



(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION
(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2017/12/14 (87) Date publication PCT/PCT Publication Date: 2018/06/21 (85) Entrée phase nationale/National Entry: 2019/05/23 (86) N° demande PCT/PCT Application No.: US 2017/066298 (87) N° publication PCT/PCT Publication No.: 2018/112139 (30) Priorité/Priority: 2016/12/14 (US62/434,033)	(51) Cl.Int./Int.Cl. <i>C12N 9/90</i> (2006.01), <i>C12P 19/02</i> (2006.01), <i>C12P 19/24</i> (2006.01) (71) Demandeur/Applicant: BONUMOSE LLC, US (72) Inventeurs/Inventors: WICHELECKI, DANIEL JOSEPH, US; ROGERS, EDWIN O., US (74) Agent: GOWLING WLG (CANADA) LLP
--	---

(54) Titre : PRODUCTION ENZYMATIQUE DE D-ALLULOSE
(54) Title: ENZYMATIC PRODUCTION OF D-ALLULOSE

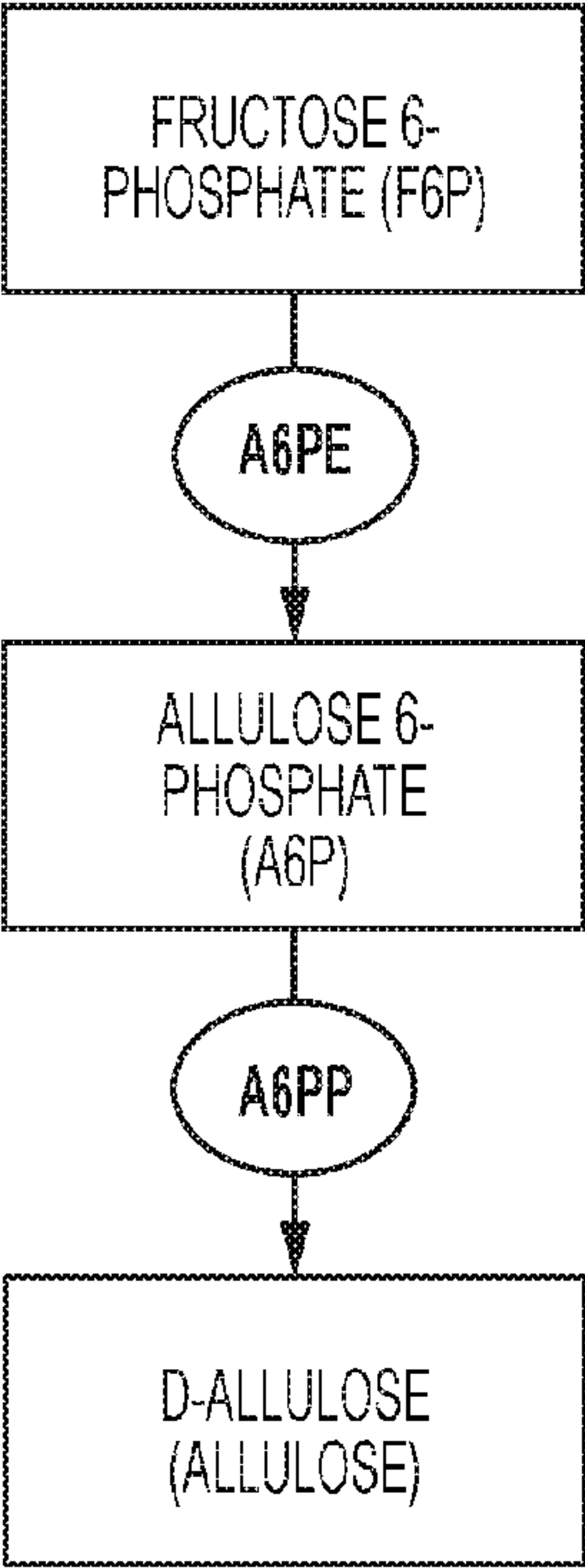


FIG. 1

(57) **Abrégé/Abstract:**
The current disclosure provides a process for enzymatically converting a saccharide into allulose. The invention also relates to a process for preparing allulose where the process involves converting fructose 6-phosphate (F6P) to allulose 6-phosphate (A6P), catalyzed by allulose 6-phosphate 3-epimerase (A6PE), and converting the A6P to allulose, catalyzed by allulose 6-phosphate phosphatase (A6PP).

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

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
21 June 2018 (21.06.2018)



(10) International Publication Number
WO 2018/112139 A1

(51) International Patent Classification:

C12N 9/90 (2006.01) C12P 19/24 (2006.01)
C12P 19/02 (2006.01)

(21) International Application Number:

PCT/US2017/066298

(22) International Filing Date:

14 December 2017 (14.12.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/434,033 14 December 2016 (14.12.2016) US

(71) Applicant: BONUMOSE LLC [US/US]; 1725 Discovery Drive, Suite 220, Charlottesville, VA 22911 (US).

(72) Inventors: WICHELECKI, Daniel, Joseph; 717 Lee Street Apartment #7, Blacksburg, VA 24060 (US).
ROGERS, Edwin, O.; 34 Canterbury Road, Charlottesville, VA 22903 (US).

(74) Agent: JHAVERI, Sulay, D.; J.A. Lindeman & Co., PLLC, 3190 Fairview Park Drive, Suite 1070, 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, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, 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.

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

(54) Title: ENZYMATIC PRODUCTION OF D-ALLULOSE

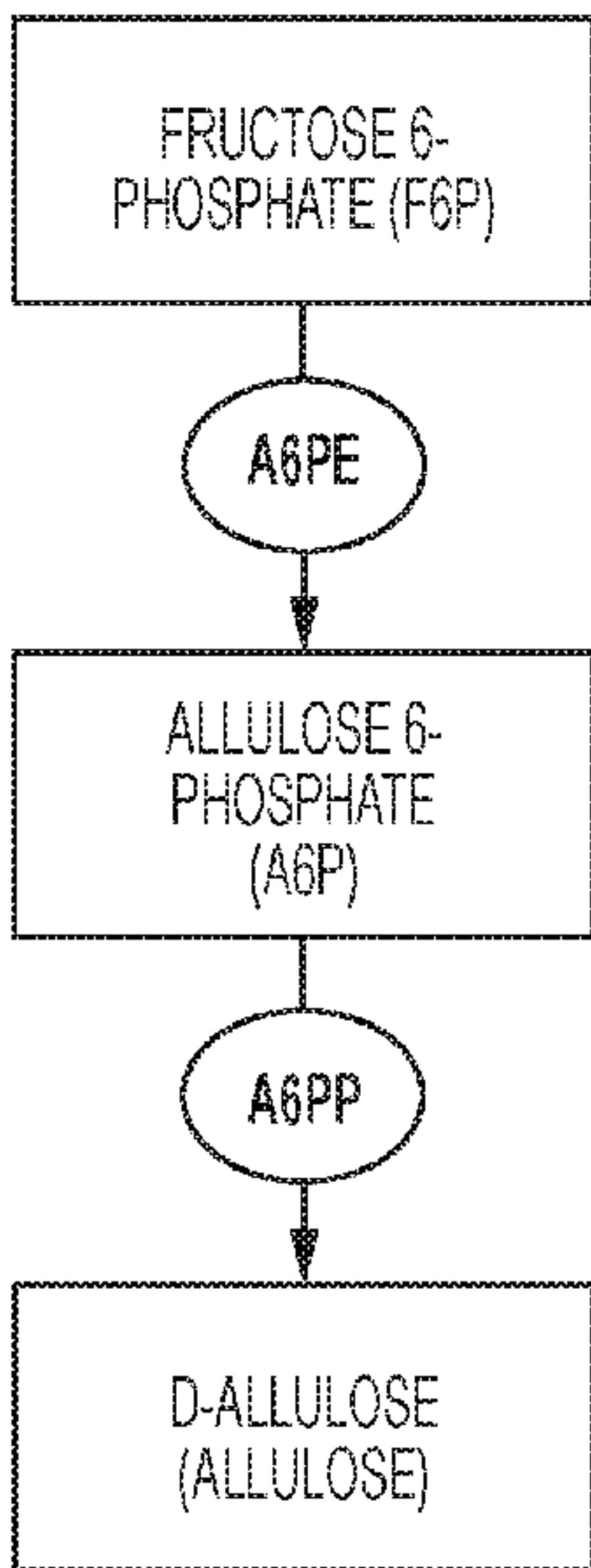


FIG. 1

(57) Abstract: The current disclosure provides a process for enzymatically converting a saccharide into allulose. The invention also relates to a process for preparing allulose where the process involves converting fructose 6-phosphate (F6P) to allulose 6-phosphate (A6P), catalyzed by allulose 6-phosphate 3-epimerase (A6PE), and converting the A6P to allulose, catalyzed by allulose 6-phosphate phosphatase (A6PP).

