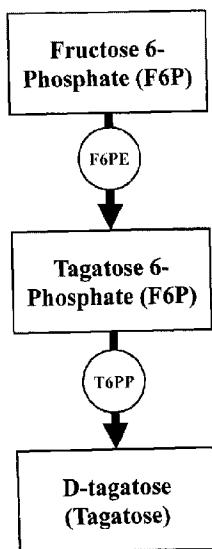




(86) Date de dépôt PCT/PCT Filing Date: 2016/09/30
(87) Date publication PCT/PCT Publication Date: 2017/04/06
(45) Date de délivrance/Issue Date: 2022/06/14
(85) Entrée phase nationale/National Entry: 2018/03/28
(86) N° demande PCT/PCT Application No.: US 2016/054838
(87) N° publication PCT/PCT Publication No.: 2017/059278
(30) Priorité/Priority: 2015/10/02 (US62/236,226)

(51) Cl.Int./Int.Cl. *C12N 9/90* (2006.01),
C12P 19/24 (2006.01)
(72) Inventeur/Inventor:
WICHELECKI, DANIEL JOSEPH, US
(73) Propriétaire/Owner:
BONUMOSE LLC, US
(74) Agent: GOWLING WLG (CANADA) LLP

(54) Titre : SYNTHÈSE ENZYMATIQUE DU D-TAGATOSE
(54) Title: ENZYMATIC PRODUCTION OF D-TAGATOSE



(57) Abrégé/Abstract:

The current disclosure provides a process for enzymatically converting a saccharide into tagatose. The invention also relates to a process for preparing tagatose where the process involves converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P), catalyzed by an epimerase, and converting the T6P to tagatose, catalyzed by a phosphatase.

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

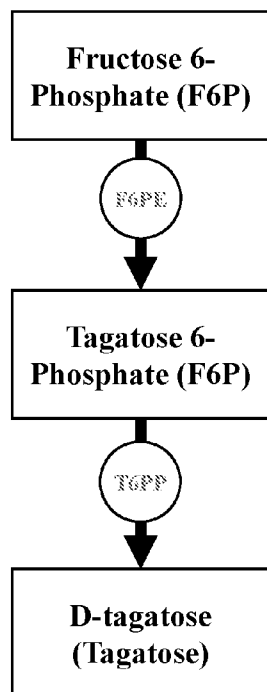
(19) World Intellectual Property
Organization
International Bureau(10) International Publication Number
WO 2017/059278 A1(43) International Publication Date
6 April 2017 (06.04.2017)

- (51) International Patent Classification:
C12N 9/90 (2006.01) *C12P 19/24* (2006.01)
- (21) International Application Number:
PCT/US2016/054838
- (22) International Filing Date:
30 September 2016 (30.09.2016)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
62/236,226 2 October 2015 (02.10.2015) US
- (71) Applicant: **BONUMOSE BIOCHEM LLC** [US/US];
722 Preston Avenue, Suite 107, Charlottesville, VA 22903 (US).
- (72) Inventors: **WICHELECKI, Daniel, Joseph**; 717 Lee Street Apartment #7, Blacksburg, VA 24060 (US).
ZHANG, Yi Heng, Percival; 210 A, Seitz Hall, Virginia Tech, Blacksburg, VA 24061 (US).
- (74) Agent: **GUNNELS, Zarema, E.**; J.A. Lindeman & Co., PLLC, 3190 Fairview Park Drive, Suite 480, Falls Church, VA 22042 (US).
- (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, DJ, DK, DM,

[Continued on next page]

(54) Title: ENZYMATIC SYNTHESIS OF D-TAGATOSE

(57) Abstract: The current disclosure provides a process for enzymatically converting a saccharide into tagatose. The invention also relates to a process for preparing tagatose where the process involves converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P), catalyzed by an epimerase, and converting the T6P to tagatose, catalyzed by a phosphatase.

**FIG. 1**

WO 2017/059278 A1

WO 2017/059278 A1

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, KW, 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.

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:

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH,

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

ENZYMATIC PRODUCTION OF D-TAGATOSE

CROSS-REFERENCE TO RELATED APPLICATIONS

- [1] This application claims priority to U.S. Application Ser. No. 62/236,226, filed on October 2, 2015.

FIELD OF THE INVENTION

- [2] The invention relates to preparation of the sugar D-tagatose. More specifically, the invention relates to methods of preparing D-tagatose by enzymatically converting saccharides (e.g., polysaccharides, oligosaccharides, disaccharides, sucrose, D-glucose, and D-fructose) into D-tagatose.

BACKGROUND OF THE INVENTION

- [3] D-tagatose (tagatose hereafter) is a low-calorie, natural sweetener that has 92% the sweetness of sucrose, but only 38% of the calories. It is a naturally occurring monosaccharide hexose that is present in only small amounts in fruits, cacao, and dairy products. Tagatose was approved as a food additive by the Food and Drug Administration (FDA) in 2003, which designated it as generally recognized as safe (GRAS). However, due to tagatose's 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 raising 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 the pharmaceutical, biotechnological, academic, food, beverage, dietary supplement, and grocer industries.
- [4] Currently tagatose is produced predominantly through the hydrolysis of lactose by lactase or acid hydrolysis to form D-glucose and D-galactose (WO 2011150556, CN 103025894, US 5002612, US 6057135, and US 8802843). 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 process is performed in several tanks or bioreactors. Overall, the method suffers because of the costly separation of other sugars (e.g., D-glucose, D-galactose, and unhydrolyzed lactose) 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.

- [5] There is a need to develop a cost-effective synthetic pathway for high-yield tagatose production where at least one step of the process involves an energetically favorable chemical reaction. Furthermore, there is a need for a tagatose production process where the process steps can be conducted in one tank or bioreactor. There is also a need for a process of tagatose production that can be conducted at a relatively low concentration of phosphate, where phosphate can be recycled, and/or the process does not require using adenosine triphosphate (ATP) as a source of phosphate. There is also a need for a tagatose production pathway that does not require the use of the costly nicotinamide adenosine dinucleotide (NAD(H)) coenzyme in any of the reaction steps.

SUMMARY OF THE INVENTION

- [6] The inventions described herein relate to processes for preparing tagatose. In various aspects, the processes involve converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P), catalyzed by an epimerase; and converting the T6P to tagatose, catalyzed by a phosphatase. The inventions also relate to tagatose prepared by any of the processes described herein.
- [7] In some aspects of the invention, a 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). In other aspects, a process for tagatose synthesis also includes the step of converting glucose 1-phosphate (G1P) to the G6P, and this conversion step is catalyzed by phosphoglucomutase (PGM).
- [8] In various aspects, a process for preparing tagatose can involve converting a saccharide to the G1P, catalyzed by at least one enzyme; converting G1P to G6P, catalyzed by

phosphoglucumutase (PGM); converting G6P to F6P, catalyzed by phosphoglucose isomerase (PGI); converting F6P to tagatose 6-phosphate (T6P), catalyzed by an epimerase; and converting the T6P produced to tagatose, catalyzed by a phosphatase.

- [9] 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, amyloextrin, maltodextrin, maltose, or glucose. In some aspects of the invention, a process for preparing tagatose involves converting starch to a starch derivative by enzymatic hydrolysis or by acid hydrolysis of starch. In other aspects, a starch derivative can be is prepared by enzymatic hydrolysis of starch catalyzed by isoamylase, pullulanase, alpha-amylase, or a combination of two or more of these enzymes. A process for preparing tagatose, in certain aspects, can also involve adding 4-glucan transferase (4GT).
- [10] In various aspects, a process for preparing tagatose can involve converting fructose to the F6P, catalyzed by at least one enzyme; converting F6P to tagatose 6-phosphate (T6P) catalyzed by an epimerase; and converting the T6P produced to tagatose, catalyzed by a phosphatase. In other embodiments, a tagatose production process involves converting sucrose to the fructose, catalyzed by at least one enzyme; converting fructose to the F6P, catalyzed by at least one enzyme; converting F6P to tagatose 6-phosphate (T6P) catalyzed by an epimerase; and converting the T6P produced to tagatose, catalyzed by a phosphatase.
- [11] In other aspects of the invention, G6P to be used in a process for preparing tagatose can 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.
- [12] In some aspects of the invention, epimerase used to convert F6P to T6P is fructose 6-phosphate epimerase. The fructose 6-phosphate epimerase can be encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 1, 3, 5, 7, 9, or 10. In various aspects, the fructose 6-phosphate epimerase comprises an amino acid sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 2, 4, 6, 8, or 11.

- [13] In various aspects of the invention, the phosphatase used to convert T6P to tagatose is tagatose 6-phosphate phosphatase. The tagatose 6-phosphate phosphatase can be encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NO.: 12, 14, or 16. In some aspects of the invention, the tagatose 6-phosphate phosphatase comprises an amino acid sequence having at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NO.: 13, 15, or 17.
- [14] In various aspects, a process of the invention are be conducted at a temperature ranging from about 40°C to about 70°C, at a pH ranging from about 5.0 to about 8.0, and/or for about 8 hours to about 48 hours. In some aspects, the steps of a process for preparing tagatose are conducted in one bioreactor. In other aspects, the steps are conducted in a plurality of bioreactors arranged in series.
- [15] In other aspects of the invention, the steps of a process for preparing tagatose are conducted ATP-free, NAD(H)-free, at a phosphate concentration from about 0 mM to about 150 mM, the phosphate is recycled, and/or at least one step of the process involves an energetically favorable chemical reaction.
- [15a] There is provided an enzymatic process for preparing tagatose from a starch derivative, the process comprising the steps of: (i) converting a saccharide to glucose 1-phosphate (G1P) using alpha glucan phosphorylase (α GP), wherein the saccharide is selected from one or more derivatives of starch; (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 fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P) catalyzed by a fructose 6-phosphate epimerase (F6PE); and (v) converting the T6P produced to tagatose catalyzed by a tagatose 6-phosphate phosphatase (T6PP), wherein steps (i)-(v) are conducted in a single reaction vessel.

BRIEF DESCRIPTION OF THE FIGURES

- [16] These drawings illustrate certain aspects of some of the embodiments of the invention, and should not be used to limit or define the invention.

- [17] FIG. 1 is a schematic diagram illustrating an enzymatic pathway converting fructose 6-phosphate to tagatose 6-phosphate and then to D-tagatose (tagatose).
- [18] FIG. 2 is a schematic diagram illustrating an enzymatic pathway converting starch or its derived products to tagatose. The following abbreviations are used: α GP, 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.
- [19] FIG. 3 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.

[20] FIG. 4 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.

[21] FIG. 5 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.

[22] FIG. 6 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.

[23] FIG. 7 shows the Reaction Gibbs Energy between intermediates based on formation Gibbs energy for the conversion of glucose 1-phosphate to tagatose.

DETAILED DESCRIPTION OF THE INVENTION

[24] The invention provides enzymatic pathways, or processes, for synthesizing tagatose with a high product yield, while greatly decreasing the product separation costs and tagatose production costs.

[25] The invention relates to a process for preparing tagatose where the process involves converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P) catalyzed by an epimerase and converting the T6P produced to tagatose catalyzed by a phosphatase (e.g., tagatose 6-phosphate phosphatase, T6PP). This process is generally shown in FIG. 1. In certain embodiments, the epimerase that catalyzes the conversion of F6P to T6P is fructose 6-phosphate epimerase (F6PE).

