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(71) Applicant: KEMBIOTIX LLC [US/US]; 54 Mill Street #3, Woburn, MA 01801 (US).

(72) Inventors: MILAN, Jay, L.; 54 Mill Street #3, Woburn, MA 01801 (US). MANNAN, Ramasamy, Mannar; 159 Pleasant Street, Lexington, MA 01801 (US).

(74) Agent: MANNAN, Ramasamy, Mannar; 64 Arrowhead Road, Weston MA 02493 (US).

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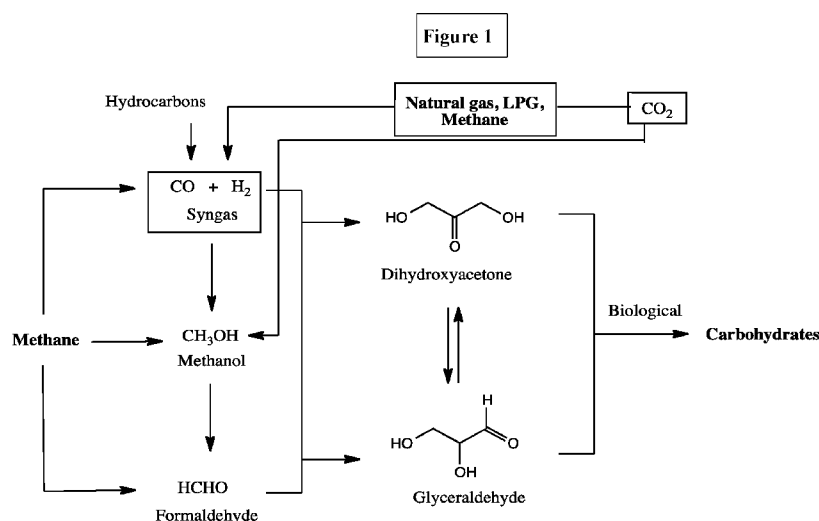
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(54) Title: METHOD FOR PRODUCING CARBOHYDRATES FROM DIHYDROXYACETONE



(57) Abstract: The present invention relates to the use of dihydroxyacetone ("DHA") in the preparation of a number of natural and rare carbohydrates. The present invention comprises three stages. In the first stage of the present invention, syngas and formaldehyde are produced from natural gas, biogas, biomass and CO₂ from industrial plants including electricity generating plants, steel mills, cement factories and bio refineries. In the second stage of the present invention, formaldehyde and syngas from first stage are condensed to produce DHA. In the third stage of the present invention, DHA serves as a starting material for the synthesis of natural and rare carbohydrates using enzymes belonging to isomerase, aldolases, epimerase and transketolase groups.

METHODS FOR PRODUCING CARBOHYDRATES FROM DIHYDROXYACETONE

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the priority to the U.S. Provisional Application Serial No. 62/173,199, filed on June 9, 2015.

FIELD OF THE INVENTION

[0002] This invention is in the field of carbohydrate biochemistry and more specifically in the field of manufacturing dihydroxyacetone (“DHA”) from fossil hydrocarbons, biomass, CO₂ released from industrial operations and glycerol from biodiesel industry and manufacturing natural and rare carbohydrates from DHA using enzyme and chemical catalysts.

Background of the Invention

[0003] Carbohydrate is a molecule consisting of carbon, hydrogen and oxygen atoms with the empirical formula C_m(H₂O)_n (where m could be different from n). Saccharide is the most commonly used term for carbohydrates in the field of biochemistry. The saccharides are divided into four chemical groups: monosaccharides, disaccharides, oligosaccharides and polysaccharides. In general monosaccharides and disaccharides are referred as sugars. Monosaccharides include glucose and fructose. Sucrose, the granulated table sugar, is a disaccharides made up of glucose and fructose.

[0004] Glucose, is produced commercially via enzymatic hydrolysis of starch derived from plants. In the United States, cornstarch is used almost exclusively for glucose manufacturing. Fructose has relatively high sweetness. It is sweetest of all naturally occurring carbohydrates. In general, fructose is regarded as being 1.73 times as sweet as sucrose and 2.33 times as sweet as glucose. As a result, the high fructose corn syrup (HFCS) has become the most widely used sugar and it is commonly found in most processed foods and beverages. In the commercial manufacturing process for HFCS, glucose solution is passed through an immobilized xylose isomerase enzyme column to isomerize glucose to fructose. In HFCS, the amount of fructose

can be increased to 90% by repeating the isomerization process. There is a desire in the sugar industry to manufacture 100% pure fructose through a simple process in a cost-effective way. The present invention provides a cost-effective manufacturing process for producing 100% pure fructose without the need for the glucose derived from cornstarch.

[0005] The international society of rare sugars (ISRS) has classified monosaccharides and their derivatives according to their abundance in nature (Granstrom et al., 2004). In this classification, of all possible hexoses and pentoses, only seven were considered to be present in significant amount, whereas 20 hexoses and nine pentoses were described as rare or unnatural sugars. Rare sugars cannot be extracted from natural sources and thus have to be produced by biochemical reactions. Despite their low abundance, rare sugars hold enormous potential for practical applications. L-ribose, for example, can be used as a building block for drugs against cancers and viral infections. L-sugars can also be used as active compound on their own, for instance as glycosidase inhibitors or as insecticides. Other rare sugars, such as D-tagatose, can serve as low-calorie sweeteners, replacing classical table sugar in the food industry. Another major advantage is that D-tagatose has a low glycemic index, making it suitable for diabetic patients (Lu et al., 2008). Similar effects have been attributed to D-psicose, which shows potential as a no-calorie sweetener as well as diabetic and obesity control agent (Baek et al., 2010). In contrast, D-allose, an isomer of D-psicose, displays rather different properties. In addition to its inhibitory effect on both carcinogenesis and cancer proliferation, it is also useful in surgery and transplantation as an anti-inflammatory agent, immunosuppressant and cryoprotectant (Lim et al., 2011). At this time some of these rare sugars such as D-tagatose and D-sorbose are available at higher costs as specialty chemicals and are used mainly in high-value applications. It can be expected that more efficient production methods for rare sugars will increase their availability for research purposes, resulting in the discovery of new applications and yet unidentified characteristics.

[0006] Based on many years of research at Rare Sugar Research Center, Prof. Ken Izumori and coworkers designed a novel strategy called Izumoring for producing any rare monosaccharides from the cheap natural carbohydrates. (Izumori, 2006). A number of monosaccharides have been produced based on the Izumoring strategy (Beerens et al., 2012).

However, enzymes used in the Izumoring strategy yields at best 1:1 mixture of starting material and the desired product. If the desired rare sugar theoretically requires a two-step enzymatic process from a natural sugar, Izumoring strategy at best yields 25% of the desired rare sugar and requires exhaustive downstream separation technology to remove desired products from the mixture. If the desired rare sugar theoretically requires three-step enzymatic process or more, Izumoring strategy is not practical. Thus there is a need in the field of carbohydrates, to develop a new strategy to produce rare sugars in a cost-effective way. The present invention, besides providing a cost-effective fructose manufacturing process, discloses novel methods to synthesize a number of rare sugars in an economically acceptable way.

SUMMARY OF THE INVENTION

[0007] The invention relates to a process for preparing natural and rare carbohydrates from dihydroxyacetone (“DHA”). The DHA useful in the present invention is derived from fossil hydrocarbons (including natural gas and shale gas), biogas, biomass and CO₂ released from industrial plants including power generation plants, steel mills and cement factories. The present invention comprises three stages. In the first stage of the present invention, syngas and formaldehyde are produced from fossil hydrocarbons, biogas, biomass and CO₂ released from industrial plants. In the second stage of the present invention, formaldehyde and syngas are condensed to produce DHA. In one aspect of the present invention, the DHA useful for practicing this invention can also be derived from glycerol which is produced in large volume as a byproduct of biodiesel industry. In the third stage of the present invention, natural and rare carbohydrates are synthesized using enzymes belonging to the groups namely isomerase, aldolases, epimerase and transketolase.

[0008] In one embodiment, the present invention provides a method for producing fructose, a natural sugar and a number of rare sugars. In the first stage of this embodiment for producing fructose and rare sugars according to this embodiment, DHA is subjected to isomerization reaction using an isomerase enzyme to yield glyceraldehyde (“GA”). GA occurs in two isomeric forms namely D-glyceraldehyde (“DGA”) and L-glyceraldehyde (“LGA”). In one aspect, this invention uses a wide range of isomerases including but not limited to triose isomerase, L-

arabinose isomerase, xylose isomerase, L-rhamnose isomerase, ribose isomerase and mutant derivatives thereof to produce either DGA or LGA from DHA.

[0009] In the second stage of this embodiment, the DHA is condensed either with DGA or LGA using an aldolase enzyme to yield a hexose sugar. The list of aldolase enzymes suitable for catalyzing the condensation reaction between DHA and DGA includes but not limited to fructose aldolase, fucose aldolase, rhamnulose aldolase, tagatose aldolase and mutant derivatives thereof. The list of hexoses that can be synthesized from aldolase catalyzed condensation reaction involving DHA and DGA includes but not limited to D-fructose, D-psicose, D-sorbose and D-tagatose. D-tagatose is also produced by the epimerization of D-fructose at C4 position catalyzed by L-ribulose-5P-4-epimerase. The list of aldolase enzymes suitable for catalyzing the condensation of DHA and LGA includes but not limited to rhamnulose aldolase, tagatose aldolase, fructose aldolase, fucose aldolase and mutant derivatives thereof. The list of hexoses that can be synthesized from aldolase catalyzed condensation reaction involving DHA and DGA includes but not limited to L-fructose, L-psicose, L-sorbose and L-tagatose.

[0010] In another embodiment, the present invention the hexose sugars derived from DHA through isomerization and aldol condensation reactions are subjected to enzyme reaction involving one or other type of isomerase enzyme to yield another isomeric form of hexose sugar. In one aspect of this embodiment, isomerization reaction involving D-fructose and either L-ribose isomerase or D-mannose isomerase or lyxose isomerase yields D-mannose. In another aspect of this embodiment, isomerization reaction involving D-fructose and xylose isomerase yields D-glucose. In yet another aspect of this embodiment, D-altrose is derived from D-psicose in an isomerase reaction involving D-arabinose isomerase or L-fucose isomerase. In yet another aspect of this embodiment, D-allose is derived from D-psicose through an isomerization reaction involving L-rhamnose isomerase or ribose isomerase. Similarly, an isomerization reaction involving D-sorbose and xylose isomerase yields D-idose while an isomerization reaction involving D-sorbose and L-rhamnose isomerase produces D-gulose. In another aspect of this embodiment, D-talose is derived from D-tagatose in an isomerization reaction involving D-lyxose isomerase or L-ribose isomerase. In yet another aspect of this embodiment, D-galactose is derived from D-tagatose through an isomerization reaction involving L-arabinose isomerase. In another aspect of this embodiment, L-fructose is isomerized to L-mannose using L-rhamnose

isomerase while L-fructose is isomerized to L-glucose using xylose isomerase. In another aspect of this embodiment, L-psicose is isomerized to L-altrose using L-arabinose isomerase or galactose-6P isomerase while L-allose is derived from L-psicose through an isomerization reaction involving L-ribose isomerase or D-lyxose isomerase. Similarly, L-sorbose is isomerized to L-idose using D-glucose isomerase while L-gulose is derived from L-sorbose through an isomerase reaction -involving either D-mannose isomerase or L-ribose isomerase or D-lyxose isomerase. In yet another aspect of this embodiment, L-talose is derived from L-tagatose through an isomerization reaction involving L-rhamnose isomerase or ribose isomerase while L-tagatose is isomerized to L-galactose through an isomerase reaction involving L-fucose isomerase or D-arabinose isomerase.

[0011] In another embodiment of the present invention, DGA (a triose) derived from isomerization of DHA is subjected to transketolase reaction with D-fructose (a hexose) produced by the aldol condensation of DGA and DHA to yield D-xylulose (a pentose) and D-erythrose (a tetrose). In yet another aspect of this embodiment, mixture of DGA and DHA produced by the isomerization of DHA is subjected to a transketolase reaction catalyzed by dihydroxyacetone synthase to produce D-xylulose and formaldehyde. D-xylulose is epimerized at C3 position by D-tagatose-3-epimerase to produce D-ribulose and epimerized at C4 position by L-ribulose-5P-4-epimerase to produce L-ribulose.

[0012] In another embodiment, the present invention subjects the pentose sugars derived from DHA through isomerization, aldol condensation and transketolase reactions are subjected to enzyme reaction involving one or other type of isomerase enzyme to yield another isomeric form of pentose sugar. In one aspect, isomerization reaction involving D-xylulose with either D-lyxose isomerase or D-mannose isomerase yields D-lyxose. Similarly, isomerization using xylose isomerase yields D-xylose. In another aspect, isomerization reaction involving D-ribulose with either L-fucose isomerase or D-arabinose isomerase yields D-arabinose. Similarly, isomerization of D-ribulose using ribose isomerase yields D-ribose. In yet another aspect, isomerization reaction involving L-ribulose with either D-lyxose isomerase, D-mannose isomerase or L-ribose isomerase yields L-ribose. Similarly, isomerization reaction involving L-ribulose using L-arabinose isomerase yields L-arabinose.

[0013] In yet another embodiment of the present invention, the ketose and aldose sugars derived from DHA are subjected to a hydrogenation reaction involving chemical hydrogenation catalysts or enzymes to yield corresponding sugar alcohol. In one aspect of this embodiment, D-erythrose is hydrogenated to D-erythritol using a chemical hydrogenation catalyst. In another aspect of this embodiment, D-fructose is hydrogenated using mannitol 2-dehydrogenase to yield mannitol while hydrogenation reaction involving sorbitol 2-dehydrogenase and D-fructose yields sorbitol. In yet another aspect of this embodiment, hydrogenation reaction involving D-sorbose using iditol 2-dehydrogenase yields iditol while hydrogenation of D-tagatose using galacitol 2-dehydrogenase yields galacitol. In yet another aspect, enzymatic hydrogenation of D-xylulose using D-xylulose reductase yields D-xylitol while using D-arabitol 4-dehydrogenase yields D-arabitol. Enzymatic reduction of D-ribulose using D-arabitol 2-dehydrogenase yields D-arabitol while using ribitol 2-dehydrogenase yields D-ribitol. In another aspect, ketohexoses and ketopentoses are chemically hydrogenated to produce a mixture of sugar alcohols. The mixture is further separated through column chromatography to produce enantiomerically pure sugar alcohols. In one example, D-fructose is chemically hydrogenated to produce mixture of sorbitol and mannitol. In another example, D-xylulose is chemically hydrogenated to produce mixture of D-xylitol and D-lyxitol. In yet another example, D-ribulose is chemically hydrogenated to produce a mixture of D-arabitol and D-ribitol.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The following figures are included to illustrate certain aspects of the present invention, and should not be viewed as exclusive embodiments. The subject matter disclosed is capable of considerable modifications, alterations, combinations, and equivalents in form and function, as will occur to those skilled in the art and having the benefit of this disclosure.

[0015] FIG. 1. Carbohydrate production from methane through syngas, methanol, formaldehyde, dihydroxyacetone (DHA) and glyceraldehyde (GA). Methane useful for the present invention is derived from natural gas, bio-gas, shale gas, town gas, liquefied refinery gas (LRG) and crystalized natural gas such as methane clathrates and hydrates. Syngas useful for the present invention is produced from methane via oxidation reaction using chemical catalyst. Syngas could also be produced from biomass and other fossil hydrocarbon sources. Syngas

comprising carbon monoxide and hydrogen reacts over chemical catalyst to produce methanol. Methanol could also be produced from methane through partial oxidation process. In another method, CO₂ is reduced with hydrogen gas to produce methanol. Formaldehyde useful for the present invention is produced from methanol through a catalytic oxidation method or catalytic dehydrogenation methods. Formaldehyde could also be directly produced through a partial oxidation of methane. DHA is produced from formaldehyde, glycolaldehyde and syngas as described in Figure 2. Glycolaldehyde is produced from the condensation of two formaldehyde molecules. GA can also be derived from formaldehyde. GA is also derived from DHA through isomerization reaction catalyzed by isomerase enzyme. DHA and GA can undergo aldol condensation reaction catalyzed by aldolase enzyme to yield a hexose sugar.

[0016] FIG. 2. Multiple routes for the production of DHA. Formaldehyde useful for the present invention is produced from methanol through a catalytic oxidation or catalytic dehydrogenation methods. Formaldehyde could also be directly produced through a partial oxidation of methane. Condensation of three molecules of formaldehyde over a chemical catalyst yields DHA. Condensation of two molecules of formaldehyde yields glycolaldehyde. Condensation of a molecule of glycolaldehyde with a molecule of formaldehyde could also yield DHA. DHA could also be produced by reacting formaldehyde and syngas over a catalyst. In another method, syngas comprising carbon monoxide and hydrogen is passed over glycolaldehyde in the presence of a catalyst to produce DHA.

[0017] FIG. 3. Enzyme routes for the production of D-aldohexoses and D-ketohexoses from DHA. In the first stage of this process, DHA is isomerized using one or more of the enzymes belonging to isomerase class (EC 5.3.1) to produce DGA. In the second stage of this process, DHA and DGA are condensed using enzymes belonging to the aldolase class (EC 4.1.2) to produce a variety of D-ketohexoses. In the third stage of this process, D-ketohexoses resulting from the second stage are treated with aldose-ketose isomerase enzymes belonging to the isomerase class (EC 5.3.1) to produce D-aldohexoses. Non-limiting examples of the enzymes that are suitable for each stage of this process are provided for the purpose of illustrating the routes for the synthesis of D-aldohexoses and D-ketohexoses according to the present invention.

[0018] FIG. 4. Enzyme routes for the production of L-aldohexoses and L-ketohexoses from DHA as sole carbon source. In the first stage of this process, DHA is isomerized using one or more of the enzymes belonging to isomerase class (EC 5.3.1) to produce LGA. In the second stage of this process, DHA and LGA are condensed using enzymes belonging to the aldolase class of enzymes (EC 4.1.2) class to produce a variety of L-ketohexoses. In the third stage of this process, L-ketohexoses resulting from the second stage are treated with aldose-ketose isomerase enzymes belonging to the isomerase class of enzymes (EC 5.3.1) to produce L-aldohexoses. Non-limiting examples of the enzymes that are suitable for each stage of this process are provided for the purpose of illustrating the routes for the synthesis of L-aldohexoses and L-ketohexoses according to the present invention.

[0019] FIG. 5. Enzyme routes for the production of tetrose and pentose sugars from DHA. In the first stage of this process, DHA is isomerized using one or more of the enzymes belonging to isomerase class (EC 5.3.1) to produce DGA. In the second stage of this process, DHA and DGA are condensed using enzymes belonging to the aldolase class of enzymes (EC 4.1.2) to produce D-fructose, a D-ketohexose sugar. In the third stage of this process, D-fructose is reacted with DGA using a transketolase enzyme belonging to transferase class of enzymes (EC.2.2.1) to produce D-xylulose (a ketopentose) and D-erythrose (an aldotetrose). D-erythrose can be subjected to chemical hydrogenation reaction to produce D-erythritol, a sugar alcohol.

[0020] FIG. 6. Enzyme routes for the production of D-xylulose, D-lyxose, D, ribulose, D-arabinose and D-ribose. D-xylulose, a ketopentose sugar is produced using an enzyme route as illustrated in the Figure 5 and Figure 7. In one aspect of this invention, D-xylulose is treated with D-tagatose-3-epimerase, an enzyme belonging to the racemase class of enzymes (EC 5.1.3) to yield D-ribulose. When treated with xylose isomerase, D-xylulose yields D-xylose. On the other hand when treated with D-mannose isomerase or D-lyxose isomerase, D-xylulose yields D-lyxose. D-ribulose is treated with ribose isomerase to yield D-ribose. Alternately, D-ribulose is treated with L-fucose isomerase or D-arabinose isomerase to yield D-arabinose. In another aspect, D-fructose is derived from DHA using an enzyme route as illustrated in Figure 3. Upon treatment with L-ribulose-5P-4-epimerase belonging to racemase (EC 5.1.3) class, D-fructose is converted into D-tagatose.

[0021] FIG. 7. Enzyme route for the production of D-xylulose, a ketopentose sugar from DHA. In the first stage of this enzyme route, DHA is isomerized using one or more of the enzymes belonging to isomerase class of enzymes (EC 5.3.1) to produce DGA. In the second stage of this enzyme route, DHA and DGA are reacted in the presence of dihydroxyacetone synthase belonging to the transferases class of enzymes (EC 2.2.1) to yield D-xylulose and formaldehyde. In one aspect of the present invention, D-xylulose is converted into D-ribulose using D-tagatose-3-epimerase. In another aspect of the present invention, D-xylulose is reacted with L-ribulose-5P-4-epimerase belonging to the racemase class of enzymes (EC 5.1.3) to yield L-ribulose. L-ribulose is further converted to L-arabinose using L-arabinose isomerase or to L-ribose using an enzyme selected from a group consisting of D-lyxose isomerase, mannose-6P isomerase and L-ribose isomerase.

[0022] FIG. 8. Process flow diagram for large scale industrial production of methanol from natural gas.

[0023] FIG. 9. Process flow diagram for large scale industrial production of formaldehyde from methanol.

Detailed Description

[0024] The present invention relates to the field of manufacturing DHA from fossil hydrocarbons, biomass, CO₂ released from industrial operations and glycerol from biodiesel industry and manufacturing natural and rare carbohydrates from the DHA using enzyme and chemical catalysts.

[0025] The term “fossil hydrocarbon” as used in the present invention includes coal, petroleum and natural gas. The term “coal” as used in the present invention refers to coal and coal related products such as peat, lignite, sub-bituminous coal, bituminous coal, stream coal, anthracite, graphite, coke, tar sand, coal gas, coal to liquid products, refined coal and other coal derived products that can be gasified under heat and pressure. The term “petroleum” as used in this invention includes liquid fuel products derived from methane, ethane, propane, butane, pentane, hexane, heptane, octane, nonane, and decane, liquefied petroleum gas (LPG) and other liquid

fuels including fuel oil, gasoline, jet fuel, diesel fuel, heavy fuel oil, kerosene, liquefied refinery gas, still gas, coke, heating oil, lubricants, wax, asphalt, petroleum coke and petrochemical tar sand and petroleum products derived from crude oil that can be gasified under heat and pressure. The term “natural gas” as used in the present invention refers to gaseous fuels such as Natural gas, methane, bio-gas, shale gas, town gas, gaseous fuel obtained during crude oil drilling process, liquid refinery gas (LRG) and crystalized natural gas such as methane clathrates, hydrates. The term “Methane” as used in the present invention refers to hydrocarbon with the chemical structure “CH₄”.

[0026] The term “biomass” as used in the present invention refers to any plant derived renewable material that can be processed to yield syngas which can be used in the production of DHA used as the starting material in the production of a variety of carbohydrates according to the present invention. The list of biomass materials useful for the present invention include (1) Virgin wood from forestry, arboricultural activities or from wood processing; (2) Energy crops grown specifically for energy applications; (3) Agricultural residues from agricultural harvesting or processing; (4) Food waste from food and drink manufacture, preparation and processing and post-consumer waste; and (5) Industrial waste including co-products from manufacturing and industrial process.

[0027] The term “biogas” as used in the present invention refers to mixture of gases formed during the anaerobic digestion of organic matters in the absence of oxygen. Biogas consists of methane, carbon dioxide, hydrogen sulfide, nitrogen, hydrogen, oxygen and moisture. Biogas useful for the present invention is produced from organic matters including agricultural waste, manure, municipal waste, plant material, sewage, green waste and food waste.

[0028] The carbon dioxide (“CO₂”) useful for the production of DHA according to the present invention is obtained from one or more of the sources including but not limited to utility industry using fossil hydrocarbons, steel industry, cement industry and biorefineries conducting large scale fermentation processes.

[0029] The raw materials for the synthesis of biodiesel can include vegetable oil, animal fats and discarded cooking oil. Large scale industrial processes have been developed and are being used commercially to convert glycerol esters of fatty acids (also known as glycerides, mono-glycerides, diglycerides, and tri-glycerides) to glycerol esters of methanol, ethanol, or other alcohols. The resulting fatty acid esters (also known as FAME for fatty acid methyl ester or FAEE for fatty acid ethyl ester) are commonly known as “biodiesel”, because they can be used by themselves or in blends with conventional hydrocarbons as fuel for diesel engines. A major volume byproduct of the biodiesel process is glycerol (also known as glycerin or glycerin). For each kilogram of biodiesel produced, about 0.1 kg of glycerol byproduct is produced. Glycerol can be subjected to chemical or biological oxidation process to yield DHA which can be used as a raw material in the manufacture of natural and rare sugars according to the present invention.

[0030] The term “Syngas” used in the present invention refers to a “blend” of gaseous products carbon monoxide, carbon dioxide and hydrogen with chemical structure “(CO + H₂ + CO₂)”. The ratio of carbon monoxide, Carbon dioxide and hydrogen in the “blend” varies from 1:1 to 1:4 depending on the feedstock used to produce syngas. Syngas used in the present invention is derived from fossil hydrocarbon, industrial CO₂ emissions and biomass sources.