WO 2018/112139 A1

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*

ENZYMATIC PRODUCTION OF D-ALLULOSE

CROSS-REFERENCE TO RELATED APPLICATIONS

- [1] This application claims priority to U.S. Application Ser. No. 62/434,033, filed on December 14, 2016, which is incorporated herein by reference.

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

- [3] D-allulose (also known as D-psicose) (allulose hereafter) is a low-calorie, natural sweetener that has 70% the sweetness of sucrose, but only 10% of the calories. It is a naturally occurring monosaccharide hexose that is present in only small amounts in wheat and other plants. Allulose was approved as a food additive by the Food and Drug Administration (FDA) in 2012, which designated it as generally recognized as safe (GRAS). However, due to allulose's high selling prices, its use as a sweetener has been limited. Allulose boasts a myriad of health benefits: it is low-calorie (10% of sucrose); it has a very low glycemic index of 1; it is fully absorbed in the small intestine but not metabolized and instead secreted in urine and feces; it helps regulate blood sugar by inhibiting alpha-amylase, sucrase and maltase; and it has similar functionality in foods and beverages as sucrose. As such, allulose clearly has a variety of applications in the food and beverage industries.
- [4] Currently allulose is produced predominantly through the enzymatic isomerization of fructose (WO 2014049373). Overall, the method suffers because of higher feedstock cost, the costly separation of allulose from fructose, and relatively low product yields.
- [5] There is a need to develop a cost-effective synthetic pathway for high-yield allulose production where at least one step of the process involves an energetically favorable chemical

reaction. Furthermore, there is a need for production process where the process steps can be conducted in one tank or bioreactor. There is also a need for a process of allulose 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 an allulose 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 allulose. In various aspects, the processes involve converting fructose 6-phosphate (F6P) to allulose 6-phosphate (A6P), catalyzed by allulose 6-phosphate 3-epimerase (A6PE); and converting the A6P to allulose, catalyzed by allulose 6-phosphate phosphatase (A6PP). The inventions also relate to allulose prepared by any of the processes described herein.
- [7] In some aspects of the invention, a process for preparing allulose also involves the step of converting glucose 6-phosphate (G6P) to the F6P, where the step is catalyzed by phosphoglucisomerase (PGI). In other aspects, a process for allulose 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 allulose can involve converting a saccharide to the G1P, catalyzed by at least one enzyme; converting G1P to G6P, catalyzed by phosphoglucomutase (PGM); converting G6P to F6P, catalyzed by phosphoglucisomerase (PGI); converting F6P to allulose 6-phosphate (A6P), catalyzed by A6PE; and converting the A6P produced to allulose, catalyzed by A6PP.
- [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 allulose involves converting starch to a starch derivative by enzymatic hydrolysis or by acid hydrolysis of starch. In other aspects, a starch derivative can be 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 allulose, in certain aspects, can also involve adding 4-glucan transferase (4GT).

[10] In various aspects, a process for preparing allulose can involve converting fructose to the F6P, catalyzed by at least one enzyme; converting F6P to allulose 6-phosphate (A6P) catalyzed by A6PE; and converting the A6P produced to allulose, catalyzed by A6PP. In other embodiments, allulose 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 allulose 6-phosphate (A6P) catalyzed by A6PE; and converting the A6P produced to allulose, catalyzed by A6PP.

[11] In other aspects of the invention, G6P to be used in a process for preparing allulose 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 other aspects of the invention, the steps of a process for preparing allulose 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.

BRIEF DESCRIPTION OF THE FIGURES

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

[14] FIG. 1 is a schematic diagram illustrating an enzymatic pathway converting fructose 6-phosphate to allulose 6-phosphate and then to allulose.

[15] FIG. 2 is a schematic diagram illustrating an enzymatic pathway converting starch or its derived products to allulose. The following abbreviations are used: α GP, alpha-glucan phosphorylase or starch phosphorylase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; IA, isoamylase; PA, pullulanase; MP, maltose phosphorylase; PPGK, polyphosphate glucokinase.

[16] FIG. 3 shows an enzymatic pathway converting cellulose or its derived products to allulose. CDP, cellodextrin phosphorylase; CBP, cellobiose phosphorylase; PPGK, polyphosphate glucokinase ; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase.

- [17] FIG. 4 is a schematic diagram illustrating an enzymatic pathway converting fructose to allulose. PPFK, polyphosphate fructokinase.
- [18] FIG. 5 is a schematic diagram illustrating an enzymatic pathway converting glucose to allulose. PPGK, polyphosphate glucokinase; PGI, phosphoglucoisomerase.
- [19] FIG. 6 shows an enzymatic pathway converting sucrose or its derived products to allulose. SP, sucrose phosphorylase; PPFK, polyphosphate fructokinase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase.
- [20] FIG. 7 shows the Reaction Gibbs Energy between intermediates based on formation Gibbs energy for the conversion of glucose 1-phosphate to allulose.

DETAILED DESCRIPTION OF THE INVENTION

- [21] The invention provides enzymatic pathways, or processes, for synthesizing allulose with a high product yield, while greatly decreasing the product separation costs and allulose production costs.
- [22] The invention relates to a process for preparing allulose where the process involves converting fructose 6-phosphate (F6P) to allulose 6-phosphate (A6P) catalyzed by an epimerase and converting the A6P produced to allulose catalyzed by a phosphatase (e.g., allulose 6-phosphate phosphatase, A6PP). This process is generally shown in FIG. 1. In certain embodiments, the epimerase that catalyzes the conversion of F6P to A6P is allulose 6-phosphate 3-epimerase (A6PE).
- [23] Epimerases that convert F6P to A6P may be used in a process of the invention. Epimerases are also capable of converting A6P to F6P. In some aspects of the invention, epimerases suitable for use in the processes to convert F6P to A6P comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NOs.: 3 or 6, of at least 45%, more preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more 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%, at least 91%, at least 92%, at least 93%, or at least 94%, 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, 2, 4, and 5; of at least 30%, preferably at least 35%, more

preferably at least 40%, more preferably at least 45%, more preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more 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%.

[24] Examples of A6PEs include, but are not limited to the following proteins, identified by UNIPROT ID numbers: D9TQJ4, A0A090IXZ8, and P32719. Of these, D9TQJ4 and A0A090IXZ8 are obtained from thermophilic organisms. P32719 is obtained from a mesophilic organism. P32719 is 53% identical to A0A090IXZ8 and 55% identical to D9TQJ4, and each protein catalyzes the epimerization of F6P to A6P. Furthermore, A0A090IXZ8 is 45% identical to D9TQJ4. Conversely, other epimerase proteins identified by UNIPROT ID numbers: A0A101D823, R1AXD6, A0A150LBU8, A0A023CQG9, and H1XWY2, which have a degree of identity to D9TQJ4 of 45% or less do not catalyze the epimerization of F6P to A6P.