[26] Epimerases that convert F6P to T6P may be used in a process of the invention. In some aspects of the invention, epimerases suitable for use in the processes to convert F6P to T6P comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NOS.: 2, 4, 6, 8, or 11 (shown below), of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and

even most preferably at least 96%, 97%, 98%, 99%, or 100%. The suitable epimerases are encoded by a polynucleotide comprising a nucleotide sequence that has a degree of identity to the nucleotide sequence of SEQ ID NOS.: 1, 3, 5, 7, 9, or 10 (shown below), of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%.

[27] The invention also relates to epimerases that comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NOS.: 2, 4, 6, 8, or 11, of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%. In other aspects, the invention relates to epimerases that are encoded by a polynucleotide comprising a nucleotide sequence that has a degree of identity to the nucleotide sequence of SEQ ID NOS.: 1, 3, 5, 7, 9, or 10, of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%.

[28] Phosphatases that convert T6P to tagatose (D-tagatose) may be used in a process of the invention. In some aspects of the invention, phosphatases that can be used in to convert T6P to tagatose (D-tagatose) comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NOS.: 12, 14, or 16 (shown below), of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%. The tagatose phosphatases are encoded by a polynucleotide comprising a nucleotide sequence that has a degree of identity to the nucleotide sequence of SEQ ID NOS.: 13, 15, or 17 (shown below), of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%.

- [29] The invention also relates to phosphatases that convert T6P to tagatose (D-tagatose) and comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NOS.: 12, 14, or 16, of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%. In various aspects, the invention relates to phosphatases that convert T6P to tagatose (D-tagatose) and are encoded by a polynucleotide comprising a nucleotide sequence that has a degree of identity to the nucleotide sequence of SEQ ID NOS.: 13, 15, or 17, of at least 60%, preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, 97%, 98%, 99%, or 100%.
- [30] In some embodiments, a process for preparing tagatose according to the invention also includes the step of enzymatically converting glucose 6-phosphate (G6P) to the F6P, and this step is catalyzed by phosphoglucose isomerase (PGI). In other embodiments, the process for preparing tagatose additionally includes the step of converting glucose 1-phosphate (G1P) to the G6P, where the step is catalyzed by phosphoglucomutase (PGM). In yet further embodiments, tagatose production process also includes the step of converting a saccharide to the G1P that is catalyzed at least one enzyme.
- [31] Therefore, a process for preparing tagatose according to the invention can, 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). An example of the process where the saccharide is starch is shown in FIG. 2.
- [32] Typically, the ratios of enzyme units used in the disclosed process are 1:1:1:1:1 (α GP:PGM:PGI:F6PE:T6PP). To optimize product yields, these ratios can be adjusted in any number of combinations. For example, a ratio of 3:1:1:1:1 can be used to maximize the concentration of phosphorylated intermediates, which will result in increased activity of the downstream reactions. Conversely, a ratio of 1:1:1:1:3 can be used to maintain a robust supply

of phosphate for α GP, which will result in more efficient phosphorolytic cleavage of alpha-1,4-glycosidic bonds. A ratio of enzymes, for example, 3:1:1:1:3 can be used to further increase the reaction rate. Therefore, the enzyme ratios, including other optional enzymes discussed below, can be varied to increase the efficiency of tagatose production. For example, a particular enzyme may be present in an amount about 2 x, 3 x, 4 x, 5 x, etc. relative to the amount of other enzymes.

- [33] One of the important advantages of the processes is that the process steps can be conducted in one bioreactor or reaction vessel. Alternatively, the steps can also be conducted in a plurality of bioreactors, or reaction vessels, that are arranged in series.
- [34] 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.
- [35] 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.
- [36] Therefore, low phosphate concentration results 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.
- [37] 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. 7).

- [38] Examples of the enzymes used to convert a saccharide to G1P include alpha-glucan phosphorylase (α GP, EC 2.4.1.1), maltose phosphorylase (MP, EC 2.4.1.8), cellodextrin phosphorylase (CDP, EC 2.4.1.49), cellobiose phosphorylase (CBP, EC 2.4.1.20), cellulose phosphorylase, sucrose phosphorylase (SP, EC 2.4.1.7), and a combination thereof. The choice of the enzyme or enzyme combination depends on the saccharide used in the process.
- [39] The saccharides used for generating G1P can be polysaccharides, oligosaccharides, and/or disaccharides. For example, the saccharide can be starch, one or more derivatives of starch, cellulose, one or more derivatives of cellulose, sucrose, one or more derivatives of sucrose, or a combination thereof.
- [40] 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, amylopectin, 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.
- [41] The derivatives of starch can be prepared by enzymatic hydrolysis of starch or by acid hydrolysis of starch. Specifically, the enzymatic hydrolysis of starch can be catalyzed or enhanced by isoamylase (IA, EC 3.2.1.68), which hydrolyzes α -1,6-glucosidic bonds; pullulanase (PA, EC 3.2.1.41), which hydrolyzes α -1,6-glucosidic bonds; 4- α -glucanotransferase (4GT, EC 2.4.1.25), which catalyzes the transglycosylation of short maltooligosaccharides, yielding longer maltooligosaccharides; or alpha-amylase (EC 3.2.1.1), which cleaves α -1,4-glucosidic bonds.
- [42] Furthermore, derivatives of cellulose can be prepared by enzymatic hydrolysis of cellulose catalyzed by cellulase mixtures, by acids, or by pretreatment of biomass.
- [43] In certain embodiments, the enzymes used to convert a saccharide to G1P contain α GP. In this step, when the saccharides include starch, the G1P is generated from starch by α GP; when the saccharides contain soluble starch, amylopectin, or maltodextrin, the G1P is produced from soluble starch, amylopectin, or maltodextrin by α GP.
- [44] 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.

[45] In yet another embodiment, when the saccharides include cellobiose, and the enzymes contain cellobiose phosphorylase, the G1P is generated from cellobiose by cellobiose phosphorylase.

[46] In an additional embodiment, when the saccharides contain cellodextrins and the enzymes include cellodextrin phosphorylase, the G1P is generated from cellodextrins by cellodextrin phosphorylase.

[47] In an alternative embodiment of converting a saccharide to G1P, when the saccharides include cellulose, and enzymes contain cellulose phosphorylase, the G1P is generated from cellulose by cellulose phosphorylase.

[48] According to the invention, tagatose can also be produced from fructose. An example of the process is shown in FIG. 4. For example, the process involves generating F6P from fructose and polyphosphate catalyzed by polyphosphate fructokinase (PPFK); converting F6P to T6P catalyzed by F6PE; and converting T6P to tagatose catalyzed by T6PP. The fructose can be produced, for example, by an enzymatic conversion of sucrose.

[49] In other embodiments, tagatose can be produced from sucrose. An example of such process is shown in FIG. 6. The process provides an *in vitro* synthetic pathway that includes the following enzymatic steps: generating G1P from sucrose and free phosphate catalyzed by sucrose phosphorylase (SP); converting G1P to G6P catalyzed by PGM; converting G6P to F6P catalyzed by PGI; converting F6P to T6P catalyzed by F6PE; and converting T6P to tagatose catalyzed by T6PP.

[50] The phosphate ions generated when T6P is converted to tagatose can then be recycled in the step of converting sucrose to G1P. Additionally, as shown in FIG. 6, PPFK and polyphosphate can be used to increase tagatose yields by producing F6P from fructose generated by the phosphorolytic cleavage of sucrose by SP.

[51] In some embodiments, a process for preparing tagatose includes the following 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.

- [52] In other embodiments, G6P is produced from glucose and sodium polyphosphate by polyphosphate glucokinase.
- [53] The present disclosure provides processes for converting saccharides, such as polysaccharides and oligosaccharides in starch, cellulose, sucrose and their derived products, to tagatose. In certain embodiments, artificial (non-natural) ATP-free enzymatic pathways are provided to convert starch, cellulose, sucrose, and their derived products to tagatose using cell-free enzyme cocktails.
- [54] As shown above, several enzymes can be used to hydrolyze starch to increase the G1P yield. Such enzymes include isoamylase, pullulanase, and alpha-amylase. Corn starch contains many branches that impede α GP action. Isoamylase can be used to de-branch starch, yielding linear amyloextrin. 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.
- [55] As shown in FIG. 2, maltose phosphorylase (MP) can be used to increase tagatose yields by phosphorytically 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 phosphorytically cleaved by α GP to yield G1P.
- [56] Additionally, 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.
- [57] In certain embodiments, cellulose and its derived products can be converted to tagatose through a series of steps. An example of such process is shown in FIG. 3. The process

provides an *in vitro* synthetic 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 T6P catalyzed by F6PE; and converting T6P to tagatose catalyzed by T6PP. In this process, the phosphate ions can be recycled by the step of converting cellodextrin and cellobiose to G1P.

- [58] 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).
- [59] 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.
- [60] In some embodiments, polyphosphate and polyphosphate glucokinase (PPGK) can be added to the process, thus increasing yields of tagatose by phosphorylating the degradation product glucose to G6P, as shown in FIG. 3.
- [61] In other embodiments, tagatose can be generated from glucose. An example of such process is shown in FIG.5. The process involves the steps of generating G6P from glucose and polyphosphate catalyzed by polyphosphate glucokinase (PPGK); converting G6P to F6P catalyzed by PGI; converting F6P to T6P catalyzed by F6PE; and converting T6P to tagatose catalyzed by T6PP.
- [62] 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 5.0-8.0. 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.
- [63] The reaction buffer can also contain key metal cations. Examples of the metal ions include Mg^{2+} and Zn^{2+} .

- [64] 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 40°C to about 70°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.
- [65] The reaction time of the disclosed processes can be adjusted as necessary, and can range from about 8 hours 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.
- [66] The processes according to the invention can achieve high yields due to the very favorable equilibrium constant for the overall reaction. For example, FIG. 7 shows the Reaction Gibbs Energy between intermediates based on formation Gibbs energy for the conversion of glucose 1-phosphate to tagatose. Reaction Gibbs Energies were generated using <http://equilibrator.weizmann.ac.il/>. Theoretically, up to 99% yields can be achieved if the starting material is completely converted to an intermediate.
- [67] 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.
- [68] 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 while effectively minimizing the subsequent product separation costs.
- [69] 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. It also has a final product free of nutrient-rich fermentation media/cellular metabolites.

EXAMPLES

Materials and Methods

[70] Chemicals

[71] All chemicals, including corn starch, soluble starch, maltodextrins, maltose, glucose, filter paper were reagent grade or higher and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Restriction enzymes, T4 ligase, and Phusion DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotides were synthesized either by Integrated DNA Technologies (Coralville, IA, USA) or Eurofins MWG Operon (Huntsville, AL, USA). Regenerated amorphous cellulose used in enzyme purification was prepared from Avicel PH105 (FMC BioPolymer, Philadelphia, PA, USA) through its dissolution and regeneration, as described in: Ye et al., *Fusion of a family 9 cellulose-binding module improves catalytic potential of Clostridium thermocellum cellodextrin phosphorylase on insoluble cellulose*. Appl. Microbiol. Biotechnol. 2011; 92:551-560. *Escherichia coli* Sig10 (Sigma-Aldrich, St. Louis, MO, USA) was used as a host cell for DNA manipulation and *E. coli* BL21 (DE3) (Sigma-Aldrich, St. Louis, MO, USA) was used as a host cell for recombinant protein expression. ZYM-5052 media including either 100 mg L⁻¹ ampicillin or 50 mg L⁻¹ kanamycin was used for *E. coli* cell growth and recombinant protein expression. Cellulase from *Trichoderma reesei* (Catalog number: C2730) and pullulanase (Catalog number: P1067) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and produced by Novozymes (Franklinton, NC, USA). Maltose phosphorylase (Catalog number: M8284) was purchased from Sigma-Aldrich.