[0031] Sugars or carbohydrates as used in this invention refer to a biological molecule consisting of carbon, hydrogen and oxygen atoms, usually with hydrogen: oxygen atom ratio of 2:1; in other words with the empirical formula C_m(H₂O)_n (where m could be different from n). Saccharide is the most commonly used term for carbohydrate and four types of saccharides namely monosaccharide, disaccharide, oligosaccharide and polysaccharide are generally recognized. In general monosaccharides and disaccharides, which are smaller carbohydrates, are commonly referred to as sugars. Monosaccharides refers to single molecules of sugar units. Monosaccharides exist in cyclic or acyclic form. Some non-limiting examples of monosaccharides includes aldoses and ketoses sugars belonging to diose, triose, tetrose, pentose, hexose and heptose groups. Disaccharides refers to two molecules of sugar bound together by a glycosidic linkage. Disaccharides may be homodisaccharides, formed between two of the same aldoses or two of the same ketoses. Disaccharides are also hetrodisaccharides, formed between

two of the different aldoses, two of the different ketoses or between an aldose and a ketose. Some non-limiting examples of disaccharides includes sucrose, maltose and lactose. Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages. Some non-limiting examples of polysaccharides include starch, glycogen, cellulose and chitin.

[0032] The term “aldoses” as used herein refers to monosaccharides that contain one aldehyde group per molecule of carbohydrate. Examples of aldoses includes but not limited to glycoldehyde, D-glyceraldehyde, L-glyceraldehyde, D-erythrose, L-erythrose, D-throes, L-throes, D-ribose, L-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-lyxose, L-lyxose, D-allose, L-allose, D-altrose, L-altrose, D-glucose, L-glucose, D-mannose, L-mannose, D-gulose, L-gulose, D-idose, L-idose, D-galactose, L-galactose, D-talose and L-talose. The term “ketoses” as used herein refers to monosaccharides that contain one keto group per molecule of carbohydrates. Examples of ketoses includes but not limited to dihydroxyacetone, D-erythrulose, L-erythrulose, D-ribulose, L-ribulose, D-xylulose, L-xylulose, D-fructose, L-fructose, D-sorbose, L-sorbose, D-psicose, L-psicose, D-tagatose, L-tagatose, D-sedoheptulose, L-sedoheptulose, D-mannoheptulose, L-mannoheptulose, D-mannooctulose and L-mannooctulose.

[0033] The term “aldohexose” as used herein refers to one or more of the carbohydrates including but not limited to D-mannose, D-glucose, D-altrose, D-allose, D-idose, D-gulose, D-talose, D-galactose, L-mannose, L-glucose, L-altrose, L-allose, L-idose, L-gulose, L-talose and L-galactose.

[0034] The term “ketohexose” as used herein refers to one or more of the carbohydrates including but not limited to D-fructose, D-psicose, D-sorbose, D-tagatose, L-fructose, L-psicose, L-sorbose and L-tagatose. Fructose or fruit sugar is a simple ketonic monosaccharide found in many fruits. Fructose is the most widely used sugars of all. It is sweetest of all naturally occurring carbohydrates. In general, fructose is regarded as being 1.73 times as sweet as sucrose and 2.33 times as sweet as glucose. D-tagatose is a monosaccharide sweetener. It is an isomer of D-galactose and is rarely found in nature. Many properties of D-tagatose are closer to those of

sucrose than those of other known sugar-substitute candidates. Its melting temperature is closer to sucrose and it is 92% as sweet as sucrose when compared in 10% solutions. The sweetness profile of D-tagatose is similar to that of sucrose. The caloric value of D-tagatose is nearly one-third of sucrose. Its humecting properties are similar to that of sorbitol and its bulking value is similar to that of sucrose. D-tagatose is less hygroscopic than fructose. Since tagatose is a reducing sugar, it is involved in browning reactions during heat treatment. D-psicose is an epimer of D-fructose and it is very similar to D-fructose in the aspect of intensity and type of sweetness. Unlike D-fructose, D-psicose is hardly metabolized during assimilation in the body and has a near zero caloric value. D-psicose also has a function of reducing abdominal fats by suppressing the activities of lipogenic enzymes. In addition, unnatural sugars such as D-tagatose and D-psicose has near zero glycemic index value.

[0035] The term “aldopentose” as used herein refers to one or more of the carbohydrates including but not limited to D-xylose, D-lyxose, D-ribose, D-arabinose, L-xylose, L-lyxose, L-ribose and L-arabinose. Xylose is a high value material and is used to derive xylitol which has wide range of applications as an artificial sweetener, oral care products and food additives. Conventional process to produce xylose from hemicellulose is highly corrosive and environmentally unfriendly due to the use of mineral acids and low pH. They are also expensive due to the high energy input required and the erosion faced by the reactor materials due to the super acidic nature of the reactions. L-arabinose is useful in the production of savory reaction flavors, dietary supplements and food ingredients. L-arabinose derivatives are widely employed in the developments of antiviral agents such as nucleotides. L-arabinose is a naturally occurring sugar manufactured by hydrolyzing wood based or other biomass such as Arabic gum. L-ribose and its derivatives are useful in the pharmaceutical industry due to its lack of interaction with enzymes in living systems. Some of the examples including L-ribose in the drug moieties are L-nucleoside based antiviral agents, L-ribose comprising glycoconjugates and L-ribose comprising oligonucleotides. L-ribose is conventionally manufactured from L-arabinose by epimerization reaction.

[0036] The term “ketopentose” as used herein refers to one or more of the carbohydrates including but not limited to D-xylulose, D-ribulose, L-xylulose and L-ribulose. L-xylulose is an

intermediate in certain metabolic pathways and is classified as a rare sugar. L-xylulose was demonstrated to decrease blood glucose due to the inhibition of alpha-glycosidase, an enzyme that catalyzes the degradation of disaccharides and polysaccharides to monosaccharides, which can be absorbed and utilized by human. L-xylulose is conventionally produced from xylitol by the enzyme xylitol-4-dehydrogenase.

[0037] In general two types of sugars are recognized namely “natural sugars” and “unnatural sugars”. As defined herein, the term “natural sugars” refers to those sugars that are readily obtained in large quantities from plant sources. The list of natural sugars include sucrose, glucose and fructose. The term “unnatural sugar” as used herein refers to sugars which are not readily available from plant sources. The “unnatural sugars” are also referred as “rare sugars”. Only six types of aldoses namely D-glucose, D-galactose, D-mannose, D-ribose, D-xylose and L-arabinose exist abundantly in nature. Therefore, other aldose sugars are defined as unnatural sugars or carbohydrates. Similarly, D-fructose is an abundantly occurring natural ketose sugar. The other ketose sugars including D-psicose, D-tagatose, D-sorbose, L-fructose, L-psicose, L-tagatose and L-sorbose are defined as unnatural or rare ketose sugars. Sugar alcohols are prepared by reducing monosaccharides. In the natural kingdom, D-sorbitol exist relatively abundantly and D-sorbitol is referred as a natural sugar alcohol. All other sugar alcohols are referred as unnatural or rare sugar alcohol as they exist in nature in very small amounts.

[0038] The term “dioses” or “C2 sugars” refers to monosaccharide containing two carbon atoms. Glycoldehyde is an example of diose. The term “trioses” or “C3 sugars” refers to monosaccharide containing three carbon atoms. Some non-limiting examples of trioses are D-glyceraldehyde (DGA), L-glyceraldehyde (LGA) and dihydroxyacetone (DHA). The term “tetroses” or “C4 sugars” refers to monosaccharide containing four carbon atoms. Some non-limiting examples of tetroses are D-erythrose, L-erythrose, D-threose, L-threose, D-erythrulose and L-erythrulose. The term “pentoses” or “C5 sugars” refers to monosaccharide containing five carbon atoms. Some non-limiting examples of pentoses are D-ribose, L-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-lyxose, L-lyxose, D-ribulose, L-ribulose, D-xylulose and L-xylulose. The term “hexoses” or “C6 sugars” refers to monosaccharide containing six carbon atoms. Some non-limiting examples of hexoses are D-allose, L-allose, D-altrose, L-altrose, D-

glucose, L-glucose, D-mannose, L-mannose, D-gulose, L-gulose, D-idose, L-idose, D-galactose, L-galactose, D-talose, L-talose, D-fructose, L-fructose, D-sorbose, L-sorbose, D-psicose, L-psicose, D-tagatose, and L-tagatose. The term “heptoses” or “C7 sugars” refers to monosaccharide containing seven carbon atoms. Some non-limiting examples of heptoses are D-sedoheptulose, L-sedoheptulose, D-mannoheptulose and L-mannoheptulose. The present invention relates to the process for a producing a variety of natural and rare monosaccharides from DHA (a simple ketotriose) using one or other enzymes.

[0039] As defined in this invention, the term “isomer” or “stereoisomer” refers to molecules that have same molecular formula and sequence of bonded atoms, but differ in the three-dimensional orientations of their atoms in space. Two molecules are described as stereoisomers of each other if they are made of the same atoms, connected in the same sequence, but atoms are positioned differently in the space. Stereoisomers are classified either as enantiomers or diastereoisomers.

[0040] As defined in this invention, the term “enantiomer” refers to stereoisomers that are non-superimposable mirror images. A molecule with one chiral carbon atom exists as two stereoisomers termed enantiomers. Enantiomers differ in their configuration (R or S) at the stereogenic center. Configuration is assigned by Cahn-Ingold-Prelog (CIP) rules. Enantiomers have identical chemical and physical properties in an achiral environment but interact with chiral molecules differently. Enantiomers rotate the direction of plane polarized light to equal, but opposite angles. Since, many carbohydrates are enantiomers, there is marked difference in the effects of two enantiomers on biological organisms. For example, D-fructose and L-fructose are enantiomers with identical sweetness index. D-fructose is metabolized in the body to produce 4 calories per gram, whereas L-fructose does not get metabolized at all.

[0041] As defined in this invention, the term “diastereomer” refers to stereoisomers that are not mirror images of one another and are non-superimposable on one another. Stereoisomers with two or more stereocenters can be diastereomers. For example, triose, a single stereocenter carbohydrate does not have diastereoisomer whereas, multi-stereocenter carbohydrates such as tetrose, pentose and hexoses have diastereoisomers. Diastereomers have different physical

properties and different chemical reactivity. For example, D-tagatose, a diastereoisomer of D-fructose has one-fifth of the caloric value of D-fructose.

[0042] As used in this invention, the term “enantiomerically enriched” as used herein refers to the isomeric purity of the sugar products. Isomeric purity refers to both enantiomeric purity as well as diastereomeric purity of the sugar products. Sugar products produced by the invention disclosed here generally consists of 90% or above of enantiomeric purity and 90% or above of diastereomeric purity. However, in some cases where epimerization of a ketosugar is performed by enzyme catalysts such as 3-epimerase and 4-epimerase there may be a diastereomeric mixtures of sugars. In those cases, diastereomeric purity of the resulting sugar products would be less than 90%. Relative configuration of sugar products is denoted with respect to the position of a group in relation to the position of the same group in other stereoisomer as per IUPAC (International Union of Pure and Applied Chemistry) convention and assigned by Cahn-Ingold-Prelog (CIP) rules. Absolute configuration of the isomer is denoted as R or S as per IUPAC conventions. A person skilled in the art of carbohydrate research will be able to understand the relative configuration of various carbohydrates besides being able to determine the relative configuration of various carbohydrates,

[0043] Carbohydrate synthesis has become a major focus of current biological research. Carbohydrates are structurally complex with several stereoisomers that exhibit specific orientation relative to each other. Depending on the position of the isomers and the relationship between them, several natural and unnatural carbohydrates are identified. The synthesis of such highly functionalized, complex molecules via chemical methods often requires many selective protection and deprotection steps which can be avoided using enzymes. Enzymes are increasingly recognized as useful catalysts for synthesis of complex carbohydrates. One of the main attractions for the use of enzymes is their ability to perform reactions in a stereo selective way. Additionally, the use of enzymes has several advantages over chemical methods. Because of the mild conditions required for enzymatic reactions and the regioselectivity displayed by the enzymes unprotected substrates can be used. Since most enzymes operate at room temperature in aqueous solution around pH 7, the reactions by multiples enzymes are often compatible with each other. This makes it possible to combine several enzymes in a one-pot multistep reaction

sequence. Their use in aqueous solution and their biodegradability make enzymes also an excellent environmentally acceptable options. The high regio and stereoselectivity and catalytic efficiency make enzymes especially useful for the synthesis of complex and highly functionalized carbohydrates.

[0044] The present invention relates to the use of DHA in the preparation of a number of natural and rare carbohydrates. The present invention comprises three stages. In the first stage of the present invention, syngas and formaldehyde are produced from natural gas, biogas, biomass and CO₂ from industrial plants including electricity generating plants, steel mills, cement factories and biorefineries. In the second stage of the present invention, formaldehyde and syngas from first stage are condensed to produce DHA. In another embodiment of the present invention, DHA is derived from glycerol available as a byproduct of biodiesel industry using chemical or biological approaches. In the third stage of the present invention, DHA serves as a starting material for the synthesis of natural and rare carbohydrates using enzymes belonging to isomerase, aldolases, epimerase, and transketolase groups. The list of carbohydrates that can be produced according to the present invention include a variety of natural and rare sugars including trioses, tetrose, pentoses and hexoses and should not be restricted to the examples provided herein to illustrate the present invention.

[0045] Within the process step related to the production of formaldehyde from natural gas, or biomass or CO₂, three different stages could be recognized. In the first stage of the process according to the present invention, suitable chemical catalysts are used to produce syngas from a fossil hydrocarbon, preferably methane. Methane useful for the present invention is derived from natural gas, shale gas, town gas, liquefied refinery gas (LRG) and crystalized natural gas such as methane clathrate. In one aspect of the present invention, biogas derived from anaerobic digestion of biogenic waste can also be used as a source of methane. Syngas useful for the present invention can also be obtained directly from biomass through certain chemical conversion processes. However, syngas production from methane rich natural gas is highly preferred due to the lower cost and relative operational simplicity.

[0046] Two methods are available for the production of syngas from natural gas. Steam reforming method, an endothermic process, is widely used when hydrogen rich syngas is desired. For methanol related applications an exothermic catalytic partial oxidation process is preferred due to its lower energy demand in large scale productions. CO_2 , a common by-product of partial oxidation process, is also a precursor for methanol synthesis. NiO supported on various minerals such as silica, alumina, TiO_2 or CaO is generally used as a catalyst in the partial oxidation process for the production of syngas from methane. Syngas production from natural gas is practiced in large scale by major oil companies for the gas to liquid (GTL) or methanol to gasoline (MTG) production processes.

[0047] As mentioned above, in another aspect of the present invention, syngas production from biogas is followed in the present invention. Biogas consists of methane and CO_2 along with some trace gases such as water vapor, H_2S , N_2 , H_2 and oxygen. Purification methods are required to remove impurities to prevent catalyst poisoning. H_2S levels in the biogas needs to be reduced to ppm level before its use. H_2S is readily removed by adsorption onto the iron oxide catalyst or activated carbon. Iron sulfide, a byproduct of the H_2S adsorption, is recycled by electrochemical methods for reuse. Sulfur, a byproduct of the iron sulfide electrolysis, is processed to produce sulfuric acid or SO_2 .

[0048] CO_2 reforming process known as Calcor process is a method of producing syngas from the reaction of CO_2 with hydrocarbons such as methane. Biogas produced from anaerobic digestion can also be used as a feed for CO_2 reformation. CO_2 reforming utilizes both CO_2 and CH_4 in biogas to produce CO and H_2 useful for the methanol synthesis. CO_2 reforming of methane produces synthesis gas with a high CO/ H_2 ratio, the ratio might vary based on the amount of CO_2 present in the biogas. High CO/ H_2 ratio is desirable for oxo-synthesis and acetic acid production. However, methanol synthesis requires high H_2 /CO ratio. The required hydrogen is supplied from an external source. Direct production of syngas with a wide range of H_2 /CO ratio is efficiently accomplished by controlling the raw material compositions such as steam/methane ratios and CO_2 /methane ratios. NiO supported on various minerals such as silica, alumina, TiO_2 or CaO is generally a catalyst of choice for many industrial scale CO_2 reforming

process. Large scale production of syngas from CO₂ reforming is performed by the Chiyoda Corporation in Japan.

[0049] In the second stage of the process according to the present invention, syngas derived from methane CO₂ or biomass is converted into methanol through a single step chemical process over a chemical catalyst. Methanol synthesis was first performed by BASF using ZnO/Cr₂O₃ catalyst at 250–350 bar and 350–420 °C. The ZnO/Cr₂O₃ catalyst is highly stable to sulfur and nitrogen impurities. However, chromium toxicity and high temperature / high pressure requirements for this catalyst limit its commercial viability. In 1996, a CuO/ZnO based catalyst was introduced for the methanol synthesis for sulfur free syngas containing high proportion of CO and hydrogen. Later, thermally stable CuO/ZnO/Al₂O₃ catalyst was developed and many variants of this catalyst are still being commercially used for the methanol synthesis. Copper based catalyst is extremely active and highly selective. Depending on the catalyst supplier, the reaction could be carried out at 220-250 °C at 50 bar, thereby avoiding premature aging of the catalyst. Most commercial methanol production facilities use CuO and ZnO with one or more stabilizing additives. The process is highly exothermic. Large portion of recycled H₂ is used to dilute the CO concentration at the inlet to about 10-15% to moderate the temperature rise. Methanol production from syngas is a commercially demonstrated technology, using both natural gas and coal as feedstock. The current world-class methanol plants are typically in the order of 2,000 to 2,500 metric tons per day. Large-scale (5,000 t/d) single train methanol process technologies are being offered. The list of major technology providers in this space include: (1) Toyo Engineering Corporation, (2) Lurgi Chemie GmbH, and (3) Foster Wheeler/Starchem.

[0050] Methanol useful for the present invention can also be produced from methane through partial oxidation process. This process involves reacting methane with an oxidant such as oxygen or a peroxide in the presence of a catalyst and a solvent in a reaction zone to produce an effluent stream comprising methanol as one of the products. Palladium or Copper based metal catalysts are typically used for the direct oxidation process. Selectivity and reactivity of the direct oxidation process are low and for those reasons, this process is rarely used in large scales.

[0051] In another aspect of the present invention, methanol useful for the present invention can also be produced from hydrogenation of CO₂ generated either during syngas generation process or from the operation of industrial plants using fossil hydrocarbon as a source of energy. The chemical and petrochemical industry generates 18% of the direct industrial CO₂ emission and steam cracking and ethylene production nearly accounts for 70% of CO₂ emission from petrochemical industry.

[0052] CO₂ released from industrial plants is also useful as a source of carbon for manufacturing DHA useful in the present invention. Thus the method for producing DHA from CO₂ generated at the industrial plants using fossil hydrocarbons offers a novel carbon capture and utilization (CCU) technology. Large industrial plants such as power plants, steel mills, cement factories and biorefineries represent stationary single-point sources for large volume CO₂ suitable for manufacturing DHA useful in the present invention. Electricity generation contributes over 40% of CO₂ emissions from fossil fuels in U.S. Currently, U.S. power plants do not capture large volumes of CO₂ being released and the present invention introduces a novel carbon capture and utilization approach applicable for industrial plants.

[0053] First step in CCU is to capture CO₂ at the source and produce a concentrated clean stream for downstream processing. Currently, three main CCU methods are available. (1). Post-combustion capture involves extracting CO₂ from flue gas produced from coal based power plants. This method is expensive and accounts for 80-90% of the total cost of CCU. (2). Pre-combustion capture separates CO₂ from the fuel by combining the fuel with steam to produce hydrogen and CO₂. This process is only available from limited hydrogen producing facilities. (3). Oxy-fuel combustion capture uses oxygen instead of air for combustion and produces 85-90% pure CO₂. However, use of oxygen instead of air is less preferred due to the higher cost.

[0054] CO₂ from exhaust gas contains small amounts of SO₂, CO, N₂ and NH₃. Purification methods are required to remove impurities to prevent catalyst poisoning. Currently, three main methods are available to remove the impurities in the CO₂ from exhaust gas. (1) Amine treatment methods are used trap CO₂ from flue gas and regenerate the CO₂ by thermal means. (2). Filtration membranes are designed to selectively let CO₂ pass through the membrane

when the pore sizes are appropriate. (3). Condensation methods liquefy the CO₂ from impurities like CO and N₂.

[0055] CO₂ to methanol conversion is achieved using chemical catalysts. Methanol synthesis from CO₂ was first performed by BASF using ZnO/Cr₂O₃ catalyst at 250–350 bar and 350–420 °C. This catalyst was highly stable to sulfur and nitrogen impurities present in flue gas. However, chromium toxicity and high temperature, pressure requirements limit commercial viability of this approach. In 1996, a CuO/ZnO based catalyst was introduced for the methanol synthesis for sulfur free CO₂ containing high proportion of CO and hydrogen. Later, thermally stable CuO/ZnO/Al₂O₃ catalyst was developed and many variants of this catalyst are still being commercially used for the methanol synthesis. Copper based catalyst is extremely active and highly selective. Furthermore, the reaction could be carried out at 220–250 °C at 50 bar, thereby avoiding premature aging of the catalyst due to sintering of copper. Most commercial methanol production facilities use CuO and ZnO with one or more stabilizing additives. Carbon recycling International (CRI) has developed Emission to Liquid (ETL) technology that enables cost-effective conversion of CO₂ to methanol on pilot scale. Energy and hydrogen gas for the process is derived from renewable resources such as geothermal, solar, wind and hydro power.

[0056] Conversion of natural gas, coal, CO₂ and biomass to methanol has been well documented in patent literature. For example, U. S. Pat. No. 5,496,859, U. S. Pat. No. 9,079,770, U. S. Pat. No. 7,074,347, U. S. Pat. No. 7,241,401 and U. S. Pat. No. 7,717,971 disclose improved methanol production method from methane using combined gasification and steam reforming technologies. U. S. Pat. No. 4,407,973 discloses methanol production from either coal or natural gas. International Patent Application Publication No. WO2010/041113 discloses direct oxidation of methane to methanol by cold plasma technology. International Patent Application Publication No. WO2015/066117 discloses CO₂ to methanol process via CO₂ hydrogenation to syngas. International Patent Application Publication No. WO2011/046621 discloses direct selective methane to methanol conversion. U. S. Pat. No. 7,288,684, U. S. Pat. No. 7,456,327, U. S. Pat. No. 7,578,981, U. S. Pat. No. 7,687,669, U. S. Pat. No. 7,910,787, U. S. Pat. No. 8,193,254, U. S. Pat. No. 8,202,916, U. S. Pat. No. 8,293,186, U. S. Pat. No. 4,243,613, U. S. Pat. No. 5,220,080 and U. S. Pat. No. 4,395,495 disclose direct methane to

methanol conversion. U. S. Pat. No. 8,980,961, U. S. Pat. No. 7,906,559, U. S. Pat. No. 8,133,926, U. S. Pat. No. 8,440,729 and U. S. Pat. No. 4,181,675 disclose conversion of CO₂ to methanol by CO₂ reforming with methane or natural gas. International Patent Application Publication No. WO2005/108336, U. S. Pat. No. 4,618,732, U. S. Pat. No. 5,512,599 and U. S. Pat. No. 6,100,303 disclose system and processes for the synthesis of methanol from methane. U. S. Pat. No. 8,288,446 discloses catalytic hydrogenation of CO₂ into syngas mixture used for the production of methanol. U. S. Pat. No. 4,927,857, U. S. Pat. No. 6,736,955, U. S. Pat. No. 6,881,758, U. S. Pat. No. 7,091,251, U. S. Pat. No. 8,188,322 and U. S. Pat. No. 3,940,428 disclose method of methanol production from hydrocarbon. U. S. Pat. No. 5,431,855, U. S. Pat. No. 8,388,864, U. S. Pat. No. 4,999,123, U. S. Pat. No. 5,496,859 and U. S. Pat. No. 5,310,506 disclose conversion of methane to syngas. U. S. Pat. No. 7,932,296 discloses syngas production process suitable for methanol synthesis. U. S. Pat. No. 9,321,655, U. S. Pat. No. 4,888,130, U. S. Pat. No. 4,999,133; U. S. Pat. No. 7,008,967 and U. S. Pat. No. 5,310,506 discloses systems and methods for producing syngas suitable for methanol synthesis. U. S. Pat. No. 8,142,530, U. S. Pat. No. 8,153,027, U. S. Pat. No. 4,592,762, U. S. Pat. No. 4,699,632, U. S. Pat. No. 6,133,328, U. S. Pat. No. 7,638,070, U. S. Pat. No. 6,790,383, U. S. Pat. No. 6,808,543, U. S. Pat. No. 8,173,044, U. S. Pat. No. 8,372,311, U. S. Pat. No. 7,803,845, U. S. Pat. No. 8,946,307, U. S. Pat. No. 9,133,027, U. S. Pat. No. 9,242,907 and U. S. Pat. No. 4,497,637 disclose method of producing syngas from cellulosic biomass. U. S. Pat. No. 6,875,794 and U. S. Pat. No. 6,894,080 disclose method of manufacturing methanol from either syngas or CO₂. U. S. Pat. No. 6,991,769 and U. S. Pat. No. 6,645,442 disclose method of producing methanol from syngas produced from biomass. U. S. Pat. Application Publication No. 2004/0006917, U. S. Pat. No. 4,017,272, U. S. Pat. No. 3,607,157, U. S. Pat. No. 3,840,353, U. S. Pat. No. 4,422,857, U. S. Pat. No. 5,092,984, U. S. Pat. No. 6,117,199, U. S. Pat. No. 6,863,878, U. S. Pat. No. 7,666,383, U. S. Pat. No. 8,328,890 and U. S. Pat. No. 8,647,402 disclose syngas made by gasification of coal. U. S. Pat. No. 6,849,573, U. S. Pat. No. 4,203,915, U. S. Pat. No. 4,218,389, U. S. Pat. No. 4,219,492, U. S. Pat. No. 4,395,495, U. S. Pat. No. 4,464,483, U. S. Pat. No. 4,628,066, U. S. Pat. No. 5,063,250, U. S. Pat. No. 5,173,513, U. S. Pat. No. 5,512,599, U. S. Pat. No. 5,998,489, U. S. Pat. No. 6,191,174, U. S. Pat. No. 6,218,439, U. S. Pat. No. 4,271,086, U. S. Pat. No. 4,289,709, U. S. Pat. No. 4,289,710, U. S. Pat. No. 4,312,955, U. S. Pat. No. 4,366,260, U. S. Pat. No. 4,540,712, U. S. Pat. No. 4,614,749, U. S. Pat. No. 4,623,668, U. S. Pat. No. 4,628,065,

U. S. Pat. No. 5,631,302, U. S. Pat. No. 6,028,119, U. S. Pat. No. 6,300,380 and U. S. Pat. No. 4,994,093 discloses process for the condensation of syngas to methanol under catalyst. All the patent documents listed in this paragraph are incorporated herein by reference. Based on the disclosures in the paragraphs above and in the patent documents listed in this paragraph, a person skilled in the art will be able to produce methanol from fossil hydrocarbons including natural gas, biomass and CO₂.

