim 17, wherein phosphate is recycled, and wherein phosphate ions produced by T6PP dephosphorylation of T6P are used in the process step of converting a saccharide to G1P.

19. The process of claim 17, wherein the step of converting T6P to tagatose is an energetically favorable, irreversible phosphatase reaction.

20. The process of any one of claims 14-19, further comprising the step of separating recovering the tagatose produced, wherein the separation recovery is not via chromatography separation.

21. The process of claim 14 or 15, wherein the derivatives of starch are selected from the group consisting of amylose, amylopectin, soluble starch, amylodextrin, maltodextrin, maltotriose, maltose, and glucose.

22. Tagatose produced from a process of any one of claims 1-21.

23. A consumable product containing tagatose produced from a process of any one of claims 1-21.

24. The process of claim 1, wherein the F6PE has a higher activity compared to that of F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7).

25. The process of claim 2, wherein the T6PP has a higher activity compared to that of T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805).

26. The process of claim 24, wherein the F6PE has at least a 10% higher activity compared to that of F6PE from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7).

27. The process of claim 25, wherein the T6PP has at least a 10% higher activity compared to that of T6PP from *Archaeoglobus fulgidus* (Uniprot ID O29805).

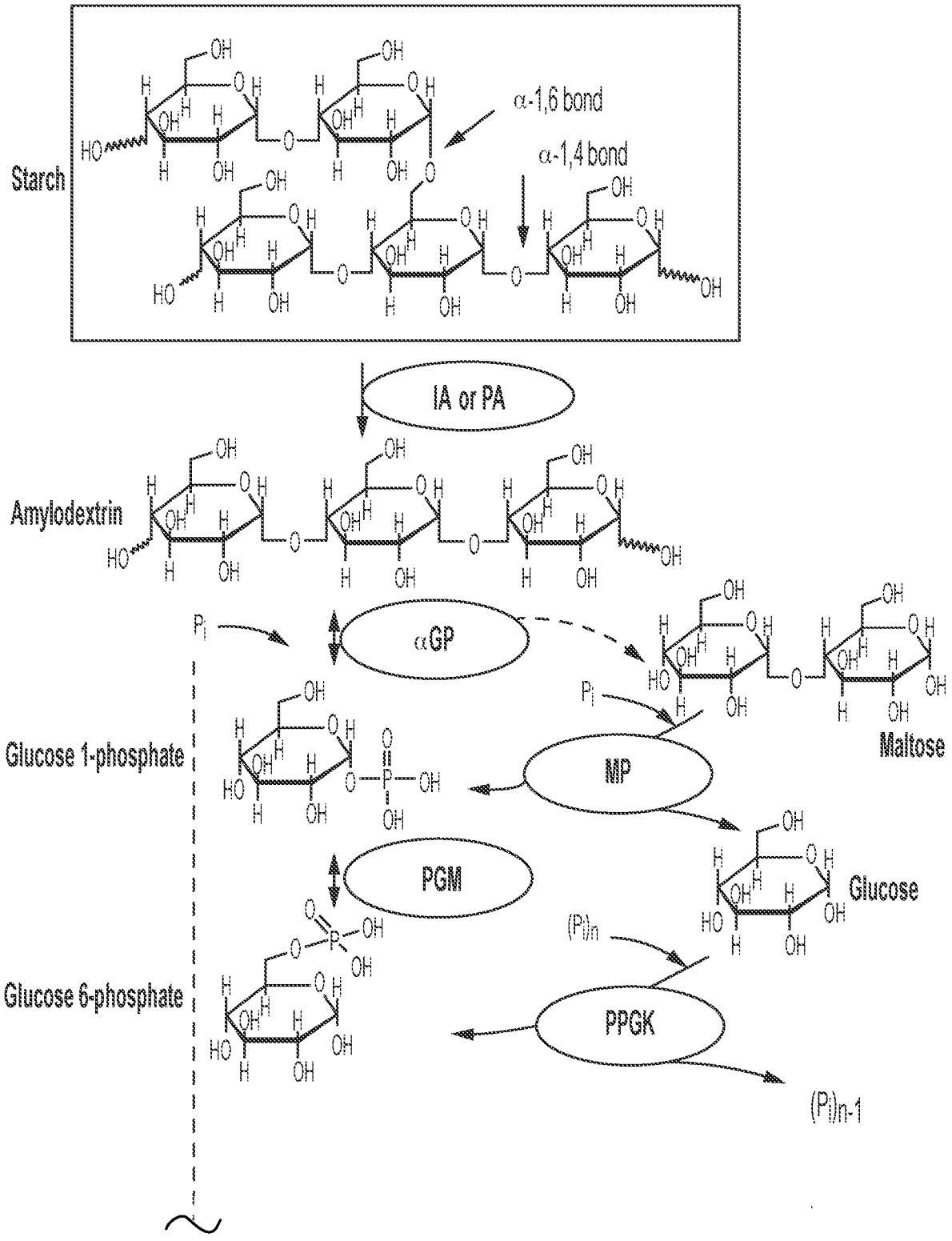

Starch
α-1,6 bond
α-1,4 bond
IA or PA
Amylodextrin
Pi
αGP
Maltose
Glucose 1-phosphate
MP
PGM
Glucose
(Pi)n
Glucose 6-phosphate
PPGK
(Pi)n-1