[25] In some aspects of the invention, epimerases suitable for use in the processes to convert F6P to A6P utilize a divalent metal cofactor: preferably, but not limited to, cobalt. In further aspects of the invention the epimerase contains but is not limited to containing an (α/β)₈-barrel domain for catalysis; additionally but not limited to containing a phosphate binding domain including a Ser at the end of the 7th β -strand of the barrel, a Ser at the end of the 8th β -strand of the barrel, and a Gly in the active site loop; additionally but not limited to containing a metal binding domain including a His in the 2nd and 3rd β -strands of the barrel; additionally but not limited to containing an Asp in the 2nd and 7th β -strand of the barrel to act as the acid/base catalyst for 1,1 proton transfer, and additionally but not limited to containing a His-hydrophobic residue-Asp signature in the 2nd β -strand of the barrel where the His is utilized in metal binding and the Asp for acid/base catalysis. These features are known in the art, and are referenced in, for example, Chan *et al.*, Structural Basis for Substrate Specificity in Phosphate Binding (β / α)₈-Barrels: D-Allulose 6-Phosphate 3-Epimerase from *Escherichia coli* K-12. *Biochemistry*. 2008; 47 (36); 9608 – 9617. Preferably, an epimerase for use in the processes of the invention contains an (α/β)₈-barrel domain for catalysis, a Ser at the end of the 7th β -strand of the barrel, a Ser at the end of the 8th β -strand of the barrel, a Gly in the active site

loop, a His in the 2nd and 3rd β -strands of the barrel, an Asp in the 2nd and 7th β -strand of the barrel, and a His-hydrophobic residue-Asp signature in the 2nd β -strand of the barrel.

[26] Processes of the invention use phosphatases that convert A6P to allulose (D-allulose). In some aspects of the invention, phosphatases suitable for the process to convert A6P to allulose comprise an amino acid sequence that has a degree of identity to the amino acid sequence of SEQ ID NO.: 9, of at least 45%, more preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more 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%, at least 91%, at least 92%, at least 93%, or at least 94%, 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.: 7 and 8; of at least 30%, preferably at least 35%, more preferably at least 40%, more preferably at least 45%, more preferably at least 50%, more preferably at least 55%, more preferably at least 60%, more 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] Examples of A6PPs include, but are not limited to the following proteins, identified by UNIPROT ID numbers: A3DC21, Q5LGR4, and Q89ZR1. A3DC21 is 46% identical to Q5LGR4 and 45% identical to Q89ZR1, and each protein catalyzes the specific dephosphorylation of A6P to allulose. Conversely, other phosphatases from the haloacid dehydrogenase super family, proteins identified by UNIPROT ID numbers: H0UQ29, Q67LU4, A0A0K6IPM3, C8WSJ0, A0A151YX61, and others, which are less than 45% identical to A3DC21 do not catalyze the specific dephosphorylation of A6P to allulose.

[28] Phosphatases to convert A6P to allulose, suitable for use in the processes of the invention are specific to allulose 6-phosphate. As used herein, specific to allulose 6-phosphate refers to having a higher specific activity on allulose 6-phosphate compared to glucose 1-phosphate, glucose 6-phosphate, or fructose 6-phosphate.

[29] Phosphatases to convert A6P to allulose utilize a divalent metal cofactor: preferably magnesium. In further aspects of the invention the phosphatase contains but is not limited to containing a Rossmannoid fold domain for catalysis; additionally but not limited to containing

a C1 capping domain for substrate specificity; additionally but not limited to containing a DxD signature in the 1st β -strand of the Rossmannoid fold for coordinating magnesium where the second Asp is a general acid/base catalyst; additionally but not limited to containing a Thr or Ser at the end of the 2nd β -strand of the Rossmannoid fold that helps stability of reaction intermediates; additionally but not limited to containing a Lys at the N-terminus of the α -helix C-terminal to the 3rd β -strand of the Rossmannoid fold that helps stability of reaction intermediates; and additionally but not limited to containing a GDxxxD signature at the end of the 4th β -strand of the Rossmannoid fold for coordinating magnesium. These features are known in the art and are referenced in, for example, 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. Preferably, a phosphatase to convert A6P to allulose used in the processes of the invention contains a Rossmannoid fold domain for catalysis, a C1 capping domain, DxD signature in the 1st β -strand of the Rossmannoid fold, a Thr or Ser at the end of the 2nd β -strand of the Rossmannoid fold, a Lys at the N-terminus of the α -helix C-terminal to the 3rd β -strand of the Rossmannoid fold, and a GDxxxD signature at the end of the 4th β -strand of the Rossmannoid fold.

- [30] In some embodiments, a process for preparing allulose according to the invention also includes the step of enzymatically converting glucose 6-phosphate (G6P) to the F6P, and this step is catalyzed by phosphoglucosomerase (PGI). In other embodiments, the process for preparing allulose 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, allulose 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 allulose 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 phosphoglucosomerase (PGI, EC 5.3.1.9); (iv) converting F6P to A6P via A6PE, and (v) converting A6P to allulose via A6PP. 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:A6PE:A6PP). 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 allulose 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 dephosphorylation of A6P 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 allulose 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 the

A6PP 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 allulose 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 which also includes maltodextrin phosphorylase, starch phosphorylase, glycogen phosphorylase, and other α -1,4 glycosidic bond degrading phosphorylases), 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, amyloextrin, 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, amyloextrin, or maltodextrin, the G1P is produced from soluble starch, amyloextrin, 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, allulose 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 A6P catalyzed by A6PE; and converting A6P to allulose catalyzed by A6PP. The fructose can be produced, for example, by an enzymatic conversion of sucrose.
- [49] In other embodiments, allulose 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 A6P catalyzed by A6PE; and converting A6P to allulose catalyzed by A6PP.

- [50] The phosphate ions generated when A6P is converted to allulose 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 allulose yields by producing F6P from fructose generated by the phosphorolytic cleavage of sucrose by SP.
- [51] In some embodiments, a process for preparing allulose 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 allulose. In certain embodiments, artificial (non-natural) ATP-free enzymatic pathways are provided to convert starch, cellulose, sucrose, and their derived products to allulose 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 amylopectin. 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 allulose.
- [55] As shown in FIG. 2, maltose phosphorylase (MP) can be used to increase allulose yields by phosphorolytically cleaving the degradation product maltose into G1P and glucose. Alternatively, 4-glucan transferase (4GT) can be used to increase allulose yields by recycling the degradation products glucose, maltose, and maltotriose into longer maltooligosaccharides; which can be phosphorolytically 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 allulose 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 A6P catalyzed by A6PE; and converting A6P to allulose catalyzed by A6PP. 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 allulose by phosphorylating the degradation product glucose to G6P, as shown in FIG. 3.
- [61] In other embodiments, allulose 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 A6P catalyzed by an enzyme; and converting A6P to allulose catalyzed by A6PP.

- [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+} , Co^{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. 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 some of their derivatives are less expensive feedstocks than, for example, fructose. When allulose is produced from fructose, yields are lower than in the present invention, and allulose must be separated from fructose via chromatography, which leads to higher production costs.
- [68] Also, the step of converting A6P to allulose according to the invention is an irreversible phosphatase reaction, regardless of the feedstock. Therefore, allulose 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 allulose, 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). The nucleotide sequence, SEQ ID NO 1, encodes for Thermophilic A6PE from *Thermoanaerobacterium thermosaccharolyticum* (UNIPROT ID D9TQJ4). SEQ ID NO 2 is a codon optimized version of that nucleotide sequence. SEQ ID NO 3 is the amino acid sequence for the enzyme. The nucleotide sequence SEQ ID NO 4 encodes for Thermophilic A6PE from *Bacillus thermoamylovorans* (UNIPROT ID A0A090IXZ8). SEQ ID NO 5 is a codon optimized version of that nucleotide sequence. SEQ ID NO 6 is the amino acid sequence for the enzyme. The nucleotide sequence SEQ ID NO 7 encodes for Thermophilic A6PP from *Clostridium thermocellum* (UNIPROT ID A3DC21). SEQ ID NO 8 is a codon optimized version of the nucleotide sequence. SEQ ID NO 9 is the amino acid sequence corresponding to the enzyme. 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 30° 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). A6PE was purified with 80 µM CoCl₂ present in growth media, elution buffers, dialysis buffer, and protein storage buffer.