[72] Production and purification of recombinant enzymes

[73] The *E. coli* BL21 (DE3) strain harboring a protein expression plasmid was incubated in a 1-L Erlenmeyer flask with 100 mL of ZYM-5052 media containing either 100 mg L⁻¹ ampicillin or 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 MgCl₂ (heat precipitation and cellulose-binding module) 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 ultra-sonication (Fisher Scientific Sonic Dismembrator Model 500; 5 s pulse on and

10 s off, total 21 min at 50% amplitude). After centrifugation, the target proteins in the supernatants were purified.

[74] Three approaches were used to purify the various recombinant proteins. His-tagged proteins were purified by the Profinity IMAC Ni-Charged Resin (Bio-Rad, Hercules, CA, USA). Fusion proteins containing a cellulose-binding module (CBM) and self-cleavage intein were purified through high-affinity adsorption on a large surface-area regenerated amorphous cellulose. Heat precipitation at 70-95° C for 5-30 min was used to purify hyperthermostable enzymes. The purity of the recombinant proteins was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

[75] Enzymes used and their activity assays

[76] *Alpha-glucan phosphorylase* (α GP) from *Thermotoga maritima* (Uniprot ID G4FEH8) was used. Activity was assayed in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM $MgCl_2$, 5 mM DTT, and 30 mM maltodextrin at 50° C. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO) (Vivaproducts, Inc., Littleton, MA, USA). Glucose 1-phosphate (G1P) was measured using a glucose hexokinase/G6PDH assay kit (Sigma Aldrich, Catalog No. GAHK20-1KT) supplemented with 25 U/mL phosphoglucomutase. A unit (U) is described as μ mol/min.

[77] *Phosphoglucomutase* (PGM) from *Thermococcus kodakaraensis* (Uniprot ID Q68BJ6) was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM $MgCl_2$ and 5 mM G1P at 50°C. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product glucose 6-phosphate (G6P) was determined using a hexokinase/G6PDH assay kit (Sigma Aldrich, Catalog No. GAHK20-1KT).

[78] Two different sources of phosphoglucoisomerase (PGI) were used from *Clostridium thermocellum* (Uniprot ID A3DBX9) and *Thermus thermophilus* (Uniprot ID Q5SLL6). Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM $MgCl_2$ and 10 mM G6P at 50°C. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, fructose 6-phosphate (F6P), was determined using a fructose 6-phosphate kinase (F6PK)/pyruvate dehydrogenase (PK)/lactate dehydrogenase (LD) coupled enzyme assay where a decrease in absorbance at 340 nm indicates production of F6P. This 200 μ L reaction contained 50 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 10 mM G6P, 1.5 mM ATP, 1.5 mM phosphoenol pyruvate, 200 μ M NADH, 0.1 U PGI, 5 U PK, and 5 U LD.

[79] Fructose 6-phosphate epimerase (F6PE) from *Dictyoglomus thermophilum* (Uniprot ID B5YBD7) was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl₂, and 10 mM F6P at 50°C. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, tagatose 6-phosphate (T6P), was determined using tagatose 6-phosphate phosphatase and detecting free phosphate release. To detect free phosphate release, 500 µL of a solution containing 0.1 M zinc acetate and 2 mM ammonium molybdate (pH 5) was added to 50 µL of reaction. This was mixed and followed by 125 µL of 5% ascorbic acid (pH 5). This solution was mixed then incubated at 30°C for 20 min. The absorbance at 850 nm was read to determine free phosphate release.

[80] **Thermophilic F6PE from *Anaerolinea thermophila* UNI-1** (Uniprot ID: E8N0N6)

[81] Nucleotide Sequence (SEQ ID NO.: 1)

[82] ATGTTCTGGCTCGCCTGCTCCCTGCTGGATATGGTCACCGCGCAGAAACAGG
GCATGGCGCGGGGTATCCCATCCATTTGTTCTGGCACATCCGGTGGTGCTGAGTGC
CGCCTGCCATCTTGCCCGCCGAGCGGCGCGCCCTGCTCATCGAAACCACCTGC
AATCAGGTCAACCACCAAGGTGGGTACAGCGGCATGACCCCCGCCGATTTTGTC
CGCTTTCTGCGCGAAATTCTGGAACGGGAAGGTATTCCCCCGCAACAGGTCATCC
TGGGCGGGGATCACCTGGGTCTTACCCCTGGCGGAAAGAGCCTGCCGAAACCG
CCATAGCACAAGCGCTGGAAATGGTGCGGGCATACTGTCAGGCAGGCTACACCA
AAATTCATCTGGACGCTTCCATGCCCTGCGCCGATGACGACCCCGAGCGTCCCCT
GCCGCTGGAGCGCATAGCCCGACGGGCGGCGCAGTTGTGCGCCGCCGCCGAAGC
CGCCGCGGGAGCGGTTTCAGCCGGTGTACGTAATTGGCAGTGAGGTGCCCCCGCC
CGGCGGCGCGCAGGGTCAGGAGGCAAGACTTCACGTCACCACTCCGCAGGAAGC
CCAAGCCGCGCTGGATGCCTTTTCGGGAAGCCTTTCTGCAGGCAGGCTTGACTCCC
GTTTGGGAGCGGGTCATTGCGCTGGTAGTCCAGCCGGGGGTGGAGTTTGGCGTG
GACAGCATTCACGCCTATCAGCGCGAAGCCGCCCGCCCGCTGAAGACCTTCATC
GAGGGCGTGCCCGGCATGGTGTATGAAGCCCACTCGACCGATTACCAGACCCGT
GCCTCCCTGCGTGCGCTGGTGGAAAGACCACTTTTCCATTCTCAAGGTTGGTCCGG
CACTAACCTTTGCCTACCGCGAAGCCGTGTTTCGCCCTGGAACACATCGAACGGG
AAATATTGGGCAGGCAGGATATGCCTCTCTCCCGCCTGAGTGAAGTCCTCGACG
AGGTGATGCTGAACGATCCACGCCACTGGCAGGGATACTTTGCCGGCGCTCCCG
CCGAACAGGCGCTGGCGCGCCGCTACAGTTTCAGCGACCGCATTCGCTATTACTG

GCACCATCCCGCCGCGCAGGAAGCCGTGCGGAGACTGCTCGCCAACCTGATCGA
AACCCCGCCGCGCTGAGTTTGCTCAGCCAGTACCTGCCGCGCGAGTATGAGAT
GGTGCGCGCGGGGAAATCTCCAGCCACCCGCGAGGACCTGATTGCGGCACATAT
CCAGCACACGCTGGAAGATTACGCTGCGGCGTGCGGGTAA

[83] Amino acid sequence (SEQ ID NO.: 2)

[84] MFGSPAPLLDMVTAQKQGMARGIPSICSAHPVVLSAACHLARRSGAPLLIETTCN
QVNHQGGYSGMTPADFVRFLREILEREGIPPQQVILGGDHLGPYPWRKEPAETAIAQ
ALEMVRAYVQAGYTKIHLDASMPCADDDPERPLPLERIARRAAQLCAAAEAAAGA
VQPVYVIGSEVPPPGGAQGGQEARLHVTTTPQEAQAALDAFREAFQAGLTPWVERVI
ALVVQPGVEFGVDSIHAYQREARPLKTFIEGVPGMVYEAHSTDYQTRASLRALVE
DHFSILKVGPALTFAFREAVFALEHIEREILGRQDMPLSRLSEVLDEVMLNDPRHWQ
GYFAGAPAEQALARRYSFSDRIRYYWHHPAAQEA VRRLLANLIETPPPLSLLSQYLP
REYEMVRAGEISSHPQDLIRAHIQHTLEDYAAACG

[85] **Thermophilic F6PE from *Caldicellulosiruptor kronotskyensis*** (Uniprot ID: E4SEH3)

[86] Nucleotide sequence (SEQ ID NO.: 3)

[87] ATGAGTCCTCAAAATCCATTGATTGGTTTATTTAAGAATAGAGAAAAAGAGT
TTAAGGGTATTATTTTCAGTTTGTCTTCAAATGAAATAGTCTTAGAAGCAGTTTTA
AAAAGAATGAAAGATACAAACCTACCAATTATTATTGAAGCCACAGCGAACCAG
GTAAATCAATTTGGCGGGTATTCTGGGTTGACACCGTCTCAGTTCAAAGAACGAG
TTATAAAAATTGCTCAAAAAGTTGATTTTCCACTTGAGAGAATAATTCTTGGTGG
GGACCATCTTGGACCATTTGTGTGGCGTGACCAGGAACCAGAAATTGCTATGGA
GTATGCTAAGCAAATGATAAAAGAATACATAAAAGCAGGTTTTACCAAATTCA
CATCGACACGAGTATGCCTTTAAAAGGGGAGAACAGCATAGATGATGAAATAAT
TGCTAAAAGAACTGCTGTGCTCTGCAGGATTGCGGAGGAGTGTTTTGAGAAGAT
TTCTATAACAATCCCTATATTACAAGGCCAGTTTATGTGATAGGAGCTGATGTG
CCACCTCCCGCGGAGAGTCTTCTATTTGTCAAACAATTACTACTAAAGATGAAT
TAGAAAGAAGTTTAGAATATTTCAAAGAAGCATTTAAAAGGAAGGAATTGAGC
ATGTATTCGATTATGTAGTTGCTGTTGTTGCAAATTTTGGAGTTGAATTTGGGAG
CGATGAAATTGTTGATTTTGATATGGAAAAAGTAAAGCCGCTAAAAGAACTTTT
GGCAAAGTACAATATAGTATTTGAAGGCCATTCTACAGATTATCAAACAAAAGA
AAACTTAAAAGAAATGGTCGAATGTGGTATTGCAATTTTAAAGGTTGGTCCTGCT

CTAACATTTACATTGCGCGAAGCGTTAGTAGCACTTAGTCATATTGAAGAAGAA
ATTTATAGCAATGAAAAGGAGAACTGTCAAGATTTAGAGAAGTTTTATTGAAT
ACTATGCTAACATGCAAAGATCACTGGAGTAAATATTTTGATGAGAATGATAAG
TTAATTAAGTCAAAGCTCCTATATAGCTATCTTGACAGATGGAGATACTATTTTG
AAAACGAGAGTGTGAAAAGTGCTGTTTATTCTCTTATTGGAAATTTAGAGAATGT
TAAAATTCCACCTTGGCTTGTAAGTCAGTATTTTCCTTCTCAGTACCAAAAGATG
AGAAAAAAGATTTAAAAAACGGTGCTGCCGACCTAATATTGGATAAAATAGGG
GAAGTCATTGACCATTATGTTTATGCGGTAAAAGAATAA

[88] Amino acid sequence (SEQ ID NO.: 4)

[89] MSPQNPLIGLFKNREKEFKGIISVCSSNEIVLEAVLKRMKDTNLPPIIEATANQVNVQ
FGGYSGLTPSQFKERVIAQKVDFPLERILGGDHLGPFVWRDQEPEIAMEYAKQMI
KEYIKAGFTKIHIDTSMPLKGENSIDDEIIAKRTAVLCRIAECEFEKISINNPYITRPVYV
IGADVPPPGGESSICQTITTKDELESLLEYFKEAFKKEGIEHVFDYVAVVANFVVEF
GSDEIVDFDMEKVKPLKELLAKYNIVFEHSTDYQTKENLKRMVECGIAILKVGPAL
TFTLREALVALSHIEEEIYSNEKEKLSRFREVLNTMLTCKDHW SKYFDENDKLIKSK
LLYSYLDWRWRYFENESVKS AVYSLIGNLENVKIPPWLVSQYFPSQYQKMRKKDLK
NGAADLILDKIGEVIDHYVYAVKE