FIG. 1

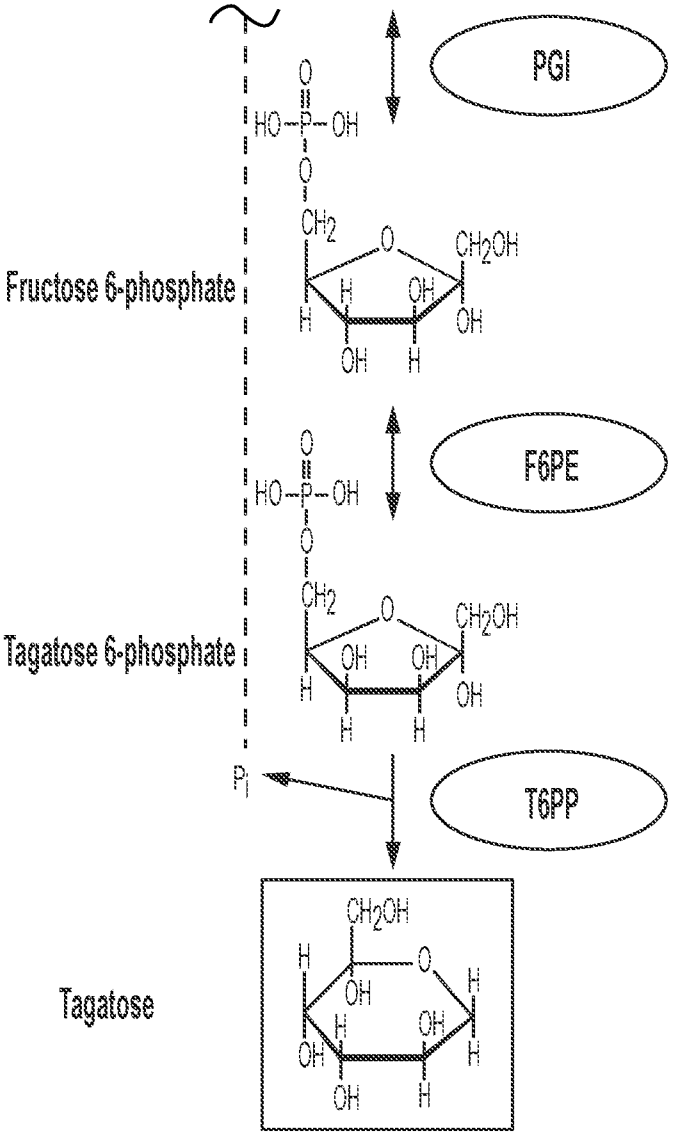


FIG. 1 - CONTINUED

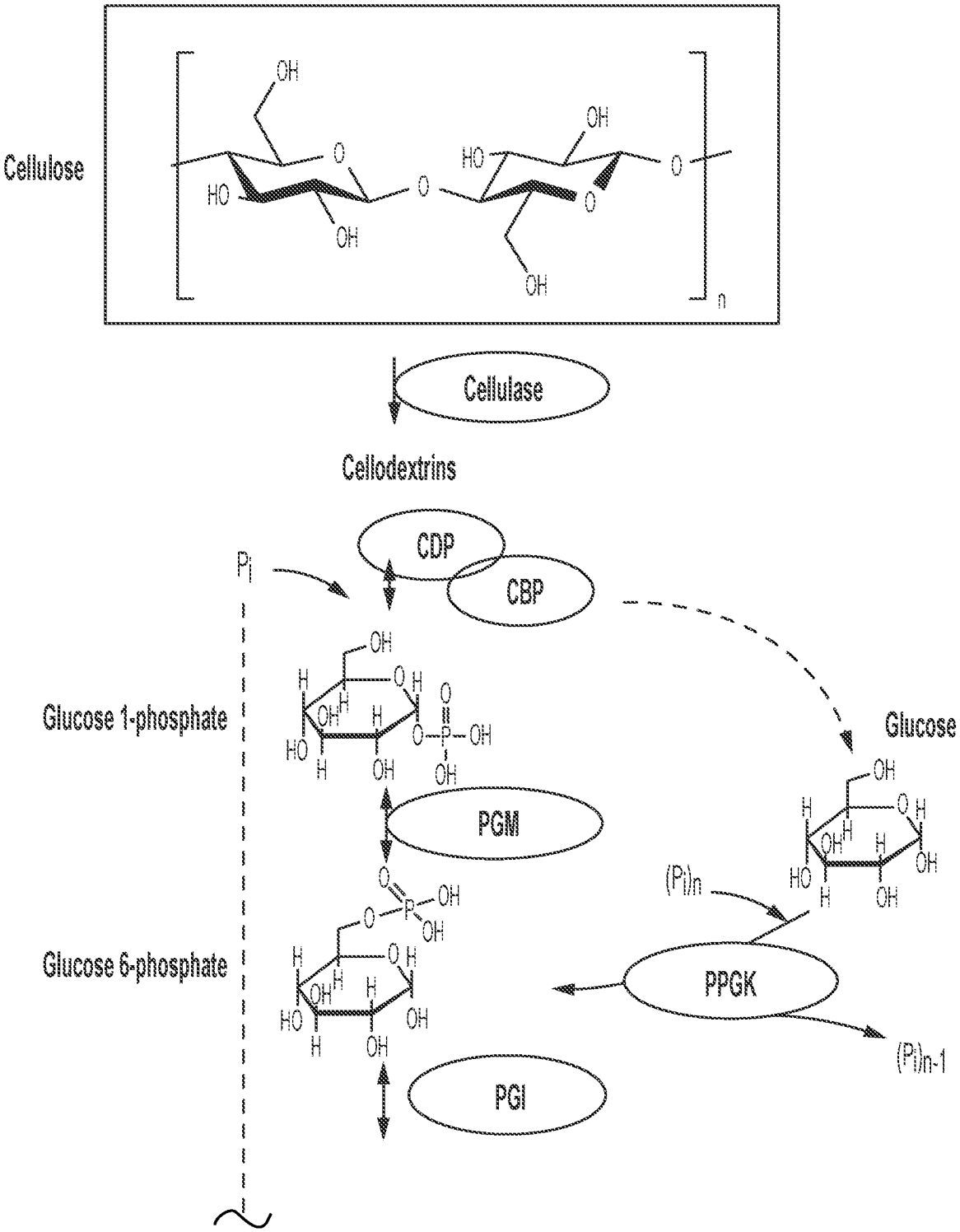

Cellulose
Cellulase
Cellodextrins
CDP
CBP
Pi
Glucose 1-phosphate
PGM
Glucose 6-phosphate
PGI
Glucose
(Pi)n
PPGK
(Pi)n-1