[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 MgCl₂, 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 $\mu\text{mol}/\text{min}$.

- [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 μL 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 μM NADH, 0.1 U PGI, 5 U PK, and 5 U LD.
- [79] *Allulose 6-phosphate 3-epimerase* (A6PE) from *Thermoanaerobacterium thermosaccharolyticum* (UNIPROT ID D9TQJ4), SEQ ID NO 3, was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl_2 , 80 μM CoCl_2 , 1 U/mL A6PP, 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, allulose 6-phosphate (A6P), was determined using allulose 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] *Allulose 6-phosphate phosphatase* (A6PP) from *Clostridium thermocellum* (UNIPROT ID A3DC21), SEQ ID NO 9, was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl_2 , 80 μM CoCl_2 , 1 U/mL A6PE, 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, allulose, was determined through detecting free phosphate release as described for A6PE.

- [81] 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.
- [82] 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.
- [83] 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.
- [84] 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.
- [85] 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.
- [86] 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.

[87] Example 1

[88] To validate the technical feasibility of the enzymatic biosynthesis of fructose 6-phosphate from starch, three enzymes were recombinantly expressed: alpha-glucan phosphorylase from *T. maritima* (UNIPROT ID G4FEH8), phosphoglucomutase from *Thermococcus kodakaraensis* (UNIPROT ID Q68BJ6), and phosphoglucoisomerase from *Clostridium thermocellum* (UNIPROT ID A3DBX9). The recombinant proteins were over-expressed in *E. coli* BL21 (DE3) and purified as described above.

[89] 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.

[90] Example 2

[91] 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.

[92] Example 3

[93] It was found that, at 80°C, an enzyme unit 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.

[94] Example 4

[95] 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.

[96] Example 5

[97] 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 MgCl₂ and 0.5 mM ZnCl₂. 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.

[98] Example 6

[99] 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 MgCl₂ and 0.5 mM ZnCl₂. 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.

[100] Example 7

[101] 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 MgCl₂ and 0.5 mM ZnCl₂. 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.

[102] Example 8

[103] To validate allulose production from F6P, 2 g/L F6P was mixed with 1 U/ml A6PE and 1 U/ml A6PP in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCl₂ and 80 μ M CoCl₂. The reaction was incubated for 6 hours at 50°C. 99% conversion of F6P to allulose was seen via HPLC (Agilent 1100 series) using an Agilent Hi-Plex H-column and refractive index detector. The sample was run in 5 mM H₂SO₄ at 0.6 mL/min.

[104] Example 9

[105] To validate production of allulose from maltodextrin, a 0.20 mL reaction mixture containing 20 g/L maltodextrin, 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 80 μ M CoCl₂, 0.05 U of α GP, 0.05 U PGM, 0.05 U PGI, 0.05 U A6PE and 0.05 U A6PP is incubated at 50°C for 24 hours. The reaction is stopped via filtration of enzyme with a Vivaspin 2 concentrator (10,000 MWCO). Allulose is detected and quantified using an Agilent 1100 series HPLC with refractive index detector and an Agilent Hi-Plex H-column. The mobile phase is 5 mM H₂SO₄, which runs at 0.6 mL/min. Standards of various concentrations of allulose are used to quantify our yield.

[106] Example 10

A reaction mixture containing 200 g/L maltodextrin, 10 mM acetate buffer (pH 5.5), 5 mM MgCl₂, 80 μM CoCl₂, and 0.1 g/L isoamylase is incubated at 80°C for 24 hours. This is 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 αGP, 0.05 U PGM, 0.05 U PGI, 0.05 U A6PE, and 0.05 U A6PP is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[107] Example 11

A reaction mixture containing 200 g/L maltodextrin, 10 mM acetate buffer (pH 4.5), 5 mM MgCl₂, and 1:200 dilution of Novozymes D6 pullulanase is incubated at 50°C for 4 hours. This is used to create another reaction mixture containing 20 g/L pullulanase treated maltodextrin, 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 80 μM CoCl₂, 0.05 U of αGP, 0.05 U PGM, 0.05 U PGI, 0.05 U A6PE, and 0.05 U A6PP is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[108] Example 12

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

[110] 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, 80 μM CoCl₂, 0.05 U of αGP, 0.05 U PGM, 0.05 U PGI, 0.05 U A6PE, 0.05 U A6PP, and 0.05 U 4GT is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[111] Example 13

[112] 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 αGP; 0.1 U PGM; and 0.1 U PGI is incubated at 50°C for 6 hours. The short duration ensures completion is not reached, and therefore differences in efficiency can be clearly seen. Production of F6P is quantified 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. 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 F6P is 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).

Table 1

Concentration of PBS pH 7.2 (mM)	g/L of F6P
6.25	4.5
12.5	5.1
25	5.6
37.5	4.8
50	4.9

[113] Example 14

[114] 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; and 0.02 U PGI is incubated at 50°C for 16 hours. The units are lowered to ensure completion is not reached, and therefore differences in efficiency can be clearly seen. Production of F6P is quantified as in example 12. 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 F6P 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.

Table 2

pH of PBS	g/L of F6P
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

[115] Example 15

[116] To investigate scale-up, a 20 mL reaction mixture containing 50 g/L isoamylase treated maltodextrin (see Example 10), 50 mM phosphate buffered saline pH 7.2, 5 mM MgCl₂, 80 μM CoCl₂, 10 U of αGP, 10 U PGM, 10 U PGI, 10 U A6PE, and 10 U A6PP is incubated at 50°C for 24 hours. Production of allulose was quantified as in Example 9.

[117] Example 16

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

[119] Example 17

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

[121] Example 18

[122] To produce allulose from fructose, a reaction mixture containing 10 g/L fructose, 50 mM Tris buffer pH 7.0, 75 mM polyphosphate, 5 mM MgCl₂, 80 μM CoCl₂, 0.05 U fructose polyphosphate kinase, 0.05 U A6PE, and 0.05 U A6PP is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[123] Example 19

[124] To produce allulose from glucose, a reaction mixture containing 10 g/L glucose, 50 mM Tris buffer pH 7.0, 75 mM polyphosphate, 5 mM MgCl₂, 80 μM CoCl₂, 0.05 U glucose polyphosphate kinase, 0.05 U PGI, 0.05 U A6PE, and 0.05 U A6PP is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[125] Example 20

[126] To produce allulose from sucrose, a reaction mixture containing 10 g/L sucrose, 50 mM phosphate buffered saline pH 7.0, 5 mM MgCl₂, 80 μM CoCl₂, 0.05 U sucrose phosphorylase, 0.05 PGM, 0.05 U PGI, 0.05 U A6PE, and 0.05 U A6PP is incubated at 50°C for 24 hours. Production of allulose is quantified as in Example 9.

[127] Example 21

[128] To further increase yields of allulose from sucrose, 75 mM polyphosphate and 0.05 polyphosphate fructokinase is added to the reaction mixture in example 20. Production of allulose is quantified as in Example 9.

What is claimed is:

1. A process for preparing allulose, the process comprising:
converting fructose 6-phosphate (F6P) to allulose 6-phosphate (A6P), catalyzed by an epimerase; and
converting the A6P produced to allulose, catalyzed by a phosphatase.
2. The process of claim 1, further comprising a step of converting glucose 6-phosphate (G6P) to the F6P, wherein the step is catalyzed by phosphoglucosomerase (PGI).
3. The process of claim 2, further comprising the step of converting glucose 1-phosphate (G1P) to the G6P, wherein the step is catalyzed by phosphoglucomutase (PGM).
4. The process of claim 3, 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.
5. The process of claim 4, wherein at least one enzyme is selected from the group consisting of alpha-glucan phosphorylase (α GP), maltose phosphorylase, sucrose phosphorylase, cellodextrin phosphorylase, cellobiose phosphorylase, and cellulose phosphorylase.
6. The process of claim 4, wherein the saccharide is starch or a derivative thereof selected from the group consisting of amylose, amylopectin, soluble starch, amylopectin, maltodextrin, maltose, and glucose.
7. The process of claim 6, 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.
8. The process of claim 6, wherein 4-glucan transferase (4GT) is added to the process.

