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

Fructose 6-Phosphate (F6P)

D-tagatose (Tagatose)
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ENZYMATIC SYNTHESIS 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, which is incorporated herein by reference.

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.

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

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.

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 phosphoglucone 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).

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
phosphoglucomutase (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, amylodextrin, 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.
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.

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.

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.

**BRIEF DESCRIPTION OF THE FIGURES**

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

FIG. 1 is a schematic diagram illustrating an enzymatic pathway converting fructose 6-phosphate to tagatose 6-phosphate and then to D-tagatose (tagatose).

FIG. 2 is a schematic diagram illustrating an enzymatic pathway converting starch or its derived products to tagatose. The following abbreviations are used: αGP, alpha-glucan phosphorylase or starch phosphorylase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; F6PE, fructose 6-phosphate epimerase; T6PP, tagatose 6-phosphate phosphatase; IA, isoamylase; PA, pullulanase; MP, maltose phosphorylase; PPGK, polyphosphate glucokinase.

FIG. 3 shows an enzymatic pathway converting cellulose or its derived products to tagatose. CDP, cellobextrin 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. PPK, 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; PPK, 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%.
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%.

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 phosphogluco 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.

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.

Typically, the ratios of enzyme units used in the disclosed process are 1:1:1:1:1 (aGP: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 aGP, which will result in more efficient phosphorolytic cleavage of alpha-1,4-glycosidic bonds. A ratio of enzymes, for example, 3:1:1:3 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).
Examples of the enzymes used to convert a saccharide to GIP include alpha-glucan phosphorylase (aGP, EC 2.4.1.1), maltose phosphorylase (MP, EC 2.4.1.8), cellobextrin 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.

The saccharides used for generating GIP 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.

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 amylpectin. Examples of starch derivatives include amylase, amylpectin, soluble starch, amylodextrin, maltodextrin, maltose, fructose, and glucose. Examples of cellulose derivatives include pretreated biomass, regenerated amorphous cellulose, cellobextrin, cellobiose, fructose, and glucose. Sucrose derivatives include fructose and glucose.

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-a-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.

Furthermore, derivatives of cellulose can be prepared by enzymatic hydrolysis of cellulose catalyzed by cellulase mixtures, by acids, or by pretreatment of biomass.

In certain embodiments, the enzymes used to convert a saccharide to GIP contain aGP. In this step, when the saccharides include starch, the GIP is generated from starch by aGP; when the saccharides contain soluble starch, amylodextrin, or maltodextrin, the GIP is produced from soluble starch, amylodextrin, or maltodextrin by aGP.

When the saccharides include maltose and the enzymes contain maltose phosphorylase, the GIP 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, PPK 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 aGP 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 tagatose.

[55] As shown in FIG. 2, maltose phosphorylase (MP) can be used to increase tagatose yields by phosphorolytically cleaving the degradation product maltose into G1P and glucose. Alternatively, 4-glucan transferase (4GT) can be used to increase tagatose yields by recycling the degradation products glucose, maltose, and maltotriose into longer maltooligosaccharides; which can be phosphorolytically cleaved by aGP 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 a 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.

Several enzymes may be used to hydrolyze solid cellulose to water-soluble cellodextrins and cellobiose. Such enzymes include endoglucanase and cellobiohydrolase, but not including beta-glucosidase (cellobiase).

Prior to cellulose hydrolysis and G1P generation, cellulose and biomass can be pretreated to increase their reactivity and decrease the degree of polymerization of cellulose chains. Cellulose and biomass pretreatment methods include dilute acid pretreatment, cellulose solvent-based lignocellulose fractionation, ammonia fiber expansion, ammonia aqueous soaking, ionic liquid treatment, and partially hydrolyzed by using concentrated acids, including hydrochloric acid, sulfuric acid, phosphoric acid and their combinations.

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.

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.

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.

The reaction buffer can also contain key metal cations. Examples of the metal ions include Mg$^{2+}$ and Zn$^{2+}$. 

[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$^{2+}$. 

12
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.

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.

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.

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.

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.

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 PHI 05 (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 cellodextrinphosphorylase on insoluble cellulose. Appl. Microbiol. Biotechnol. 2011; 92:551-560. Escherichia coli SigI0 (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

[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

ampicillin or 50 mg L

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 MgCh (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.

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).

**Enzymes used and their activity assays**

*Alpha-glucan phosphorylase* (aGP) from *Thermotoga maritima* (Uniprot ID G4FEH8) was used. Activity was assayed in 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM MgCh, 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 μmol/μg.

*Phosphoglucomutase* (PGM) from *Thermococcus kodakaraensis* (Uniprot ID Q68BJ6) was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCh 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).

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 MgCh 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 MgCh, 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.
Fructose 6-phosphate epimerase (F6PE) from Dictyoglomus thermophilum (Uniprot ID: B5YBD7) was used. Activity was measured in 50 mM FIEPES buffer (pH 7.2) containing 5 mM MgCh, 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.