[0057] In the third stage of the process according to the present invention, methanol is converted to formaldehyde through a single-step chemical process over a chemical catalyst. Formaldehyde has been produced commercially since 1889 by the catalytic oxidation of methanol following two different methods. In one method, methanol is subjected to partial oxidation and dehydrogenation reactions in the presence of silver crystals and steam at 680-720 °C under atmospheric pressure (Beuhler et al., 2001). Methanol conversion in this silver conversion process is 77-87% and formic acid, methyl formate, CO and CO₂ appear as typical byproducts. In the second process referred as Formox process, the oxidation of methanol is carried out with excess amount of air in the presence of Fe-Mo-V oxide catalyst at 250-400 °C under atmospheric pressure. Methanol conversion in the Formox process is 98-99% and the typical byproducts are formic acid, dimethyl ether and CO₂. The global production capacity of formaldehyde is 55 million metric tons a year. Major players in the formaldehyde markets are BASF, Dynea, Perstop, Georgia-Pacific Corp, Celanese and Ercros S.A. Major engineering and deployment firms such as Haldor Topsoe, Linde or Dynea provide engineering as well as operational support for building and operating pilot as well as demo scale methanol to formaldehyde production plants.

[0058] Formaldehyde useful for the present invention can also be produced from methane through partial oxidation process. This process involves reacting methane with an oxidant such as oxygen in the presence of a catalyst such as PbO and a solvent such as water or methanol in a reaction zone maintained at 750 °C to produce an effluent stream comprising formaldehyde as one of the products. Selectivity and reactivity of the direct methane oxidation to formaldehyde process are low and for those reasons, this process is rarely used in large scales.

[0059] Conversion of methanol to formaldehyde has been well documented in patent literature. For example; U. S. Pat. No. 6,147,263 and U. S. Pat. No. 4,348,540 disclose a two-step process for methanol to formaldehyde conversion using silver and copper catalyst. U. S. Pat. No. 2,462,413, U. S. Pat. No. 3,965,195, U. S. Pat. No. 4,072,717, U. S. Pat. No. 4,208,353, U. S. Pat. No. 4,306,089 and U. S. Pat. No. 3,928,461 disclose process for the conversion of methanol to formaldehyde. U. S. Pat. No. 2,519,788, U. S. Pat. No. 4,343,954 and U. S. Pat. No. 3,987,107 disclose a two-step process catalyzed by silver and metal oxide catalyst for the production of formaldehyde. U. S. Pat. No. 3,959,383, U. S. Pat. No. 4,076,754, U. S. Pat. No. 7,109,382 and U. S. Pat. No. 4,450,301 disclose two-stage oxidation process catalyzed by different types of silver catalyst. U. S. Pat. No. 4,167,527 and U. S. Pat. No. 4,474,996 disclose methanol oxidation over silver/gold alloy. U. S. Pat. No. 4,198,351 discloses an improvement in oxidation process. U. S. Pat. No. 4,010,208 discloses methanol oxidation using multi-layer silver catalyst. U. S. Pat. No. 7,468,341 discloses catalytic mixture of Fe/Mo oxides for the oxidation of methanol. U. S. Pat. No. 6,875,724, U. S. Pat. No. 6,552,233 and U. S. Pat. No. 6,518,463 disclose dual catalyst bed containing MoO_3 and V_2O_5 for the methanol oxidation. U. S. Pat. No. 3,678,139 disclose two-stage methanol oxidation using silver and bismuth molybdate catalyst system. U. S. Pat. No. 4,390,730 disclose a lead/silver catalyst for the methanol oxidation. U. S. Pat. No. 3,334,143 discloses methanol oxidation in silver/cadmium alloy. U. S. Pat. No. 3,152,997, U. S. Pat. No. 3,716,497, U. S. Pat. No. 3,846,341, U. S. Pat. No. 3,459,807, U. S. Pat. No. 4,829,042, U. S. Pat. No. 3,978,136, U. S. Pat. No. 7,803,972, U. S. Pat. No. 4,181,629, U. S. Pat. No. 4,000,085 and U. S. Pat. No. 4,024,074 disclose Mo/Fe oxide catalyst system for methanol oxidation. U. S. Pat. No. 3,408,309 discloses Fe/Mo/Co/W oxide system for the methanol oxidation. U. S. Pat. No. 2,439,880 discloses W/Mo catalyst for the oxidation of methanol. U. S. Pat. No. 3,198,753 and U. S. Pat. No. 3,440,180 disclose Mo/Fe/Co catalyst for the oxidation of methanol. U. S. Pat. No. 5,217,936 discloses partial oxidation catalyst for Mo and metal oxide catalyst system. U. S. Pat. No. 3,014,969, U. S. Pat. No. 3,032,588, U. S. Pat. No. 3,232,991, U. S. Pat. No. 1,697,106, U. S. Pat. No. 2,196,188, U. S. Pat. No. 2,384,028, U. S. Pat. No. 2,467,997 and U. S. Pat. No. 1,697,105 disclose direct oxidation of methane to formaldehyde. All the patent documents listed in this paragraph are incorporated herein by reference. Figure 11 provides a process flow diagram for the production of methanol from natural gas. Figure 12 provides a process flow diagram for the production of formaldehyde from

methanol. Based on the disclosures in the paragraphs above, the details for industrial scale production of formaldehyde provided in the Figures 11 and 12, and the teachings in the patent documents listed in this paragraph, a person skilled in the art will be able to produce formaldehyde from methanol, methane and syngas.

[0060] In the next stage of the process according to the present invention, formaldehyde is subjected to self-condensation reaction to yield DHA or glyceraldehyde. In the presence of bases, formaldehyde undergoes self-condensation reaction called formose reaction. However, formose reaction produces a complex mixture of racemic carbohydrates. DHA is a non-chiral three carbon sugar molecule. Chemical production of DHA from formaldehyde is a relatively simple and scalable process due to the lack of stereoisomers. Moreover, the mild reaction conditions required for the formaldehyde self-condensation reaction required for the production of DHA prevents the formation of other complex sugar based byproducts. Thiazolium catalyst has been shown to catalyze the self-condensation of formaldehyde to DHA with up to 98% selectivity (Matsumoto et al., 1984). From the economic stand point, formalin (37% formaldehyde in water) is a better feedstock than anhydrous paraformaldehyde. However, thiazolium based catalysts are deactivated in the presence of water. Several reports have recently appeared where continuous water removal methods are used for the improved production of DHA from 37% formalin solution. Using this process, 96% selectivity was achieved with the single pass conversion of around 30%. Similar DHA production methods are possible in industrial scale but were never practiced due to the lack of large market opportunity for DHA.

[0061] The production of DHA from syngas and formaldehyde can be achieved through four different pathways as illustrated in Figure 2. Self-condensation of formaldehyde in the presence of a NHC (N-heterocyclic carbene) catalyst is the most widely used method to produce DHA. Other methods such as cross-aldol condensation between glycoldehyde and formaldehyde or glycoldehyde and syngas can be used but are not well practiced in the industry.

[0062] Thiazolium catalyzed conversion of formaldehyde to DHA has been reported in patent literature. For example U. S. Pat. No. 5,410,089 discloses two-phase liquid reaction mixture system for the production of DHA from aqueous formalin. U. S. Pat. No. 5,166,450

discloses a continuous process for producing DHA by condensation of formaldehyde in the presence of a thiazolium or imidazolium type catalyst. International Patent Application Publication No. WO2013/157651 discloses a multi-stage water/solvent extraction system for the thiazolium promoted condensation of formaldehyde to DHA. International PCT Application No. WO2015/112096 discloses polymer bound thiazolium catalyst for the condensation of formaldehyde to DHA. U. S. Pat. No. 4,782,186 discloses process condition for the thiazolium catalyzed condensation of formaldehyde to dihydroxyacetone. U. S. Pat. No. 5,097,089 and European Pat. No. EP 0274226 disclose the production of glycerol from formaldehyde through formaldehyde condensation to DHA. U. S. Pat. No. 5,087,761 discloses method of production of DHA using thiazolium catalyst in the absence of any base. European Pat. No. EP 0474387 discloses thiazolium catalyst reactivation by continuous removal of water from the reaction system. U. S. Pat. No. 4,775,448 discloses an isolation process for the production of dihydroxyacetone from the organic solvent mixture. U. S. Pat. Application No. 2014/0235867 discloses various high reactive thiazolium salts for the production of hydroxyacetone from aldehydes. U. S. Pat. No. 4,301,310, U. S. Pat. No. 4,326,086, U. S. Pat. No. 4,358,619, U. S. Pat. No. 4,156,636, U. S. Pat. No. 4,219,508, and U. S. Pat. No. 4,247,653 disclose method of production of low-molecular weight carbohydrates by formose reaction. Deng *et al* has recently proposed a schematic diagram for the large scale production of DHA from formaldehyde wherein the DHA used for the production of fuel (Deng J., Pan T., Xu Q., Chen M., Zhang Y., Guo Q., Fu Y. *Nature*, **2013**, 3, 1244). All the patent documents listed in this paragraph are incorporated herein by reference. Based on the disclosures in the paragraphs above and in the patent documents listed in this paragraph, a person skilled in the art will be able to produce DHA from formaldehyde.

[0063] In an another method for the preparation of DHA, selective C2 oxidation of glycerol obtained as a byproduct of biodiesel industry is used for the production of DHA. Glycerol used for the process is derived from the renewable resources such as but not limited to vegetable oil produced from plant resources. Two methods are commonly used for the selective oxidation of glycerol are: (1) In the microbial process, conversion of glycerol to DHA is carried out with the aid of a microorganism which has dehydrogenase activity using external oxidant such as air. (2) In the chemical oxidation process, homogeneous or heterogeneous metal catalyst is used along

with oxidant such as air, hydrogen peroxide or other metal oxidants. Chemical process generally yields low selectivity and mixture of products. Microbial processes are generally preferred due to the high selectivity and minimal byproducts formation. Conversion of glycerol to DHA is well studied process and has been reported extensively in the scientific and patent literature. For example; U. S. Pat. No. 4,076,589 and U. S. Pat. No. 2,948,658 disclose the production of DHA from glycerol by *Acetobacter suboxydans* at an acidic pH under aerobic conditions. Zheng *et al* disclose immobilized glycerol dehydrogenase for the production of DHA (Zheng M., Zhang S. *Biocatalysis and biotransformation* **2011**, 29(6), 278-287). Joshi *et al* disclosed *Gluconobacter oxydans* for the production of DHA (Joshi P. A., Bhalerao A. *Int. J. Pharm. Biol. Sci.* **2010**, 4(2), 121-126). Nabe *et al* disclosed immobilized *Acetobacter xylinum* for the production of DHA (Nabe K. *Appl. Environ. Microbiol.* **1979**, 38(6), 1056-1060). Chung *et al* reported chemoselective palladium catalyzed oxidation of glycerol (Chung K., Banik S. M., Crisci A. G., Waymouth R. M. *J. Am. Chem. Soc.* **2013**, 135(20), 7593-7602). Liu *et al* reported a gold catalyzed oxidation of glycerol to DHA (Liu S. S., Sun K. Q., Xu B. Q. *ACS catalysis* **2014**, 4(7), 2226-2230). Hu *et al* reported a Pt-Bi/C catalyst for the selective conversion of glycerol to DHA (Hu W., Knight D., Lowry B., Varma A. *Ind. Eng. Chem. Res.* **2010**, 49(21), 10876-10882). All the patent documents listed in this paragraph are incorporated herein by reference. Based on the disclosures in the paragraphs above and in the patent documents listed in this paragraph, a person skilled in the art will be able to produce DHA from glycerol and use it to produce natural and rare carbohydrates according to the present invention.

[0064] The current industrial uses of dihydroxyacetone include the use in sunless tanning products, winemaking process, production of glycerol, production of propylene glycol and production of racemic lactic acid. U. S. Pat. No. 8,455,668 discloses methods and processes for the use of DHA derived from formaldehyde for the production of hydroxymethylfurfural and oxygenated liquid fuels. Prior to the present invention, no effort has been taken to manufacture natural and rare carbohydrates using DHA as a sole starting material and enzyme catalysts.

[0065] In the present invention related to the synthesis of natural and rare carbohydrates, the following three categories of enzymes are used for the production of

variety of natural and rare sugars from DHA as a starting material. (1) Isomerase, epimerase and oxidoreductase enzymes involved in the interconversion of monosaccharides; (2) Aldolase enzymes involved in the C-C bond formation between two monosaccharides; and (3) Transferase enzymes, including transketolase and transaldolase enzymes capable of moving the carbon groups from one monosaccharide to another monosaccharide. These enzymes suitable for the present invention are used either as homogenous catalysts in an aqueous solutions or as immobilized enzymes on a solid support. Immobilization of enzymes on the solid surfaces and their use in industrial scale are well known in the field of industrial biotechnology. A person skilled based on the disclosures in this patent application and the patent documents cited and incorporated herein will be able to immobilize the enzymes useful for practicing the instant invention and use them in industrial scale.

[0066] There are two requirements for the use of an enzyme in the present invention. (1) The enzyme suitable for the present invention should be able to use non-phosphorylated substrates. The isomerase, aldolase and epimerase enzymes are known to work with phosphorylated substrates within the biological systems. For example, the triose isomerase functioning in the energy metabolism within the biological systems requires phosphorylated DHA (dihydroxyacetone phosphate “DHAP”) to produce phosphoglyceraldehyde (P-GA). Similarly, the fructose-1, 6-diphosphate (“FDP”) aldolase functioning in the biological systems requires DHAP and P-GA to produce FDP. Although it is possible to phosphorylate any monosaccharide using appropriate kinase enzyme and ATP as a phosphoryl donor, such an approach would be cost-prohibitive in the present invention related to the production of natural and rare sugars in bulk quantities. As a result the present invention is based on the use of enzymes which use non-phosphorylated substrates in the isomerization, C-C bond formation and epimerization reactions. As explained in detail below, with the current state of our knowledge about the biochemistry and molecular biology of the isomerase, aldolase and epimerase enzymes, a person will be able to identify and use appropriate isomerase, aldolase and epimerase enzymes which would work with non-phosphorylated substrates in the synthesis of natural and rare sugars from DHA as taught in this present invention. (2) The enzymes suitable for the present invention should

not require any specific cofactors for its function. The oxidoreductase enzymes including polyol dehydrogenases and aldose reductase are known to require expensive cofactor NAD(P)H within the biological system. Although it is possible to supply a specific cofactor regeneration systems such as formic acid and formate dehydrogenase along with oxidoreductase enzymes (Demir et al., 2011), such an approach would be cost-prohibitive in the present invention. Under the circumstance where there is a need for cofactor for enzyme action, the present invention would prefer the use of a chemical catalyst in place of an enzyme catalyst.

[0067] The enzymes useful in the present invention are either wild-type enzymes or that have undergone certain genetic modifications that improves the reactivity towards non-phosphorylated DHA and GA as substrates. The enzymes with desirable properties, such as ability to use non-phosphorylated substrates, which are derived from the wild type enzymes through recombinant technology are referred as “mutants”, “mutant derivatives” or “mutants thereof from the corresponding wild type enzymes”. In general, the wild-type enzymes are very specific towards phosphorylated substrates and show lack of selectivity or reactivity towards non-phosphorylated substrates. Industrially, phosphorylated substrates such as DHAP are expensive to prepare. In addition, the resulting phosphorylated products from phosphorylated substrates needs to be dephosphorylated to obtain desired biochemicals which leaves behind undesirable phosphate waste. In the industrial scale production of bulk commodity biochemical such as fructose and tagatose, it is necessary to work with an enzyme that demonstrates reactivity and selectivity towards non-phosphorylated substrates. This objective of using non-phosphorylated substrates for the production of non-phosphorylated bulk commodity biochemical in an industrial scale using an enzyme is achieved by subjecting the selected enzyme to specific genetic modifications. As explained in detail below, through specific genetic modifications, it is possible to redesign the binding sites of an enzyme and modify its catalytic properties making the enzyme more versatile to non-phosphorylated substrate so that the enzyme is tailored to produce one specific biochemical product in high yield and stereoselectivity without the production of undesired phosphate byproducts. With the information provided below for each of the enzyme classes, namely isomerases, aldolases, epimerases and transferases useful in practicing the present

invention, a person skilled in the art of carbohydrate biochemistry will be able to genetically engineer the required enzymes to use the non-phosphorylated substrates.

[0068] Isomerase enzymes (E.C. 5.3.1) catalyze an intermolecular redox reaction causing the exchange of carbonyl functionality between the C1 and C2 positions. For example, the triose phosphate isomerase (a key keto-aldo isomerase involved in the cellular metabolism of monosaccharides) converts DHAP into P-GA. In contrast, the enzyme epimerase (E.C. 5.1.3) catalyzes the reorientation of a hydroxyl group, converting the substrate into one of its epimers. For example, L-ribulose-5P 4-epimerase converts D-fructose to its epimer D-tagatose. Similarly, the enzyme D-Tagatose-3-epimerase converts D-xylulose to D-ribulose. The third group of enzymes involved in the interconversion of the monosaccharides are known as oxidoreductases (E.C. 1.1) and are responsible for converting carbohydrates into their corresponding polyols and vice versa. Oxidoreductases acting on ketoses are known as polyol dehydrogenases while those oxidoreductases acting on aldolase are known as aldose reductases.

[0069] Aldolases are part of a large group of enzymes called lyases and are present in all organisms. Aldolases are mainly involved in the metabolism of carbohydrates. However, aldol reactions are reversible and by choosing the proper conditions, C-C bond formation can be favored leading to the synthesis of hexose sugars from triose sugars. Over 30 aldolases have been identified and isolated so far, the majority of which catalyzes the reversible stereospecific addition of a ketone donor to an aldehyde acceptor. Aldolases can be divided into four main groups namely, DHAP dependent aldolases, pyruvate or phosphoenolpyruvate aldolases, acetaldehyde dependent aldolases and glycine dependent aldolases. DHAP dependent aldolases has been extensively used in the production of carbohydrates. DHAP dependent aldolases catalyze the reversible aldol addition of DHAP to P-GA or L-acetaldehyde and each reaction generates one unique carbohydrate whose stereochemistry at C3 and C4 position is complementary to each other. Fructose 1,6-diphosphate aldolase (FDP aldolase) condenses DHAP and P-GA to form 3S/4R D-fructose 1,6-diphosphate. By using the same substrates, tagatose 1,6-diphosphate (TDP) aldolase produces 3S/4S tagatose 1,6-diphosphate. Fuculose 1-phosphate aldolase and rhamnulose 1-phosphate aldolase catalyze the condensation between

DHAP and L-lactaldehyde to give 3R/4R fucose 1-phosphate and 3R/4S rhamnose 1-phosphate. DHAP aldolases have been cloned and overexpressed, and the bacterial strains harboring a specific aldolase gene are available from ATCC including the ATCC 77472 strain harboring FDP aldolase, ATCC 86984 strain harboring fucose 1-phosphate aldolase, ATCC 87025 strain harboring TDP aldolase and ATCC strain 86983 strain harboring rhamnose 1-phosphate aldolase.

[0070] Transferase enzyme (E.C. 2.2.1) facilitates the exchange of carbon groups between two monosaccharides leading to the synthesis of monosaccharides which are different in their carbon number when compared to the starting monosaccharides. Thus a transferase reaction between DGA, (a triose) and D-fructose (a hexose) involving transketolase results in the formation of D-xylulose (a pentose) and D-erythrose (a tetrose). In one aspect of the present invention, transferase enzyme, transketolase (EC 2.2.1.1), that is capable of converting D-fructose and D-glyceraldehyde to D-xylulose and D-erythrose is used. Thermostable transketolase from *Geobacillus stearothermophilus* has been used for the conversion of D-fructose to D-xylulose (Zaber J. A. et al., 2013). D-xylulose has been produced from D-glyceraldehyde using hydroxypyruvate using a transketolase (Solovjeva O. N. et al., 2008). Enzymatic synthesis of D-sedoheptulose from D-ribose and hydroxypyruvic acid has been performed by using transketolase (Charmantray F. et al., 2009). Single point mutation leading to the enhancement in transketolase selectivity towards the formation of erythrulose from DHA have been demonstrated (Smith M. E. B. et al., 2008). Transketolase has been evolved to enhance the substrate specificity towards aliphatic aldehyde (Hibbert, E. G. et al., 2008). Genetically modified transketolase R526Q/S525T has been developed for the transaldol reaction using non-phosphorylated substrates (Ranoux A. et al., 2012). A person skilled in the art may use one or more of the transketolase or transketolase mutants and the process methods and conditions as mentioned in publications cited above for carrying out the transaldolase reaction of D-glyceraldehyde and D-fructose to produce D-xylulose and D-erythrulose. In another aspect of this embodiment, D-erythrulose produced from D-fructose and D-glyceraldehyde is subjected to chemical hydrogenation reaction to produce erythritol as described in U. S. Pat. Application No. 20130210099.

[0071] Isomerases that are conventionally used for the aldose-ketose isomerization are generally suitable for the isomerization of DHA to DGA. In addition, substrate specific triose phosphate isomerase can be genetically engineered to utilize DHA as a substrate for the production of DGA. The present invention is based on the premises that by using wild-type or genetically engineered enzymes from one or more of classes of isomerases, aldolases and epimerases, it is possible to utilize DHA as a substrate for the enzymatic production of various natural and unnatural carbohydrates. By choosing appropriate isomerase enzymes for the intramolecular isomerization of DHA to either DGA or LGA, treating the mixture of DHA and DGA or DHA and LGA with either wild-type or genetically modified aldolase enzyme and optionally treating the resulting ketohexoses with an appropriate isomerase, it is possible to produce all possible isomers of carbohydrates that includes all natural and unnatural C6-aldoheptoses and C6-ketohexoses.

[0072] It is ideal to design a single enzyme with dual functional capacity namely the ability to carry out both isomerization and aldol condensation reactions for the one-step production of ketohexoses from DHA. While waiting to develop such an ideal enzyme to carry out the present invention, one can use isomerase and aldolase enzymes in a single pot reaction. These two different enzymes can be used in a homogeneous liquid medium or both these enzymes may be immobilized on a single solid surface. The resulting product mixture after a consecutive isomerization and aldol condensation would contain a mixture of original C3 sugars and the newly produced C6 sugar which can be subjected to chromatographic separation techniques to isolate C6 sugars from C3 sugars. The remaining C3 sugars can be recycled to complete the isomerization and aldol condensation reactions.

[0073] The terms isomerase, aldo-keto isomerase and aldose-ketose isomerase are used herein interchangeably to refer enzymes that are useful in the synthesis of various aldoses and ketoses. Aldose-ketose isomerases catalyze the interconversion of isomeric aldo and keto sugars by causing the migration of carbon-bound hydrogen between carbons 1 and 2. Isomerases may be classified in two groups according to their action on phosphorylated and non-phosphorylated sugar substrates. The isomerase using non-phosphorylated sugar substrates is preferred in the present invention. Isomerases are highly regio and stereospecific and are generally very

promiscuous with substrates. Among a large number of naturally-occurring and recombinant isomerase, a thermostable isomerase enzyme capable of immobilization on a solid surface and shows a high specific rate for the conversion of non-phosphorylated DHA substrate into DGA or LGA is the preferred isomerase to carry out the present invention

[0074] The names glucose isomerase and xylose isomerase (EC 5.3.1.5) are used herein interchangeably to refer enzymes that catalyzes the reversible isomerization of 3S/4R ketose to 2R/3S/4R aldose and 3R/4S ketose to 2S/3R/4S aldose. The isomerization reactions catalyzed by xylose isomerase includes but not limited to isomerization of D-glucose to D-fructose, isomerization of D-xylose to D-xylulose, isomerization of D-sorbose to D-idose and isomerization of L-sorbose to L-idose. Xylose isomerase exist in a wide range of microorganisms as a key enzyme participating in the D-xylose metabolism. Xylose isomerase is extensively used in food industry due to its wide spread use in the isomerization of D-glucose to D-fructose to produce high fructose corn syrup. Immobilized xylose isomerase has been in industrial use for decades and currently it is the largest volume commercial immobilized enzyme product worldwide.