[90] **Thermophilic F6PE from *Caldilinea aerophila*** (Uniprot ID: I0I507)

[91] Nucleotide sequence (SEQ ID NO.: 5)

[92] ATGTCAACACTTCGCCACATCATTTTGC GACTGATCGAGCTGCGTGAACGAG
AACAGATCCATCTCACGCTGCTGGCCGTCTGTCCCAACTCGGCGGCGGTGCTGGA
GGCAGCGGTGAAGGTCGCCGCGCGCTGCCACACGCCGATGCTCTTCGCTGCCAC
GCTCAATCAAGTCGATCGCGACGGCGGCTACACCGGTTGGACGCCTGCGCAATT
CGTCGCCGAGATGCGTCGCTATGCCGTCCGCTATGGCTGCACCACCCCGCTCTAT
CCTTGCCTGGATCACGGCGGGCCGTGGCTCAAAGATCGCCATGCACAGGAAAAG
CTACCGCTCGACCAGGCGATGCATGAGGTCAAGCTGAGCCTCACCGCCTGTCTG
GAGGCCGGCTACGCGCTGCTGCACATCGACCCACGGTCGATCGCACGCTCCCG
CCCGGAGAAGCGCCGCTCGTGCCGATCGTCGTCGAGCGCACGGTCGAGCTGATC
GAACATGCCGAACAGGAGCGACAGCGGCTGAACCTGCCGGCGGTGCCTATGAA
GTCGGCACCGAAGAAGTACATGGCGGGCTGGTGAATTTGACAATTTTGTGCGCT
TCTTGATTTGCTCAAGGCAAGGCTTGAACAACGTGCCCTGATGCACGCCTGGCC

CGCCTTCGTGGTGGCGCAGGTCGGCACTGACCTGCATACAACGTATTTTGACCCC
AGTGCGGCGCAACGGCTGACTGAGATCGTGCGCCCTACCGGTGCACTGTTGAAG
GGGCACTACACCGACTGGGTGAAAAATCCCGCCGACTATCCGAGGGTAGGCATG
GGAGGCGCCAACGTTGGTCCAGAGTTTACGGCGGCCGAGTTCGAGGCGCTGGAA
GCGCTGGAACGGCGGGAACAACGGCTGTGCGCCAACCGGAAATTGCAGCCCGCC
TGTTTTTTGGCTGCACTGGAAGAGGCAGTAGTCGCTTCAGATCGTTGGCGGAAGT
GGCTCCAGCCCGATGAGATCGGCAAGCCCTTTGCAGAATTAACGCCCCGCACGCC
GGCGCTGGCTCGTGCAGACCGGGGCACGCTACGTCTGGACTGCGCCGAAAGTTA
TCGCCGCACGCGAACAGCTCTATGCGCACCTCTCCCTTGTGCAGGCGGATCCACA
TGCCTACGTGGTAGAGTCAGTCGCCCCGTCAATCGAGCGCTATATCGATGCCTTC
AACTTATACGACGCCGCTACATTGCTTGGATGA

[93] Amino acid sequence (SEQ ID NO.: 6)

[94] MSTLRHILRLIELREREQIHL TLLAVCPNSAAVLEAAVKVAARCHTPMLFAATLN
QVDRDGGYTGWTPAQFVAEMRRYAVRYGCTTPLYPCLDHGGPWLKDRHAQEKL
LDQAMHEVKLSLTACLEAGYALLHIDPTVDRTLPPGEAPLVPIVVERTVELIEHAEQ
RQRLNLPVAVYEVGTEEVHGGGLVNFDNFVAFLDLLKARLEQRALMHAWPAFVVAQ
VGTDLHTTYFDPSAAQRLTEIVRPTGALLKGHYTDWVENPADYPRVGMGGANVGP
EFTAAEFEALEALERREQRLCANRKLQPACFLAALEEAVVASDRWRKWLQPDEIGK
PFAELTPARRRWLVQTGARYVWTAPKVIAAREQLYAHLSLVQADPHAYVVESVAR
SIERYIDAFNLYDAATLLG

[95] **Thermophilic F6PE from *Caldithrix abyssi*** (Uniprot ID: H1XRG1)

[96] Nucleotide sequence (SEQ ID NO.: 7)

[97] ATGAGTCTGCATCCTTTAAATAAATTAATCGAGCGACACAAAAAAGGAACGC
CGGTCGGTATTTATTCCGTCTGTTGCGCCAATCCCTTTGTTTTGAAAGCGGCCATG
CTACAGGCGCAAAAGGATCAGTCTTTGCTACTTATTGAGGCCACTTCCAACCAGG
TAGATCAATTCGGCGGTTACACCGGCATGCGGCCCCGAAGATTTTAAACAATGA
CGCTTGAACCTGGCAGCCGAAAACAATTACGATCCACAGGGATTAATCCTGGGCG
GCGACCATCTGGGGCCCAACCGCTGGACAAAACCTGAGCGCCTCCCGGGCCATGG
ACTACGCCAGAGAGCAGATTGCCGCTTATGTAAAGCCGGCTTTTCCAAAATCCA
CTTAGACGCCACCATGCCCTTGCAAAACGATGCCACAGATTCCGCCGGCCGCCTT
CCAGTCGAAACAATCGCTCAACGTACCGCAGAATTATGCGCCGTGGCCGAACAA

ACTTACCGGCAGAGCGACCAACTCTTTCCGCCGCTGTTTACATTGTCGGCAGCG
ACGTGCCCATCCCGGGCGGCGCGCAAGAAGCGCTGAACCAGATCCATATTACGG
AGGTAAAAGAGGTTCAACAGACCATTGATCACGTGCGGCGGGCCTTTGAAAAAA
ACGGCCTGGAAGCGGCTTACGAAAGAGTTTGCGCCGTTGTCGTGCAGCCAGGCG
TTGAATTCGCCGATCAAATCGTTTTTGAATACGCTCCCGACAGAGCGGCGGCCTT
AAAAGATTTTATTGAAAGCCATTTCGCAGCTGGTTTATGAAGCGCACTCTACTGAT
TACCAGACCGCACCTCTTTTGCGCCAGATGGTAAAAGATCACTTTGCCATTTTAA
AGGTCGGGCCTGCGCTCACCTTTGCCCTGCGCGAAGCCATTTTTGCTCTGGCCTT
TATGGAAAAAGAGCTTTTGCCATTGCACAGAGCGCTCAAACCTTCTGCCATTCTG
GAAACGCTGGACCAAACGATGGACAAAAACCCTGCTTACTGGCAAAGCATTAC
GGCGGAACAAAGGAAGAAGTACGCTTTGCGCAGCGGTTTAGCCTGAGCGACCGC
ATTCGTTACTACTGGCCGTTTCCAAAGGTTCAAAAGGCCCTGCGCCAATTGCTAA
AAAAC TTGCAACAAATTTCCATTCTCTAACTTTGGTAAGCCAGTTCATGCCAGA
GGAATACCAACGTATTTCGCCAAGGAACGTTAACCAACGATCCGCAGGCGCTGAT
TTTGAACAAAATTCAAAGCGTATTAAAGCAATACGCGGAGGCGACGCAAATTCA
AAACTCTTTGACATTCACGCAAAATCAAATTCATTAGCAATGGAGCGACTATG
A

[98] Amino acid sequence (SEQ ID NO.: 8)

[99] MSLHPLNKLIERHKKGTPVGIYSVCSANPFVLKAAMLQAQKDQSLLLIEATSNQV
DQFGGYTGMRPEDFKTMTLELAAENNYDPQGLILGGDHLGPNRWTKLSASRAMDY
AREQIAAYVKAGFSKIHLDATMPLQNDATDSAGRLPVETIAQRTAELCAVAEQTYR
QSDQLFPPPVYIVGSDVIPGGAQEALNQIHTEVKEVQQTIDHVRRAFEKNGLEAAY
ERVCAVVVQPGVEFADQIVFEYAPDRAAALKDFIESHSQLVYEAHSTDYQTAPLLRQ
MVKDHFALIKVGPALTFALREAIFALAFMEKELLPLHRALKPSAILETLDQTMCKNP
AYWQKHYYGGTKEEVRFARFSLSDRIRYYWPFQKALRQLLKNLQQISIPLTLS
QFMPEEYQIRIRQGTLTNDPQALILNKIQSVLKQYAEATQIQNSLTFTQNQNSLAMER
L

[100] **Thermophilic F6PE from *Dictyoglomus thermophilum*** (Uniprot ID: B5YBD7)

[101] Nucleotide sequence (SEQ ID NO.: 9)

[102] ATGTGGCTTAGTAAAGATTATTTGAGAAAAAAGGGAGTTTATTCTATATGTA
GCTCTAATCCATATGTGATTGAGGCAAGTGTTGAATTTGCTAAGGAGAAGAATG

ATTATATTTTAATTGAGGCGACACCTCATCAGATAAACCAGTTTGGTGGATATTC
AGGTATGACTCCCGAAGATTTTAAAACTTTGTAATGGGAATAATAAAAGAAAA
GGGAATAGAAGAGGATAGGGTGATTCTTGGAGGGGACCATTTAGGCCCTCTCCC
TTGGCAAGATGAACCTTCTTCTTCTGCAATGAAAAAGGCAAAAGACCTTATAAG
GGCCTTTGTGGAGAGTGTTTATAAGAAGATACACCTTGATTGTAGTATGTCTCTT
TCTGATGATCCTGTAGTGCTCTCTCCCGAGAAGATAGCAGAAAGGGAGAGGGAA
CTTCTTGAGGTTGCAGAAGAGACTGCTAGAAAGTACAATTTTCAGCCTGTGTATG
TGGTGGGAACTGATGTACCGGTAGCTGGAGGAGGCGAAGAGGAAGGTATTACCT
CAGTGGAGGATTTTAGAGTAGCAATCTCCTCTTTAAAAAATATTTTGAGGATGT
TCCAAGGATATGGGATAGGATAATTGGTTTTGTAATAATGCTTGGTATAGGTTTT
AATTATGAAAAAGTGTTTGAGTATGACAGGATTAAGGTGAGAAAAATTTTAGAG
GAGGTAAAGAAAGAGAATCTTTTTGTTGAAGGTCACTCTACTGACTATCAGACA
AAACGTGCATTGAGAGATATGGTAGAGGATGGAGTAAGAATTCTTAAGGTTGGT
CCTGCTTTAACAGCAAGTTTTAGAAGGGGAGTATTTTTATTAAGTAGCATTGAGG
ATGAGCTTATATCGGAAGATAAAAGGTCTAATATTAAGAAAGTTGTGCTTGAGA
CTATGTTAAAAGATGATAAATATTGGAGAAAGTATTATAAGGATTCAGAAAGAT
TAGAATTAGATATTTGGTACAACCTTACTTGATAGGATTAGATATTATTGGGAATA
TAAAGAGATAAAAATAGCTTTAAATAGGCTTTTTGAAAATTTTTCGGAAGGGGTT
GATATTAGATACATCTATCAATATTTTTATGATTCGTATTTTAAAGTAAGAGAAG
GAAAAATAAGAAATGATCCAAGGGAGCTAATAAAGAATGAAATAAAGAAGGTC
TTGGAGGACTATCACTATGCTGTAAACTTATAA

[103] Codon optimized nucleotide sequence (SEQ ID NO.: 10)