**Mutual Section [80]**

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

**Nucleotide Sequence** (SEQ ID NO.: 1)

ATGTTCGGCTCGCCTGCTCCCCTGCTGGATATGGTCACCGCGCAGAAACAGG
GCATGGGCAGGGGTATCCCATCCATTTTGTTCGGCACATCCGGTGGTGCTGAGTGCG
CCCTGTCATCTTGGCCGCCGGAGCCGGCGGCCCTGCTCATCGAAACCACCTGCT
AATCAGGTCAACCACCAAGGGTGACATCCGGTGGTGCTGAGTCGCTG
CGCTTCCTTGCGCAAAATTCTGGGAAACGGGAAGGTATTTCCCGCAACAGGCGAATCCCGG
CCATAGCAACAGCGCTGGAAAATGGTGCGGGCAGACGTGACGGCAGGCTACACCA
AAATTCATCTGGACGTGCTCCATGGCGACGAGCGACCAGCGAAGCTCTGCACTCCGT
CCGCTGGAGGCAGCGCAGCCGCAAGCGAAGCTTCACGTCACCTCCGCCAGGAAGC
CCAAAGCCGCAGCTGGATGCTCTTGGGAAAGCCTCTTGCAGCCGAGGCTTGGACTCCG
GGTGGGAGCGGCTTGGAGGCGAAGACTTCACTGCACACTCCGCAAGGAGGC
CCCAAGCCGCAGCTGGATGCTCTTGGGAAAGCCTCTTGCAGCCGAGGCTTGGACTCCG
GGTGGGAGCGGCTTGGAGGCGAAGACTTCACTGCACACTCCGCAAGGAGGC
CCCAAGCCGCAGCTGGATGCTCTTGGGAAAGCCTCTTGCAGCCGAGGCTTGGACTCCG
GGTGGGAGCGGCTTGGAGGCGAAGACTTCACTGCACACTCCGCAAGGAGGC

GCACCATCCCGCCGCGCAGGAAGCCGTGCGGAGACTGCTCGCCAACCTGATCGA
AACCCCGCCGCCGCTGAGTTTGCTCAGCCAGTACCTGCCGCGCGAGTATGAGAT
GGTGCGCGCGGGGGAAATCTCCAGCCACCCCGCAGGACCTGTATCCGGGCACATAT
CCAGCACACGCTGGAGATTACGGTGCCTGCCTGGGCTTGCTTAA

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

[84] MFGSPAPLLDMVTAAQKGMARGIPSICSAHPVVLASSAHLARRSGAPLLIETTCN
QVNHQGGYSMTAPDFVRLREIREGIPQQVILGGDHLPYPWRKEPAETIAQ
ALEMVRAYVQAGYTKHLDASMPACDIDPERPLPLIERAAQLCAAEEAAAGA
VQPVYVIGSEVPPPPGGAQGQEARLHVTTPQEAQAALDAFREAFLQAGLTPWVERVI
ALVQVQPGVEFVDSIHYQREAARPLKTFIEGVMPYEAHSTDYQTRASRALVE
DHFSILKVGPAFTFAREAVFALEHIEREILGRQDMPLSRLSEVLDENVNLNDPRHWQ
GWAGAPAEQALARRYFSFRIRYVWHHPAAQEAVRLLANLIEPTPLSLLSQYLP
REYEMVRAGEISSHHPQDLIRAHQHTLEDYAAACG

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

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

[87] ATGAGTCCTCAAAATCCATTGATGGTTTTATTTAAGAATAGAGAAAAAGGT
TTAAGGGTATTATTTTCAGGTGGTTTCTTCAAAATAGAATAGGCGATTTTA
AAAAAGAAATGAAAAATACAAAAACCTACCAATTATATTGAAAGCCACAGCGAACCAG
GTAATCATTTGCGGGTATTCTGGGTTGACACCGTCTCAGTTCAAAAGCAGAG
TTATAAAATTGCTCAAAAGATGTATTTTCCACTTGAAGAAATAATTCTTTGTTG
GGACCACCTCTGGACACATTGTGTTGGGCTGAACCAGAACCACAAAATTTGCTATGGA
GTATGCTAAGCAAGAATGATAAAAAGAATACATAAAAAAGCAGGTTTTTACCAAAAATCTCA
CATCGACACGAGATGTGCTTTAATAAGGGGGAAGAAGCAGATGGTAGGATGAAAATAT
TGCTAAAGAACGCTTGCTGCTGCTGCAAGGATGGAGATGATTGGAAGAGAT
TTCTATTTACATACATCTACTACAAAAGAGCCAGTTTATGTGATAGGACGTGATGTG
CCACCTCCCAGCCGGAAGAGTCTTCTATTTGTCACAACATATTACTACTAAAGATGAA
TAGAAAAAGGTTTGAATATTTCACAAAAAGCAAGCTTTTAAAGAGGAAGGAAATGGAC
ATGTATTTAGATGTGCTTTGCTTGGCTAATAATTTTGAGATTTGGGA
CGATGAAATTTGTTTATATGCAAAAAAGTAAAGGCCGCTAAAGAAGCTTTT
GGCAAGATCACATTAGTATTTGGAAGGACCTTTCTACAGATTACAAACAAGAG
AAACTAAAAAAGATGCTGAATGTGATGTTTGAATTTTAAAGGTTGCTTGC
CTAACATTTACATTGCGCGAAGCGTTAGTAGCACTTTGATCTATTGGAAGAAGAA
ATTATAGCAATGAAAAAGGAGAAACTGTCAGATTAGGAAGGTATTTATTGAAT
ACTATGCTAAACATGCAAAGATCACTGGAGTAAATATTTTGATGAGAATGATAAG
TTAATTAAGTCAAAGCTCTCATATATAGCTATCTTGACAGATGGAGATCATATT
AAAACGAGAGCTGTAAAGTCTGTTTTATTCCTATTTGGAAAAATTTAGAGATGT
TAAAAATTCCACTTGAGTGAAGTGCAAGATTTAGAGAATGTAAAATTCCACCTT
GGCTTGTAAGTCAGTATTTTCCTTCTCAGTACCAAAAAGATGAGAAAAAGATTTAA
AAGCGTGCTGCCGACCTAATATTGGGAAAATAATAGGGGAGGTCATTGACCATTAT
TTATTTGACGGTTAAAAGAAT