9. The process of claim 7, wherein the starch derivative is prepared by enzymatic hydrolysis of starch catalyzed by isoamylase, pullulanase, alpha-amylase, or a combination thereof.
10. The process of claim 1, further comprising:
the step of converting fructose to the 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.
11. The process of claim 2, further comprising:
the step of converting glucose to the G6P, wherein the step is catalyzed by at least one enzyme, and optionally, the step of converting sucrose to the glucose, wherein the step is catalyzed by at least one enzyme.
12. The process of any one of claims 1-11, wherein the epimerase is an allulose 6-phosphate 3-epimerase.
13. The process of claim 12, wherein the allulose 6-phosphate 3-epimerase comprises an amino acid sequence having at 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% sequence identity with SEQ ID NOs.: 3 or 6, wherein said epimerase catalyzes the conversion of F6P to A6P.
14. The process of claim 12, wherein the allulose 6-phosphate 3-epimerase contains an (α/β)₈-barrel domain for catalysis, a Ser at the end of the 7th β -strand of the barrel, a Ser at the end of the 8th β -strand of the barrel, a Gly in the active site loop, a His in the 2nd and 3rd β -strands of the barrel, an Asp in the 2nd and 7th β -strand of the barrel, and a His-hydrophobic residue-Asp signature in the 2nd β -strand of the barrel.
15. The process of any one of claims 1-11, wherein the phosphatase is an allulose 6-phosphate phosphatase.

16. The process of claim 15, wherein the allulose 6-phosphate phosphatase comprises an amino acid sequence having at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% sequence identity with SEQ ID NO.: 9, wherein said phosphates catalyzes the conversion of A6P to allulose.
17. The process of claim 15, wherein the allulose 6-phosphate phosphatase is specific for allulose 6-phosphate.
18. The process of claim 15, wherein the allulose 6-phosphate phosphatase contains a Rossmannoid fold domain for catalysis, a C1 capping domain, Dx D signature in the 1st β -strand of the Rossmannoid fold, a Thr or Ser at the end of the 2nd β -strand of the Rossmannoid fold, a Lys at the N-terminus of the α -helix C-terminal to the 3rd β -strand of the Rossmannoid fold, and a GDxxx D signature at the end of the 4th β -strand of the Rossmannoid fold.
19. The process of any one of claims 1-11, wherein the process steps are 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.
20. The process of any one of claims 1-11, wherein the process steps are conducted in one bioreactor or in a plurality of bioreactors arranged in series.
21. The process of any one of claims 1-11, wherein the process steps 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.
22. Allulose prepared by a process of any one of claims 1-11.