[0075] Mannose isomerase (EC 5.3.1.7) as used herein refers to enzymes that catalyzes the reversible isomerization of 3S/4R ketose to 2S/3S/4R aldose. The isomerization reactions that are catalyzed by mannose isomerase includes but not limited to isomerization of D-mannose to D-fructose, isomerization of D-xylulose to D-lyxose and isomerization of L-sorbose to L-gulose. Mannose isomerase exist in a wide range of microorganisms including *Agrobacterium radiobacter*, *E.coli*, *Mycobacterium sp.*, *Pseudomonas sp.*, *Salmonella enterica* and *Streptomyces aerocoligines*. Immobilized mannose isomerase from *Pseudomonas cepacia* is used for the production of D-fructose from D- mannose. Continuous production of D-mannose from D-fructose has been performed with immobilized mannose isomerase containing *A. radiobacter* cells (Hirose J. et al., 2003).

[0076] D-lyxose isomerase (EC 5.3.1.15) as used herein refers to enzymes that catalyzes the reversible isomerization of 3S/4R ketose to 2S/3S/4R aldose and 3S/4S ketose to 2S/3S/4S aldose. The isomerization reactions catalyzed by D-lyxose isomerase includes but not limited to

isomerization of D-mannose to D-fructose, isomerization of D-xylulose to D-lyxose, isomerization of L-psicose to L-allose, isomerization of L-sorbose to L-gulose, isomerization of L-ribulose to L-ribose and isomerization of tagatose to L-talose. D-lyxose isomerase exist in a wide range of microorganisms including *Agrobacter aerogenes*, *Cohnella laevoribosii*, *Serratia Proteamaculans*, *Providencia stuartii*, *Bacillus licheniformis* and *Dictyoglomus turgidum*. (Park C. S. et al., 2010).

[0077] L-rhamnose isomerase (EC 5.3.1.14) as used herein refers to enzymes that catalyzes the reversible isomerization of 3R/4S ketose to 2R/3R/4S aldose. Rhamnose isomerase is also found to catalyze the reversible isomerization of 3R/4R ketose to 2R/3R/4R aldose. The isomerization reactions catalyzed by L-rhamnose isomerase includes but not limited to isomerization of D-sorbose to D-gulose, isomerization of D-psicose to D-allose, isomerization of L-fructose to L-mannose and isomerization of L-tagatose to L-talose. L-rhamnose isomerase exist in a wide range of microorganisms as a key enzyme participating in the metabolism of some monosaccharides such as D-mannose and D-fructose (Moralejo P. et al., 1993). This isomerase has broad substrate specificity, and the L-rhamnose isomerases acting on D-allose have been identified from *Pseudomonas sp.*, *Pseudomonas stutzeri*, *Bacillus pallidus*, *Thermotoga maritima*, *Thermoanaerobacterium saccharolyticum*, *Caldicellulosiruptor saccharolyticus* and *Bacillus subtilis*. Immobilized L-rhamnose isomerase from *P.stutzeri* has been used for efficient D-allose production from D-psicose (Menavuvu B. T. et al., 2006).

[0078] L-arabinose isomerase (EC 5.3.1.4) as used herein refers to enzymes that catalyzes the reversible isomerization of 3S/4S ketose to 2R/3S/4S aldose. The isomerization reactions catalyzed by L-arabinose isomerase includes but not limited to isomerization of D-tagatose to D-galactose, isomerization of L-psicose to L-altrose and isomerization of L-ribulose to L-arabinose. L-arabinose isomerase exist in a wide range of microorganisms participating in the L-arabinose metabolic pathway (Lee Y. J. et al., 2014). Recently L-arabinose isomerase has attracted increasing attention as this enzyme is found to catalyze the isomerization of D-galactose to D-tagatose (Xu Z. et al., 2014). L-arabinose isomerase is expected to become the second largest volume industrial enzyme next to xylose isomerase. Thermostable L-arabinose isomerases have been characterized from thermophilic or hyper thermophilic microorganisms such as *Thermotoga*

neapolitana, *Yhermotoga maritima*, *Anoxybacillus flavithermus*, *Alicyclobacillus hesperidum*, *Acidothermus cellulolyticus* and *Bacillus stearothermophilus*. Continuous production of D-tagatose using alginate immobilized *G. stearothermophilus* L-arabinose isomerase has shown the productivity of 54g/L/h (Ryu S. A. et al., 2003).

[0079] D-arabinose isomerase (EC 5.3.1.3) as used herein refers to enzymes that catalyzes the reversible isomerization of 3R/4R ketose to 2S/3R/4R aldose. The isomerization reactions catalyzed by D-arabinose isomerase includes but not limited to isomerization of D-psicose to D-altrose, isomerization of L-tagatose to L-galactose and isomerization of D-ribulose to D-arabinose. D-arabinose isomerase exists in a wide range of microorganisms participating in the D-arabinose metabolic pathway. In general, D-arabinose isomerase shows specificity towards D-arabinose; however, because of the structural similarities, this enzyme also shows broad substrate specificities towards other monosaccharides. D-arabinose isomerase from *Klebsiella pneumonia* has been used to produce D-altrose from D-psicose with the conversion yield of 13% (Menavuvu B. T. et al., 2006).

[0080] L-fucose isomerase (EC 5.3.1.25) as used herein refers to enzymes that catalyzes the reversible isomerization of 3R/4R ketose to 2S/3R/4R aldose. The isomerization reaction catalyzed by L-fucose isomerase includes but not limited to isomerization of D-psicose to D-altrose, isomerization of L-tagatose to L-galactose and isomerization of D-ribulose to D-arabinose. L-fucose isomerase exists in a wide range of microorganisms participating in the D-arabinose metabolic pathway. In general, L-fucose isomerase shows specificity towards D-arabinose; however, because of the structural similarities, this enzyme also shows substrate specificities towards various other monosaccharides. L-fucose isomerases from *C. saccharolyticus* have been used to produce D-altrose from D-psicose with the conversion yield of 24% (Ju Y.H. et al., 2010).

[0081] Ribose isomerase (EC 5.3.1.20) as used herein refers to enzymes that catalyzes the reversible isomerization of 3R/4R ketose to 2R/3R/4R aldose. The isomerization reaction catalyzed by ribose isomerase includes but not limited to isomerization of D-ribose to D-ribulose, isomerization of L-tagatose to L-talose and isomerization of D-psicose to D-allose.

Ribose isomerase exists in a wide range of microorganisms participating in the D-ribose metabolic pathway. In general, ribose isomerase shows specificity towards D-ribose; however, because of the structural similarities, this enzyme also shows broad substrate specificities towards various other monosaccharides. Ribose isomerase from *Mycobacterium smegmatis* has been used to produce D-ribulose from D-ribose with the conversion yield of 18% (Izumori K. et al., 1975.).

[0082] Ribose-5-phosphate isomerase (EC 5.3.1.6) as used herein refers to enzymes that catalyzes the reversible isomerization of 3R/4R phosphorylated ketose to 2R/3R/4R phosphorylated aldose. The isomerization reactions catalyzed by Ribose-5-phosphate isomerase includes but not limited to isomerization of D-ribose to D-ribulose, isomerization of L-tagatose to L-talose and isomerization of D-psicose to D-allose. Ribose-5-phosphate isomerase is an important enzyme participating in pentose phosphate pathway and widely exists in various organism (Zhang R. et al., 2003). Ribose-5-phosphate isomerase generally shows specificity towards D-ribose phosphate; however, because of the structural similarities, this enzyme also shows broad substrate specificities towards various other phosphorylated monosaccharides. Ribose-5-phosphate isomerase from *Clostridium thermocellum* and *Clostridium difficile* have been used to produce D-allose from D-psicose with the conversion yield of 32% (Yeom S. J. et al., 2009).

[0083] D-galactose-6-phosphate isomerase (EC 5.3.1.26) as used herein refers to enzymes that catalyzes the reversible isomerization of 3S/4S phosphorylated ketose to 2R/3S/4S phosphorylated aldose. The isomerization reactions catalyzed by D-galactose-6-phosphate includes but not limited to isomerization of D-tagatose to D-galactose, isomerization of L-psicose to L-altrose and isomerization of L-ribulose to L-arabinose. D-galactose-6-phosphate isomerase from *Lactococcus lactis* reacts with a large number of aldoses and ketose (Park H. Y. et al., 2007). D-galactose-6-phosphate generally shows very close specificity towards D-galactose phosphate; however, because of the structural similarities, this enzyme also show broad substrate specificities towards various other phosphorylated monosaccharides. D-galactose-6-phosphate isomerase from *Lactococcus Lactis* has been used to produce D-altrose from D-psicose with the conversion yield of 35%.

[0084] DHA and DGA are structural isomers wherein it has the same chemical formula but with different chemical structure. Conversion of DHA to DGA is achieved by the intramolecular hydride transfer from the C1 to C2. The hydride transfer generates chirality at C2 center and thus the conversion of DHA to DGA is an enantioselective reaction. Although chemical catalyst could be developed for the conversion of DHA to DGA (Cho H. J. et al., 2014), enzymatic transformations are preferred due to their high substrate specificity, stereoselectivity and lack of side products. Moreover, isomerases class enzymes require no co-factors, no co-substrates, no metal ions, no external oxidant or reductants and some requiring no phosphorylation of the substrates. Triose phosphate isomerase (TIM) catalyzes the stereospecific and reversible conversion of DHAP to P-GA, by a proton transfer mechanism. Glu-165 abstracts a proton to begin the isomerization reaction leading to the formation of enediol intermediate stabilized by Lys12. His 95 plays acid and base roles to permit resolution of the enediol (O'Donoghue A. C. et al., 2005). TIM shows remarkable reactivity and selectivity towards phosphorylated DGA (10^8 times higher) over the non-phosphorylated DGA. However, recent efforts in TIM engineering has revealed that a simple phosphite dianion metal salts in the medium can eliminate the need for the phosphorylated substrates (Amyes T. L. et al., 2007). It has also been discovered that the turnover of $K_{cat}/K_m = 185 \text{ M}^{-1} \text{ s}^{-1}$ is possible for the isomerization of glycoldehyde by TIM that is saturated with phosphite dianion (Go M. K. et al., 2009). Other metal salts such as borates, arsenates and sulfates can also be used in the place of phosphates. Using the current state of knowledge about TIM, a person skilled in the art could make TIM to isomerize non-phosphorylated DHA to non-phosphorylated DGA or LGA either by following genetic engineering techniques well known in the art or by manipulating the composition of the medium for enzyme reaction. X-ray crystallographic analysis of TIM (Jogl G. et al., 2003) complexed with DHAP has revealed interaction between DHAP phosphodianion group with Gly-232 and Gly-233, hydrogen bonding interaction with Lys-12, NH groups of Ser-211 and flexible interaction with Gly-171 (Knowless J. R. 1991). Recent developments in computational modeling of enzyme structure and function along with precise protein engineering methods now provide access to study the active site of the TIM and redesign the phosphorylate binding site for the improved affinity towards non-phosphorylated DHA. Redesigning the active site of TIM by replacing Lys-12 with Asp, Asn, Glu or Gln can provide more hydrogen bonding interactions

with free hydroxyl group of DHA and can lead to greater substrate binding and better stabilization of DHA without requiring the presence of phosphate group. Moreover, by replacing the Gly-232, Gly-233, Ser-211 and Gly-171 that show large interaction with phosphate dianion group (Kursula I., Wierenga R. K. *J. Biol. Chem.* **2003**, 278, 9544-9551) with His, Glu and Asn can lead to hydrogen bonding interactions that are similar to the interaction demonstrated at the hydroxyl end of the DHAP. A person skilled in the art could perform genetic modifications of a wild-type TIM as described here for the utilization of DHA as a substrate in the production of DGA or LGA.

[0085] The equilibrium of the wild-type TIM catalyzed reaction favors the isomerization of P-GA to DHAP. Isomerization reaction catalyzed by TIM favors P-GA as a substrate 20 times over the DHAP (O'Donoghue A. C. et al., 2005). Although this phenomenon may disfavor the DHA utilization and hence the DGA formation, tying the isomerization product to aldol reaction catalyzed by aldolase enzymes can facilitate continuous removal of DGA towards the formation of C6-sugars. In such a cases, it is possible to facilitate the simultaneous consumption of DGA by condensation with DHA to form D-fructose to favor the DHA to DGA forward process and improve the DHA utilization. Moreover, protein engineering methods can be effectively used to overcome this specificity hurdle faced by TIM. By redesigning the active site by substituting Glu-165 responsible for the formation of enediol intermediate with Asp and by substituting Lys-12 responsible for the stabilization of enediol intermediate with Ser or Thr, it is possible to favor the isomerization towards the production of DGA. In addition, methyl glyoxal is produced as one of the cytotoxic side-product of the TIM catalyzed isomerization by the non-enzymatic elimination of phosphate group from DHAP. In the absence of the phosphoryl substrate such as DHAP, formation of methyl glyoxal will be substantially reduced and a reduced formation of methyl glyoxal would improve the selectivity for the isomerization of DHA to GA by TIM.

[0086] Genetically engineered xylose isomerase type enzymes are also useful for the production of DGA from DHA. Xylose isomerase catalyzes the isomerization of D-xylose to D-xylulose or the isomerization of D-glucose to D-fructose. Xylose isomerase catalyzes the isomerization through metal-dependent hydride transfer mechanism as opposed to the base catalyzed proton-transfer mechanism of TIM (Asboth B. et al., 2000). Xylose isomerase does not require

phosphorylated substrates for substrate binding and are able to achieve binding and stabilization through hydrogen bonding with hydroxyl group present in the sugar substrates and thus xylose isomerase is able to catalyze the isomerization of non-phosphorylated DGA to DHA (Toteva M. M. et al., 2011). Moreover, due to the intrinsic mechanistic differences between xylose isomerase and TIM, xylose isomerase catalyzed isomerization reactions generally produce an equal mixture of aldose and ketose while TIM favors the aldose as substrate and produce ketose as predominant product in the mixture. Moreover, x-ray crystal structure of the DGA bound xylose isomerase is strikingly similar to that of the cyclic form of glucose bound xylose isomerase (Fenn T. D. et al., 2004) and it is closer to match to that of the linear xylulose bound xylose isomerase (Kovalevsky A. Y. et al., 2008). Glucose and other aldoses exhibit a six membered cyclic coordination between C2 and C4 hydroxyl group of the sugars with Mg metal atom, which leads to the nucleophilic activation of the C2 hydride towards C2 to C1 hydride transfer. Similarly, xylulose and other ketoses exhibit a six-member coordination between C2 keto group and C4 hydroxyl group with Mg₁ and a stronger five-member coordination between C1 hydroxyl and C2 keto group with Mg₂ metal atoms leading to the nucleophilic activation of the C1 hydride towards C1 to C2 transfer. Due to the lack of C4 hydroxyl group in DHA, it is possible that the DHA exhibit a five-member coordination between C2 keto and C3 hydroxyl group with Mg₁ and an another five-membered coordination between C1 hydroxyl and C2 keto group with Mg₂. Since it has been previously demonstrated that the free hydroxyl bound Mg atom coordinated between His-219, Asp-254 and Asp-256 responsible for the nucleophilic activation of hydride transfer between C1 and C2 carbon (Allen K. N. et al., 1995), one of the two Mg atom has the ability to perform the hydride shift whereas the another one provides substrate stabilization through coordinative binding. Although xylose isomerase catalyzed isomerization reaction is much less efficient with C3 sugars than C6 sugars (k_{cat}/K_m differs by 10^4 fold), protein engineering of XI to better position the C3 sugars for hydride transfer and increase reactivity is feasible. For instance, mutation of the His54Tyr and Asp57Ala could allow the side chain to occupy the space where C4-C6 of the sugar bind in the native enzymes (Woodyear R. et al., 2009). Furthermore, mutation of Glu217Asp can lead to the better positioning of the metal atom for DHA binding. A person skilled in the art could perform genetic modifications of a wild-type xylose isomerase as described here for the utilization of DHA as a substrate for the production of DGA.

[0087] Xylose isomerase and TIM produce GA wherein the absolute configuration of the hydroxyl functionality at the C2 position is 2R. Other enzymes from the class isomerases (EC 5.3.1) that are known to produce aldoses with 2R hydroxyl group at C2 positions includes but not limited to L-arabinose isomerase, L-rhamnose isomerase, ribose isomerase, ribose-5P-isomerase, galactose-6P-isomerase. A person skilled in the art could perform genetic modifications in any one of these enzymes mentioned above for the utilization of DHA as a substrate for the production of DGA. For example: (a) L-arabinose isomerase known for the isomerization of L-ribulose to L-arabinose has been modified to produce D-tagatose from D-galactose (Manjasetty B. A. et al., 2006). Crystal structure of L-fucitol bound L-arabinose isomerase has shown a single point five-membered coordination between Mn^{2+} center and C1, C2 hydroxyl groups of L-fucitol; (b) Substrate bound crystal structure of L-rhamnose isomerase also demonstrate similar single point binding between Mn^{2+} center and C1, C2 hydroxyl group of L-rhamnitol in the presence of $MnCl_2$ in the system (Korndorfer I. P. et al., 2000). L-rhamnose isomerase binds to C2 and C3 hydroxyl group similar to xylose isomerase. Both Zn^{2+} and Mn^{2+} in L-rhamnose isomerase are capable of promoting nucleophilic hydride shift similar to xylose isomerase. Moreover, mutation of Ser329Phe in L-rhamnose isomerase (Yoshida H. et al., 2010) could possibly reduce the hydrogen bonding interaction with C4 hydroxyl group that is not required when DHA is used as a substrate; (c) crystal structure of ribose isomerase or ribose-5P-isomerase has also shown a single point substrate binding with the metal catalyst via C1 and C2 hydroxyl group of the sugars. In addition, ribose isomerase also shows strong hydrogen bonding stabilization between C3 hydroxyl group with Asp8A and Gly66A and another hydrogen bonding between C4 hydroxyl group with His98B (Jung J. et al., 2011). Similar type of hydrogen bonding stabilization is also possible when DHA is used as a substrate; (d) crystal structure of galactose-6P-isomerase has also shown unique hydrogen bonding stabilization between C2 ketone with His96 and Thr67, C1 hydroxyl group with Thr67, Gly70 and Asn97, C3 hydroxyl group with Gly66 and Asp8 (Jung W. S. et al., 2013). Galactose-6P-isomerase has also showed a single point substrate binding with metal catalyst via C1 and C2 hydroxyl group of the sugar substrate. A person skilled in the art having knowledge about the crystal structure of these isomerase enzymes and binding properties of the sugar substrates to these enzymes would be in a position to genetically engineer an enzyme that accepts dihydroxyacetone as a substrate by

modulating stability and binding required for the highly stereo selective conversion of dihydroxyacetone to GA.

[0088] DHA can be converted to LGA by isomerases class (EC 5.3.1) enzymes. Conversion of DHA to LGA is achieved by the intramolecular hydride transfer from the C1 to C2. The hydride transfer from C1 carbon generates hydroxyl group at C2 center wherein the absolute configuration of the hydroxyl functionality at the C2 position is 2S. There are no known chemical or biological catalyst for the conversion of DHA to LGA. However, enzymes from the isomerases class (EC 5.3.1) are known to produce aldoses with 2S hydroxyl group at C2 positions from keto sugars via hydride transfer from C1 to C2. Those enzymes that produce 2S aldo sugars from keto sugar via isomerization includes but not limited to D-arabinose isomerase, D-lyxose isomerase, L-fucose isomerase, D-mannose isomerase and L-ribose isomerase. A person skilled in the art could perform genetic modifications in any one of the enzymes mentioned above for the utilization of DHA as a substrate for the production of LGA. For example: (a) D-arabinose isomerase known to produce D-arabinose from D-ribulose has been modified to produce D-altrose from D-psicose (Menavuvu B. T. et al., 2006). Crystal structure of active site of the D-arabinose isomerase has shown a single point coordination between Zn^{2+} center and with sugars including hydrogen bonding interaction with Ala59, His88, Glu111 and Glu152 (Goulay L. J. Et al., 2010); (b) D-lyxose isomerase known to produce D-lyxose from D-xylulose has been modified to produce D-mannose from D-fructose (Patel D. H. et al., 2011). Crystal structure of lyxose isomerases are unknown but the relative activities against several metal salts has shown highest activity against $MnCl_2$ (Anderson R. L. et al., 1965). suggesting that the lyxose isomerase is an Mn^{2+} dependent enzyme like other isomerases belonging to the class including L-arabinose isomerase and L-rhamnose isomerase; (c) crystal structure of L-fucitol bound L-fucose isomerase has also demonstrated a single point substrate binding with the Mn^{2+} catalyst via C1 and C2 hydroxyl group of the sugars, wherein hydrogen bonding from Asp 361, Glu337 and Asn 527 to C1 and C2 oxygens further stabilize the substrate binding. It has also been found that the Glu 337 acts as a base to facilitate the hydride transfer from C1 center to C2 center, whereas Asp 361 protonates either one of the C1 or C2 oxygen depending on the direction of the hydride transfer (Seemann J. E. et al., 1997). An identical mechanism would be suitable when DHA is used as substrate and such a mechanism would also selectively produce

LGA from DHA; (d) L-ribose isomerase, an isolated mutant from *Acinetobacter* sp. *DL-28* (Shimonishi T. et al., 1996) has also been shown to catalyze the isomerization of L-ribulose to L-ribose. Crystal structure of ribitol bound L-ribose isomerase has also demonstrated a single point substrate binding with the Mn^{2+} catalyst via C1 and C2 hydroxyl group of the sugars, wherein hydrogen bonding from Lys111, Glu204 and Glu113 to C1 and C2 oxygens further stabilize the substrate binding. It was also found that Glu113 and Glu204 could either act as base or acid or participate in the concerted hydride transfer between ketose and aldose depending on the direction of hydride migration (Yoshida H. et al., 2014). An identical mechanism would be suitable when DHA is used as a substrate and such a mechanism would also selectively produce LGA from DHA; (e) mannose isomerase known to convert D-fructose to D-mannose is also used for the conversion of xylulose to D-lyxose (Hirose J. et al., 2001). Crystal structure of the mannose phosphate isomerase has demonstrated substrate binding with the Zn^{2+} catalyst via C1 and C2 hydroxyl group of the sugars. In addition, His248 and His383 also participate in a concerted manner to shuffle the hydride between C1 and C2 carbon (Cleasby A. Et al., 1996). An identical mechanism would be suitable when DHA is used as substrate and such a mechanism would also selectively produce LGA from DHA. A person skilled in the art will study the crystal structure of these isomerase enzymes and binding properties of the sugar substrates to this enzyme and can easily genetically engineer an enzyme that accepts dihydroxyacetone as a substrate by modulating stability and binding required for the highly stereo selective conversion of dihydroxyacetone to L-glyceraldehyde.

[0089] Epimerases or ketohexose-3-epimerases as used herein interchangeably refers to enzymes that can be applied in the synthesis of various ketoses. Ketohexose-3-epimerase catalyzes the interconversion of stereochemistry of the keto sugars by causing the migration of carbon-bound hydrogen within the same carbon but to an opposite configuration. Epimerases may be classified in two groups according to their action on phosphorylated or non-phosphorylated sugar substrates. Epimerases are highly regio and stereospecific and are generally very promiscuous with substrates. Epimerases yields a 1:1 mixture of ketoses and thus extensive purification technologies are required for the isolation of products from the mixture. Several epimerases that are naturally active on phosphorylated sugars have also been shown to use non-phosphorylated carbohydrates. Epimerases are effective in epimerizing the C3 hydroxyl group of both D and L

sugars. Broad substrate specificities demonstrated by ketohexose-3-epimerases has made them an attractive class of enzymes for the production of several rare sugars.

[0090] D-tagatose 3-epimerase (EC 5.1.3.31) as used herein refers to enzymes that catalyzes the epimerization of various ketoses at the C3 position, making it a very useful enzyme for rare sugar production. D-tagatose 3-epimerase (DTE) has already been used for the synthesis of various carbohydrates, both in single and multiple enzyme reactions. At the laboratory scale, DTE has been used to produce all possible ketohexoses (Itoh H. et al., 1996. Furthermore, DTE is a highly promiscuous enzyme that can accept a large range of unnatural substrates, such as methylated pentoses, methylated hexoses and various deoxyketohexoses. In addition, immobilized DTE has been applied in the mass production of D-psicose from D-fructose (Takeshita K. et al., 2000). Moreover, DTE has been combined with dehydrogenases or isomerases for the multistep enzymatic production of allitol and D-arabinose starting from fructose and xylose respectively (Takeshita K. et al 2000).

[0091] D-psicose 3-epimerase (EC 5.1.3.30) as used herein refers to enzymes that catalyzes the epimerization of various ketoses at the C3 position, making it a very useful enzyme for rare sugar production. D-psicose 3-epimerase has already been used for the production of D-psicose from D-fructose. Unfortunately, due to its short half-time (63 min at 50°C), this enzyme is inefficient for the industrial production of D-psicose from D-fructose. However, 30-fold increase in half-life time at 50°C is observed when double mutants were introduced. In a continuous production process with an immobilized double mutant, no decrease in activity could be observed even after 30 days (Choi J. G. et al., 2011).

[0092] L-ribulose-5P-4-epimerase (EC 5.1.3.4) as used herein refers to enzymes that catalyzes the epimerization of various ketoses at the C4 position, making it a very useful enzyme for rare sugar production. L-ribulose-5P-4-epimerase has already been used for the production of L-ribulose-5P from D-xylulose-5P. L-ribulose-5P-4-epimerase requires phosphorylated substrates and are inactive against non-phosphorylated sugars. L-ribulose-5P-4-epimerase has shown to work by means of aldolase like carbon-carbon bond cleavage making it as another member of aldolase class (Johnson A. E and Tanner M. E. 1998).