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

MSPQNPLGLFKNREKEFKGIISVCSNEIVLEAVLKRMRKDTNLPIIIIEATANQVNQ
FGGYSLTPSOFKERVIKAIQKVDPLERIIILGDHLPFVWRQPEIAMEYAYKQMI
KEYIKAGFTIKHIDSMLKGENSEIDDEIARAKRTAVLCRIAECEFKISNNPYIPRYVY
IGADVPPPGGESSICQITITTKDELSLEYKEAFKEKIEHVFDFYYVAVVANFGVEF
GSDEIVDFDKMKPLKELLAKYNIVNFEGHTDYQTKENLRMVECGIAILKVGFPAL
TFTLREALVALSHIEEIEYSNEKEKLSRFREVVLLMTLTCKDHSKYFDEENDKLI
LLYSYLDRWRYFENESVKSAYSLIGNLENVKIPPLVSQYPFSQYQKMRKDDLKN
GAADLILDKIGEVIDHYVYAVKE

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

ATGTCAACACTTCCACACATACATTGTCGACTGATCGAGCTGCGTGAGACGAG
AACAGATCCATCTCACGCTGCTGCCGCTCTCTGATCAACTCGCCGCGGCTTGAGG
GCCAGCGGTGAAGGTCGCCGCTGCCACACCGCATGCTTCCGGTCCTGACAC
GCTCAATCAATGCATCCGACGCGGCGGCTGCTCAGCCGGGTTGGAGCTCGCAATT
CGTTCGCGAGATGCTCTGCTATGCGCTGCTGCCACCACCCGGTCTCTAT
CCTGCGCTGCCATAGCCGGGCGCGCTGGCTCAAGATGACGACAGGACAGGAAAGG
CTACCGGCTCGACAGGGAGATGCTAGCTGGCCTCAAGAGCTGAGCTGAGGCTC
GAGGCGCGCTACGCGCTGCTGCACATCGACCCAGCGTGCCACTGACCTGGCCTC
GCCAGAAGCGGCCGCTCCGATCGTGGGCATGAGCAGCGGCGCCGCTTCGATGAC
GAGACATGGCCGACAGGGGACCGGCGTGAACCTCGCGCGGCGTCGCTATGAA
GTCGCCACCGAAGGAGATGACGACGACGCGCTGGAATTTCGAGGCTTGGATTT
GCTCAAGGCAAGGCTGAGCTGAGATTCGATGAGCCCTACCGGCTCCGGCCTCG
TCTGCGAGCTCCAGCGAAGCGGCCGCTCGTGGCAGCTGAGCGAAGCGGCTCGATG
GATCACGAGCTGAGGTGGGATCGAGCCGCCCGCGCCCGCGCGCGCCCGCGCCCGCG

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

ATGTCAACACTTCCACACATACATTGTCGACTGATCGAGCTGCGTGAGACGAG
AACAGATCCATCTCACGCTGCTGCCGCTCTCTGATCAACTCGCCGCGGCTTGAGG
GCCAGCGGTGAAGGTCGCCGCTGCCACACCGCATGCTTCCGGTCCTGACAC
GCTCAATCAATGCATCCGACGCGGCGGCTGCTCAGCCGGGTTGGAGCTCGCAATT
CGTTCGCGAGATGCTCTGCTATGCGCTGCTGCCACCACCCGGTCTCTAT
CCTGCGCTGCCATAGCCGGGCGCGCTGGCTCAAGATGACGACAGGACAGGAAAGG
CTACCGGCTCGACAGGGAGATGCTAGCTGGCCTCAAGAGCTGAGCTGAGGCTC
GAGGCGCGCTACGCGCTGCTGCACATCGACCCAGCGTGCCACTGACCTGGCCTC
GCCAGAAGCGGCCGCTCCGATCGTGGGCATGAGCAGCGGCGCCGCTTCGATGAC
GAGACATGGCCGACAGGGGACCGGCGTGAACCTCGCGCGGCGTCGCTATGAA
GTCGCCACCGAAGGAGATGACGACGACGACGACCGCGCTGGAATTTCGAGGCTTGGA