1/7

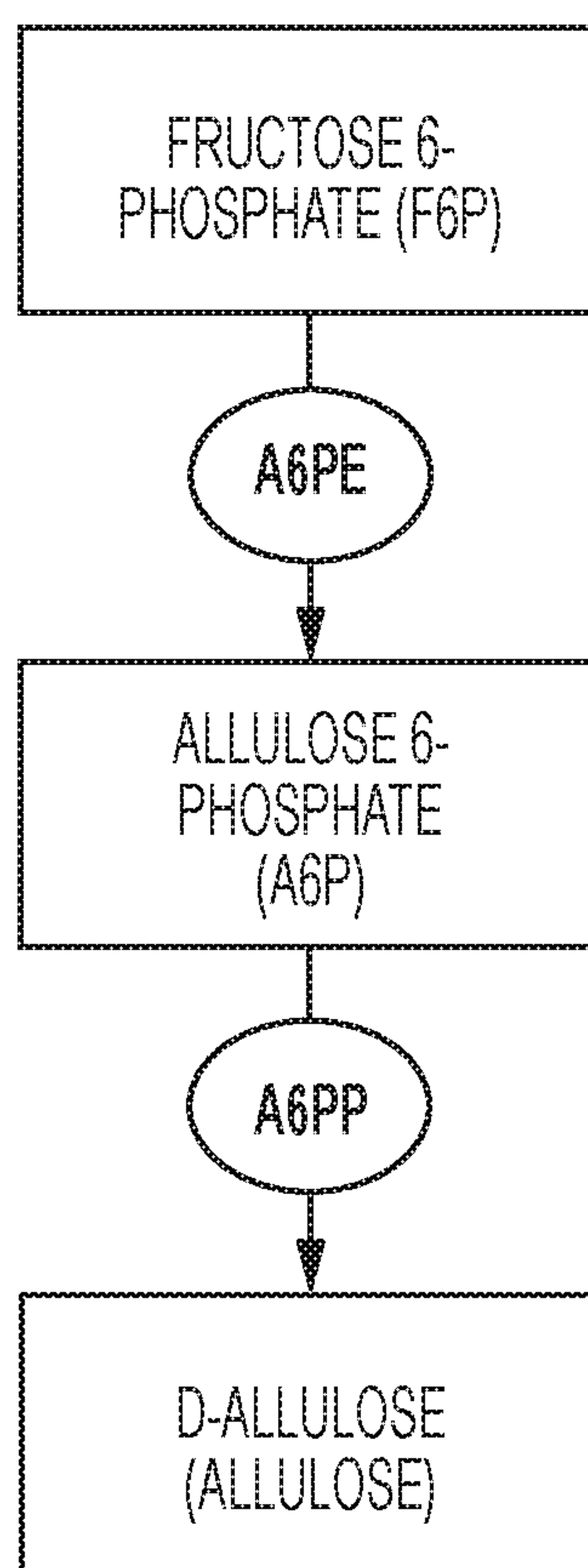


FIG. 1

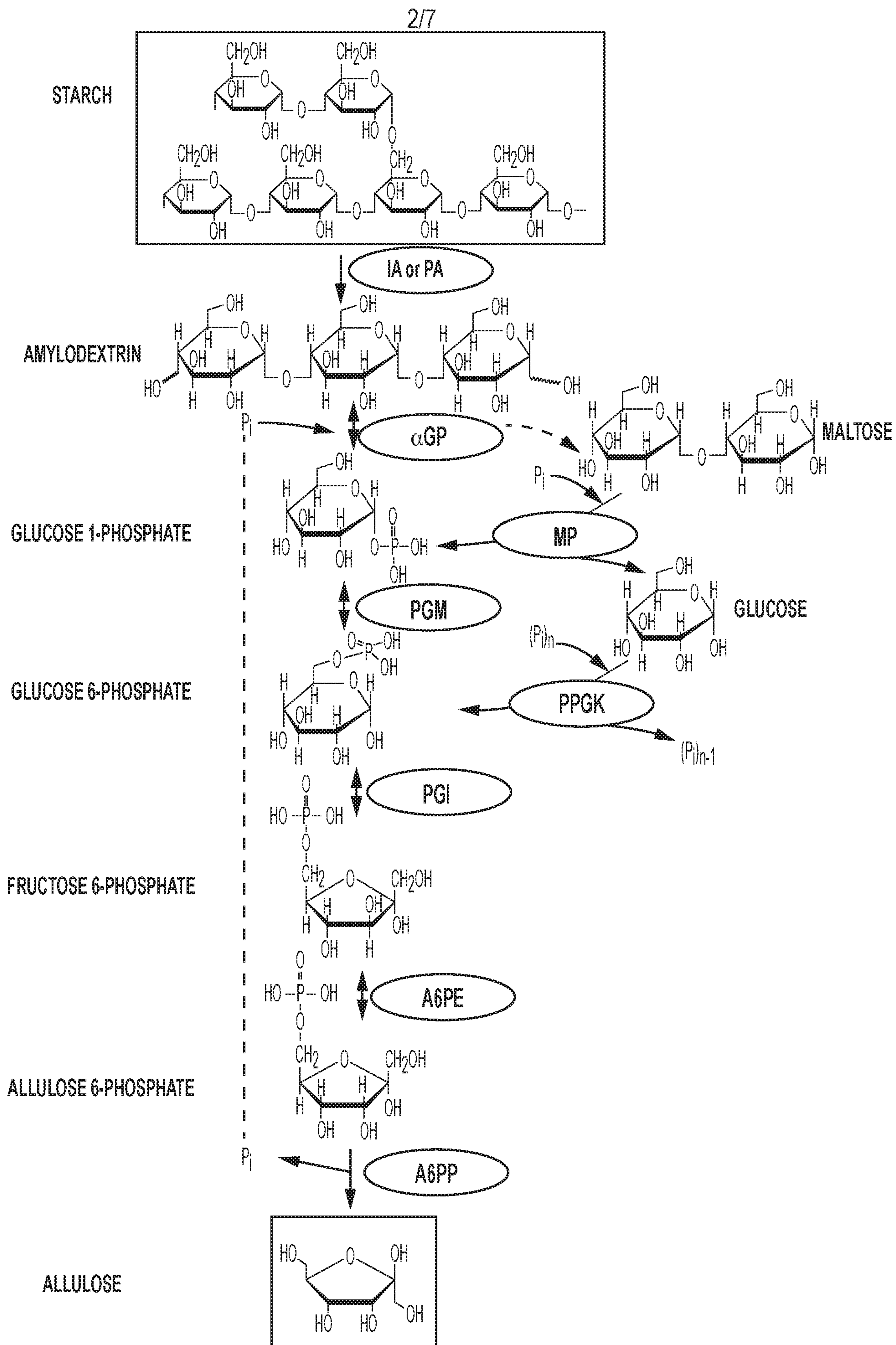


FIG. 2

3/7

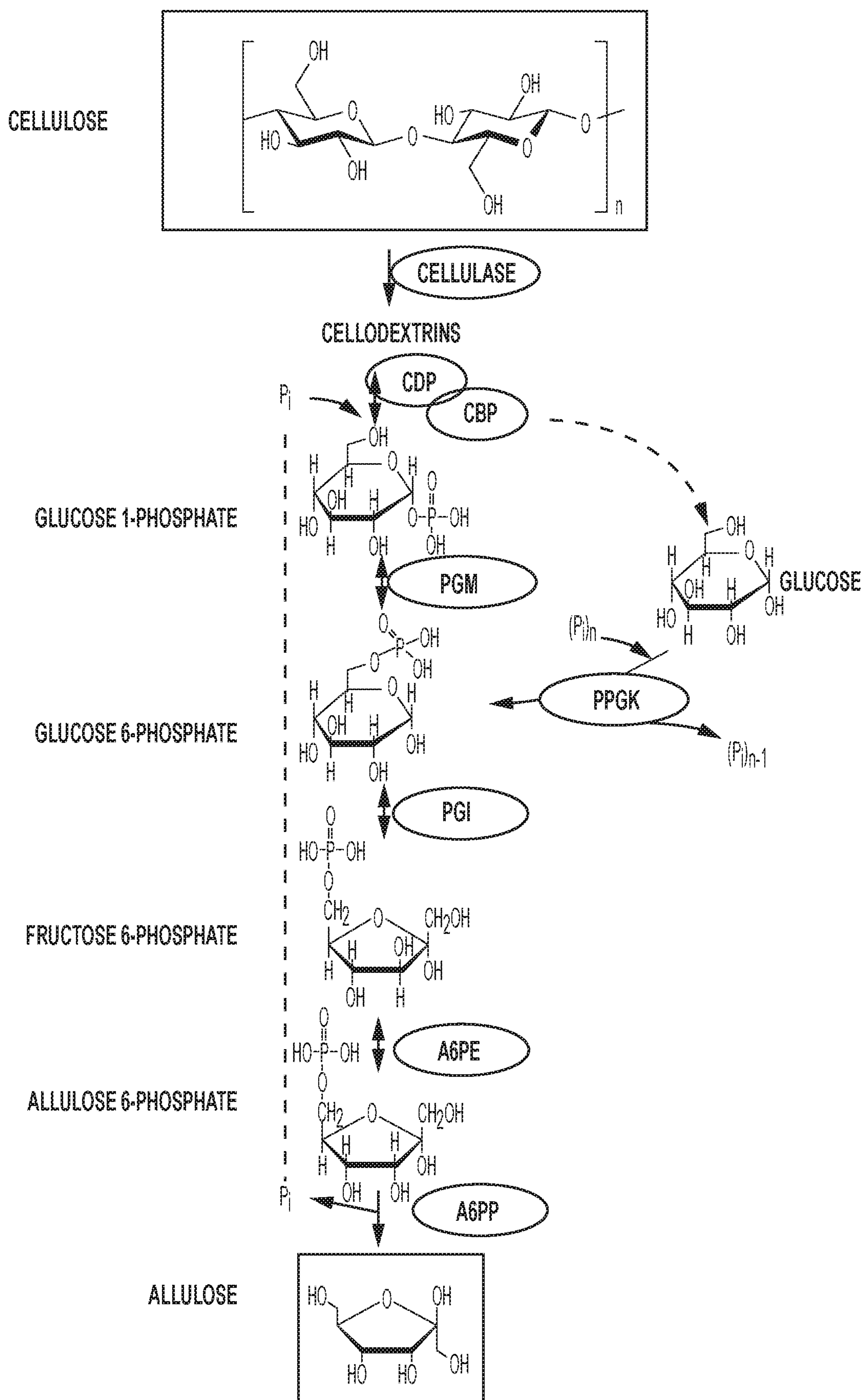