FIG. 2

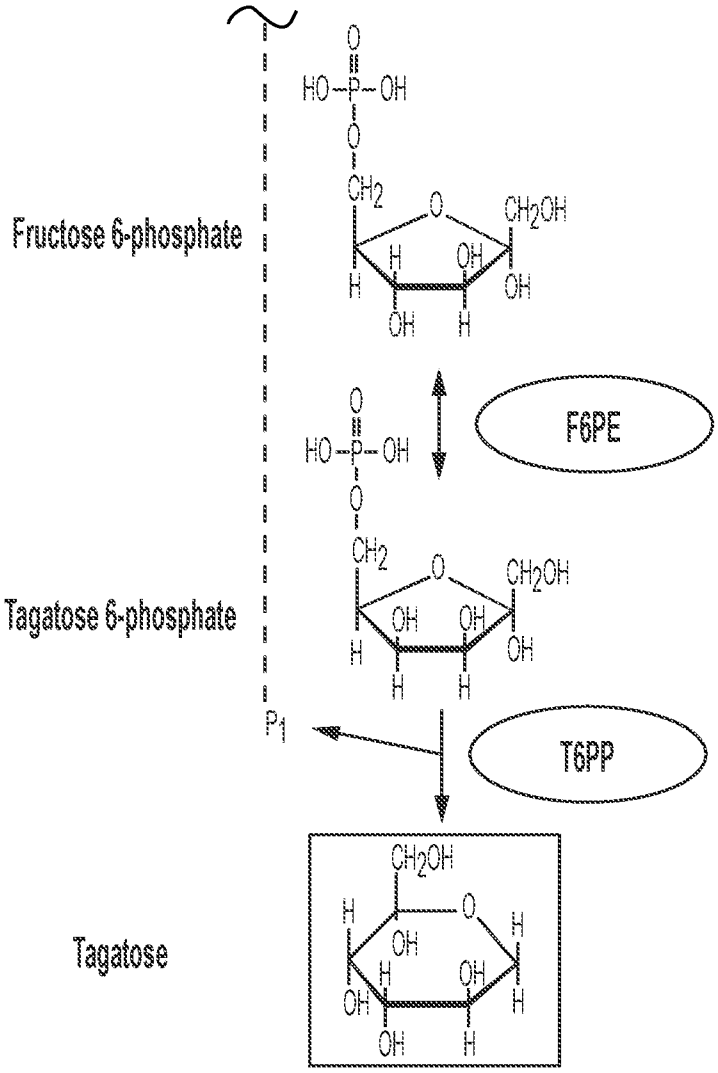


FIG. 2 - CONTINUED

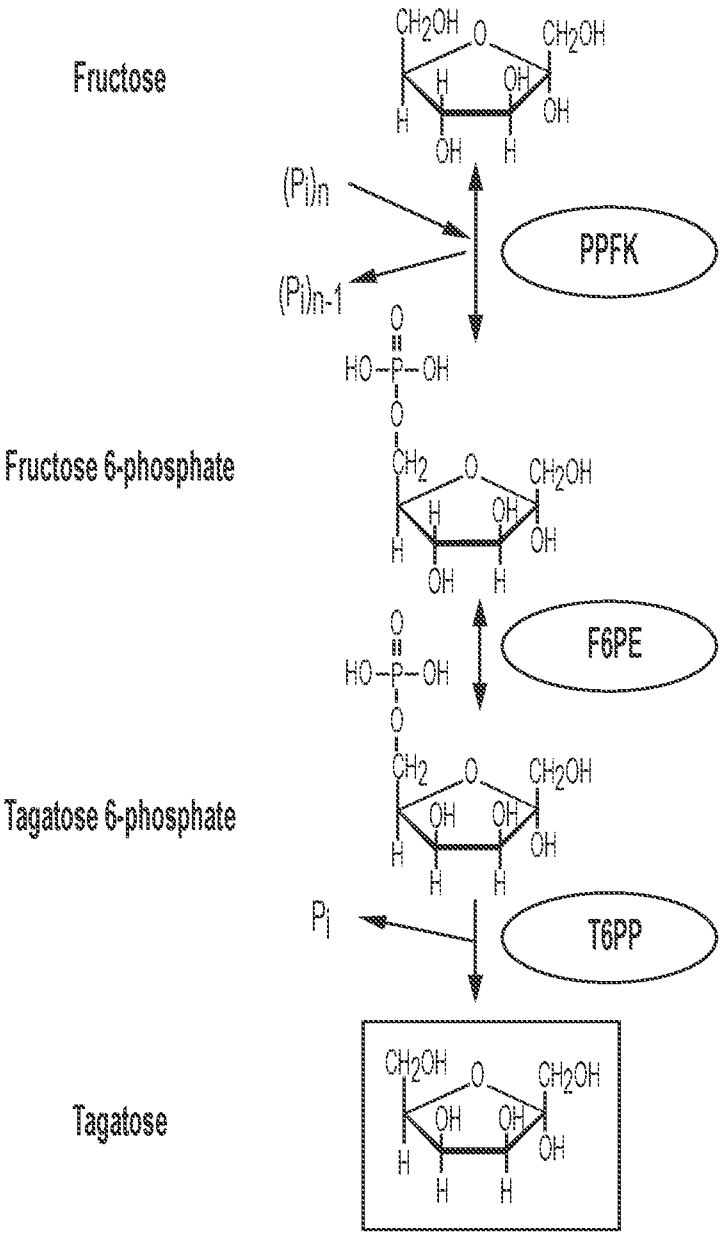

Fructose
(Pi)n
(Pi)n-1
PPFK
Fructose 6-phosphate
F6PE
Tagatose 6-phosphate
Pi
T6PP
Tagatose