18
CGCCTTCTGTGGTGCGCAGTGGCTCAGTGACTGCATGACGTAACGTTTATGACCCC
AGTGCGGCGCAACGGCTGACTGAGATCGTGCGCCCTACCGGTGCACTGTTGAAG
GGGCACTACCGCCACTGGGAAATCCCGCGACTATCGGAGGGTAGGCATG
GGAGGCGCCACGTGAGCAGTGGCAACCGGAAATCGAGCGGGAAGT
GGCTCCAGGCCGTAGATCGGCCAAGGCCACGCTTTGCAAGAATACGCACCGCC
GGCCGCTGGTGCAGACCGGGGCACGCTACGTCTGGACTGCGCCGAAAGTTA
TCGCCGCACGCGAACAGCTCTATGCGCACCTCTCCCTTGTGACGCGGATCCACA
TGACTACGTGGTAGAGTCAGTCGCCCGGTCAATCGAGCGCTATATCGATGCCTTC
AACTTATACGCAGCCCGCTACATGGTGGATGA

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

[94] MSTLRHIILRLIILRERQIHLTLAVCPNSAVLAEAVKVAARCTHPMLFAATLN
QVDRDGGYTGWTPAQFVAEMRYAVRYGCTTPLYPCDHGGPWKDRHAQEKLP
LDQAMHEVKLSTACLEAGYALLHDIPVDRTPLPGPEAPLVPIVVERTVELIEHAEQE
RQRNLPAVAYEVTGEHHGLVFNDNFVAFDLLKARLEQRALMHAWPAFVVAQ
VGTDLHHTYFDPAAQRLTEIVRPYKGLKHYTDWVENPADYPRVXMGGANGVGP
EFTAEFEALEALERREQRLCANKLQPAFLAALAEAVVASRWRKWLQPDIEIGK
PFAELTPARRWLVQTYVWTAPKIAAREQLYAHLSLVQADPHAYVVESVAR
SIERYDANFYDAATLLG

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

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

[97] ATGAGTCTGTACATCCTTAAAATAATAATTATCTCAGGCACACAAAAAGGAACGC
CGGTCGGTATTTATATCCGTCTGTCGGCAACACTCTTCTTGTGAAGCGGCGCATG
CTACAGGGCGAAAAAGGATCAGTTTTTGCTACTTTATGAGGCGCACCACCTCAACAGG
TAGATCAATTCGGCGGTTACACCGGCATCGCCGCGCGGAATTTAATAAACATTAG
CGCTTGAAACTGCGACCGAAAACAATTACAGATCCACAGGGATTAATCCTTGCGC
GCGACCCTCTGCGGCCCCAAACCCGTGGACAAAACCTGACGCGCCTCCCGGCGCGATTG
ACTACGCCCAGAGAGCAGATCGGCGCTTTATGTAAAGCGGCCTTTCTAAAAATCCA
CTTAGCAGCCACCACATGCCCCCTTGCAAAACGATGCCACAGATCCCGCAGCCGCCCTT
CCAGTCGAAACAGATCGACTCCAGCAGATCCGCCGAATATTATGCAGCGTGCGCGCAACAA
ACTTACCGGCAGAGCGACCAACTCTTTCCGCCGCCTGTTTACATTGTCGGCAGCG
ACGTGCCCATCCCGGGCGGCGCGCAAGAAGCGCTGAACCAGATCCATATTACGG
AGGTAAAAGAGGTTCAACAGACCATTGATCACGTGCGGCGGGCCTTTGAAAAAA
ACGGCCTGGAAGCGGCTTACGAAAGAGTTTGCGCCGTTGTCGTGCAGCCAGGCG
TTGAATTCGCCGATCAAATCGTTTTGAATAGCTCCCACAGAGCGCGGCCGCCTT
AAAAAGATTTTATGGAAAGGCCATTTGCCAGCTGCTGCTCAAACCTTCTGCAATTG
TACCAGACCCGACCTCTTTTGCGCCAGATGGTAAAAGATCACTTTGCCATTTTAA
AGGTCGCGCCTGCGCATCTTTGCCCTGCGCGAAGCCATTTTTGCTCTGGCCTT
ATATGGAAAAAGAGCTTTTGCACTTGCACAGAGCGCTCACAACCTTTCTGCCATTCTG
GAAACGCCTGGGACCAAGAGTGACAAAAACCTGCTTTACTGCAAAAACAGATTAC
GGCGGAACAAAGGAAGAGTACGCTTTGCGCAGCGCGTTAGCTGACAGCGACCAC
ATTCTGTACTACTGCGCGTTTCCAAGAGGGCTGCGCAATTGCTAA
AAAACCTGCCAAAAATTTCCATTCCTCTAACTTTGGTAAGCCAGTTCATGCCAGA
GGAATACCAACGTATTCGCCAAGGAACGTACCAACGATCCGCAGGGCGCTGAT
TTTGAACAAAATTTCAAGCGTATTTAAGCAATACGCGAGGCGACGCAAATTCA
AAACTCTTTGACATTCACGCAAAATCAAAATTCATTAGCAATGGAGCGACTATG
A