FIG. 3

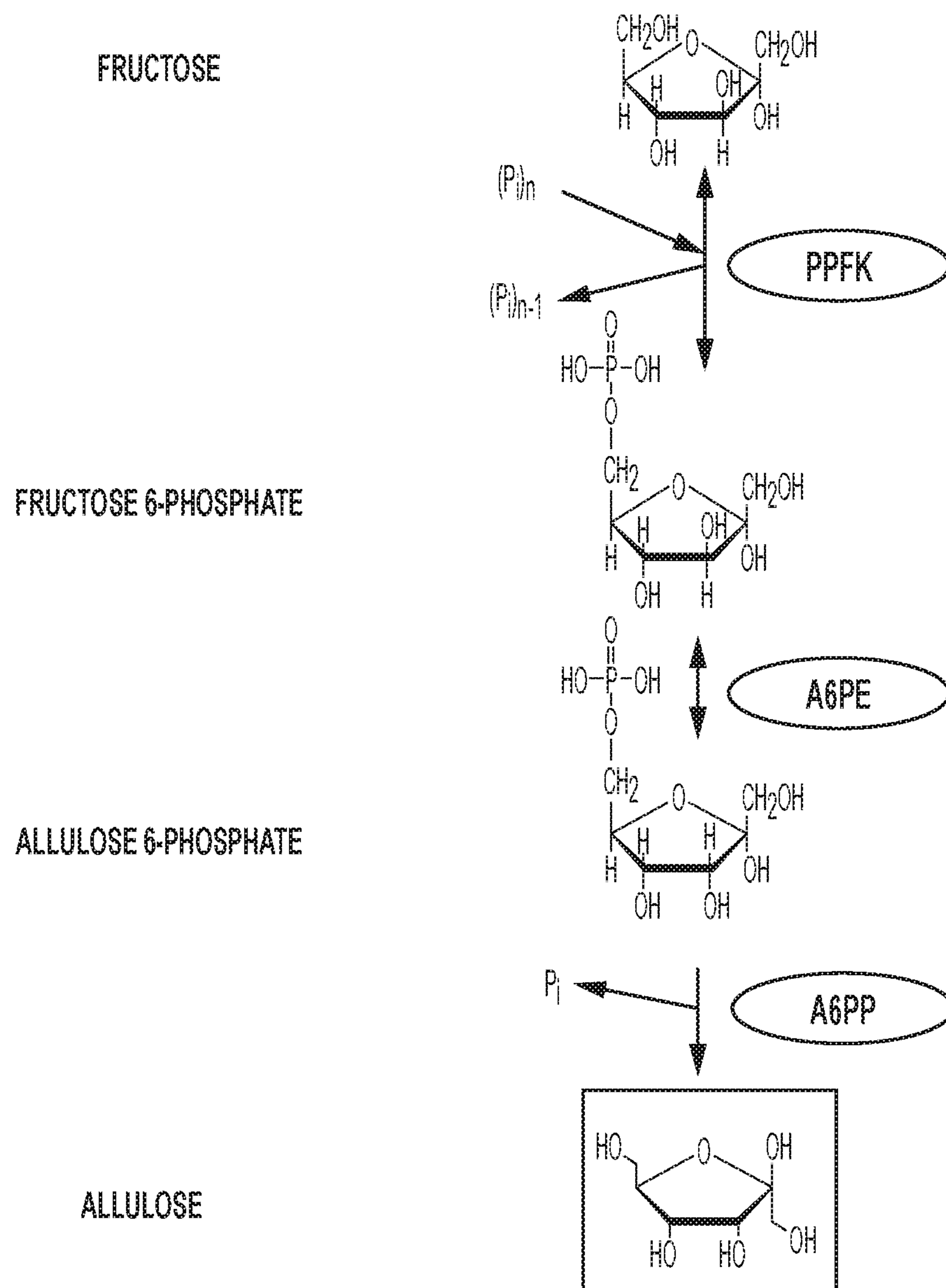


FIG. 4

5/7

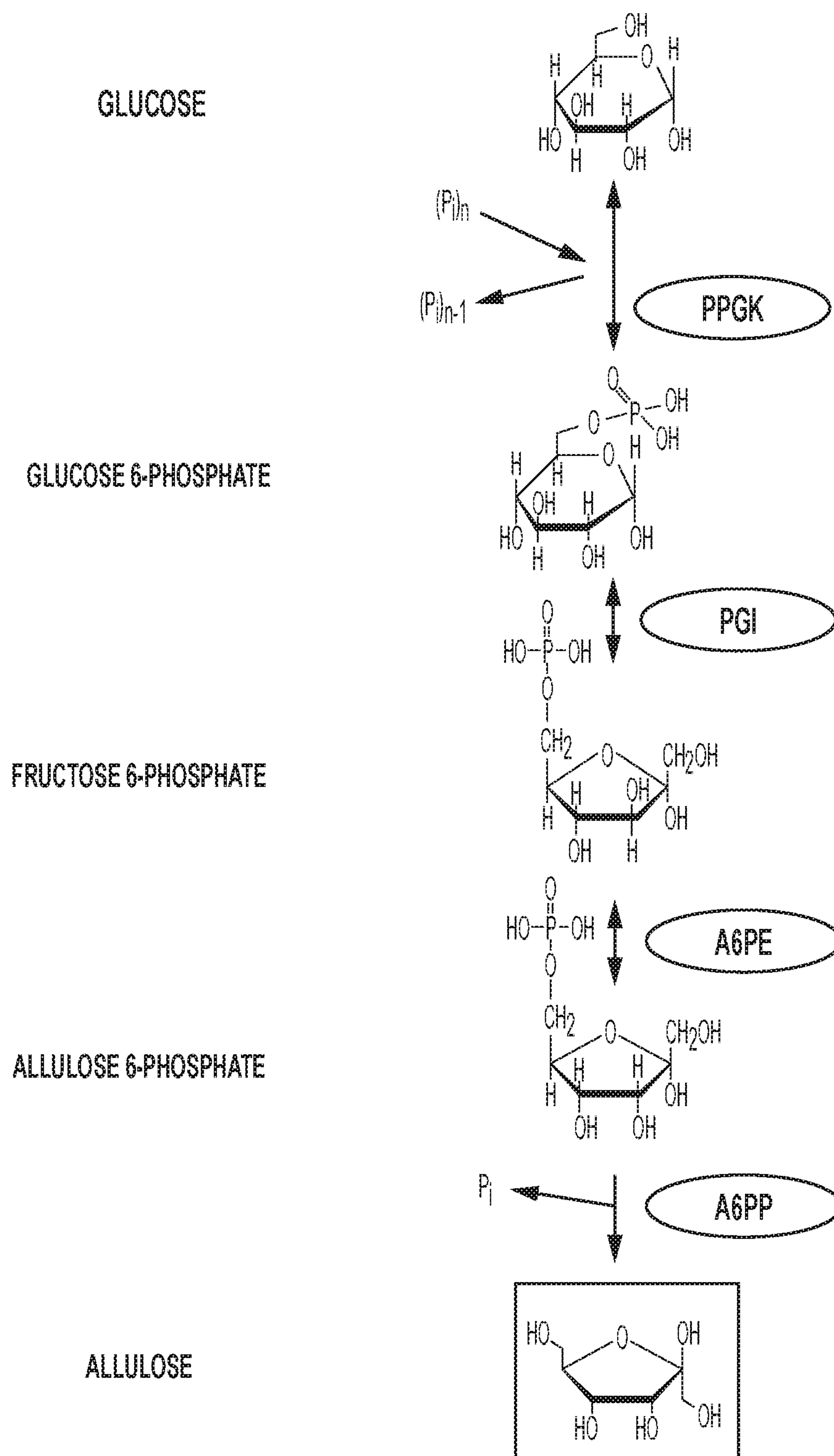


FIG. 5

6/7