[0093] Aldolases are the another class of enzymes that demonstrates excellent reactivity towards DHA or DHAP. Aldolases are the key enzymes in the asymmetric aldol reaction of DHA with various aldehydes. Aldolases are responsible for the production of various natural and unnatural carbohydrates and their analogs. Wild-type aldolases such as D-fructose 1,6-bisphosphate aldolase (FruA) and D-tagatose 1,6-bisphosphate aldolase (TagA) use DHAP as ketone donors and P-GA as aldehyde acceptor and leads to the formation of C6-ketosugars with (3*S*, 4*R*) and (3*S*, 4*S*) configuration. Moreover, aldolases show excellent substrate promiscuity towards various phosphorylated or non-phosphorylated aldehyde acceptors leading to the formation of high diversity of carbohydrate related products from DHA.

[0094] Aldolases as used herein refers to enzymes that can be applied in the synthesis of various C6 ketoses from their respective C3 sugar fragments. Aldolases are part of a large group of enzymes called lyases and are present in all organisms. They are involved in the metabolism of carbohydrates, amino acids and hydroxyl acids. Although aldolases function in vivo is often related to the degradative cleavage of carbohydrates, the aldol reactions are reversible and by choosing the proper conditions, C-C bond formation can become the favored reaction pathway. Over 30 aldolases have been identified and isolated so far, the majority of which catalyzes the reversible stereospecific addition of ketone donor to an aldehyde acceptor. Aldolases are classified in four groups based on the structure of the donor substrate and the resulting formation of ketose 1-phosphate after reaction with an aldehyde. Aldolases are highly regio and stereospecific and demonstrate limited promiscuity with substrates. Aldolases yields a 1:1 mixture of desired sugars and their respective ketone and aldehyde fragments and a separation and purification technologies would be required for the isolation of desired products from the mixture. Some aldolases that are naturally active on phosphorylated sugars have been mutated for the use of non-phosphorylated donors and acceptors. Aldolases are chosen based on the availability and the specificity towards the desired sugar products. If the enzyme is not specific enough, mixtures will be obtained containing three sugars, namely substrate, the desired sugar product and the undesired sugar product.

[0095] Fructose 1,6-bisphosphate (FDP) aldolase (EC 4.1.2.13) as used herein refers to enzymes that catalyzes the reversible aldol condensation of C3-ketone donor dihydroxyacetone phosphate

with various chiral C-3 acceptors. FDP aldolases leads to the formation of C6-ketosugar having 3S/4R configuration. Substrates that are useful for the aldol reaction catalyzed FDP aldolases includes but not limited to D-glyceraldehyde-3-phosphate, L-lactaldehyde, L-glyceraldehyde-3-phosphate, glycoldehyde. Products produced by FDP aldolase includes but not limited to D-fructose, L-sorbose and D-threose FDP aldolases that are naturally active on phosphorylated C3-sugars have also been shown to convert non-phosphorylated C3-sugars.

[0096] Tagatose 1,6-bisphosphate aldolase (EC 4.1.2.40) as used herein refers to enzymes that catalyzes the reversible aldol condensation of C3-ketone donor dihydroxyacetone phosphate with various chiral C-3 acceptors. TDP aldolases leads to the formation of C6-ketosugar having 3S/4S configuration. Substrates that are useful for the aldol reaction catalyzed TDP aldolases includes but not limited to D-glyceraldehyde-3-phosphate, L-lactaldehyde, L-glyceraldehyde-3-phosphate, glycoldehyde. Products produced by TDP aldolase includes but not limited to D-tagatose, L-psicose and D-erythrose. TDP aldolases that are naturally active on phosphorylated C3-sugars can be genetically engineered to act on non-phosphorylated C3-sugars.

[0097] L-Fuculose 1-phosphate aldolase (EC 4.1.2.17) as used herein refers to enzymes that catalyzes the reversible aldol condensation of C3-ketone donor dihydroxyacetone phosphate with various chiral C-3 acceptors. FP aldolases leads to the formation of C6-ketosugar having 3R/4R configuration. Substrates that are useful for the aldol reaction catalyzed FP aldolases includes but not limited to D-glyceraldehyde-3-phosphate, L-lactaldehyde, L-glyceraldehyde-3-phosphate, glycoldehyde. Products produced by FP aldolase includes but not limited to D-psicose and L-tagatose. FP aldolases that are naturally active on phosphorylated C3-sugars can be genetically engineered to act on non-phosphorylated C3-sugars.

[0098] L-Rhamnulose 1-phosphate aldolase (EC 4.1.2.19) as used herein refers to enzymes that catalyzes the reversible aldol condensation of C3-ketone donor dihydroxyacetone phosphate with various chiral C-3 acceptors. RP aldolases leads to the formation of C6-ketosugar having 3R/4S configuration. Substrates that are useful for the aldol reaction catalyzed RP aldolases includes but not limited to D-glyceraldehyde-3-phosphate, L-lactaldehyde, L-glyceraldehyde-3-phosphate, glycoldehyde. Products produced by RP aldolase includes but not limited to D-

sorbose and L-fructose. RP aldolases that are naturally active on phosphorylated C3-sugars can be genetically engineered to act on non-phosphorylated C3-sugars.

[0099] Fructose aldolase is used for the production of D-fructose by the aldol condensation of DHA and DGA. Fructose aldolase as defined in this invention is an enzyme that combines a ketose and an aldose and produces two new (3S, 4R) hydroxyl stereo centers. Although chemical catalyst could be developed for the production of fructose derivatives from DHA (Trost B. M., Brindle and C. S. **2010**) enzymatic transformations are preferred due to their high substrate specificity, stereoselectivity and lack of side products. In one aspect of the production of D-fructose, fructose 1,6-bisphosphate (FDP) aldolase is used as an enzyme. DHAP dependent FDP aldolase combines DHAP and P-GA to produce fructose 1,6-bisphosphate (Henderson I. et al., 1994). FDP accepts wide range of aldehydes as the acceptor, but are quite specific to the donor substrate DHAP (Bednarski M. D. et al., 1989). To accommodate the DHAP requirement, DHAP is prepared by the chemical phosphorylation of DHA. In another method, phosphorylation of DHA is performed by biological means using acetyl phosphate and phosphoenol pyruvate as a co-substrate. A person skilled in the art may use one or more the phosphorylation methods for the selective phosphorylation of DHA in "DHA + DGA" mixture to produce "DHAP + DGA" and utilize FDP aldolase to combine DHAP and DGA for the production of D-fructose 1-phosphate (for example: Moreno I. S. et al., 2004 and Iturrate L. et al., 2009). Phosphatase enzyme are used to hydrolyze the phosphate from fructose phosphate to produce D-fructose (Wen L et al., 2015). In another method, metal salts such as borates, phosphites, arsenates are used as a phosphate ester mimic for the production of D-fructose from FDP aldolase without the phosphorylation steps (Sugiyama M. et al., 2006). In another aspect of the production of D-fructose, fructose 6-phosphate aldolase (FSA) is used as an enzyme. FSA combines DHA and PGA for the production of fructose 6-phosphate (Schurmann M. and Sprenger G. A. **2001**). FSA has a striking advantage of using non-phosphorylated DHA and its analogues as donors instead of DHAP needed for FDP aldolase. In addition, FSA is also found to be moderately effective in taking various aldehyde acceptors such as amino aldehydes (Concia A. L. et al., 2007) and azido aldehyde (Sugiyama M. et al., 2007). FSA has been mutated at A129S and been shown to produce D-fructose up to 81% conversion and 67% yield (Castillo J. A. et al. 2010). A two-point double mutation of FSA at A129S/A165G has been shown to

further improve the reactivity and selectivity towards various aldehydes (Gutierrez M. et al., 2011). It is possible to use A129S/A165G FSA for the improved production of D-fructose from DHA and DGA. A double mutation of FSA at L107A/L163A has been shown to further improve the substrate promiscuity towards various aldehydes (Guclu D. et al., 2016). It is possible to use L107A/L163A FSA for the improved production of D-fructose from DHA and DGA. Structure guided redesign of FSA resulted mutations A129S/R134X/A165G/S166G and L107Y/A129G/R134X/A165G/S166G, where X=R, V, P or S has been shown as suitable catalyst for DHA addition to various aldehydes (Soler A., Gutierrez M. L., Bujons J., Parella T., Minguillon C., Joglar J., Clapes P. *Adv. Synth. Catal.*, **2015**, 5, 1787-1807). It is possible to use A129S/R134X/A165G/S166G and L107Y/A129G/R134X/A165G/S166G FSA for the improved production of D-fructose from DHA and DGA. A person skilled in the art may use one or more the wild-type FSA or FSA mutants as mentioned above for the aldol condensation of DHA with DGA. In addition, it is also possible to redesign the FSA enzymes guided by its structural features and develop a mutant to exhibit enhanced reactivity and selectivity towards DHA as a donor and DGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity.

[00100] In another aspect of the production of D-fructose, transaldolase (TalB) is used as an enzyme. TalB are specific group of lyases that catalyze the reversible addition of donor compounds onto acceptor compounds. TalB is very specific to DHA or DHAP as donors whereas wide range of aldehydes are tolerated as acceptors. Replacement of phenylalanine by tyrosine in the active site was found to confer FSA activity in TalB. Mutant TalB F1786Y has been shown to be effective for the production of fructose from sedoheptulose (Schneider S., et al., 2008). Engineered TalB F178Y has also been shown to be effective against various donors and acceptor including for the production of deoxy sugars (Rale. M. et al., 2011). TalB F178Y has been further mutated at the phosphate binding site. A new mutant containing TalB F178Y/R181E has been found to exhibit at least five-fold increase in reactivity and selectivity towards the production of D-fructose from non-phosphorylated substrates (Schneider S. et al., 2010). A high-throughput screening based on genome mining has resulted in new enzymes such as UniprotKB ID # P78055, B7NAG9, A0A0D6J3Z8, A0A0E4G3U3, A0A0E4C393,

A0A0D6H018, A0A0E4C363 and Q8E738 having dihydroxyacetone aldolase activity (Helaine C et al., 2015). A person skilled in the art may use one or more the wild-type TalB or TalB mutants as mentioned above in the aldol condensation reaction to yield fructose using DHA and DGA as the starting materials. In addition, it is also possible to redesign the TalB enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards DHA as a donor and DGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving reactivity to DHA.

[00101] Fuculose aldolase is used for the production of D-psicose by the aldol condensation of DHA and DGA. Fuculose aldolase as defined in this invention is an enzyme that combines a ketose and an aldose to produce two new (3R, 4R) hydroxyl stereo centers. In one aspect of the production of D-psicose, L-fuculose 1-phosphate aldolase (FucA) is used as an enzyme. DHAP dependent FucA combines DHAP and L-lactaldehyde to produce L-fuculose 1-phosphate. FucA accepts wide range of aldehydes as the acceptor, but are quite specific to the donor substrate DHAP (Fessner W. et al., 1991). FucA accepts DGA at 83% relative rate compared to L-lactaldehyde to produce D-psicose 1-phosphate (Ozaki A. et al., 1990). Wild-type FucA has been used with DHAP and amino aldehyde (Espelt L. et al., 2005). Genetically engineered FucA F131A has been used for the improved production of hydroxylated pyrrolizidines (Garrahou X. et al., 2010). Similarly, it is possible to use FucA F131A for the production of D-psicose from DHA and DGA. It is also disclosed that arsenate salts used in the medium could mimic phosphate esters and promote aldol reaction between DHA and amino aldehyde (Garrahou X. et al., 2011). It is possible to use metal salts such as borates, arsenates and vanadates for utilizing non-phosphorylated DHA as substrates. Furthermore, structural analysis of DHAP binding at the FucA active site has revealed that the phosphate binds to Asn29, Ser71, Gly44, Thr43, Ser72 and C2 oxygen binds with Gly28 (Joerger A. C. et al., 2000). It is possible to further mutate FucA by replacing the binding amino acid acids in the phosphate binding site with non-binding amino acids such as Asn29Gln and Ser71Gln can lead to the improved reactivity towards DHA instead of DHAP (Joerger, A.C. et al., 2000b). A person skilled in the art may use one or more the wild-type FucA or FucA mutants as mentioned above for the aldol condensation of DHA with DGA. In addition, it is also possible to redesign the FucA enzymes to develop a mutant that

exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and DGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity.

[00102] In another method to produce D-psicose, D-fructose produced by the aldol condensation of DHA and DGA is subjected to C3 epimerization by epimerase class (EC 5.1.3) enzymes. In one aspect, tagatose-3-epimerase is used as an enzyme. Tagatose-3-epimerases show very high substrate promiscuity against several non-phosphorylated ketohexoses. Tagatose-3-epimerase show highest reactivity against D-fructose and moderate reactivity against D-tagatose. In one aspect, D-tagatose-3-epimerase is used for the production of D-psicose from D-fructose. U. S. Pat. No. 8,030,035; U. S. Pat. No. 8,216,818; U. S. Pat. Application No. 2015/0210996 discloses successful use of D-tagatose-3-epimerase for the production of D-psicose from D-fructose. U. S. Pat. No. 8,735,106 discloses immobilization of tagatose-3-epimerase and its use in the production of D-psicose from D-fructose. Mass production of D-tagatose from D-fructose using continuous bioreactor system has also been demonstrated (Takeshita K. et al. 2000). A person skilled in the art may use one or more of the methods as described above for the production of D-psicose from D-fructose. The patent documents referenced herein in this paragraph are incorporated in their entirety by reference.

[00103] In another aspect of this embodiment for the production of carbohydrates from methane, biomass, carbon dioxide and other hydrocarbon sources, mixture of DHA and DGA produced by one or more of isomerization methods is subjected to aldol condensation by aldolases class (EC 4.1.2) enzymes. In one aspect, rhamnulose aldolase is used for the production of D-sorbose by the aldol condensation of DHA and DGA. Rhamnulose aldolase as defined in this invention is an enzyme that combines a ketose and an aldose and produces two new (3R, 4S) hydroxyl stereo centers. In one aspect of this embodiment, the production of D-sorbose, L-rhamnulose 1-phosphate aldolase (RhuA) is used as an enzyme. DHAP dependent RhuA combines DHAP and L-lactaldehyde to produce L-rhamnulose 1-phosphate. RhuA accepts wide range of aldehydes as the acceptor, but are quite specific to the donor substrate DHAP (Fessner W. et al., 1991). Wild-type RhuA accepts DHA in the presence of inorganic

arsenate salts as substrate for the production of D-sorbose (Drueckhammer D. G. et al., 1989). Wild-type RhuA produces a mixture of D-sorbose and D-psicose when DGA is used as substrate with DHAP (Li Z., Cai L. et al., 2011). Redesigning of the phosphate binding site of RhuA and the development of RhuA N29D allows the use of non-phosphorylated DHA as a substrate for the production of L-rhamnulose (Garrabou X. et al., 2011). It is also possible to use RhuA N29D for the production of D-sorbose from DHA and DGA. In addition, crystal structure of the RhuA wild type active center shows N29, N32, S75, T115 and S116 in the phosphate binding site (Kromer, M. et al., 2003). Further mutations to remove S75, S116 and T115 with non-coordinating amino acids such as alanine, phenylalanine, valine and leucine may improve enzyme reactivity and selectivity towards the DHA and DGA. A person skilled in the art may use one or more the wild-type RhuA or RhuA mutants as mentioned above for the aldol condensation of DHA with DGA. In addition, it is also possible to redesign the RhuA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and DGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity. In another method to produce D-sorbose, D-tagatose produced by the aldol condensation of DHA and DGA is subjected to C3 epimerization by epimerase class (EC 5.1.3) enzymes. In one aspect, D-tagatose-3-epimerase is used for the production of D-sorbose from D-tagatose. Tagatose-3-epimerases show very high substrate promiscuity against several non-phosphorylated ketohexoses. Tagatose-3-epimerase show highest reactivity against D-tagatose and D-fructose and moderate reactivity against other sugars. Production of D-sorbose from D-tagatose by immobilized D-tagatose 3-epimerase from *Pseudomonas* has been demonstrated (Itoh H. et al., 1995). U. S. Pat. No. 8,008,058 discloses methods of producing ketose-3-epimerase and its uses in making D-sorbose from D-tagatose. A person skilled in the art may use one or more of the methods as described above for the production of D-sorbose from D-tagatose.

[00104] Tagatose aldolase is used for the production of D-tagatose by the aldol condensation of DHA and DGA. Tagatose aldolase as defined in this invention is an enzyme that combines a ketose and an aldose and produces two new (3S, 4S) hydroxyl stereo centers. In one aspect of the production of D-tagatose, D-tagatose 1,6-bisphosphate aldolase (TagA) is used

as an enzyme. DHAP dependent TagA combines DHAP and P-GA to produce D-tagatose 1,6-bisphosphate. Highly diastereoselective production of D-tagatose 1,6-bisphosphate is produced by the aldol reaction of DHAP and P-GA by TagA (Fessner W. D. and Eyrisch O. **1992**). Structure of a class I TagA shows DHAP phosphate interaction with Arg278, Ser249, Gly277 and Gln28, DHAP C3 hydroxyl group makes interaction with Asp 27, Lys 125 and a water molecule (Lowkam C. et al., 2010). Structure of class II TagA shows phosphate interaction with Gly181, Thr233, Ser211 and Ala232, C3 hydroxyl interaction with Asp82, and Asn24, a five membered coordination between C1 and C2 oxygens by a Zn^{2+} (Hall D. R. et al., 2002). It is possible to redesign the phosphate binding active site of the class I TagA or class II TagA to confer more hydrophobicity by replacing Arg278, Ser249, Gly277 and Gln28 with alanine, phenylalanine, lysine, leucine and isoleucine that may improve its reactivity and selectivity with DHA and DGA for the production of D-tagatose. In addition, it is also possible to switch the specificity of the FDP aldolase or FSA to TagA by making several changes in FDP aldolase or FSA active sites (Zgiby S. M. et al., 2000) to produce D-tagatose from enzymes that conventionally produced D-fructose. A person skilled in the art may use one or more of the wild-type TagA or TagA mutants for the aldol condensation of DHA with DGA. In addition, it is also possible to redesign the TagA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and DGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity.

[00105] L-rhamnulose 1-phosphate aldolase (RhuA) is used for the production of L-fructose by the aldol condensation of DHA and LGA. DHAP dependent RhuA combines DHAP and L-lactaldehyde to produce fructose L-rhamnulose 1-phosphate. RhuA accepts wide range of aldehydes as the acceptor, but are quite specific to the donor substrate DHAP (Fessner W. et al., 1991). One-pot synthesis of L-fructose from DHAP and LGA produced from the oxidation of glycerol is achieved with 55% overall yield using RhuA (Franke D. et al., 2003). L-fructose was synthesized using DHA as donor and racemic glyceraldehyde as an acceptor using RhuA in the presence of borate buffer (Sugiyama M. et al., 2006). It is possible that an identical conditions developed by Sugiyama *et al* would produce L-fructose when DHA and LGA is used as substrate. In addition, directed evolution methods are used to alter the substrate specificity of the

RhuA from DHAP to DHA (Sugiyama M et al., 2007). Up to 87% yield of L-fructose was obtained by a one-step synthesis from LGA and DHAP using RhuA (Alajarin R. et al., 1995). Redesigning of the phosphate binding site of RhuA and the development of RhuA N29D allows the use of non-phosphorylated DHA as a substrate for the production of L-rhamnulose (Garrahou X. et al., 2011). It is also possible to use RhuA N29D for the production of L-fructose from DHA and LGA. In addition, crystal structure of the RhuA wild type active center shows N29, N32, S75, T115 and S116 in the phosphate binding site. Further mutations to remove S75, S116 and T115 with non-coordinating amino acids such as alanine, phenylalanine, valine and leucine may improve enzyme reactivity and selectivity towards the DHA and LGA. A person skilled in the art may use one or more the wild-type RhuA or RhuA mutants as mentioned above for the aldol condensation of DHA with LGA. In addition, it is also possible to redesign the RhuA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and LGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity.

[00106] D-tagatose 1,6-bisphosphate aldolase (TagA) is used for the production of L-psicose by the aldol condensation of DHA and LGA. DHAP dependent TagA is known for the highly diastereoselective production of (3S, 4S) D-tagatose 1,6-bisphosphate from DHAP and G3P (Fessner W. D. and Eyrisch O. 1992). Enzymatic synthesis of L-psicose or L-psicose 1,6-bisphosphate from phosphorylated or non-phosphorylated trioses are unknown. However, since L-psicose has identical (3S, 4S) configuration as D-tagatose, it is possible to expect TagA to exhibit similar configurational preferences when LGA is used as an acceptor in the place of DGA. In addition, it is possible to redesign the phosphate binding active site of the class I TagA or class II TagA to confer more hydrophobicity by replacing Arg278, Ser249, Gly277 and Gln28 with alanine, phenylalanine, lysine, leucine and isoleucine that may improve its reactivity and selectivity with DHA and LGA for the production of L-psicose. A person skilled in the art may use one or more the wild-type TagA or TagA mutants for the aldol condensation of DHA with LGA. In addition, it is also possible to redesign the TagA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and LGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain

one or more of the mutations that has already been shown to be effective in improving DHA reactivity. In another method to produce L-psicose, L-fructose produced by the aldol condensation of DHA and LGA is subjected to C3 epimerization by epimerase class (EC 5.1.3) enzymes. In one aspect, tagatose-3-epimerase is used as an enzyme. Tagatose-3-epimerases show very high substrate promiscuity against several non-phosphorylated ketohexoses. Tagatose-3-epimerase show highest reactivity against D-fructose and modest reactivity against L-fructose. A person skilled in the art may use one or more of the methods as described above for the production of L-psicose from L-fructose.

[00107] Fructose 6-phosphate aldolase (FSA) is used for the production of L-sorbose by the aldol condensation of DHA and LGA. FSA combines DHA and P-GA for the production of fructose 6-phosphate (Schurmann M. and Sprenger G. A. **2001**). FSA has a striking advantage of using non-phosphorylated DHA as donors. In addition, FSA is also found to be moderately effective in taking various aldehyde acceptors. FSA has been used for the two-step production of L-sorbose from glycoldehyde (Helaine V. et al., 2015). In the first step, FSA is used for the production of LGA from glycoldehyde. In the second step LGA is combined with DHA to produce L-sorbose. FSA has been mutated at A129S/A165G and at L107A/L163A for the improved substrate promiscuity and specificity. It is possible to use the mutated FSA that has been modified for the improved D-fructose production for the production of L-sorbose from DHA and LGA. A person skilled in the art may use one or more the wild-type FSA or FSA mutants for the aldol condensation of DHA with LGA. In addition, it is also possible to redesign the FSA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated LGA as an acceptor and DHA as a donor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity. In another method to produce L-sorbose, L-tagatose produced by the aldol condensation of DHA and LGA is subjected to C3 epimerization by epimerase class (EC 5.1.3) enzymes. In one aspect, tagatose-3-epimerase is used as an enzyme. Tagatose-3-epimerases show very high substrate promiscuity against several non-phosphorylated ketohexoses. A person skilled in the art may use one or more of the methods as described above for the production of L-sorbose from L-tagatose. In another aspect L-sorbose produced from DHA and LGA as described in the methods above is used as a precursor for the

chemical synthesis of L-ascorbic acid. L-sorbose conventionally prepared by the microbial oxidation of sorbitol by *Acetobactor* (Wulf P. D. et al., 2000). It is possible to substitute L-sorbose produced from DHA and LGA for the chemical synthesis of L-ascorbic acid that conventionally use L-sorbose produced by microbial oxidation process. A person skilled in the art may use one or more of the methods as described in U. S. Pat. No. 5,998,634; U. S. Pat. No. 6,610,863; U. S. Pat. No. 5,637,734; U. S. Pat. No. 6,197,977; U. S. Pat. No. 5,744,618; U. S. Pat. No. 5,391,770 and U. S. Pat. No. 4,491,668 for the production of L-ascorbic acid from L-sorbose.

[00108] Fuculose 6-phosphate aldolase (FucA) is used for the production of L-tagatose by the aldol condensation of DHA and LGA. DHAP dependent FucA combines DHAP and L-lactaldehyde to produce L-fuculose 1-phosphate. FucA accepts wide range of aldehydes as the acceptor, but are quite specific to the donor substrate DHAP (Fessner W. et al., 1991). FucA produces a mixture of L-fructose and L-tagatose when LGA was used as acceptor along with DHAP (Li Z. et al., 2011). Genetically engineered FucA F131A has been used for the improved production of aldol products. Furthermore, structural analysis of DHAP binding at the FucA active site has revealed that the phosphate binds to Asn29, Ser71, Gly44, Thr43, Ser72 and C2 oxygen binds with Gly28. It is possible to mutate FucA by replacing the binding amino acid acids in the phosphate binding site with non-binding amino acids such as alanine, phenylalanine, valine, leucine and isoleucine. A person skilled in the art may use one or more of the wild-type FucA or FucA mutants as mentioned above for the aldol condensation of DHA with LGA. In addition, it is also possible to redesign the FucA enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated DHA as a donor and LGA as an acceptor. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving DHA reactivity.