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

[99] MSLHPLNLIERHKKGTPVGYSVCSANPFVLAAMQLAQKDKQSLLIEATSNQV
DQFGGYTGMREPDKTMNLAAENNYDPQGLILGDLHGNPRWTKLASRAMDY
AREQIAAVKYAKSGFVHLDMATDLDADSMGRPVTIAEQTAECLAQEAQTYR
QSDQLFPPPVYGVSDVPQPIGGAQELNQHIETVEKIVQQTIDHVRAFEKNGLEAAY
ERVCAVWQQPGVEADFQIVFEYPADRAAALKDFFIESHSVLYEAHSTDYQTAPLRRQ
MVKHAILKVPGALFTALreaIflaFMEKELLPLHRAKPSAILETLDQTMDKNP
AYWQKHYGGTKEEVFARQFSLSDRIRYWPFPKVQALKRLQDLNLQPSIPLTLVS
QFMPEEYQRIRQGLTLNDPQALNKLINQSLKQYEATQIQNSLFTQTNQNSLAMER
L

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

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

[102] ATGTGCGTCAGTAAAGATTATTTGAGAAAAAGGGAGTTTATTCTATATGTA
GCTCTAATCCATATGTAGTTGACAGCAAGTGTTTATTTGCTAAGAGGAAAGAATG
Codon optimized nucleotide sequence (SEQ ID NO.: 10)

ATGTGGCTGAGCAAGGACTACCTGCGTAAGAAGGGCGTTTACAGCATTTGCA
GCAGAACCCTGTATGTTTATGAAGGCGAGCAGCGTGGAGTTCGCAAGGAGAAAGC
ATTACATCTGATTGAACGCCACCCGCACCCACGATCAACCAATTGTTGGCTATAG
CGGCACTGGACCCGGAGAGCTTCAGAAACTTTGTTATGGGCATCAATTGAAGAAG
AGGTATCGAGGAAGACCTGTTCTTGAGGCTAACCTGCGGATCACTCTGGCTGCGC
GTGGCCAGGATGAGCCAGGCGAGCAGCGGAGTAAGAAGGCGAGAAAGCACCTGAC
GTAGCGCTCCGTTGAAACCGGTGGATTACAAGAAATTCACCTGGAATTGCAGCAGC
TGAGCGACGATCCGGTGGTTCTGAGCCCGGAGAAGATCGCGGAACGTGAGCGTG
AACTGCTGGAAGTTGCGGAGGAAACCGCGCGTAAATACAACTTTCAACCGGTGT
TTGGGAGGACATCAGACATGCTGAAAACTTACAGCATTGCA
AGGTATGACCTCAGAAGGAGATTTTAACTTTGTAAAATGGAAATATAGGAAAAAA
GGGAAATAGAAGAGGAGTGGATCTTTGAGGGAACCTATAGGCCCTCTCCC
TTGGCAAGATGAAACCTCTTCTCTTCTGCAATGAAGAAAGCAGACACCTATAAG
GGGCTTTTGAGGAGTGGATTATAAAGGACATACCTTGAGTTAGTATGCTCTTT
CTGATGATCTCTGTAGCTCTCTCTCCGAGAAGATAGCAGAAAGGGAGAGGGAA
CTTCTTGGATCGAGAAGGACTACCTGCGTAAGAAGGGCGTTTACAGCATTTGCA
GCAGCAACCCGTATGGTTATGAAGCAGCGTGGAGTTGCGAAGGAGAAAAACG
ATTACATCTGATTGAACGCCACCCGCACCCACGATCAACCAATTGTTGGCTATAG
CGGCACTGGACCCGGAGAGCTTCAGAAACTTTGTTATGGGCATCAATTGAAGAAG
AGGTATCGAGGAAGACCTGTTCTTGAGGCTAACCTGCGGATCACTCTGGCTGCGC
GTGGCCAGGATGAGCCAGGCGAGCAGCGGAGTAAGAAGGCGAGAAAGCACCTGAC
GTAGCGCTCCGTTGAAACCGGTGGATTACAAGAAATTCACCTGGAATTGCAGCAGC
TGAGCGACGATCCGGTGGTTCTGAGCCCGGAGAAGATCGCGGAACGTGAGCGTG
AACTGCTGGAAGTTGCGGAGGAAACCGCGCGTAAATACAACTTTCAACCGGTGT
ATGTGGTGACGATGCTCGGGTGGCAGGTAGAGGAGGATCA
CCAGCGTGGAGGACTTCCGTGTTGCGATTAGCAGCCTGAAGAAATACTTTGAAG
ACGTTCCGCGTACGATGCTGGAGAGGATGCGGTTGCTGATCCTGAAAGTG
GGTCCGGCGCTGGACCGCGAGCTTCCGTCGTGGTGTGTTTCTGCTGAGCAGCATCG
AGGACGAACTGATAGCCAGGATAAACGTAGCAACATTAAGAAAGTGGTCTGG
AAACCATGCTGAAAGGAGATAAATCTGGCGTAAGTACTATAAAGACAGCGAGC
GTCTGGAAACTGGATATCTGTGACAACCTGCTGGACCCTATTCTGTAACTCTGGGA
GTACAAGGAATCAAGATTGCCTGAACCGTCTGTGCAGAAGCTTAAGCGAAGG
CGTTGATATCCGTATACATCTACCAATACTTCTACGACAGCTACTTCAAAGTGCGT
GAGGGTAAAATCCGTAACGACCCGCGTGAACTGATTAAGAACGAGATTAAGAA
GTGCTGGAAGACTACCATTATGCGGTGAACCTGTAA