FIG. 3

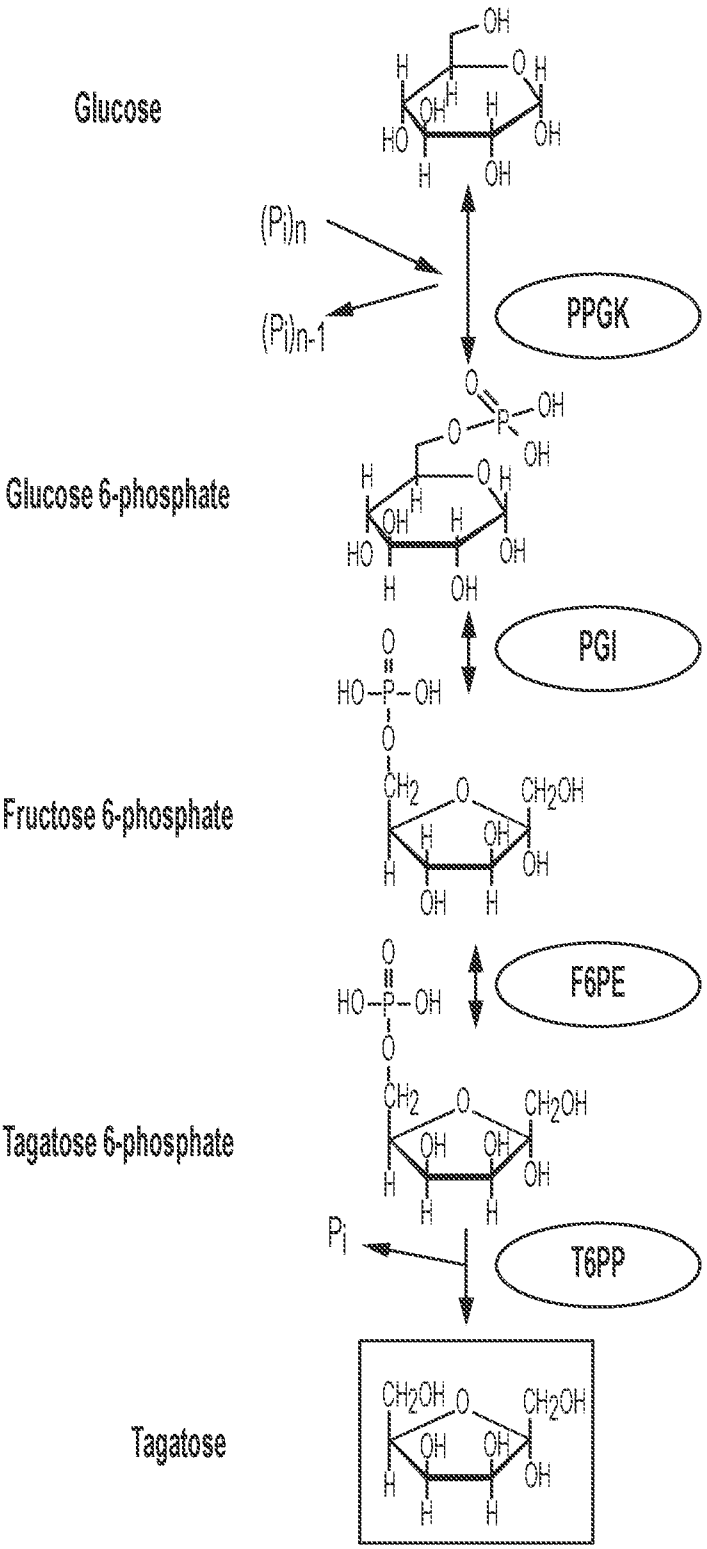

Glucose
(Pi)n
(Pi)n-1
PPGK
Glucose 6-phosphate
PGI
Fructose 6-phosphate
F6PE
Tagatose 6-phosphate
Pi
T6PP
Tagatose