[00109] In another aspect of this embodiment ketohexoses obtained by one or more of isomerization and aldol condensation reactions are subjected to isomerization by isomerase class (EC 5.3.1) enzymes. In one aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is used for the production of D-mannose from D-fructose. In one aspect of this embodiment, the isomerase enzyme that

converts 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is mannose isomerase. In another aspect of this embodiment, isomerase enzyme that converts 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is L-ribose isomerase or lyxose isomerase. Although chemical catalyst could be developed for the isomerization of fructose to mixture of glucose and mannose (Deval R. B. et al., 2014) enzymatic transformations are preferred due to their high substrate specificity, stereoselectivity and lack of side products. Moreover, isomerase class enzymes require no co-factors, no co-substrates, no external oxidant or reductants and no phosphorylation of the substrates. U. S. Pat. No. 5,240,717 discloses mannose isomerase produced from culturing the strains of *Pseudomonas sp.* for the isomerization of fructose to mannose. U. S. Pat. No. 5,049,494 discloses immobilization of mannose isomerase from *Pseudomonas cepacia* for the economical production of mannose from fructose. Thermostable mannose isomerase from *Pseudomonas sp.* is used for the high temperature isomerization of D-fructose to D-mannose (Takasaki Y. et al., 1993). Immobilized *Agrobacterium radiobacter* cells are used for the continuous isomerization of D-fructose to D-mannose (Hirose J. et al., 2003). A person skilled in the art may use one or more the mannose isomerase from *Pseudomonas sp.*, L-ribose isomerase or lyxose isomerase as mentioned above for the isomerization of D-fructose to D-mannose. In another aspect of this embodiment, D-mannose produced from D-fructose is subjected to chemical hydrogenation to produce mannitol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514.

[00110] In another aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4R) hexose to 2R-hydroxy (3S, 4R) aldohexose is used for the production of D-glucose from D-fructose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4R) hexose to 2R-hydroxy (3S, 4R) aldohexose is xylose isomerase. Xylose isomerase, also called glucose isomerase, has been extensively used in the isomerization of D-glucose to D-fructose. It is also reported that xylose isomerase is equally effective in producing nearly a 1:1 mixture of D-glucose and D-fructose whether the substrate is D-glucose or D-fructose (Takasaki Y. 1967). U. S. Pat. No. 4,373,025 discloses xylose isomerase based isomerization, where D-glucose is extracted by ion-exchange resin and removed from the reaction mixture. U. S. Pat. No.

3,689,362 discloses enzymatic methods for the production of D-fructose from D-glucose. U. S. Pat. No. 3,868,304 discloses immobilization of xylose isomerase for the isomerization of D-fructose from D-glucose. U. S. Pat. No. 4,060,456 discloses microbial cells having the ability to isomerize D-glucose to D-fructose. U. S. Pat. No. 3,694,314 discloses methods and processes for the isomerization of D-glucose to D-fructose. U. S. Pat. No. 4,008,124 discloses immobilized glucose isomerase containing iron or magnesium for the isomerization of D-glucose to D-fructose. It is possible to use xylose isomerase and the process described above for the isomerization of D-glucose to D-fructose for the purpose of carrying out isomerization of D-fructose to D-glucose. A person skilled in the art may use one or more the enzyme xylose isomerase and the process methods and conditions as mentioned in patent documents above for the isomerization of D-fructose to D-glucose. In another aspect of this embodiment, D-glucose produced from D-fructose is subjected to chemical hydrogenation to produce sorbitol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. All the patent documents listed in this paragraph are herein incorporated by reference in their entirety.

[00111] In yet another aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is used for the production of D-galactose from D-tagatose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is L-arabinose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is galactose isomerase or glucose phosphate isomerase. L-arabinose isomerase has been extensively used in the isomerization of D-galactose to D-tagatose. It is also reported that L-arabinose isomerase is equally effective in producing nearly a 1:1 mixture of D-galactose and D-tagatose whether the substrate is D-tagatose or D-galactose (Schleif R. et al., 1973). U. S. Pat. No. 8,481,720 discloses high yield production of tagatose from galactose using L-arabinose isomerase and borate salts. U. S. Pat. No. 6,057,135 discloses process of manufacturing tagatose from cheese wherein one of the key steps utilizes L-arabinose isomerase for the conversion of galactose to tagatose. U. S. Pat. Application No. 2003/0175909 discloses thermostable galactose isomerase for the production of tagatose from galactose. *Pseudomonas aeruginosa* glucose

phosphate isomerase is used for the conversion of galactose to tagatose (Patel M. J. et al., 2016). *Pediococcus pentosaceus* L-arabinose isomerase is used for the enzymatic conversion of galactose to tagatose (Men Y., et al. 2014). Immobilized thermostable L-arabinose isomerase is used for the continuous production of D-tagatose from D-galactose (Liang M. et al., 2012). It is possible to use L-arabinose isomerase and the methods, process described above for the isomerization of D-galactose to D-tagatose for the isomerization of D-tagatose to D-galactose. A person skilled in the art may use one or more the enzyme L-arabinose isomerase and the process methods and conditions as mentioned in the patent documents cited above for the isomerization of D-tagatose to D-galactose. In another aspect of this embodiment, D-galactose produced from D-tagatose is subjected to chemical hydrogenation to produce galacitol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. All the patent documents listed in this paragraph are herein incorporated by reference in their entirety.

[00112] In yet another aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is used for the production of D-talose from D-tagatose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is L-ribose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is Lyxose isomerase. L-ribose isomerase from *Cellulomonas parahominis* is used for the conversion of D-tagatose to D-talose (Terami Y., Uechi K., Nomura S., Okamoto N., Morimoto K., Takata G. *Bioscience Biotechnology Biochemistry* **2015**, 70, 1725-1729). U. S. Pat. No. 6,037,153 discloses enzyme L-ribose isomerases for the conversion of D-talose to D-tagatose. A person skilled in the art may use one or more the enzyme L-ribose isomerase and the process methods and conditions as mentioned in the patent document cited above for the isomerization of D-tagatose to D-talose.

[00113] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4S) hexose to 2R-hydroxy (3R, 4S) aldohexose is used for the production of D-gulose from D-sorbose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4S)

hexose to 2R-hydroxy (3R, 4S) aldohexose is L-rhamnose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is D-lyxose isomerase. L-rhamnose isomerase from *Pseudomonas sp.* is immobilized and used for the conversion of D-sorbose to D-gulose (Bhuiyan S. H. et al., 1999). A person skilled in the art may use L-rhamnose isomerase for the isomerization of D-gulose from D-sorbose.

[00114] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4S) hexose to 2S-hydroxy (3R, 4S) aldohexose is used for the production of D-idose from D-sorbose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4S) hexose to 2R-hydroxy (3R, 4S) aldohexose is xylose isomerase. Enzymatic synthesis of D-idose from D-sorbose or D-sorbose from D-idose is unknown. However, since D-idose has a mirror image (2S, 3R, 4S) configuration to D-glucose (2R, 3S, 4R), it is possible to expect xylose isomerase that catalyze the isomerization of D-fructose to D-glucose to exhibit similar configurational preferences when D-sorbose is used as a substrate in the place of D-fructose. In addition, it is possible to redesign the active site of the xylose isomerase to confer its ability to accept D-sorbose as substrate for the production of D-idose. A person skilled in the art may use one or more the wild-type xylose isomerase or xylose isomerase mutants for the isomerization of D-sorbose to D-idose. In another aspect of this embodiment, D-idose produced from D-sorbose is subjected to chemical hydrogenation to produce iditol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. All the patent documents listed in this paragraph are herein incorporated by reference in their entirety.

[00115] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is used for the production of D-altrose from D-psicose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is D-arabinose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is L-fucose isomerase. Enzymatic synthesis of D-altrose from D-psicose or D-psicose from D-altrose is unknown. However, since D-altrose has an identical (2S, 3R, 4R) configuration to D-arabinose

(2S, 3R, 4R) and L-fuculose (2S, 3R, 4R), it is possible to expect D-arabinose isomerase that catalyze the isomerization of D-ribulose to D-arabinose and L-fucose isomerase that catalyze the isomerization of L-fuculose to L-fucose to exhibit similar configurational preferences when D-psicose is used as a substrate in the place of D-ribulose for D-arabinose isomerase and L-fuculose for L-fucose isomerase. In addition, it is possible to redesign the active site of the D-arabinose isomerase and L-fucose isomerase to confer its ability to accept D-psicose as substrate for the production of D-altrose. A person skilled in the art may use one or more the wild-type D-arabinose isomerase or D-arabinose isomerase mutants for the isomerization of D-psicose to D-altrose.

[00116] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is used for the production of D-allose from D-psicose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is L-rhamnose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is ribose isomerase. Immobilized L-rhamnose isomerase from *Pseudomonas sp.* is used for the production of D-allose from D-psicose (Bhuiyan S. H. et al., 1998). L-rhamnose isomerase crosslinked with glutaraldehyde is used for the isomerization D-psicose to D-allose (Menavuvu B. T. et al., 2006). Large scale production of D-allose from D-psicose using *Pseudomonas stutzeri* LL172 is reported with continuous bioreactor and separation systems (Morimoto K. et al., 2006). U. S. Pat. Application No. 2015/0361473 discloses D-tagatose 3-epimerase and L-rhamnose isomerase for the production of D-allose from D-fructose. U. S. Pat. Application No. 2015/0284759 discloses enzyme protein produced from *Streptomyces* for the isomerization of D-psicose to D-allose. U. S. Pat. No. 8,748,589 discloses crystallization process for the separation of D-allose from D-psicose. A person skilled in the art may use one or more the enzyme L-rhamnose isomerase and the process methods and conditions as mentioned in the patent documents cited above for the isomerization of D-allose from D-psicose.

[00117] In yet another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4S) hexose to 2R-hydroxy (3R, 4S) aldohexose is used for the production of L-mannose from L-fructose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4S)

hexose to 2R-hydroxy (3R, 4S) aldohexose is L-rhamnose isomerase. L-rhamnose isomerase produced from *Pseudomonas sp.* is immobilized on chitopearl beads BCW 2603 and used for the production of L-mannose from L-fructose (Bhuiyan S. H. et al., 1997). A person skilled in the art may use one or more the enzyme L-rhamnose isomerase and the process methods and conditions as mentioned in publications above for the isomerization of L-mannose from L-fructose.

[00118] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4S) hexose to 2S-hydroxy (3R, 4S) aldohexose is used for the production of L-glucose from L-fructose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4S) hexose to 2S-hydroxy (3R, 4S) aldohexose is xylose isomerase. Enzymatic synthesis of L-glucose from L-fructose or L-fructose from L-glucose is unknown. However, since L-glucose has an identical (2S, 3R, 4S) configuration to D-idose (2S, 3R, 4S), it is possible to expect xylose isomerase that catalyze the isomerization of D-sorbose to D-idose to exhibit similar configurational preferences when L-fructose is used as a substrate in the place of D-sorbose. In addition, it is possible to redesign the active site of the xylose isomerase to confer its ability to accept L-fructose as substrate for the production of L-glucose. A person skilled in the art may use one or more the wild-type xylose isomerase or xylose isomerase mutants for the isomerization of L-fructose to L-glucose.

[00119] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is used for the production of L-altrose from L-psicose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is L-arabinose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2R-hydroxy (3S, 4S) aldohexose is galactose 6P isomerase. Enzymatic synthesis of L-altrose from L-psicose or L-psicose from L-altrose is unknown. However, since L-altrose has an identical (2R, 3S, 4S) configuration to D-galactose (2R, 3S, 4S), it is possible to expect L-arabinose isomerase and galactose 6P isomerase that are known to catalyze the isomerization of D-galactose to D-tagatose to exhibit similar configurational preferences when L-psicose is used as a substrate in the place of D-tagatose. In addition, it is possible to redesign the active site of the L-arabinose isomerase to

confer its ability to accept L-psicose as substrate for the production of L-altrose. A person skilled in the art may use one or more the wild-type L-arabinose isomerase or L-arabinose isomerase mutants for the isomerization of L-psicose to L-altrose.

[00120] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is used for the production of L-allose from L-psicose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is L-ribose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) hexose to 2S-hydroxy (3S, 4S) aldohexose is D-lyxose isomerase. L-ribose isomerase produced from *Cellulomonas parahominis* MB426 is immobilized on Diaion HPA25L and used for the production of L-allose from L-psicose (Terami Y. et al. 2015). U. S. Pat. No. 6,037,153 discloses L-ribose isomerase obtained from *Acinetobacter* for the production of L-allose from L-psicose. A person skilled in the art may use one or more the enzyme L-ribose isomerase or D-lyxose isomerase and the process methods and conditions as mentioned in patent document cited above for the isomerization of L-allose from L-psicose.

[00121] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3S, 4R) hexose to 2R-hydroxy (3S, 4R) aldohexose is used for the production of L-idose from L-sorbose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4R) hexose to 2R-hydroxy (3S, 4R) aldohexose is xylose isomerase. Enzymatic synthesis of L-idose from L-sorbose or L-sorbose from L-idose is unknown. However, since L-idose has an identical (2R, 3S, 4R) configuration to D-glucose (2R, 3S, 4R), it is possible to expect xylose isomerase that is known to catalyze the isomerization of D-glucose to D-fructose to exhibit similar configurational preferences when L-sorbose is used as a substrate in the place of D-fructose. In addition, it is possible to redesign the active site of the xylose isomerase to confer its ability to accept L-sorbose as substrate for the production of L-idose. A person skilled in the art may use one or more of the wild-type xylose isomerase or xylose isomerase mutants for the isomerization of L-idose to L-sorbose.

[00122] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is used for the production

of L-gulose from L-sorbose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is L-ribose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4R) hexose to 2S-hydroxy (3S, 4R) aldohexose is D-mannose isomerase or D-lyxose isomerase. U. S. Pat. No. 6,037,153 discloses L-ribose isomerase obtained from *Acinetobacter* for the production of L-gulose from L-sorbose. A person skilled in the art may use one or more the enzyme L-ribose isomerase or D-mannose isomerase and the process methods and conditions as mentioned in patent document cited above for the isomerization of L-gulose from L-sorbose. The patent document cited in this paragraph is herein incorporated by reference in their entirety.

[00123] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is used for the production of L-talose from L-tagatose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is L-rhamnose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2R-hydroxy (3R, 4R) aldohexose is ribose isomerase. L-rhamnose isomerase isolated from *Pseudomonas sp.* LL173 immobilized on BCW 2603 chitopearl beads was used to produce L-talose from L-tagatose (Bhuiyan S. H. et al., 1999). A person skilled in the art may use one or more the wild-type L-rhamnose isomerase or ribose isomerase and the process methods and conditions as mentioned in publication above for the isomerization of L-talose from L-tagatose.

[00124] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is used for the production of L-galactose from L-tagatose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is D-arabinose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) hexose to 2S-hydroxy (3R, 4R) aldohexose is L-fucose isomerase. Enzymatic synthesis of L-galactose from L-tagatose or L-tagatose from L-galactose is unknown. However, since L-galactose has an identical (2S, 3R, 4R) configuration to D-altrose (2S, 3R, 4R), it is possible to expect D-arabinose isomerase that is known to catalyze the isomerization of D-psicose to D-altrose to exhibit similar configurational preferences when L-tagatose is used as a substrate in the place of D-psicose. In addition, it is possible to

redesign the active site of the D-arabinose isomerase to confer its ability to accept L-tagatose as substrate for the production of L-galactose. A person skilled in the art may use one or more the wild-type D-arabinose isomerase or D-arabinose isomerase mutants for the isomerization of L-tagatose to L-galactose.

[00125] In another aspect of this embodiment ketohexoses obtained by or more of isomerization and aldol condensation methods are subjected to transfer reaction by transferase class (EC 2.2.1) enzymes. In one aspect, transferase enzyme that is capable of converting D-fructose and D-glyceraldehyde to D-xylulose and D-erythrose is used. In one aspect, transferase enzyme that is capable of converting D-fructose and D-glyceraldehyde to D-xylulose and D-erythrose is transketolase (EC 2.2.1.1). Thermostable transketolase from *Geobacillus stearothermophilus* is used for the conversion of D-fructose to D-xylulose (Zaber J. A et al., 2013). D-xylulose is produced from D-glyceraldehyde using hydroxypyruvate by transketolases (Solovjeva O. N. et al., 2008). Enzymatic synthesis of D-sedoheptulose from D-ribose and hydroxypyruvic acid is performed by transketolase (Charmantray F. et al., 2009). Single point mutation leads to the enhancement in transketolase selectivity towards the formation of erythrulose from DHA (Smith M. E. B. Et al., 2008). Transketolase is evolved to enhance substrate specificity towards aliphatic aldehyde (Hibbert, E. G. et al., 2008). Genetically modified transketolase R526Q/S525T is developed for the transaldol reaction of non-phosphorylated substrates (Ranoux A. et al., 2012). A person skilled in the art may use one or more the transketolase or transketolase mutant and the process methods and conditions as mentioned in publications above for the transaldol reaction of D-glyceraldehyde and D-fructose to produce D-xylulose and D-erythrulose. In another aspect of this embodiment, D-erythrulose produced from D-fructose and D-glyceraldehyde is subjected to chemical hydrogenation to produce erythritol as described in U. S. Pat. Application No. 2013/0210099.

[00126] In another aspect of this embodiment ketohexoses obtained by or more of isomerization and aldol condensation methods are subjected to transfer reaction by transferase class (EC 2.2.1) enzymes. In one aspect, transferase enzyme that is capable of converting DHA and D-glyceraldehyde to D-xylulose and formaldehyde is used. In one aspect, transferase enzyme that is capable of converting DHA and D-glyceraldehyde to D-xylulose and

formaldehyde is dihydroxyacetone synthase (EC 2.2.1.3). Enzymatic synthesis of D-xylulose from D-glyceraldehyde has not been performed in vitro systems. However, dihydroxyacetone synthase has been isolated and characterized from *Acinetobacter* sp. strain JC1 DSM 3803 (Ro Y. T. et al., 1997). Transketolase synthesized during methylotrophic growth of *Candida boidinii* is also capable of using formaldehyde and glyceraldehyde as an acceptor (Waites M. J. and Quayle J. R. 1981). Dihydroxyacetone synthase is an abundant constituent of the methylotrophic yeasts. A person skilled in the art may isolate one or more of the dihydroxyacetone synthase enzymes from one or more methanol, formaldehyde and methane utilizing organisms. In addition, dihydroxyacetone synthase is also mutated to utilize non-phosphorylated DHA and DGA as substrate. One or more either wild-type dihydroxyacetone synthase or dihydroxyacetone synthase mutants is used for the transaldol reaction between of L-glyceraldehyde and DHA.

[00127] In another aspect of this embodiment ketopentoses obtained by one or more of isomerization, aldol condensation and transferase methods are subjected to aldose-ketose isomerization by isomerase class (EC 5.3.1) enzymes. In one aspect, isomerase enzyme that is capable of converting 2-keto (3S, 4R) pentose to 2R-hydroxy (3S, 4R) aldopentose is used for the production of D-xylose from D-xylulose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4R) pentose to 2R-hydroxy (3S, 4R) aldopentose is xylose isomerase. Xylose isomerase is also called glucose isomerase has been extensively used in the isomerization of D-glucose to D-fructose. It is also reported that xylose isomerase is equally effective in producing nearly a 1:1 mixture of D-xylose and D-xylulose whether the substrate is D-xylose or D-xylulose (Aida T. M. et al., 2010). U. S. Pat. No. 5,238,826 discloses process for manufacturing D-xylose by subjecting D-xylulose to an enzymatic isomerization in the presence of enzyme glucose isomerase. Xylose isomerase has been expressed in the host cells to improve cells ability to metabolize xylose as sole carbon and energy source for the production of biochemicals. U. S. Pat. No. 7,622,284 discloses eukaryotic cells transformed by the expression of xylose isomerase that has ability to isomerize xylose to xylulose for the production of ethanol. U. S. Pat. No. 9,187,743 discloses bacterial xylose isomerase active in yeast cells. U. S. Pat. Application No. 2011/0244525 discloses microorganism expressing xylose isomerase and comprising an ability to metabolize xylose. U. S. Pat. No. 8,586,336 discloses nucleic acid molecule encoding xylose

isomerase. A person skilled in the art may use one or more the enzyme xylose isomerase and the process methods and conditions as mentioned in patents above for the isomerization of D-xylulose to D-xylose. In another aspect of this embodiment, D-xylose produced from D-xylulose is subjected to chemical hydrogenation to produce xylitol using one or more methods described in China Pat. No. 101,285,082; China Pat. No. 1,699,587; U. S. Pat. Appl. Publ. No. 2005/0203291; China Pat. No. 1,446,784; China Pat. No. 101,829,574; Japan Pat. No. 2001/1079411; Japan Pat. No. 2000/236900; German Pat. No. 2,418,800; German Pat. No. 1,935,934; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. The patent documents cited in this paragraph are herein incorporated by reference in their entirety.

[00128] In another aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4R) pentose to 2S-hydroxy (3S, 4R) aldopentose is used for the production of D-lyxose from D-xylulose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4R) pentose to 2S-hydroxy (3S, 4R) aldopentose is D-lyxose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4R) pentose to 2S-hydroxy (3S, 4R) aldopentose is mannose isomerase. Thermostable D-lyxose isomerase from *Dictyoglomus turgidum* has been used for the production of lyxose from xylulose (Choi J. G. et al, ,2012). Lyxose isomerase isolated from *Bacillus licheniformis* has been used as in vitro catalyst for the bio production of lyxose from xylulose (Patel D. H. et al., 2011). D-lyxose isomerase from *Serratia proteamaculans* produces D-lyxose from D-xylulose (Park C. S.,2015). D-lyxose isomerase isolated from *Providencia stuartii* has highest specific activity and catalytic efficiency for the conversion of D-xylulose to D-lyxose (Kwon H. J. et al., 2010). A person skilled in the art may use one or more the enzyme D-lyxose isomerase or mannose isomerase and the process methods and conditions as mentioned in publications above for the isomerization of D-xylulose to D-lyxose.

[00129] In another aspect of this embodiment epimerase enzyme that is capable of converting 2-keto (3S, 4R) pentose to 2-keto (3R, 4R) pentose is used for the production of D-ribulose from D-xylulose. In one aspect, enzyme that is capable of converting 2-keto (3S, 4R) pentose to 2-keto (3R, 4R) pentose is tagatose-3-epimerase. In another aspect, enzyme that is

capable of converting 2-keto (3S, 4R) pentose to 2-keto (3R, 4R) pentose is D-ribulose 5-phosphate 3-epimerase. Tagatose 3-epimerase from *Rhodobacter sphaeroides* that converted D-fructose to D-psicose is also capable of catalyzing D-ribulose to D-xylulose (Zhang L. et al., 2009862). U. S. Pat. No. 8,030,035 discloses that D-psicose epimerase from *Agrobacterium tumefaciens* capable of producing D-ribulose from D-xylulose. U. S. Pat. No. 6,911,565 discloses xylitol production process from xylulose by epimerization of D-ribulose. U. S. Pat. No. 5,411,880 discloses D-ketohexose 3-epimerase also useful for the production of D-ribulose from D-xylulose. Xylulose 5P 3-epimerase from *E.coli* has been used for the production of D-ribulose from D-xylulose (Liang W. et al., 2011). A person skilled in the art may use one or more the enzyme tagatose 3-epimerase or xylulose 5P 3-epimerase and the process methods and conditions as mentioned in publications and patent documents cited above for the epimerization of D-xylulose to D-ribulose. The patent documents cited in this paragraph are herein incorporated by reference in their entirety.

[00130] In another aspect of this embodiment ketopentoses obtained by one or more of isomerization, aldol condensation, transferase reaction and epimerization methods are subjected to aldose-ketose isomerization by isomerase class (EC 5.3.1) enzymes. In one aspect, isomerase enzyme that is capable of converting 2-keto (3R, 4R) pentose to 2R-hydroxy (3R, 4R) aldopentose is used for the production of D-ribose from D-ribulose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) pentose to 2R-hydroxy (3R, 4R) aldopentose is D-ribose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) pentose to 2R-hydroxy (3R, 4R) aldopentose is L-rhamnose isomerase. D-ribose isomerase from *Mycobacterium smegmatis* is used for the isomerization of D-ribulose to D-ribose (Izumori K. et al 1975). D-ribose isomerase from *E.coli* is used for the isomerization of D-ribulose to D-ribose (Ross A. K. et al., 2008). A person skilled in the art may use one or more the enzyme D-ribose isomerase or L-rhamnose isomerase and the process methods and conditions as mentioned in publications above for the isomerization of D-ribulose to D-ribose. In another aspect of this embodiment, D-ribose produced from D-ribulose is subjected to chemical hydrogenation to produce ribitol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S.

Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. The patent documents cited in this paragraph are herein incorporated by reference in their entirety.

[00131] In another aspect of this embodiment isomerase enzyme that is capable of converting 2-keto (3R, 4R) pentose to 2S-hydroxy (3R, 4R) aldopentose is used for the production of D-arabinose from D-ribulose. In one aspect, isomerase enzyme that converts 2-keto (3R, 4R) pentose to 2S-hydroxy (3R, 4R) aldopentose is D-arabinose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3R, 4R) pentose to 2S-hydroxy (3R, 4R) aldopentose is L-fucose isomerase. Enzymatic conversion of D-ribulose to D-arabinose catalyzed by D-arabinose isomerase has been disclosed (Cohen S. S. J. Biol. Chem. 1953, 201, 71-84). The metabolism of D-arabinose by *Salmonella typhimurium* proceed through isomerization of D-arabinose to D-ribulose catalyzed by D-arabinose isomerase (Old D. C., et al. 1977). A person skilled in the art may use one or more the enzyme D-arabinose isomerase or L-fucose isomerase and the process methods and conditions as mentioned in publications above for the isomerization of D-ribulose to D-arabinose. In another aspect of this embodiment, D-arabinose produced from D-ribulose is subjected to chemical hydrogenation to produce arabitol using one or more methods described in U. S. Pat. No. 4,503,274; U. S. Pat. No. 4,487,980; U. S. Pat. No. 4,471,144; U. S. Pat. No. 4,413,152; U. S. Pat. No. 4,382,150; U. S. Pat. No. 4,380,680; U. S. Pat. No. 4,292,451; and U. S. Pat. No. 4,173,514. The patent documents cited in this paragraph are herein incorporated by reference in their entirety.