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

[106] MWLSKDYLRKKGVYSICSSNPYVIEASVEFAKEKNDYILIEATPHQINQFGGYSG
MTPEDFKNFVMGIIKEKIEEDRVLGGDHLGPLPWQDEP SSSAMKKAKDLIRAFVE
SGYKKIHLDCMSLSDDPVVLSEPKIAERERELAVEETARKYNFQPVYVVGTVDVP
VAGGGEEEGITISVEDFRVAISLKKYFEDVPRIWDRIGFVIMLIGFYEKVFEDRI
KVRKILEEVKKNLFVEGHSTDQTJKRALRDMVEDGVRLKVGPALTASRRGFL
LSSIEDELISEDKSNIKKVVELETMLKDDKYWRKYTEKDSELERLDLWNLIRYY
WEYKEKIALNRLFENFSEGVDIRYIQYFYDSYFKVREGKIRNDPRELIKNEIKKVE
DYHYAVNL

[107] Tagatose 6-phosphate phosphatase (T6PP) from Archaeoglobus fugidis (Uniprot ID 029805) was used. Activity was measured in 50 mM HEPES buffer (pH 7.2) containing 5 mM MgCh 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.
Thermophilic T6PP from *Archaeoglobus fulgidus* (Uniprot ID: O29805)

Nucleotide sequence (SEP ID NO.: 12)

ATGTTCAAAACCAAGGCCCATCGCAGTTGACATAGATGGCACCCTCAACCAGACA GAAAGAGGGCTCTGAACTGCGAGGGCTTGAAGCTCTCCGAAGATTAAAATTCC CCGTGAATTTGGCCACTTGGAAACATATCTTTGGCGAGGGCTGAGCAAAGCT GATTGGACTCTCAGACCTGGTAAATCTCTGGGAGAAATGGGGCGGTGGTGAGGTTGA GTACGATGGGGAGGATATTGTTTTAGGAGATAAAGAGAAATGCGTTGAGGCTGT GAGGGTGCTTTGAGAAACACTATAGGATGCTGCTGATCCTGAGAATACAGGAA GTCCGAAGTGTGCTAGGAGAGAGTCTTGGACATCTCAAGGAGGAGAAAGCTCAT TGAGGGGATGGGTTAAGCTTGTGATTCAGGCTTTGGCTTACCACATTATGGAT AGGCTCAGGCGAGGTTTCGACAGTACGAGAAACACTATAGGAAATGGGGCTGAGG TATGCTGATTTAGTTACCCAGGACGAGGACGAGGAGGTTTTGGAGGCTTTTGCA AGT TTCTGGGATGGTGCCGTTGA

Amino acid sequence (SEP ID NO.: 13)

MFKPKAIAVDIDGTLTDRKRKRALNCRVEALRKVVKIPVILATGNISCFARAAAKLI GVSDVVICENGGVVRFEYDGEDIVLGDKKKEKCVEAVRVEKHYEVELLDFAEKRSV CMRRSFIDINERALKIEGMGVLSDKGAYHIMDADVSNGKALKFVAEKLAGISSAEFA VIGDSENDIDMFRVAGFFINAVANADERLKEYADLVTPSDGEGYVEALQFGLLRT

T6PP from *Archaeoglobus profundus* (Uniprot ID D2RHV2_ARCPA)

Nucleotide sequence (SEP ID NO.: 14)