[104] ATGTGGCTGAGCAAGGACTACCTGCGTAAGAAGGGCGTTTACAGCATTGCA
GCAGCAACCCGTATGTTATTGAAGCGAGCGTGGAGTTCGCGAAGGAGAAAAACG
ATTACATCCTGATTGAAGCGACCCCGCACCAGATCAACCAATTTGGTGGCTATAG
CGGCATGACCCCGGAGGACTTCAAGAACTTTGTTATGGGCATCATTAAAGGAAAA
AGGTATCGAGGAAGACCGTGTGATTCTGGGTGGCGATCACCTGGGTCCGCTGCC
GTGGCAGGATGAGCCGAGCAGCAGCGCATGAAGAAAGCGAAAGACCTGATCC
GTGCGTTCGTTGAAAGCGGTTACAAGAAAATTCACCTGGATTGCAGCATGAGCC
TGAGCGACGATCCGGTGGTTCTGAGCCCGGAGAAGATCGCGGAACGTGAGCGTG
AACTGCTGGAAGTTGCGGAGGAAACCGCGCGTAAATACAACCTTCAACCGGTGT

ATGTGGTGGGTACCGATGTTCCGGTTGCGGGTGGCGGTGAGGAAGAGGGTATCA
CCAGCGTGGAGGACTTCCGTGTTGCGATTAGCAGCCTGAAGAAATACTTTGAAG
ACGTTCCGCGTATTTGGGATCGTATCATTGGTTTCGTGATCATGCTGGGCATTGG
TTTCAACTACGAGAAGGTGTTTGAATATGATCGTATCAAAGTGCGTAAAATTCTG
GAAGAGGTTAAGAAAGAGAACCTGTTTGTGGAAGGCCACAGCACCGACTATCAG
ACCAAGCGTGCGCTGCGTGACATGGTGGAGGATGGCGTTCGTATCCTGAAAGTG
GGTCCGGCGCTGACCGCGAGCTTCCGTCTGTGGTGTGTTTCTGCTGAGCAGCATCG
AGGACGAACTGATTAGCGAGGATAAACGTAGCAACATTAAGAAAGTGGTTCTGG
AAACCATGCTGAAGGACGATAAATACTGGCGTAAGTACTATAAAGACAGCGAGC
GTCTGGAAGTGGATATCTGGTACAACCTGCTGGACCGTATTCGTTACTACTGGGA
GTACAAGGAAATCAAGATTGCGCTGAACCGTCTGTTCGAGAACTTTAGCGAAGG
CGTTGATATCCGTTACATCTACCAATACTTCTACGACAGCTACTTCAAAGTGCGT
GAGGGTAAAATCCGTAACGACCCGCGTGAAGTATTAAGAACGAGATTAAGAAA
GTGCTGGAAGACTACCATTATGCGGTGAACCTGTAA

[105] Amino acid sequence (SEQ ID NO.: 11)

[106] MWLSKDYLRRKGVYSICSSNPYVIEASVEFAKEKNDYILIEATPHQINQFGGYSG
MTPEDFKNFVMGIIKEKGIEEDRVILGGDHLGPLPWQDEPSSSAMKKAKDLIRAFVE
SGYKKIHLDCSMLSDDPVVLSPEKIAERERELLEVAEETARKYNFQPVYVVGTDVP
VAGGGEEEGITSVEDFRVAISSLKKYFEDVPRIWDRIIGFVIMLGIGFNYEKVFEDRI
KVRKILEEVKKENLFVEGHSTDYQTKRALRDMVEDGVRILKVGPALTASFRRGVFL
LSSIEDELISEDKRSNIKKVVLETMLKDDKYWRKYKDSERLELDIWYNLLDRIRYY
WEYKEIKIALNRLFENFSEGVDIRYIYQYFYDSYFKVREGKIRNDPRELIKNEIKKYLE
DYHYAVNL

[107] *Tagatose 6-phosphate phosphatase (T6PP)* from *Archaeoglobus fujidis* (Uniprot ID O29805) was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl₂ and 10 mM T6P at 50°C. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). Tagatose production was determined by detecting free phosphate release as described for F6PE.

[108] **Thermophilic T6PP from *Archaeoglobus fulgidus*** (Uniprot ID:O29805)

[109] Nucleotide sequence (SEQ ID NO.: 12)

[110] ATGTTCAAACCAAAGGCCATCGCAGTTGACATAGATGGCACCCCTCACCGACA
GAAAGAGGGCTCTGAACTGCAGGGCTGTTGAAGCTCTCCGCAAGGTAAAAATTC
CCGTGATTTTGGCCACTGGTAACATATCTTGTGTTTTCGAGGGCTGCAGCAAAGCT
GATTGGAGTCTCAGACGTGGTAATCTGCGAGAATGGGGGCGTGGTGAGGTTCTGA
GTACGATGGGGAGGATATTGTTTTAGGAGATAAAGAGAAATGCGTTGAGGCTGT
GAGGGTGCTTGAGAAACACTATGAGGTTGAGCTGCTGGACTTCGAATACAGGAA
GTCGGAAGTGTGCATGAGGAGGAGCTTTGACATCAACGAGGCGAGAAAGCTCAT
TGAGGGGATGGGGGTTAAGCTTGTGGATTCAGGCTTTGCCTACCACATTATGGAT
GCTGATGTTAGCAAGGGAAAAGCTTTGAAGTTCGTTGCCGAGAGGCTTGGTATC
AGTTCAGCGGAGTTTGCAGTTATCGGCGACTCAGAGAACGACATAGACATGTTC
AGAGTTGCTGGATTCGGAATTGCTGTTGCCAATGCCGATGAGAGGCTGAAGGAG
TATGCTGATTTAGTTACGCCATCACCAGACGGCGAGGGGGTTGTTGAGGCTTTGC
AGTTTCTGGGATTGTTGCGGTGA

[111] Amino acid sequence (SEQ ID NO.: 13)

[112] MFKPKAIAVDIDGTLTDRKRALNCRAVEALRKVKIPVILATGNISCFARAAAKLI
GVSDVICENGGVVRFEYDGEDIVLGDKEKCVAVRVLEKHYEVELLDFFEYRKSEV
CMRRSFDINEARKLIEGMGVKLVDSGFAYHIMDADVSKGKALKFVAERLGISSAEFA
VIGDSENDIDMFRVAGFGIAVANADERLKEYADLVTPSPDGEGVVEALQFLGLLR

[113] **T6PP from *Archaeoglobus profundus*** (Uniprot ID D2RHHV2_ARCPA)

[114] Nucleotide sequence (SEQ ID NO.: 14)

[115] GTGTTCAAGGCTTTGGTAGTTGATATAGACGGAACCTTTGACGGATAAGAAGA
GGGCAATAAACTGCAGAGCGGTCTGAAGCACTTAGAAAATAAGATTCCTGTTG
TCTTGGCAACCGGAAACATTTTCATGCTTTGCAAGGGCTGTAGCTAAGATTATAGG
TGTTTCCGATATTGTAATAGCTGAGAACGGAGGTGTTGTCAGATTCAGCTACGAC
GGAGAGGACATAGTTCTGGGGGATAGAAGTAAATGCTTAAGAGCTTTGGAGACA
CTTAGAAAACGCTTCAAAGTAGAGCTTCTCGACAACGAATATAGGAAGTCTGAG
GTCTGCATGAGGAGGAACTTCCCTATAGAGGAAGCTAGAAAGATACTGCCAAAA
GATGTTAGAATAGTCGATACAGGCTTCGCATACCACATAATCGATGCAAATGTC
AGCAAGGGGAAGGCTTTGATGTTTCATAGCCGATAAGCTTGGCTTGGACGTTAAG

GATTTTCATTGCGATAGGTGATTCCGAAAACGACATTGAAATGTTGGAAGTTGCA
GGTTTTGGCGTTGCAGTTGCGAATGCGGATGAAAAGCTTAAGGAGGTAGCGGAT
TTGGTCACATCGAAGCCTAATGGAGACGGAGTTGTCTGAAGCTCTTGAGTTCTTGG
GACTCATTTAG

[116] Amino acid sequence (SEQ ID NO.: 15)

[117] MFKALVVDIDGTLTDKRAINCRAVEALRKLKIPVVLATGNISCFARAVAKIIGV
SDIVIAENGGVVRFSYDGEDIVLGDERSKCLRAETLRKRFKVELLDNEYRKSEVCMR
RNFPIEEARKILPKDVRIVDTGFAYHIIDANVSKGKALMFIADKLGLDVKDFIAIGDSE
NDIEMLEVAGFGVAVANADEKLKEVADLVTSKPNGDGVVEALEFLGLI

[118] T6PP from *Archaeoglobus veneficus* (Uniprot ID F2KMK2_ARCVS)

[119] Nucleotide sequence (SEQ ID NO.: 16)

[120] ATGCTCCGTCCAAAGGGTCTCGCCATTGACATCGACGGAACCATAACATACA
GGAATCGAAGCCTGAACTGTAAGGCCGTTGAAGCTCTCAGGAAGGTAAAAATCC
CTGTAGTTCTTGCAACTGGCAACATATCCTGTTTCGCAAGAACTGCTGCAAAGCT
TATAGGCGTCTCAGACATTGTTATATGCGAAAATGGAGGTATTGTTTCGATTTCAGC
TACGATGGCGACGACATAGTGCTTGGGGACATAAGCAAATGCCTTAAAGCGGCT
GAAATTCTCAAAGAGTACTTTGAAATCGAATTCCTTGACGCTGAGTACAGGAAG
TCGGAGGTCTGTCTTCGCAGAACTTTCCTATTGAAGAGGCGAGGAAAATTCTTC
ACGATGCAAAGCTTGATGTTAAAATCGTCGATTACAGGTTTTGCGTACCACATAAT
GGATGCGAAGGTCAGCAAAGGAAGGGCTCTTGAGTACATAGCTGATGAACTTGG
TATAAGTCCGAAGGAGTTCGCTGCAATTGGTGATTCTGAGAACGACATAGACCT
GATTAAGGCTGCCGGCCTCGGTATTGCCGTTGGAGATGCTGACTTAAAGCTCAAA
ATGGAGGCCGACGTGGTAGTCTCGAAGAAGAATGGCGATGGAGTTGTTGAAGCA
CTTGAGCTTCTGGGCTTAATTTAA

[121] Amino acid sequence (SEQ ID NO.: 17)

[122] MLRPKGLAIDIDGTITYRNRSLNCKAVEALRKVKIPVVLATGNISCFARTAAKLIG
VSDIVICENGIVRFSYDGDDIVLGDISKCLKAAEILKEYFEIEFLDAEYRKSEVCLRR
NFPIEEARKILHDAKLDVKIVDSGFAYHIMDAKVSKGRALEYIADELGISPKEFAAIG
DSENDIDLIKAAGLGIAVGDADLKLKMEADVVSCKNGDGVVEALELLGLI

- [123] The recombinant cellodextrin phosphorylase and cellobiose phosphorylase from *C. thermocellum* are described in Ye *et al.* Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails. *ChemSusChem* 2009; 2:149-152. Their activities were assayed as described.
- [124] The recombinant polyphosphate glucokinase from *Thermobifida fusca* YX is described in Liao *et al.*, One-step purification and immobilization of thermophilic polyphosphate glucokinase from *Thermobifida fusca* YX: glucose-6-phosphate generation without ATP. *Appl. Microbiol. Biotechnol.* 2012; 93:1109-1117. Its activities were assayed as described.
- [125] The recombinant isoamylase from *Sulfolobus tokodaii* is described in Cheng *et al.*, Doubling power output of starch biobattery treated by the most thermostable isoamylase from an archaeon *Sulfolobus tokodaii*. *Scientific Reports* 2015; 5:13184. Its activities were assayed as described.
- [126] The recombinant 4- α -glucanotransferase from *Thermococcus litoralis* is described in Jeon *et al.* 4- α -Glucanotransferase from the Hyperthermophilic Archaeon *Thermococcus Litoralis*. *Eur. J. Biochem.* 1997; 248:171-178. Its activity was measured as described.
- [127] Sucrose phosphorylase from *Caldithrix abyssi* (Uniprot H1XT50) was used. Its activity was measured in 50 mM HEPES buffer (pH 7.5) containing 10 mM sucrose and 12 mM organic phosphate. Glucose 1-phosphate (G1P) was measured using a glucose hexokinase/G6PDH assay kit supplemented with 25 U/mL phosphoglucomutase as with α -glucan phosphorylase.
- [128] Enzyme units used in each Example below can be increased or decreased to adjust the reaction time as desired. For example, if one wanted to perform Example 9 in 8 h instead of 24 h, the units of the enzymes would be increased about 3-fold. Conversely, if one wanted perform example 9 in 48 h instead of 24 h the enzyme units could be decreased about 2-fold. These examples illustrate how the amount of enzyme units can be used to increase or decrease reaction time while maintaining constant productivity.