[00132] In another aspect of this embodiment epimerase enzyme that is capable of converting 2-keto (3S, 4R) pentose to 2-keto (3S, 4S) pentose is used for the production of L-ribulose from D-xylulose. In one aspect, epimerase enzyme that converts 2-keto (3S, 4R) pentose to 2-keto (3S, 4S) pentose is L-ribulose-5-phosphate 4-epimerase (RPE). RPE from *Aerobacter aerogens* has been shown to convert D-xylulose 5P to L-ribulose 5P (Sal, et al., 1972). Mechanistic studies of RPE catalyzed 4-epimerization has revealed an aldol type C-C bond cleavage (Johnson A. E., and Tanner M. E. **1998**, 37, 5746-5754). Moreover, ¹³C and deuterium isotope effect experiments have been performed to further prove the aldol cleavage mechanism for RPE (Lee L. V. et al., 2000). Catalysis and binding properties of the RPE has revealed a close similarity with L-fuculose 1-phosphate aldolase (Samuel J. et al., 2001). H97N mutant of

RPE has shown 10 times better performance compared to the wild-type when activated by Zn^{2+} (Lee L. V. et al., 2000). Structural activity analysis of RPE has revealed a five-membered coordination between L-ribulose 5P and Zn^{2+} metal ion of RPE held by H95, H97 and H171 (Luo Y. et al., 2001). Phosphate binding sites of the RPE containing Asn28, Ser44, Gly45, Ser74 and Ser75 is very similar to the binding residues of the aldolase. Due to the structural similarities between RPE and aldolases, it is possible to redesign the phosphate binding sites of the RPE by using methods that are previously established for the engineering aldolase enzymes for the improved affinity towards DHA (Guclu D. et al., 2010). In one such method, RPE phosphate binding sites are modified by substituting binding amino acids such as Ser, Gly and Asn with Phe, Ala, Leu and Ile. A person skilled in the art may use one or more the wild-type RPE or RPE mutants as mentioned above for the epimerization of D-xylulose-5P with L-ribulose-5P. In addition, it is also possible to redesign the RPE enzymes to develop a mutant that exhibit enhanced reactivity and selectivity towards non-phosphorylated D-xylulose. Such a mutant, in addition to the new mutations introduced, may contain one or more of the mutations that has already been shown to be effective in improving RPE reactivity.

[00133] In another aspect of this embodiment ketohexoses obtained by one or more of isomerization and aldol condensation are subjected to epimerization by epimerase class (EC 5.1.3) enzymes. In one aspect, epimerase enzyme that is capable of converting 2-keto (3S, 4R) hexose to 2-keto (3S, 4S) hexose is used for the production of D-tagatose from D-fructose. In one aspect, epimerase enzyme that converts 2-keto (3S, 4R) hexose to 2-keto (3S, 4S) hexose is L-ribulose-5-phosphate 4-epimerase (RPE). Epimerization of D-fructose at the C4 carbon leads to the production of D-tagatose. C4 epimerization of D-fructose to D-tagatose is unknown. However, D-fructose has an identical (3S, 4R) configuration to D-xylulose (3S, 4R), it is possible to expect RPE that catalyze the isomerization of D-xylulose-5P to L-ribulose-5P to exhibit similar configurational preferences when D-fructose-5P is used as a substrate in the place of D-xylulose-5P. In addition, it is possible to redesign the active site of the RPE to confer its ability to accept D-fructose as substrate for the production of D-tagatose. A person skilled in the art may use one or more the wild-type RPE or RPE mutants for the isomerization of D-fructose to D-tagatose.

[00134] In another aspect of this embodiment ketopentoses obtained by one or more of isomerization, aldol condensation, transferase reaction and epimerization methods are subjected to aldose-ketose isomerization by isomerase class (EC 5.3.1) enzymes. In one aspect, isomerase enzyme that is capable of converting 2-keto (3S, 4S) pentose to 2S-hydroxy (3S, 4S) aldopentose is used for the production of L-ribose from L-ribulose. In one aspect, isomerase enzyme that converts 2-keto (3S, 4S) pentose to 2S-hydroxy (3S, 4S) aldopentose is mannose isomerase. In another aspect, isomerase enzyme that converts 2-keto (3S, 4S) pentose to 2S-hydroxy (3S, 4S) aldopentose is L-ribose isomerase, L-arabinose isomerase and D-lyxose isomerase. Triple-site variant (W17Q, N90A, L129F) of mannose-6P-isomerase from *Geobacillus thermodenitrificans* has 7.1-fold higher activity over wild-type for the isomerization of L-ribulose to L-ribose (Lim Y. R. et al., 2012). Novel gene encoding L-ribose isomerase from *Acinetobacter sp.* has shown to isomerize L-ribulose to L-ribose (Mizanur R. M. et al., 2001 & U. S. Pat. No. 6,037,153). L-arabinose isomerase has shown modest L-ribose isomerase activity (Muynck C. D. et al., 2007). D-xylose isomerase from *Actinoplanes missouriensis* mutated at Val135Asn for the isomerization of L-ribose to L-ribulose (Santa H. et al., 2005). D-lyxose isomerase from *Cohnella laevoribosii* RI-39 has shown to isomerize L-ribose to L-ribulose (Cho E. A. et al., 2007). D-lyxose isomerase from *E. Coli* O157:H7 is shown to isomerize L-ribose to L-ribulose (Staalduinen L. M. et al., 2010). D-lyxose isomerase from *Providencia stuartii* shown to isomerize L-ribose to L-ribulose (Kwon H. J et al., 2010). Mannose 6P-isomerase from *Bacillus subtilis* (Yeom S. J. et al., 2009) *Geobacillus thermodenitrificans* (Yeom S. J. et al., *Biotechnol. Lett.*, 2009). *Thermus thermophiles* (Yeom S. J. et al., 2011) shown to isomerize L-ribose to L-ribulose. A person skilled in the art may use one or more of the wild-type or mutated enzymes as mentioned above for the isomerization of L-ribose to L-ribulose.

[00135] In another aspect of this embodiment, isomerase enzyme that is capable of converting 2-keto (3S, 4S) pentose to 2R-hydroxy (3S, 4S) aldopentose is used for the production of L-arabinose from L-ribulose. L-arabinose isomerase from *Geobacillus thermodenitrificans* has shown to be active for the isomerization of L-arabinose to L-ribulose (Yeom S. J et al., 2008). U. S. Pat. No. 6,140,498 disclosed continuous production of L-ribose by isomerization of L-arabinose to L-ribulose using L-arabinose isomerase. L-arabinose isomerase has been immobilized on aminopropyl glass (Wang Z. Y. et al., 2011.) alginate

(Zhang Y. W. et al., 2010) and other supports (Zhang Y. W. et al., 2009) for the enhanced activity. A person skilled in the art may use one or more of the wild-type or mutated L-arabinose isomerase and practice one or more of the methods and processes as mentioned above for the isomerization of L-arabinose to L-ribulose.

[00136] The carbohydrates produced from methane and other fossil hydrocarbon sources according to the present invention can be distinguished from plant-derived carbohydrates such as dextrose, sucrose, xylose and glycerol on the basis of their carbon 14 content of the resulting carbohydrates following the method ASTM-D6866 provided by American Society of Testing and Materials. Cosmic radiation produces ^{14}C (“radiocarbon”) in the stratosphere by neutron bombardment of nitrogen. ^{14}C atoms combine with oxygen atom in the atmosphere to form heavy $^{14}\text{CO}_2$, which, except in the radioactive decay, is indistinguishable from the ordinary carbon dioxide. CO_2 concentration and the $^{14}\text{C}/^{12}\text{C}$ ratio is homogeneous over the globe and because it is used by the plants, the ratio $^{14}\text{C}/^{12}\text{C}$ is retained by the biomass while the content of ^{14}C in the fossil materials, originally derived from photosynthetic energy conversion, has decayed due to its short half-life of 5730 years. By means of analyzing the ratio of ^{14}C to ^{12}C , it is possible to determine the ratio of fossil fuel derived carbon to biomass-derived carbon. International Patent Application Publication No. WO2009/155085 A2 and U.S. Patent No. 6,428,767 provide details about the use of ASTM-D6866 method for determining percent of biomass-derived carbon content in a chemical composition. The details related carbon dating disclosed in the U.S. Patent No. 6,428,767 is incorporated herein by reference. An application note from Perkin Elmer entitled “Differentiation between Fossil and Biofuels by Liquid Scintillation Beta Spectrometry – Direct Method” provides details about the methods involving ASTM Standard D6866.

[00137] In one embodiment of the present invention, a process for the production of D-hexose from DHA is provided. D-Hexoses are made from DHA through enzymatic means using one or more of the enzymes belong to the classes such as isomerases, aldolases and epimerases as illustrated in Figure 3. In a method to produce D-ketohexoses such as D-fructose, D-psicose, D-sorbose and D-tagatose, DHA is isomerized to DGA by the action of either a wild-type or mutated isomerase enzyme. The resulting product mixture comprising DHA and DGA is further

subjected to aldol reaction catalyzed by the action of either a wild-type or mutated aldolase enzyme. The isomerization reaction followed by aldol condensation reaction results in a mixture containing DHA, DGA and D-ketohexoses. D-ketohexose is purified by chromatography-based separation methods while DHA and DGA are recycled to the aldol condensation step. In another aspect of this embodiment, the D-ketohexose from aldolase reaction is subjected to the action of either wild-type or mutated epimerase enzyme. For example, D-fructose is epimerized to yield a mixture of D-fructose and D-psicose and vice versa by the action of 3-epimerases. Similarly, D-tagatose is epimerized to a mixture of D-tagatose and D-sorbose and vice versa by the action of 3-epimerases. In addition, D-psicose is epimerized to a mixture of D-psicose and D-sorbose and vice versa by the action of 4-epimerases. Chromatography-based separation methods are used for the separation of desired D-ketohexose from undesired D-ketohexose. The remaining D-ketohexose is recycled to epimerization reaction.

[00138] In another aspect of the present invention, a method to produce D-aldohehexoses such as D-glucose, D-mannose, D-altrose, D-allose, D-idose, D-gulose, D-talose, and D-galactose from D-hexoses is provided. D-ketohexoses such as D-fructose, D-psicose, D-sorbose and D-tagatose are isomerized by the action of either a wild-type or mutated isomerases enzyme to a mixture of D-aldohehexose and D-ketohexose. For example, D-fructose is isomerized to a mixture of D-fructose and D-glucose or a mixture of D-fructose and D-mannose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-fructose. Similarly, D-psicose is isomerized to a mixture of D-psicose and D-allose or a mixture of D-psicose and D-altrose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-psicose. D-sorbose is isomerized to a mixture of D-sorbose and D-gulose or a mixture of D-sorbose and D-idose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-sorbose. D-tagatose is isomerized to a mixture of D-tagatose and D-galactose or a mixture of D-tagatose and D-talose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-tagatose. The desired D-aldohehexose is purified by chromatography-based separation methods. The remaining D-ketohexoses are recycled for isomerization. In addition, D-mannose, D-glucose, D-idose and D-galactose produced by one or more methods as described here is used for the production of mannitol, sorbitol, iditol and galacitol by means of chemical hydrogenation.

[00139] In another embodiment of the present invention, a process for producing L-hexoses from DHA is provided. L-Hexoses are made from DHA using isomerase, aldolase and epimerase enzymes as illustrated in Figure 4. In a method to produce L-ketohexoses such as L-fructose, L-psicose, L-sorbose and L-tagatose; DHA is subjected to the action of either a wild-type or mutated isomerase enzyme catalyst to yield a mixture of DHA and LGA. The resulting mixture is further subjected to aldol reaction using a wild-type or mutated aldolase enzyme. The combination of isomerization reaction followed by aldol condensation reaction results in a mixture DHA, LGA and L-ketohexoses. L-ketohexose is purified by chromatography-based separation methods while the remaining DHA and LGA are recycled to the aldol condensation step for the further production of L-ketohexose. In another method to produce L-ketohexoses such as L-fructose, L-psicose, L-sorbose and L-tagatose; L-ketohexoses are epimerized by the action of either wild-type or mutated epimerase enzyme. For example, L-fructose is epimerized to a mixture of L-fructose and L-psicose and vice versa by the action of 3-epimerases. Similarly, L-tagatose is epimerized to a mixture of L-tagatose and L-sorbose and vice versa by the action of 3-epimerases. In addition, L-psicose is epimerized to a mixture of L-psicose and L-sorbose and vice versa by the action of 4-epimerases. Chromatography-based separation methods are used for the separation of desired L-ketohexose from undesired L-ketohexose. The remaining L-ketohexose is recycled for epimerization.

[00140] In another aspect of this embodiment, a method to produce L-aldohexoses such as L-glucose, L-mannose, L-altrose, L-allose, L-idose, L-gulose, L-talose, and L-galactose is provided. L-ketohexoses such as L-fructose, L-psicose, L-sorbose and L-tagatose are isomerized by the action of either a wild-type or mutated isomerases enzyme catalyst to a mixture of L-aldohexose and L-ketohexose. For example, L-fructose is isomerized to a mixture of L-fructose and L-glucose or a mixture of L-fructose and L-mannose by the action of isomerases that introduce 2S and 2R hydroxyl groups to L-fructose. Similarly, L-psicose is isomerized to a mixture of L-psicose and L-allose or a mixture of L-psicose and L-altrose by the action of isomerases that introduce 2S and 2R hydroxyl groups to L-psicose. L-sorbose is isomerized to a mixture of L-sorbose and L-gulose or a mixture of L-sorbose and L-idose by the action of isomerases that introduce 2S and 2R hydroxyl groups to L-sorbose. L-tagatose is isomerized to a mixture of L-tagatose and L-galactose or a mixture of L-tagatose and L-talose by the action of

isomerases that introduce 2S and 2R hydroxyl groups to L-tagatose. The desired D-aldohehexose is purified by chromatography- based separation methods. The remaining L-ketohexose sugar is recycled for isomerization.

[00141] In another embodiment of the present invention, a process for the production of D-pentose from DHA is provided. D-pentoses are made from DHA and D-glyceraldehyde using isomerase, aldolase, transketolase and epimerase enzymes as illustrated in Figure 5 and Figure 6. In a method to produce D-ketopentoses such as D-xylulose and D-ribulose, DHA is isomerized to DGA by the action of either a wild-type or mutated isomerases enzyme catalyst to yield a mixture of DHA and DGA. The resulting mixture is further subjected to aldol reaction by a wild-type or mutated aldolase enzyme. The isomerization reaction followed by aldol condensation reaction results in a mixture containing DHA, DGA and D-fructose. The resulting mixture is further subjected to transferase reaction by the action of either a wild-type or mutated transferase enzyme such as transketolase. The combination of isomerization reaction followed by transfer reaction results in a mixture of sugars containing DHA, DGA, D-fructose, D-xylulose and D-erythrose. D-xylulose is purified by chromatography-based separation methods and DHA, DGA and D-fructose are recycled to the transferase enzyme reaction step.

[00142] In another aspect of the present embodiment to produce D-xylulose; DHA is isomerized to DGA by the action of a wild-type or mutated isomerases enzyme to yield a mixture of DHA and DGA. The resulting mixture is further subjected to a transfer reaction catalyzed by either a wild-type or mutated dihydroxyacetone synthase enzyme as illustrated in Figure 7. The combination of isomerization reaction followed by transferase reaction results in a mixture of sugars containing one or more of the DHA, DGA and D-xylulose. D-xylulose is purified by chromatography-based separation methods and DHA and DGA are recycled to the transferase reaction step for the further production of D-xylulose. In another method to produce D-ketopentoses such as D-xylulose and D-ribulose, D-ketopentoses are epimerized by the action of either wild-type or mutated epimerase enzyme as illustrated in Figure 6. For example, D-xylulose is epimerized to a mixture of D-xylulose and D-ribulose and vice versa by the action of 3-epimerases. Chromatography-based separation methods are used for the separation of desired

D-ketopentose from undesired D-ketopentose. The undesired D-ketopentose is recycled for epimerization.

[00143] In a method to produce D-aldopentoses such as D-xylose, D-lyxose, D-ribose and D-arabinose, D-ketopentoses such as D-xylulose and D-ribulose, are isomerized by the action of a wild-type or mutated isomerases enzyme to yield a mixture of D-aldopentose and D-ketopentose as illustrated in Figure 6. For example, D-xylulose is isomerized to a mixture of D-xylulose and D-xylose or a mixture of D-xylulose and D-lyxose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-xylulose. Similarly, D-ribulose is isomerized to a mixture of D-ribulose and D-ribose or a mixture of D-ribulose and D-arabinose by the action of isomerases that introduce 2R and 2S hydroxyl groups to D-ribulose. The isomerization reaction results in a product mixture containing C3 sugars, C2 sugar and C5 sugar. The desired D-aldopentose is purified by chromatography- based separation methods. The undesired products are recycled for isomerization. In addition, D-xylose, D-ribose and D-arabinose produced by one or more methods as described here is used for the production of xylitol, arabitol and ribitol by means of chemical hydrogenation.

[00144] In another aspect of this embodiment a process for the production of L-pentose is provided. L-pentoses are made from DHA and DGA using isomerase, aldolase, transferase and epimerase as illustrated in Figure 7. In a method to produce L-pentoses from DHA, one or more of the enzymatic reactions from a group of isomerization, transfer reaction and epimerization are used. In a method to produce L-ketopentoses such as L-ribulose, D-xylulose produced by the method as illustrated in Figure 5 and Figure 7 is epimerized using epimerase enzyme to a mixture of D-xylulose and L-ribulose. Chromatography- based separation methods are used for the separation of desired L-ketopentose from undesired D-ketopentose. The undesired D-ketopentose is recycled for further epimerization. In a method to produce L-aldopentoses such as L-ribose and L-arabinose, L-ketopentoses such as L-ribulose, is isomerized by the action of either a wild-type or mutated isomerases enzyme to yield a mixture of L-aldopentose and L-ketopentose. For example, L-ribulose is isomerized to a mixture of L-ribulose and L-ribose or a mixture of L-ribulose and L-arabinose by the action of isomerases that introduce 2S and 2R hydroxyl groups to L-ribulose. The isomerization reaction results in a product mixture

containing one or more of C3 sugars, and C5 sugar. The desired L-aldopentose is purified by chromatography- based separation methods. The undesired products are recycled for isomerization.

[00145] In another aspect of this embodiment a process for the production of D-tetrose is provided. *D*-tetrose is prepared from DHA and DGA using isomerase, aldolase and transketolase enzymes as illustrated in Figure 5. In a method to produce D-erythrose, DHA is isomerized to yield a mixture of DHA and DGA which is subject to aldolase action to a mixture of DHA, DGA and D-fructose which is subjected to transferase reaction by the action of either a wild-type or mutated transketolase. The combination of isomerization reaction followed by aldol condensation and transferase reaction results in a mixture of sugars containing DHA, DGA, D-fructose, D-xylulose and D-erythrose. D-erythrose is purified by chromatography-based separation methods and DHA, DGA and D-fructose are recycled to the transferase reaction step for the further production of D-erythrose. In addition, D-erythrose produced by one or more methods as described here is used for the production of D-erythritol by means of chemical hydrogenation.

[00146] In another aspect of this embodiment for the production of carbohydrates from DHA using enzyme based isomerization, aldol condensation and epimerization methods; a downstream separation method is used for the isolation of desired carbohydrates from the undesired co-products or starting materials. In one method, separation process used is a chromatographic based separation process. A non-limiting example of a chromatographic based separation technology is simulated moving bed (SMB) technology. Metal imbedded polystyrene based ion-exchange resins where the metal is the one or more combination of Na^+ , K^+ and Ca^{++} have established themselves as the standard in the industry for efficient and economical ketohexose enrichment by SMB. Some non-limiting examples of sugar chromatography ion-exchange resins are Puralite's Chromalite[®] PCR series ion-exchange resins; Finex[®] CS11GC, CS12GC and CS16GC ion-exchange resins; Dow Chemicals DOWEX MONOSPHERE[®] ion-exchange resins. If a mixture of ketohexoses/DHA/DGA in water is pumped through fixed bed packed with ion-exchange resins in the metal ion form,

ketohexoses, being more strongly attracted to the metal on the resin beads, spends more time immobile inside the beads. DHA and DGA is attracted less and therefore spends more time outside the beads, in the flowing stream of liquid in the voids between the separation resin beads. The net result is that DHA and DGA moves down the resin more rapidly than the ketohexoses. If a stream of liquid is withdrawn from farther down the column, that stream will be enriched in DHA and DGA compared to the feed. A stream removed from higher in the column will be enriched in ketohexose. Raffinate typically containing DHA and DGA is sent back in the process to be condensed. Extract typically containing ketohexose is further purified by crystallization to obtain 99% pure ketosugar. For example; U. S. Pat. No. 3,044,904 discloses a calcium based ion-exchange resin; U. S. Pat. No. 3,044,905 discloses a strontium based ion-exchange resin; U. S. Pat. No. 3,044,906 discloses a silver based ion-exchange resin; U. S. Pat. No. 3,471,329 discloses a hydrazine based ion-exchange resin; U. S. Pat. No. 2,818,851 discloses a borate based ion-exchange resin and U. S. Pat. No. 3,806,363 discloses a bisulfite based ion-exchange resin. In another method, separation process used is a fractional crystallization based separation process. If a mixture of ketohexoses/DHA/DGA in water is slowly cooled in the presence of a small amount of ketohexose as a seed catalyst, at appropriate temperature and concentration ketohexoses crystallize out due to its lower solubility in water compared to DHA and DGA. In some cases solvents such as ethanol, methanol or butanol may be used to facilitate the crystallization process. Crystals containing mainly ketohexose is filtered out and mother liquor mainly containing DHA and DGA is sent back in the process to be condensed. In another method, separation process used is a reverse osmosis separation process. If a mixture of ketohexose/DHA/DGA in water is passed across a cellulose acetate (CA) based or poly (vinyl alcohol) (PVA) based membrane, due to the affinity difference between DHA/DGA and ketohexoses in water, DHA/DGA tend to be more permeable through CA or PVA based membranes compared to ketohexoses. A stream removed from permeate typically containing DHA and DGA is sent back in the process to be condensed and the stream removed from retentate typically containing ketohexoses is further purified by crystallization process. In another method, ketohexoses can be separated from DHA and DGA by converting ketohexose into a calcium ketohexose by treatment with calcium chloride or calcium hydroxide. In yet another method, U. S. Pat. No. 4,014,711 discloses a

zeolite based adsorption/desorption method for the separation of ketohexose. A person skilled in the art may use one or more method as mentioned above for the isolation of ketohexose from DHA and DGA.

ANALYTICAL METHOD

[00147] **HPLC analyses:** HPLC analyses are performed on a XBridge™ C18, 5µm, 4.6 x 250 mm column from Waters (Milford, USA). Samples (10 µL) are injected and eluted with the following conditions. The solvent system used is: Solvent (A): 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and Solvent (B): 0.095% (v/v) TFA in CH₃CN/H₂O 4/1, gradient elution from 10% to 70% B in 30 min, flow rate 1 mL min⁻¹, detection 215 nm. **NMR analysis:** High field ¹H (500 MHz and 400 MHz) and ¹³C (101 MHz) nuclear magnetic resonance (NMR) analyses are carried out using Varian Anova-500, Varian Mercury-400 spectrometers and Bruker Avance 500 and Bruker Avance 400. Infrared spectra of compounds are performed with a FTIR-8400S (Shimadzu Corporation, Kyoto, Japan). Melting points of compounds were determined with a Kofler apparatus (Reichert Austria). Specific rotations are measured with a JASCO model DIP-370 (Japan) polarimeter. Mass spectra are performed using ES (electrospray technique) with a Hewlett Packard 5989B apparatus

Example 1

Production of Methanol from Methane

[00148] A diagram of the complete formaldehyde production process, based on methane, natural gas or biogas is shown in Figure 8. Natural gas with a composition of 95% methane, 3.5% ethane, and 1.5% propane is used. In all cases the amount of natural gas feed is maintained at 1000 Nm³/hr. A small flow of hydrogen of 20 Nm³/hr. is added to the natural gas in all cases. The steam to carbon (S/C) ratio is defined as the molar ratio of steam to carbon derived from hydrocarbons in the natural gas (i.e. excluding carbon in tail gas). The tail gas used in this example has one of the two compositions containing either high carbon monoxide content or low carbon monoxide content. A desulphurised natural gas is mixed with steam and tail gas containing high carbon monoxide. The resulting mixture is heated to 430° C and fed to an adiabatic pre-reformer. The pre-reformed mixture is heated to 600° C. The resultant mixture is fed to the autothermal reformer together with an oxidant (oxidant composition: 99.5% oxygen

and 0.5% argon) in which the synthesis gas is produced. The feed temperature of the oxygen is 200° C. The amount of tail gas added is adjusted to give a hydrogen-to-carbon monoxide ratio in the synthesis gas stream equal to 2.00. The ATR effluent temperature is 1050° C. All the reactions are assumed to be in equilibrium at reactor outlet conditions. The pressure throughout the system is 2.48 MPa. The tail gas temperature is 200° C. The steam-to-carbon ratio is 0.6. The production of synthesis gas (Nm^3 syngas produced/ Nm^3 oxygen consumed) is 5.03. The production of synthesis gas (Nm^3 syngas produced/ Nm^3 natural gas consumed) is 3.14. Hydrogen-to-carbon monoxide ratio in synthesis gas (H_2 mole/CO mole) is 2.00 when high carbon monoxide tail gas is used.