GTGTTCAAGGCTTTTGATAGTTGAGATAGATAGGACGAGACTTTGACGGGATAAGAAGA GGGCAATAAAACTGCAAGAGCGCTGGCAAGACCCTTAGAAAACCTAAAGATCTCTTGG TCTTTGGCAACCCGGAACATTTTACATGCTTTGCAAGGGCTGTAGCTAGATTAGG TGTTTCCGATATTGTAATAGCTGAGAAACGGGAGGTGTTGTCAGATTCAGCAGCAC GGGAGGGACATGTTCTGGGGGATAGAAGATTTAATTGCTTACGAGCGTGTTTTGCA AGCTTAGAAACGCTTCAAAAGTAGAGCTTCTCGACACAGAATATAGGAAAGTCTGAG GTCTGCATGAGGAGGACTTCCCTATAGAGGAAGCTAGAAGATACCTGCAAAAA GATGTTTAGCAGTAGGCTACGACGACTTTGCAATACGATCGAGAGGACGAGTTTGG ACTAAGAGCGCTTGTGATGTTACAGGCAGCTTGGGAGTTAGAAGAAGACTGCTGAG AGCAAGGGAAGGGCTTTGATGTTACAGGCAGCTTGGGAGTTAGAAGAAGACTGCTGAG
GATTTCATTGCGATAGGTGATTCCGAAAACGACATTGAAATGTTGGAAGTTGCA
GGTTTTGGCGTTGCAGTTGCGAATGCGGATGAAAAGCTTAAGGAGGTAGCGGATT
TTGGTCACATCGAAGCCTAATGGAGACGGAGTTGTCGAAGCTCTTGAGTTCTTG
GACTCATTAG

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

[117] MFKALVVDIDTLDKRAINCRAVEALRKLKIPVVLATGNISCFARAVAKIIGV
SDIVIAENGGVVRFSYDGEDIVLGDREKVLRAETLRLRKFVEJDNEYRKSEVCMS
RNFPREEARKILPKDVRIVDTGFAYHiIDANVSKGKALMFIADKLGLDVDFIAIGDSE
NDIEMLEVAGFGVANADEKLKEVADLVSKPNGDGVVEALEFLGLI

[118] **T6PP** from *Archaeoglobus veneficus* (Uniprot ID F2KM2_ARCVS)

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

[120] ATGCTCCGTCCAAAGGCTCGCCATTGACATCGACGGAACCATAACATACA
GGATCGAGCCTGAACTGAAGGCCTGTGAAGCTCTCAGAGGTAAGGTTAAATCTCC
CTGATGTTCGTCAACTGCAACTTGAAATGCTTGAGCAAGAAGCTGTGACACAT
ATATCCAGTGCTCTCAGACATTGTGATTGAGCTCTCCAGAGGAAGGCTACAC
TCGGAGGATCAGCTCTTGCAGAAACCTTCTCTCTATGAGGAGAGCGAGGAAAATTC
ACGATGTGCAAGCTGTGACATTTGAGCTTTCGATACACATAT
GGAATCAGGAGGCTGCAAGGAGGAAGGCTGTGAGCTGATAGCTGAAGCTTTG
TATAAGTCAGAGAGGAGTTCCGTCAATGGTGATTCTGAGAAACGACATAGACCT
GATTAAAGCAGGCTGCAAGGCTGTGAGGATGCTGACTTAAAGCTGAAA
ATGGAGGCGACGTGTGATCTGAGGGATAGGGAGACGTGAGGAGTCTGAGGAAC
CTTTGAGCTCTCAGGCTTAATTAA

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

[122] MLRPKGLAIDGTTIYRNSLNCVKAVEALRKVKIPVVLATGNISCFARTAAKLIG
SDIVICENGIVRSYDGDIVLGDREKVLCLAEILKEYFIEIFDLDAEYRKSEVCMS
NFPIEEARKILHDALKDVDVSGFAYHIMDAKVSFKGRALEYIADELGISPKEFAAIG
DSENIDLLIKAAGLGIAYGDADLKLKMEADVVVSKKNGDGVEALELGLI
[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; 93:1 109-1 117. 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-alpha-glucanotransferase from Thermococcus litoralis is described in Jeon et al. 4-a-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 alpha-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: alpha-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 cxGP, 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 aGP: 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 aGP, 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.
Example 5

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 celllobiohydrolase 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 cellohexase phosphorylase, 5 U/L cellobiose phosphorylase, 5 U/mL of aGP, 5 U/mL PGM, and 5 U/mL PG 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 cellulose). F6P was detected using the coupled enzyme assay described above. Glucose was detected using a hexokinase/G6PDH assay kit as described above.

Example 6

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 celllobiohydrolase 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 cellohexase phosphorylase, 5 U/L cellobiose phosphorylase, 5 U/mL of aGP, 5 U/mL PGM, and 5 U/mL PG 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.
Example 7

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 celllobiohydrolase was re-suspended 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 celdodextrin phosphorylase, 5 U/mL cellulose phosphorylase, 5 U/mL of aGP, 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 MgCh and 0.5 mM ZnCh. 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.