[129] Example 1

- [130] To validate the technical feasibility of the enzymatic biosynthesis of fructose 6-phosphate from starch, three enzymes were recombinantly expressed: α -glucan phosphorylase from *T. maritima* (Uniprot ID G4FEH8), phosphoglucomutase from *Thermococcus kodakaraensis*

(Uniprot ID Q68BJ6), and phosphoisomerase from *Clostridium thermocellum* (Uniprot ID A3DBX9). The recombinant proteins were over-expressed in *E. coli* BL21 (DE3) and purified as described above.

[131] A 0.20 mL reaction mixture containing 10 g/L soluble starch, 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 0.5 mM ZnCl₂, 0.01 U of α GP, 0.01 U PGM, and 0.01 U PGI was incubated at 50°C for 24 hours. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, fructose 6-phosphate (F6P), was determined using a fructose 6-phosphate kinase (F6PK)/pyruvate dehydrogenase (PK)/lactate dehydrogenase (LD) coupled enzyme assay where a decrease in absorbance at 340 nm indicates production of F6P as described above. The final concentration of F6P after 24 hours was 3.6 g/L.

[132] Example 2

[133] Same tests as in Example 1 (other than reaction temperatures) were carried out from 40 to 80°C. It was found that 10 g/L soluble starch produced 0.9 g/L F6P at 40°C and 3.6 g/L F6P at 80°C after 40 hour reactions. These results suggest that increasing reaction temperature for this set of enzymes increased F6P yields, but too high temperature may impair some enzyme activity.

[134] Example 3

[135] It was found that, at 80°C, an enzyme ratio of α GP: PGM: PGI of approximately 1:1:1 resulted in fast F6P generation. It was noted that the enzyme ratio did not influence final F6P concentration greatly if the reaction time was long enough. However, the enzyme ratio affects reaction rates and the total cost of enzymes used in the system.

[136] Example 4

[137] A 0.20 mL reaction mixture containing 10 g/L maltodextrin, 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 0.5 mM ZnCl₂, 0.01 U of α GP, 0.01 U PGM, and 0.01 U PGI was incubated at 50°C for 24 hours. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). The product, fructose 6-phosphate (F6P), was determined using a fructose 6-phosphate kinase (F6PK)/pyruvate dehydrogenase (PK)/lactate dehydrogenase (LD) coupled enzyme assay where a decrease in absorbance at 340 nm indicates production of F6P as described above. The final concentration of F6P after 24 hours was 3.6 g/L.

[138] Example 5

[139] To test for F6P production from Avicel, Sigma cellulase was used to hydrolyze cellulose at 50°C. To remove beta-glucosidase from commercial cellulase, 10 filter paper units/mL of cellulase was mixed to 10 g/L Avicel at an ice-water bath for 10 min. After centrifugation at 4°C, the supernatant containing beta-glucosidase was decanted. Avicel that was bound with cellulase containing endoglucanase and cellobiohydrolase was resuspended in a citrate buffer (pH 4.8) for hydrolysis at 50°C for three days. The cellulose hydrolysate was mixed with 5 U/mL cellodextrin phosphorylase, 5 U/L cellobiose phosphorylase, 5 U/mL of α GP, 5 U/mL PGM, and 5 U/mL PGI in a 100 mM HEPES buffer (pH 7.2) containing 10 mM phosphate, 5 mM MgCl_2 and 0.5 mM ZnCl_2 . The reaction was conducted at 60°C for 72 hours and high concentrations of F6P were found (small amounts of glucose and no cellobiose). F6P was detected using the coupled enzyme assay described above. Glucose was detected using a hexokinase/G6PDH assay kit as described above.

[140] Example 6

[141] To increase F6P yields from Avicel, Avicel was pretreated with concentrated phosphoric acid to produce amorphous cellulose (RAC), as described in Zhang et al. *A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure*. Biomacromolecules 2006; 7:644-648. To remove beta-glucosidase from commercial cellulase, 10 filter paper units/mL of cellulase was mixed with 10 g/L RAC in an ice-water bath for 5 min. After centrifugation at 4°C, the supernatant containing beta-glucosidase was decanted. The RAC that was bound with cellulase containing endoglucanase and cellobiohydrolase was resuspended in a citrate buffer (pH 4.8) for hydrolysis at 50°C for 12 hours. The RAC hydrolysate was mixed with 5 U/mL cellodextrin phosphorylase, 5 U/L cellobiose phosphorylase, 5 U/mL of α GP, 5 U/mL PGM, and 5 U/mL PGI in a 100 mM HEPES buffer (pH 7.2) containing 10 mM phosphate, 5 mM MgCl_2 and 0.5 mM ZnCl_2 . The reaction was conducted at 60°C for 72 hours. High concentrations of F6P and glucose were recovered because no enzymes were added to convert glucose to F6P. F6P was detected using the coupled enzyme assay described above. Glucose was detected using a hexokinase/G6PDH assay kit as described above.

[142] Example 7

[143] To further increase F6P yields from RAC, polyphosphate glucokinase and polyphosphate were added. To remove beta-glucosidase from commercial cellulase, 10 filter paper units/mL of cellulase was mixed with 10 g/L RAC in an ice-water bath for 5 min. After centrifugation at 4° C, the supernatant containing beta-glucosidase was decanted. The RAC that was bound with cellulase containing endoglucanase and cellobiohydrolase was re-suspended in a citrate buffer (pH 4.8) for hydrolysis at 50°C was incubated in a citrate buffer (pH 4.8) for hydrolysis at 50°C for 12 hours. The RAC hydrolysate was mixed with 5 U/mL polyphosphate glucokinase, 5 U/mL cellodextrin phosphorylase, 5 U/mL cellobiose phosphorylase, 5 U/mL of α GP, 5 U/mL PGM, and 5 U/mL PGI in a 100 mM HEPES buffer (pH 7.2) containing 50 mM polyphosphate, 10 mM phosphate, 5 mM $MgCl_2$ and 0.5 mM $ZnCl_2$. The reaction was conducted at 50°C for 72 hours. F6P was found in high concentrations with only small amounts of glucose now present. F6P was detected using the coupled enzyme assay described above. Glucose was detected using a hexokinase/G6PDH assay kit as described above.

[144] Example 8

[145] To validate tagatose production from F6P, 2 g/L F6P was mixed with 1 U/ml fructose 6-phosphate epimerase (F6PE) and 1 U/ml tagatose 6-phosphate phosphatase (T6PP) in 50 mM HEPES buffer (pH 7.2) containing 5 mM $MgCl_2$. The reaction was incubated for 16 hours at 50°C. 100% conversion of F6P to tagatose is seen 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.

[146] Example 9

[147] To validate production of tagatose from maltodextrin, a 0.20 mL reaction mixture containing 20 g/L maltodextrin, 50 mM phosphate buffered saline pH 7.2, 5 mM $MgCl_2$, 0.05 U of α GP, 0.05 U PGM, 0.05 U PGI, 0.05 U F6PE, and 0.05 U T6PP was incubated at 50°C for 24 hours. The reaction was stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). Tagatose was detected and quantified using an Agilent 1100 series HPLC with refractive index detector and an Agilent Hi-Plex H-column. The mobile phase was 5 mM H_2SO_4 , which ran at 0.6 mL/min. A yield of 9.2 g/L tagatose was obtained. This equates to 92% of the theoretical yield due to limits of maltodextrin degradation without enzymes such

as isoamylase or 4-glucan transferase. Standards of various concentrations of tagatose were used to quantify our yield.

[148] Example 10

A reaction mixture containing 200 g/L maltodextrin, 10 mM acetate buffer (pH 5.5), 5 mM MgCl₂, and 0.1 g/L isoamylase was incubated at 80°C for 24 hours. This was used to create another reaction mixture containing 20 g/L isoamylase treated maltodextrin, 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 0.05 U of α GP, 0.05 U PGM, 0.05 U PGI, 0.05 U F6PE, and 0.05 U T6PP was incubated at 50°C for 24 hours. Production of tagatose was quantified as in Example 9. The yield of tagatose was increased to 16 g/L with the pretreatment of maltodextrin by isoamylase. This equates to 80% of the theoretical yield.

[149] Example 11

[150] To further increase tagatose yields from maltodextrin, 0.05 U 4-glucan transferase (4GT) was added to the reaction described in example 9.

[151] A 0.2 mL reaction mixture containing 20 g/L isoamylase treated maltodextrin (see example 9), 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 0.05 U of α GP, 0.05 U PGM, 0.05 U PGI, 0.05 U F6PE, 0.05 U T6PP, and 0.05 U 4GT was incubated at 50°C for 24 hours. Production of tagatose was quantified as in example 9. The yield of tagatose was increased to 17.7 g/L with the addition of 4GT to IA-treated maltodextrin. This equates to 88.5% of the theoretical yield.

[152] Example 12

[153] To determine the concentration range of phosphate buffered saline (PBS), a 0.20 mL reaction mixture containing 50 g/L maltodextrin; 6.25 mM, 12.5 mM, 25 mM, 37.5 mM, or 50 mM phosphate buffered saline pH 7.2; 5 mM MgCl₂; 0.1 U of α GP; 0.1 U PGM; 0.1 U PGI; 0.1 U F6PE; and 0.1 U T6PP was incubated at 50°C for 6 hours. The short duration ensures completion was not reached, and therefore differences in efficiency could be clearly seen. Production of tagatose was quantified as in example 9. Respectively, a yield of 4.5 g/L, 5.1 g/L, 5.6 g/L, 4.8 g/L, or 4.9 g/L tagatose was obtained for the reactions containing either 6.25 mM, 12.5 mM, 25 mM, 37.5 mM, or 50 mM phosphate buffered saline pH 7.2 (Table 1). These results indicate that a concentration of 25 mM PBS pH 7.2 is ideal for these particular reaction conditions. It is important to note that even the use of 6.25 mM PBS at pH 7.2 results in significant turnover due to phosphate recycling. This shows that the disclosed phosphate

recycling methods are able to keep phosphate levels low even at industrial levels of volumetric productivity (e.g., 200-300 g/L maltodextrin).