FIG. 4

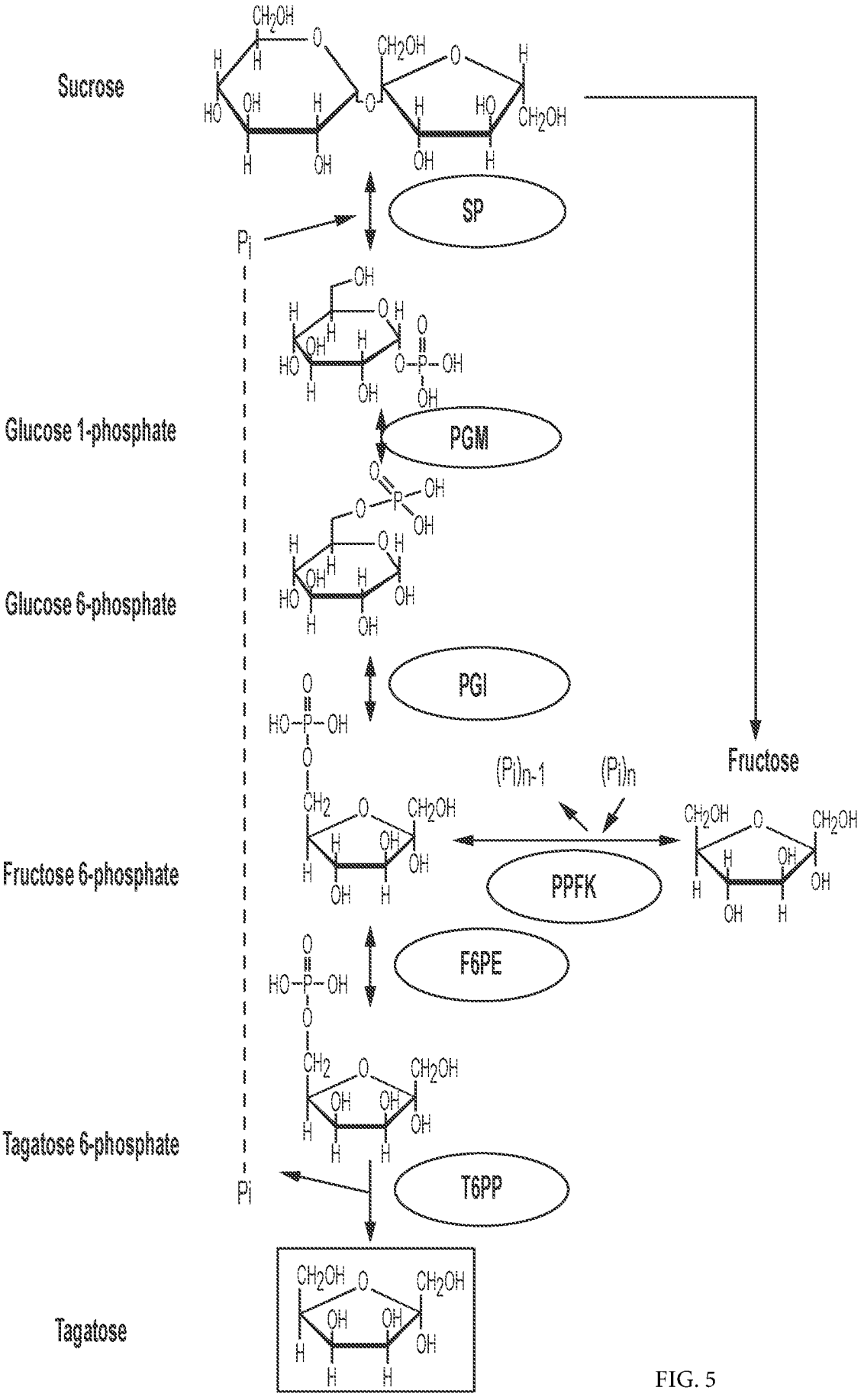

Sucrose
SP
Pi
Glucose 1-phosphate
PGM
Glucose 6-phosphate
PGI
Fructose
(Pi)n-1
(Pi)n
Fructose 6-phosphate
PPFK
F6PE
Tagatose 6-phosphate
T6PP
Pi
Tagatose