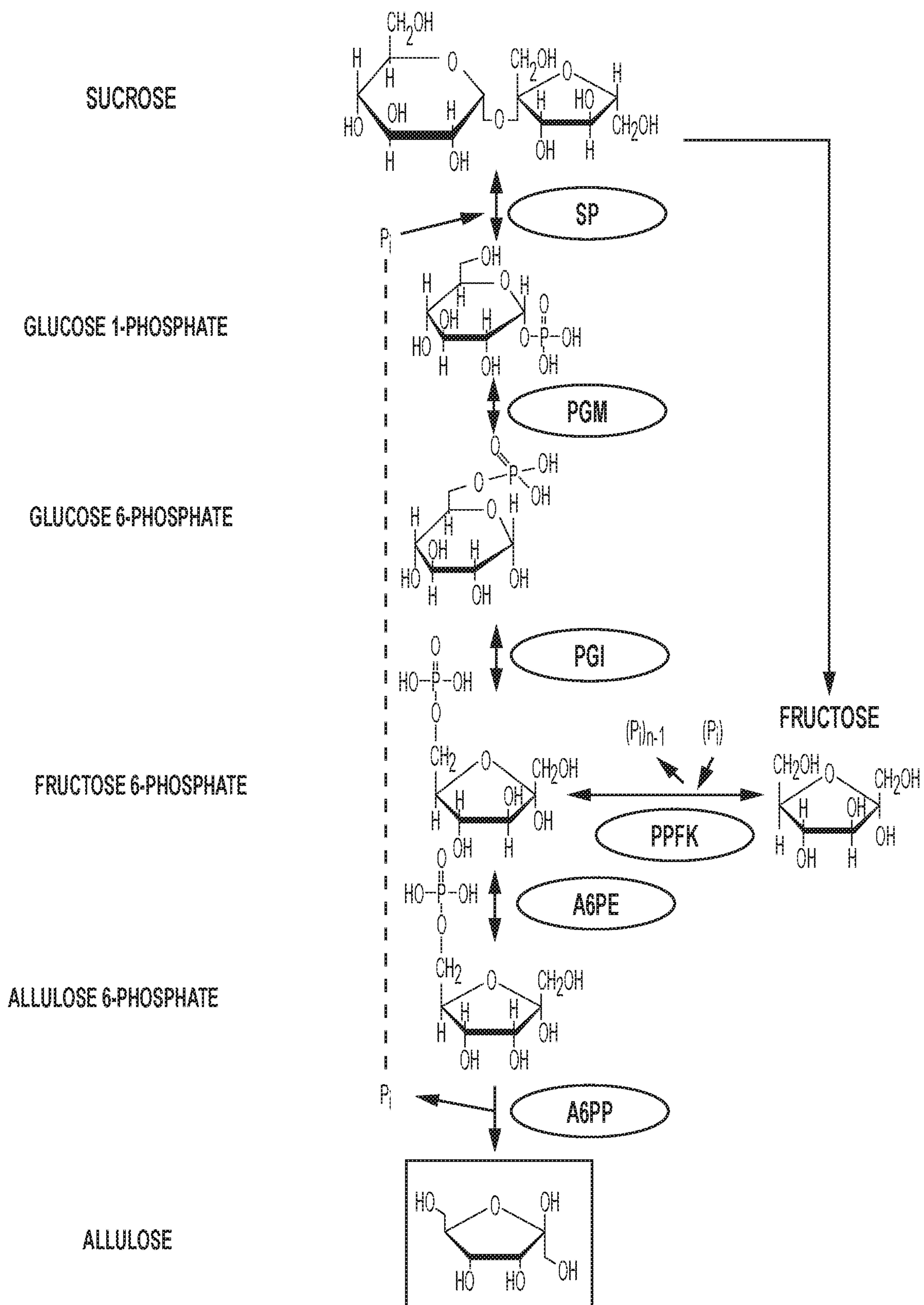


FIG. 6

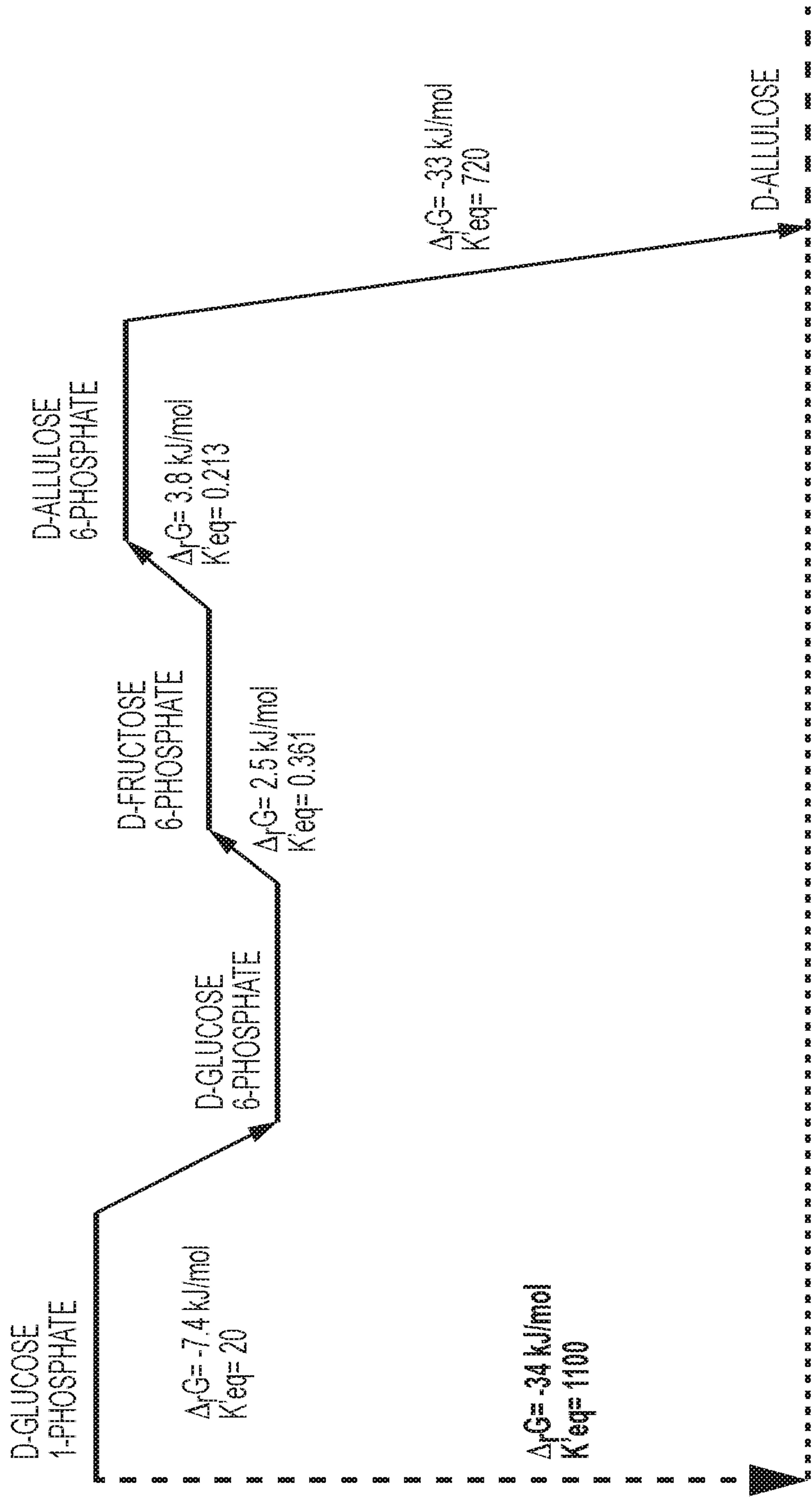


FIG. 7

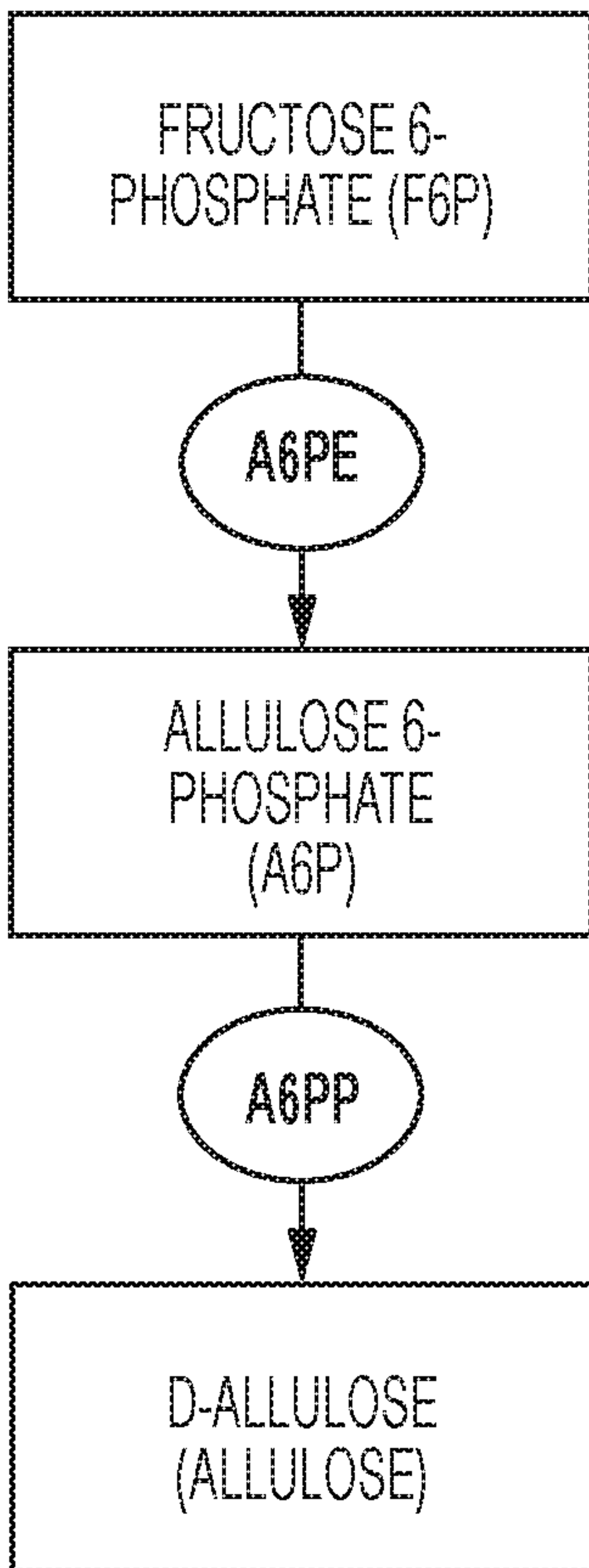


FIG. 1