[154] Table 1

Concentration of PBS pH 7.2 (mM)	g/L of Tagatose
6.25	4.5
12.5	5.1
25	5.6
37.5	4.8
50	4.9

[155] Example 13

[156] To determine the pH range of the cascade reaction, a 0.20 mL reaction mixture containing 50 g/L maltodextrin; 50 mM phosphate buffered saline pH 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 7.2, or 7.3; 5 mM MgCl₂; 0.02 U of α GP; 0.02 U PGM; 0.02 U PGI; 0.02 U F6PE; and 0.02 U T6PP was incubated at 50°C for 16 hours. The units were lowered to ensure completion was not reached, and therefore differences in efficiency could be clearly seen. Production of tagatose was quantified as in example 8. Respectively, a yield of 4.0 g/L, 4.1 g/L 4.2 g/L, 4.1 g/L, 4.4 g/L, 4.1 g/L, 3.8 g/L or 4.0 g/L tagatose was obtained for reactions containing 50 mM phosphate buffered saline at pH 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, or 7.3 (Table 2). These results indicate that a pH of 6.8 is ideal for these particular reaction conditions, although the system works through a wide pH range.

[157] Table 2

pH of PBS	g/L of Tagatose
6.0	4.0
6.2	4.1
6.4	4.2
6.6	4.1
6.8	4.4
7.0	4.1
7.2	3.8

7.3	4.0
-----	-----

[158] Example 14

[159] To investigate scale-up, a 20 mL reaction mixture containing 50 g/L isoamylase treated maltodextrin (see Example 9), 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 10 U of α GP, 10 U PGM, 10 U PGI, 10 U F6PE, and 10 U T6PP was incubated at 50°C for 24 hours. Production of tagatose was quantified as in example 8. The yield of tagatose was 37.6 g/L at the 20 mL scale and 50 g/L maltodextrin. This equates to 75% of the theoretical yield. These results indicate that scale-up to larger reaction volumes will not result in significant losses of yield.

[160] Example 15

[161] To further increase tagatose yields from maltodextrin, 0.05 U maltose phosphorylase is added to the reaction described in Example 9.

[162] Example 16

[163] To further increase tagatose yields from maltodextrin, 0.05 U polyphosphate glucokinase and 75 mM polyphosphate is added to the reaction described in Example 9.

[164] Example 17

[165] To produce tagatose from fructose, a reaction mixture containing 10 g/L fructose, 50 mM Tris buffer pH 7.0, 75 mM polyphosphate, 5 mM MgCl₂, 0.05 U fructose polyphosphate kinase, 0.05 U F6PE, and 0.05 U T6PP is incubated at 50°C for 24 hours. Production of tagatose is quantified as in Example 9.

[166] Example 18

[167] To produce tagatose from glucose, a reaction mixture containing 10 g/L glucose, 50 mM Tris buffer pH 7.0, 75 mM polyphosphate, 5 mM MgCl₂, 0.05 U glucose polyphosphate kinase, 0.05 U PGI, 0.05 U F6PE, and 0.05 U T6PP is incubated at 50°C for 24 hours. Production of tagatose is quantified as in Example 9.

[168] Example 19

[169] To produce tagatose from sucrose, a reaction mixture containing 10 g/L sucrose, 50 mM phosphate buffered saline pH 7.0, 5 mM MgCl₂, 0.05 U sucrose phosphorylase, 0.05 PGM, 0.05 U PGI, 0.05 U F6PE, and 0.05 U T6PP is incubated at 50°C for 24 hours. Production of tagatose is quantified as in Example 9.

[170] Example 20

[171] To further increase yields of tagatose from sucrose, 75 mM polyphosphate and 0.05 polyphosphate fructokinase is added to the reaction mixture in example 15. Production of tagatose is quantified as in Example 9.

What is claimed is:

1. An enzymatic process for preparing tagatose from a starch derivative, the process comprising the steps of:
 - (i) converting a saccharide to glucose 1-phosphate (G1P) using alpha glucan phosphorylase (α GP), wherein the saccharide is selected from one or more derivatives of starch;
 - (ii) converting G1P to glucose 6-phosphate (G6P) using a phosphoglucomutase (PGM);
 - (iii) converting G6P to fructose 6-phosphate (F6P) using a phosphoglucosomerase (PGI);
 - (iv) converting fructose 6-phosphate (F6P) to tagatose 6-phosphate (T6P) catalyzed by a fructose 6-phosphate epimerase (F6PE); and
 - (v) converting the T6P produced to tagatose catalyzed by a tagatose 6-phosphate phosphatase (T6PP)wherein steps (i)-(v) are conducted in a single reaction vessel.
2. The process of claim 1, 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, wherein the enzymatic hydrolysis of starch is catalyzed by isoamylase, pullulanase, alpha-amylase, or a combination thereof.
3. The process of claim 1, wherein 4-glucan transferase (4GT) is added to the process.
4. The process of any one of claims 1 to 3, wherein the F6PE is encoded by a polynucleotide comprising a nucleotide sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 1, 3, 5, 7, 9, or 10.
5. The process of any one of claims 1 to 4, wherein the F6PE comprises an amino acid sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 2, 4, 6, 8, or 11.
6. The process of any one of claims 1 to 5, wherein the T6PP is encoded by a polynucleotide comprising a nucleotide sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 12, 14, or 16.

7. The process of any one of claims 1 to 6, wherein the T6PP comprises an amino acid sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 13, 15, or 17.

8. The process of any one of claims 1 to 7, 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 8.0, or

for about 8 hours to about 48 hours.

9. The process of claim 8, 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,

where phosphate is recycled, and

where step(v) involves an energetically favorable chemical reaction.

10. The process of claim 9, wherein phosphate is recycled, wherein phosphate ions produced by T6PP dephosphorylation of T6P are used in the process step of converting a saccharide to G1P.

11. The process of claim 9 or claim 10, wherein the step of converting T6P to tagatose is an energetically favorable, irreversible phosphatase reaction.

12. The process of any one of claims 1 to 11, further comprising the step of separating tagatose, wherein the separation is not via chromatography.

13. The process of any one of claims 1 to 12 wherein the derivatives of starch are selected from the group consisting of amylose, amylopectin, soluble starch, amyloextrin, maltotriose, maltodextrin, maltose, and glucose.

14. The process of any one of claims 1 to 13, wherein the F6PE comprises an amino acid sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 2, 4, 6, 8, or 11, and wherein the T6PP comprises an amino acid sequence having at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity with SEQ ID NOS.: 13, 15, or 17.