FIG. 5

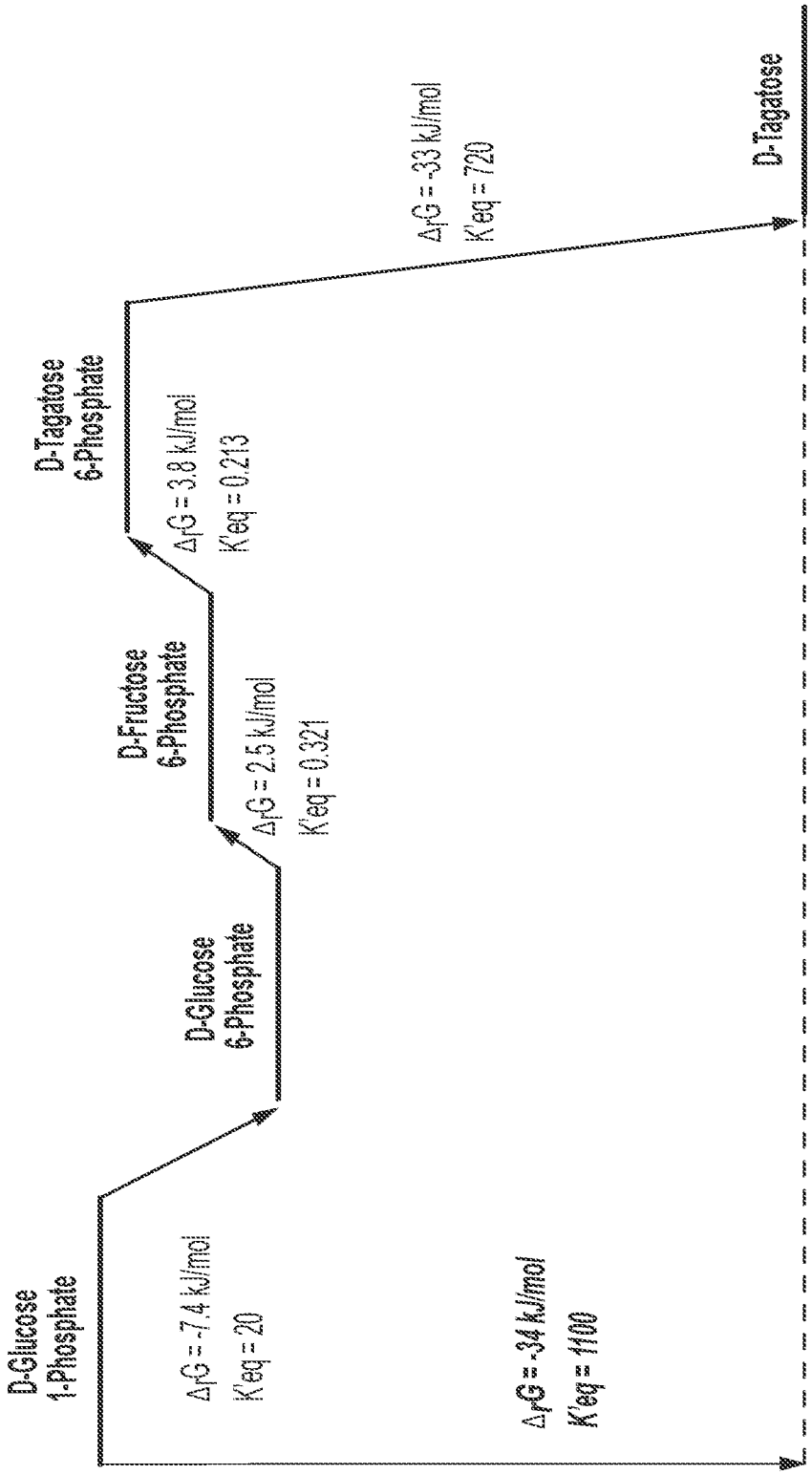

D-Glucose
1-Phosphate
ΔrG = -7.4 kJ/mol
Keq = 20
D-Glucose
6-Phosphate
ΔrG = 2.5 kJ/mol
K'eq = 0.321
D-Fructose
6-Phosphate