Example 8

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 FIEPES buffer (pH 7.2) containing 5 mM MgCh. The reaction was incubated for 16 hours at 50°C. 100% conversion of F6P to tagatose is seen via FIPLC (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.

Example 9

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 MgCh, 0.05 U of aGP, 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 H2SO4, 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 MgCh, 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 MgCk, 0.05 U of cxGP, 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 MgC12, 0.05 U of aGP, 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 aGP; 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

29
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

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<tr>
<th>Concentration of PBS pH 7.2 (mM)</th>
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Example 13

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 aGP; 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.

Table 2

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<td>7.2</td>
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[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 aGP, 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 loses 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.
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. A process for preparing tagatose, the process comprising:
   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.

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 phosphoglucose isomerase (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 (aGP), 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, amylodextrin, maltodextrin, maltose, and glucose.

7. The process of claim 4 or 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 fructose 6-phosphate epimerase.

13. The process of claim 12, wherein the fructose 6-phosphate epimerase is 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.

14. The process of claim 12, wherein 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.

15. The process of any one of claims 1-14, wherein the phosphatase is tagatose 6-phosphate phosphatase.
16. The process of claim 15, wherein the tagatose 6-phosphate phosphatase is 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.: 12, 14, or 16.

17. The process of claim 15, wherein 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 NOS.: 13, 15, or 17.

18. The process of any one of claims 1-17, 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.

19. The process of any one of claims 1-18, wherein the process steps are conducted in one bioreactor or in a plurality of bioreactors arranged in series.

20. The process of any one of claims 1-19, 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.

21. Tagatose prepared by a process of any one of claims 1-20.
FIG. 1
FIG. 2
FIG. 4
Glucose

(P_n)

(P_{n-1})

PPGK

Glucose 6-phosphate

PGI

Fructose 6-phosphate

F6PE

Tagatose 6-phosphate

P_i

T6PP

Tagatose

FIG. 5
Sucrose

Glucose 1-phosphate

Glucose 6-phosphate

Fructose 6-phosphate

Tagatose 6-phosphate

Tagatose

FIG. 6
FIG. 7

- \( \Delta G = -7.4 \text{ kJ/mol} \)
  - \( K'\text{eq} = 20 \)
- \( \Delta G = -34 \text{ kJ/mol} \)
  - \( K'\text{eq} = 1,100 \)
- \( \Delta G = 2.5 \text{ kJ/mol} \)
  - \( K'\text{eq} = 0.361 \)
- \( \Delta G = 3.8 \text{ kJ/mol} \)
  - \( K'\text{eq} = 0.213 \)
- \( \Delta G = -33 \text{ kJ/mol} \)
  - \( K'\text{eq} = 720 \)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(8) ... Halpdesk: 571-272-4300
   Facsimile No. 571-273-8300 PCT OSP: 571-272-7774
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B. FIELDS SEARCHED
   Minimum documentation searched (classification system followed by classification symbols)
   IPC(8): C12N 9/90, C12P 19/24 (2016.01)
   CPC: C12N 9/90, C12P 19/24, Y02P 20/52

   Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
   IPC(8): C12N 9/90, C12P 19/24 (2016.01)
   CPC: C12N 9/90, C12P 19/24, Y02P 20/52

   Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
   PatBase; Keyword limited: Tagatose/epimerase; Fructose 6-phosphate/fructose 6-p/6p; tagatose-6-phosphate/tagatose 6-p/6p;
   phosphoglucose isomerase/tagatose/epimerase

C. DOCUMENTS CONSIDERED TO BE RELEVANT
   Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.
   A LI et al. "Biosynthesis of rare hexoses using microorganisms and related enzymes", Beilstein J. Org. Chem. 2013. Vol. 9, pp 243472445, entire document, especially: abstract; pg 2435, scheme 1; pg 2436, Scheme 2; pg 2438, scheme 6. 1-1 1
   A WO 2005/084411 A2 (NORTH CAROLINA STATE UNIVERSITY) 15 September 2005 (15.09.2005), entire document, especially: abstract; pg 75, In 4-18. 1-1 1

   Further documents are listed in the continuation of Box C.

   • Special categories of cited documents:
     "A" document defining the general state of the art which is not considered to be of particular relevance
     "E" earlier application or patent but published on or after the international filing date
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     "O" document referring to an oral disclosure, use, exhibition or other means
     "P" document published prior to the international filing date but later than the priority date claimed
     "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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     "Z" document member of the same patent family

Date of the actual completion of the international search 22 November 2016
Date of mailing of the international search report 29 Dec 2016

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer: Lee W. Young
PCT Halpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCTASA/210 (second sheet) (January 2015)
**INTERNATIONAL SEARCH REPORT**

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<td>2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:</td>
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<td>3. □ Claims Nos.: 12-21 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
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<tr>
<td></td>
<td>3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:</td>
</tr>
<tr>
<td></td>
<td>4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:</td>
</tr>
</tbody>
</table>

**Remark on Protest**

- □ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)