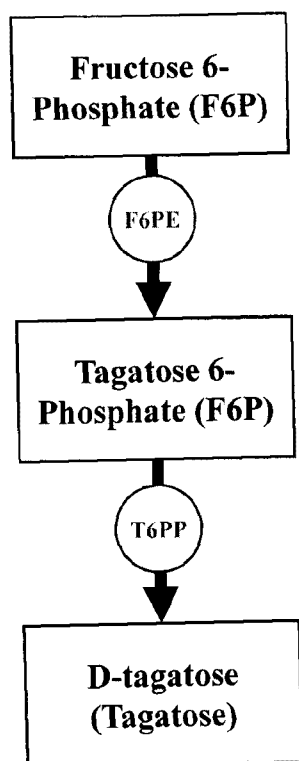


FIG. 1

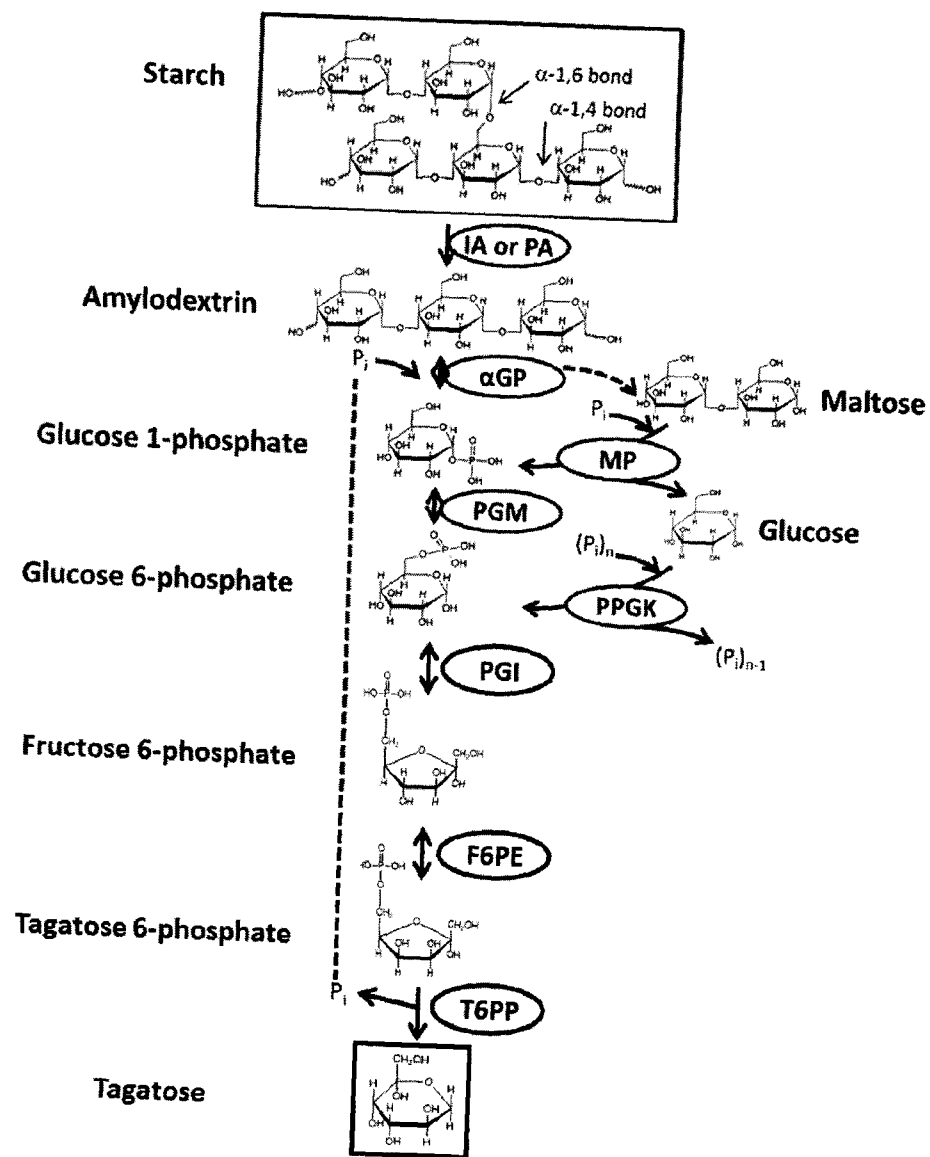


FIG. 2

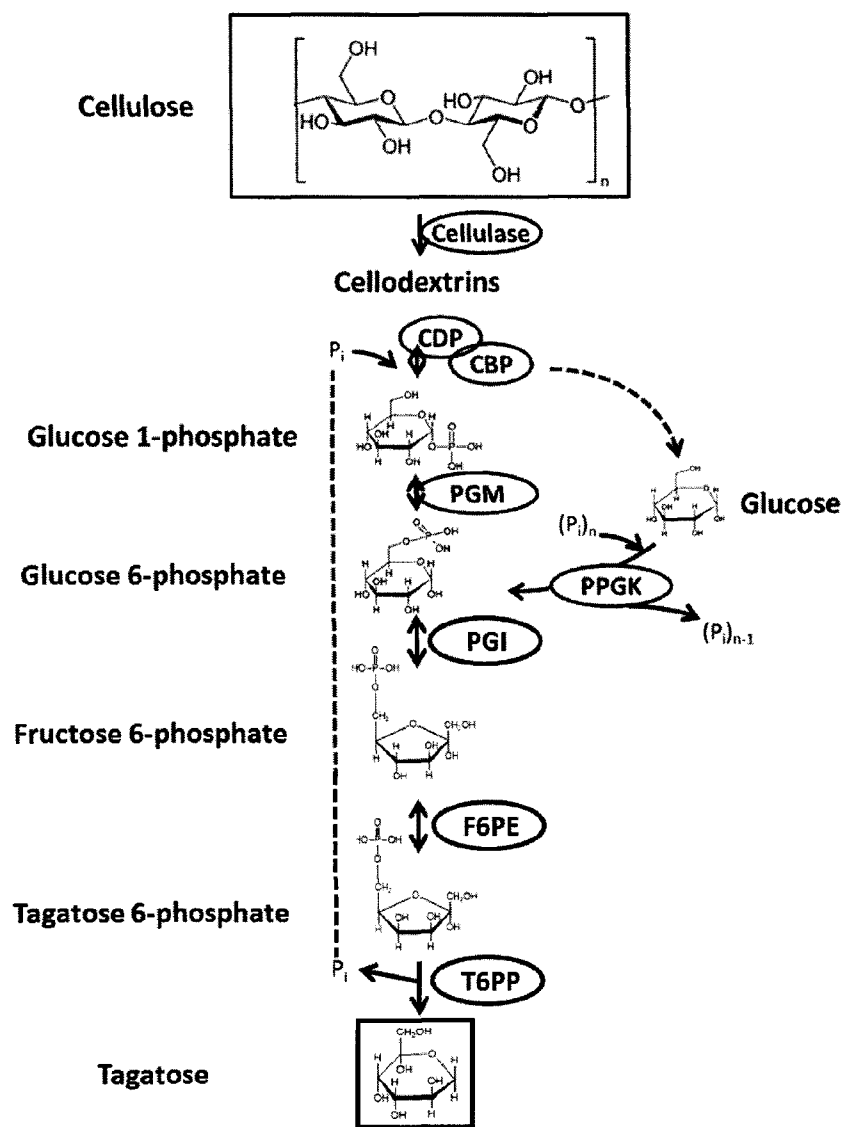


FIG. 3

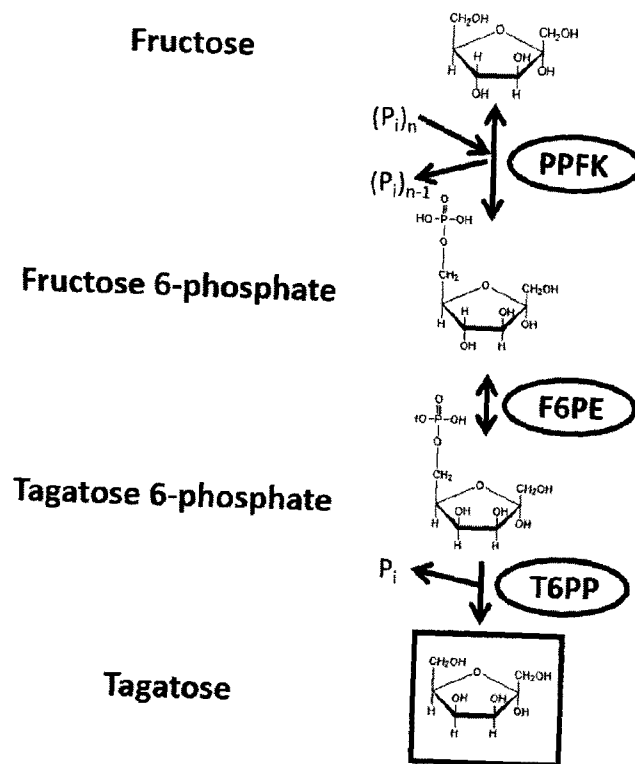


FIG. 4

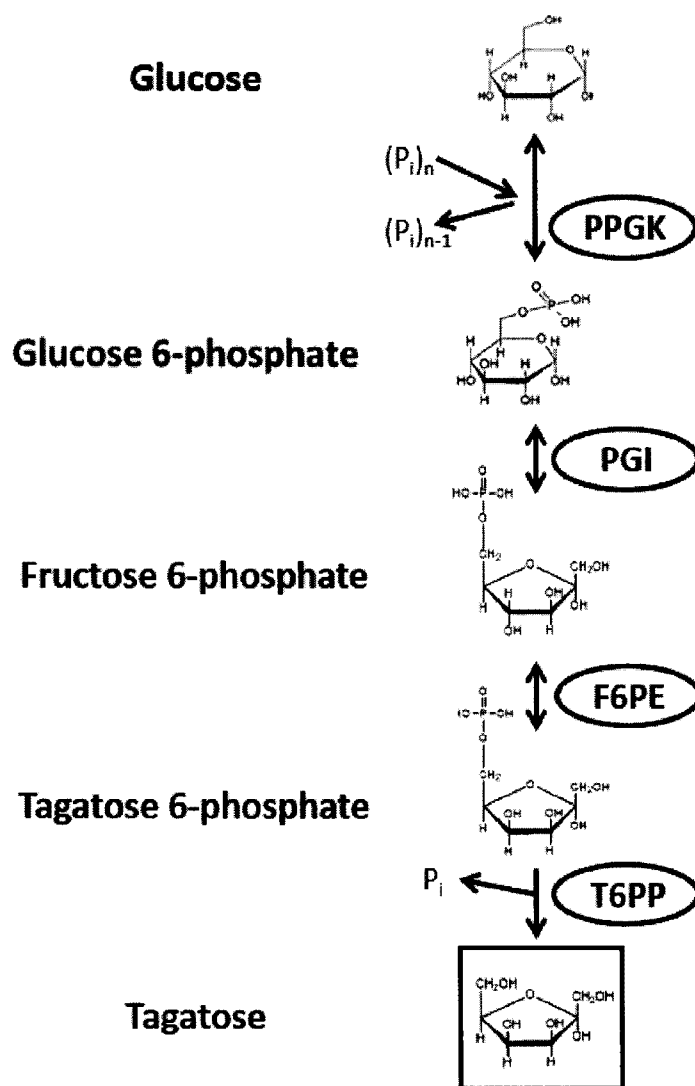


FIG. 5

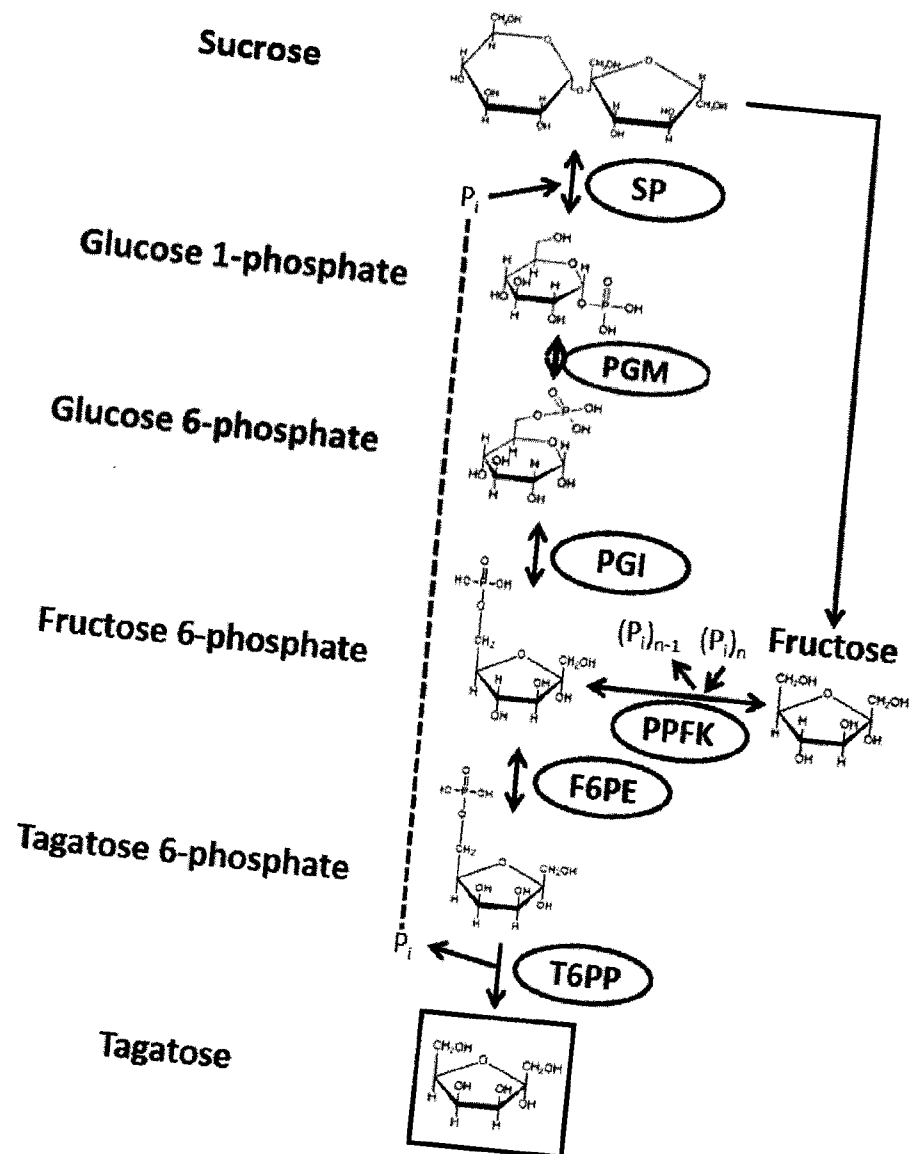


FIG. 6

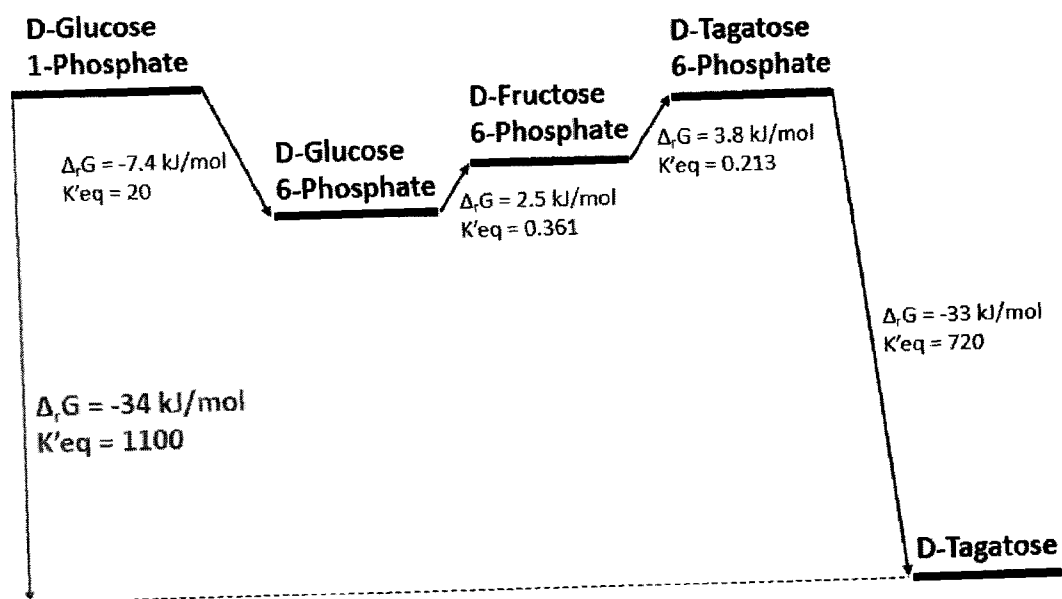


FIG. 7

**Fructose 6-
Phosphate (F6P)**

F6PE

**Tagatose 6-
Phosphate (F6P)**

T6PP

**D-tagatose
(Tagatose)**