ΔrG = 3.8 kJ/mol
K'eq = 0.213
D-Tagatose
6-Phosphate
ΔrG = -33 kJ/mol
Keq = 720
D-Tagatose
ΔrG = -34 kJ/mol
Keq = 1100

FIG. 6

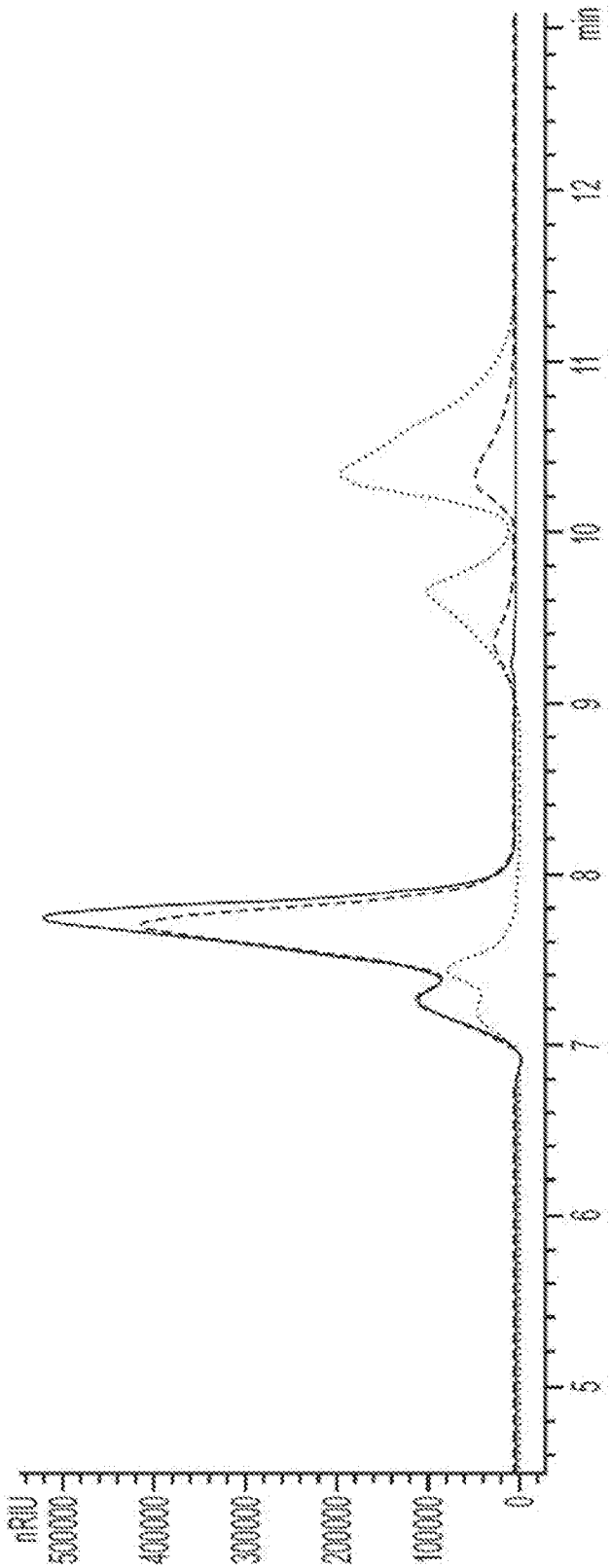


FIG. 7

FIG. 7