[00149] The catalyst used for the conversion of syngas to methanol comprises 67.4% by weight CuO, 21.4% by weight ZnO and 11.1% by weight Al_2O_3 . The catalyst composition used for this synthesis is produced by conventional manner. CuO/ZnO and Al_2O_3 catalyst (37.2 g catalyst with average particle diameter of 60 μm and particle density of 2.39 g/cm^3) described above is placed in a 260 ml fluidized or fixed bed reactor having an internal diameter of 0.31 m and a height of 20m. The reaction inlet gas temperature and pressure are set at 200°C and 80.0 $\text{kg}/\text{cm}^2\text{G}$ respectively; $\text{SV}=9,310$ (l/h); $\text{LV}=0.51$ m/sec; the boiler water temperature is set at 300°C. Under these condition, synthesis gas having of composition $\text{CO}/\text{H}_2=1/2$ (molar ratio) is introduced and a continuous reaction is initiated at a constant pressure of 80 $\text{kg}/\text{cm}^2\text{-G}$. and a temperature of 200°C, during which synthesis gas is continuously supplied into the reactor at a rate of 15 L/hr. Furthermore, a part of the supplied gas is removed as unreacted gas, while a part of the reaction liquid is removed at regular time intervals to maintain the liquid surface at a constant level. Analysis indicates that CO conversion rate and methanol selectivity are 97.9% and 94.4%, respectively and the catalyst activity is stable for 75 hours. During the reaction, an average yield of methanol [STY (space time yield)] on the basis of the solvent is 1.6 Kg-MeOH/L/hr. The appearance of the catalyst changes from black to dark reddish-brown after the reaction. Analysis indicates that its BET (Brunauer, Emmett & Teller) specific surface area was slightly reduced.

Example 2

Production of Formaldehyde from Methanol

[00150] A diagram of the complete formaldehyde production process, based on methanol is shown in Figure 9. The oxidation of methanol over the catalyst of the claimed invention is examined in a fixed-bed reactor with following dimensions: 1" outer diameter, 14 Birmingham Wire Gauge, 5' length steel tube and catalyst fillage is 18—48" (e.g., 36": the sum of 24" of Perstorp KH-44 Fe—Mo in the downstream region With 12" of V_2O_5/TiO_2 in the upstream region) with 0"—24" inert rings (e.g., inert ceramic rings from Perstorp Polyols, Inc. of Toledo, Ohio) on the bottom (i.e., between the catalyst bed and the outlet end) and 0"—24" inert rings on the top (i.e., between the inlet end and the catalyst bed). Each catalytic test consists of a gas stream of $CH_3OH/O_2/He$ with a methanol feed concentration of 9.1% and a total flow rate of 28.3 slpm (standard liters per minute). Methanol, oxygen, and nitrogen are present in a molar ratio of 9.1/9.6/81.3. In order to examine the full effect of temperature on methanol oxidation, the reactor temperature is varied from about 260° C to about 300° C under recycle conditions. The reaction products are analyzed with a gas chromatograph using the following parameters: MTI Q30H with one 14 meter OV-1 column, one 8 meter Stabilwax column and one 10 meter Molecular sieve 5A column obtained from MTI of Fremont, California, USA. The test results for a catalyst bed with 12" of V_2O_5/TiO_2 in the upstream region followed by 24" of Perstorp KH-44 Fe—Mo in the downstream region varies between the methanol conversion from 92% to 98% and formaldehyde selectivity from 78% to 90%. The best conversion and selectivity obtained from the process is about a 97% methanol conversion and a formaldehyde selectivity of 87% at 290° C in the laboratory.

Example 3

Production of DHA from Formaldehyde

[00151] A mixture of 4.86 g (60 mmol) of formalin (37% strength), 5.0 g of n-hexadecanol, 0.155 g (1.5 mmol) of triethylamine and 0.585 g of N-hexadecylnaphthothiazolium bromide is vigorously stirred at 100° C for 60 minutes. After the reaction is complete, the DHA content in the aqueous phase is determined by HPLC. After 60 min, the final concentration of formaldehyde in water is 0.20% wt/wt and the final concentration of DHA in water is 27.82% wt/wt and selectivity towards DHA is 98%.

Example 4

Production of D-fructose from DHA

Conversion of DHA to D-glyceraldehyde (DGA)

[00152] Xylose isomerase used for the production DGA from DHA is bought from commercial sources and used without further purification. The xylose isomerase catalyzed reactions of DGA and DHA in H₂O at pH 7.0 or 8.0 in the presence of 24 mM imidazole buffer and 10 mM MgCl₂ at 25 °C and I = 0.1 (NaCl) is monitored by HPLC. The reactions (10 mL volume) are initiated by making a 5-fold dilution of a 50 mM stock solution of DHA (I = 0.1, NaCl) into 30 mM imidazole buffer (I = 0.1, NaCl) containing 12.5 mM MgCl₂ to give final concentrations of 11 mM substrate, 24 mM imidazole, 10 mM MgCl₂ and 0.30 mM xylose isomerase for the reaction at pH 7.0 and 0.20 mM xylose isomerase for the reaction at pH 8.0. At timed intervals an aliquot (1 mL) is withdrawn and the solution is adjusted to pH $\approx$ 5 with 10 μ L or 25 μ L of 1 M CH₃COOH for reactions at pH 7.0 or 8.0, respectively. Control experiments shows that the drop in pH effectively quenches the slow xylose isomerase catalyzed reaction. The enzyme is removed by ultrafiltration at 5°C using a Microcon YM-10 centrifugal filtration device. The filtrate is then flash frozen over solid CO₂ and stored at -15°C. HPLC analyses of the mixture are performed within one week of freezing. HPLC analyses is performed on a XBridgeTM C18, 5 μ m, 4.6 x 250 mm column from Waters (Milford, USA). Samples (10 μ L) are injected and eluted with the following conditions. The solvent system used is: Solvent (A): 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and Solvent (B): 0.095% (v/v)TFA in CH₃CN/H₂O 4/1; gradient elution from 10% to 70% B in 30 min, flow rate 1 mL min⁻¹; detection at 215 nm. Analysis of the reaction mixture after 24h shows a 1:1 mixture of DHA and enantiomerically pure DGA.

Plasmid constructions and site-directed mutagenesis

[00153] A plasmid expressing fructose aldolase gene *fsaA* with a specific mutation is constructed following the protocol described in this paragraph. Oligonucleotides for PCR amplification and mutagenesis are custom-synthesized. The wild type *fsaA* gene of *E. coli* strain MC4100 is PCR-amplified with primers FSA1 and FSA2 (see Table 1) thereby introducing the engineered restriction sites *Sall* and *PstI*, respectively, and the DNA fragment is ligated to vector pUC18 (Boehringer Mannheim) which has been restricted with *Sall/PstI* to yield plasmid

pUC18*fsa*. The *fsaA* gene is also cloned into vector pET16b (Novagen) using primers FSA3 and FSA4 introducing restriction sites *NcoI* and *NdeI*, respectively. For expression under the control of a *Ptac-lacI* vector, *fsaA* is subcloned from pET16*fsa* into vector pJF119EH (AmpR ; using restriction sites *XbaI* and *HindIII*, respectively. The amino acid residue alanine-129 of FSA is exchanged to a serine residue (A129S) by site-directed mutagenesis (QuikChange site-directed mutagenesis Kit, Stratagene) on plasmid pUC18*fsa* according to the manufacturer's protocols with the mutagenesis primers FSAS1 and FSAS2 (Table 1). The gene *fsaA129S* is subcloned from pUC18*fsaA129S* onto vector pJF119EH using the restriction sites of *XbaI* and *HindIII* to result in vector pJF119*fsaA129S*. To avoid plasmid loss during expression in LB medium, a kanamycin resistance cassette is incorporated into pJF119*fsaA129S*. The kanamycin resistance cassette is based on vector pKD4 where two *HindIII* sites had been engineered. This 1.5 kb kanamycin resistance cassette is introduced at the single *HindIII* site on plasmid pJF119*fsaA129S*. Thus, a more stable plasmid vector pJF119*fsaA129S*-kan is constructed. Primers and plasmids in use are presented in Tables 1 and 2, respectively.

Production of FSA

[00154] Wild type *fsaA* gene and its variant, *fsaA129S* are subcloned, onto vector pJF119EH to allow its expression from an IPTG-controlled expression vector in the bacterial strain *E. coli* K-12 DH5a. Besides the wild type gene (vector pJF*fsa*), the variant *fsaA129S* is also expressed from pJF119*fsaA129S*; both expression vectors confer ampicillin-resistance on their host strain but are notorious to be lost during expression even in the presence of ampicillin. Therefore, a kanamycin resistance cassette (see Plasmid constructions and Table 1) is added to the vector. Initially, FSA wt and FSA A129S were expressed in *E. coli* to compare the enzymatic activity. The aldolase enzymes are purified in an easy and inexpensive way by a heat treatment as is well known to the one skilled in the art of protein and enzyme biochemistry. Then, centrifugation and lyophilization of the supernatant is followed to obtain a pale brown powder with required aldolase enzyme activity (FSA powder) that can be directly used in the synthesis of fructose using DHA and DGA as the substrates. The specific activity of FSA is measured using two different enzymatic assays: the formation of fructose from DHA and DGA as the substrate or the cleavage of fructose to yield DHA and DGA.

Production of D-fructose

[00155] To a 1:1 mixture of dihydroxyacetone (86.4 mg, 0.96 mmol) and D-glyceraldehyde (86 mg, 0.96 mg) produced by the isomerization of dihydroxyacetone using isomerase class enzymes is added dihydroxyacetone (86.4 mg, 0.96 mmol) and the mixture is dissolved in NaHCO_3 50mM, pH 7.5 buffer (12 ml). To this solution FSA A129S powder (21 mg, 56U, 68 mg protein) prepared as described in the paragraphs above is added and the reaction mixture placed on a reciprocal shaker (200 rpm) at 25° C. After 48h or after the complete consumption of limiting substrate (D-glyceraldehyde), the reaction is filtered through active charcoal to remove the enzyme. The filtrate is adjusted to pH 5.0 with dilute HCl and freeze-dried. The residue is purified by flash column chromatography on silica gel with ethyl acetate/chloroform (1:1) to methanol (9:1) to remove all excess dihydroxyacetone for recycle. Then, the product is eluted with a mixture of (ethylacetate/chloroform, 1:1) : methanol : water from 86:10:4 to 75:20:5. After concentration under vacuum, the residue is lyophilized to afford D-fructose as white solid with isolated yield of 140 mg (81%).

Example 5

Production of L-fructose from DHA using Borate Buffer

[00156] The RhaD gene is amplified from chromosomal DNA of *E. coli* W3110 by PCR with following two primers; RhaD5Nde, 5'-cgcgcatatgcaaacattactcagtcctgg-3', and RhaD3Xho, 5'-cggctcgagttacagcgccagcgactggcgag-3'. The amplified 650 bp fragment is cloned into the *NdeI* and *XhoI* site of pETDuet vector (Novagene) to give pETDRhaD. *E. coli* BL21 (DE3) is transformed with pETDRhaD, and transformants are cultivated in LB medium containing carbenicillin (50 mg ml⁻¹) and IPTG (10 μM) at 37°C for 16 h. The recombinant RhaD is expressed in the soluble fraction, and detected as a major band on SDS-PAGE. The cells are harvested by centrifugation, washed with saline, and used as whole cell catalysts.

[00157] To a solution of L-glyceraldehyde (0.90 g, 10 mmol) and DHA (3.60 g, 40 mmol) in water (160 mL), 1M sodium borate buffer (40 mL, pH7.6) and toluene (400 mL) are added, and *E. coli* BL21 (DE3) cells harboring pETDRhaD (2.4 g by wet weight) are suspended. The reaction mixture is shaken at 37°C for 16 h, and cells are removed by centrifugation. The reaction mixture is passed through a column of Amberlite IR-120 (H⁺) resin (50 mL) and then eluted with additional water. The resulting solution is passed through a column of Amberlite

IRA-743 resin (120 mL) to remove borate. After evaporation, the mixture is purified using silica gel chromatography with ethyl acetate/methanol/water (40/10/7) as eluent. Fractions containing L-fructose are collected and concentrated to obtain the product with a yield of 1.66 g (9.2 mmol, 92% based on L-glyceraldehyde).

Example 6

Production of D-psicose from D-fructose

Mass production of Psicose 3-epimerase

[00158] The psicose 3-epimerase gene is obtained from the DNA of *Agrobacterium Zumeifaciens* ATCC33970 by a polymerase chain reaction (PCR) using a primer designed on the basis of DNA sequence of a gene that has been suggested as a tagatose 3 -epimerase gene of *Agrobacterium Zumeifaciens* C58. The PCR amplified psicose 3-epimerase gene is inserted in an expression vector, pET-24a(+) (Novagen, Inc.), by using restriction enzymes XhoI and NdeI to produce a recombinant expression vector pET-24a(+)/psicose 3-epimerase. This recombinant expression vector is transformed into *E. coli* BL21(DE3) by a conventional transformation method. The transformed strain *E. coli* BL21(DE3) is cryogenically stored in liquid nitrogen before being cultured for mass production. An inoculum of the cryogenically stored *E. coli* BL21(DE3) strain is inoculated in a 250-ml flask containing 50 ml of LB medium, and is precultured at 37° C. until absorbance of the preculture solution at 600 nm reaches 2.0. The preculture solution is added to a 7-L fermentor (Biotron Co., Ltd., KR) containing 5 L of a fermentation medium (10 g/L of glycerol, 1 g/L of peptone, 30 g/L of yeast extract, 0.14 g/L of potassium diphosphate and 1 g/L of sodium monophosphate), and the culture is allowed to grow. When absorbance of the main culture solution at 600 nm reaches 2.0, 1 mM of IPTG is added to the main culture solution to induce mass production of the psicose 3-epimerase. During the culturing process, the rate of stirring is maintained at 500 rpm, the rate of ventilation is controlled at 1.0 vvm, and the incubation temperature is kept at 37° C. The psicose 3-epimerase is purified using an affinity HisTrap HP column (a demineralized HiPrep 16/60 column), and a gel filtration Sephacryl S-100 HR column. The molecular weight of the purified psicose 3-epimerase is measured to confirm that the psicose 3-epimerase is a monomer having a molecular

Weight of 32,600 Da. The amino acid sequence of the psicose 3-epimerase is confirmed to be identical to the amino acid sequence of NCBI accession number NP_535228.

D-psicose production by Psicose 3-epimerase

[00159] The reaction of the psicose 3-epimerase is performed in a 50 mM PIPES buffer solution containing 14 units of psicose 3-epimerase/ml, 1 mM of Mn^{2+} ions, and 700 g/L D-fructose at pH 7.0 and at temperature to 60° C. at various times to allow the reaction to proceed sufficiently. The reaction is terminated by heating the reaction solution at 100° C. for 5 minutes, and the enzyme activity is measured by analyzing the reaction mixture for the content of D-psicose and D-fructose. D-psicose production in 120 minutes reaction time is determined. In general the rare sugars are not metabolized by the biological systems and therefore to separates the rare sugars from natural sugars, a fermentation based procedure can be followed to purify the rare sugar from natural sugars. For example, in this example, to purify D-psicose from D-fructose, the mixture containing both these sugars can be mixed with certain microbial strains which can ferment D-fructose but no D-psicose. Upon complete consumption of fructose in the mixture by the microbial cells, the microbial cells are removed by centrifugation or filtration methods and the D-psicose is recovered in an aqueous or organic solution. Although this method is explained with the example of D-psicose, the approach can be used in purifying any other non-fermentable sugars including D-tagatose and L-fructose.

Production of D-psicose by enzyme immobilization

[00160] In order to investigate the efficiency of the method of producing D-psicose, the psicose 3-epimerase is immobilized during the reaction. Immobilized psicose 3-epimerase is achieved by adding a solution of psicose 3-epimerase to a 2.5% sodium alginate solution having a volume 1.5 times the volume of the psicose 3-epimerase solution and adding this mixture to a 0.2M calcium ion solution with a syringe pump and a vacuum pump. The psicose 3-epimerase enzyme reaction is performed in the same manner as described in the paragraph above, except that immobilized psicose 3-epimerase enzyme is used. The reaction mixture as described in the above paragraph is treated with the immobilized psicose 3-epimerase and the reaction mixture is analyzed for the production of D-psicose and D-fructose.

Table 1 : Primers for amplification and mutagenesis

Name	Relevant genetic characteristics
FSA 1	5'GATGTGGT <u>CTCGACT</u> GTTCAGAGAGTTTCCC 3' (<i>Sall</i> site underlined)
FSA 2	5'GAGGCTGCAGAACGTCCGGTTAAATCGACG3' (<i>PstI</i> site underlined)
FSA 3	5'GAGGATGGCCATGGAACTG3' (<i>NcoI</i> site underlined)
FSA 4	5'CCAGGCCTCATATGACGCGGC3' (<i>NdeI</i> underlined)
FSA S1	5'GCATCAATACGATTAACGTAAGG <u>GCT</u> AACATATTCC3' (bp exchange in italics)
FSA S2	5'GGTGCGGAATATGTTA <u>AGC</u> CTTACGTTAATCGTATT3' (bp exchange in italics)
Reverse Primer	5'CAGGAAACAGCTATGAC3'

Table 2: Plasmids vectors in use

Name	Relevant genetic characteristics	Reference
pUC18 <i>fsa</i>	wild-type <i>fsaA</i> gene of <i>E.coli</i> in vector pUC 18 740 bp, Ap ^R , PCR amplified with primers FSA1 and FSA2, <i>PstI/Sall</i>	M. Schürmann, G. A. Sprenger, <i>J. Biol. Chem.</i> 2001 , 276, 11055-11061
pUC18 <i>fsaA129S</i>	pUC18 vector with <i>fsa</i> mutant gene; sitedirected exchange of bp 385-387 (with primers and FSAS2), Ala129>Ser	M. Schürmann, <i>dissertation</i> , University of Düsseldorf, 2001 .
pET16 <i>fsa</i>	wild-type <i>fsaA</i> gene, PCR amplified by primers FSA3 and FSA4, <i>NcoI</i> and <i>NdeI</i> sites engineered	M. Schürmann, <i>dissertation</i> , University of Düsseldorf, 2001 .
pJF119EH	<i>Ptac/lacI^q</i> expression vector, ampicillin resistance	J.P. Fürste, W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, E. Lanka, <i>Gene</i> , 1986 , 48, 119-131.
pJF119 <i>fsa</i>	<i>fsaA</i> gene from pET16 <i>fsa</i> (<i>XbaI/HindIII</i>) cloned into pJF119EH (<i>XbaI/HindIII</i>)	T. Inoue, <i>dissertation</i> , University of Stuttgart, Germany, 2006 .
pJF119 <i>fsaA129S</i>	<i>fsaA129S</i> gene from pUC18 <i>fsaA129S</i> subcloned (<i>XbaI/HindIII</i>) into pJF119EH (<i>XbaI/HindIII</i>)	T. Inoue, <i>dissertation</i> , University of Stuttgart, Germany, 2006 .

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[00161] All scientific references cited in this section and all patent documents cited all through the sections above are listed for the convenience of the reader. All scientific reference and all patent documents cited in this patent application are incorporated by reference in its entirety.

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What is claimed is:

1. A method for producing carbohydrate from dihydroxyacetone comprising the steps of:
 - a) subjecting said dihydroxyacetone to isomerase enzyme reaction to yield glyceraldehyde;
 - b) combining said dihydroxyacetone and said glyceraldehyde and subjecting the combined mixture to aldolase enzyme reaction to yield a carbohydrate with six carbons; and
 - c) optionally subjecting the aldolase enzyme reaction mixture from step (b) to a fractionation procedure to recover the carbohydrate with a six carbons free of dihydroxyacetone and glyceraldehyde.
2. The method of claim 1, wherein said dihydroxyacetone is in a non-phosphorylated form.
3. The method of claim 1, wherein said dihydroxyacetone is derived from fossil hydrocarbons.
4. The method of claim 1, wherein said dihydroxyacetone is derived from biomass resources.
5. The method of claim 1, wherein said dihydroxyacetone is derived from glycerol.
6. The method of claim 1, wherein said dihydroxyacetone is derived from CO₂ released from industrial plants.
7. The method of claim 1, wherein said glyceraldehyde is D-glyceraldehyde and said isomerase enzyme is selected from a group consisting of triose isomerase, L-arabinose isomerase, xylose isomerase, L-rhamnose isomerase, ribose isomerase and any mutant derivatives thereof.
8. The method of claim 7, wherein said aldolase enzyme is selected from a group consisting of fructose aldolase, fuculose aldolase, rhamnulose aldolase, tagatose aldolase and any mutant derivatives thereof.
9. The method of claim 7, wherein said carbohydrate with six carbons is selected from a group consisting of D-fructose, D-psicose, D-sorbose, and D-tagatose.
10. The method of claim 8, further comprising a isomerization step wherein said carbohydrate with six carbons is subjected to an isomerization reaction using an isomerase enzyme selected from a group consisting of D-mannose isomerase, lyxose

- isomerase, xylose isomerase, D-arabinose isomerase, L-fucose isomerase, L-rhamnose isomerase, ribose isomerase, L-Arabinose isomerase and any mutant derivatives thereof to yield a carbohydrate selected from a group consisting of D-mannose, D-glucose, D-altrose, D-allose, D-idose, D-gulose, D-talose, and D-galactose.
11. The method of claim 1, wherein said glyceraldehyde in step (a) is L-glyceraldehyde and said isomerase enzyme is selected from a group consisting of L-ribose isomerase, D-arabinose isomerase, D-lyxose isomerase, L-fucose isomerase, mannose isomerase and any mutant derivatives thereof.
 12. The method of claim 11, wherein said aldolase enzyme is selected from a group consisting of fructose aldolase, fuculose aldolase, rhamnulose aldolase, tagatose aldolase and any mutant derivatives thereof.
 13. The method of claim 11, wherein said carbohydrate with six carbons is selected from a group consisting of L-fructose, L-psicose, L-sorbose, and L-tagatose.
 14. The method of claim 12, further comprising an isomerization step wherein said carbohydrate with six carbons is subjected to an isomerization reaction using an isomerase enzyme selected from a group consisting of D-arabinose isomerase, D-mannose isomerase, D-lyxose isomerase, xylose isomerase, L-arabinose isomerase, L-fucose isomerase, L-rhamnose isomerase, L-ribose isomerase, ribose isomerase, galactose-6P isomerase and any mutant derivatives thereof to yield a carbohydrate selected from a group consisting of L-mannose, L-glucose, L-altrose, L-allose, L-idose, L-gulose, L-talose, and L-galactose.
 15. The method of claim 1, wherein said glyceraldehyde is D-glyceraldehyde, said isomerase is selected from a group consisting of triose isomerase, L-arabinose isomerase, xylose isomerase, L-rhamnose isomerase, ribose isomerase and mutant derivatives thereof, said aldolase enzyme is tagatose aldolase and said carbohydrate is D-tagatose.
 16. The method of claim 1, wherein said glyceraldehyde is D-glyceraldehyde, said isomerase is selected from a group consisting of triose isomerase, L-arabinose isomerase, xylose isomerase, L-rhamnose isomerase, ribose isomerase and mutant derivatives thereof, said aldolase enzyme is fructose aldolase and said carbohydrate is D-fructose.
 17. The method of claim 16, further comprising the steps of:
 - (a) subjecting said fructose to epimerase enzyme reaction to yield D-Tagatose; and

- (b) subjecting epimerase enzyme reaction mixture from step (a) at the end of the epimerase enzyme reaction to fermentation process using a microbial biocatalyst to deplete the fructose remaining after epimerase enzyme reaction;
 - (c) removing the microbial biocatalysts at the end of the fermentation process; and
 - (d) recovering D-tagatose in a pure form through a fractionation process.
18. The method of claim 1, wherein said glyceraldehyde is L-glyceraldehyde, said isomerase is selected from a group consisting of L-ribose isomerase, D-arabinose isomerase, d-lyxose isomerase, mannose isomerase, L-fucose isomerase and mutant derivatives thereof, said aldolase enzyme is rhamnulose aldolase and said carbohydrate is L-fructose.
19. The method of claim 1, wherein said glyceraldehyde is L-glyceraldehyde, said isomerase is selected from a group consisting of L-ribose isomerase, D-arabinose isomerase, d-lyxose isomerase, mannose isomerase, L-fucose isomerase and mutant derivatives thereof, said carbohydrate with six carbons is L-tagatose and said aldolase enzyme is fuculose aldolase.
20. The method of claim 16 further comprising a step of subjecting said D-fructose to a transketolase reaction with D-glyceraldehyde to yield a molecule of D-xylulose and a molecule of D-erythrose.
21. The method of claim 20 further comprising a step of hydrogenation of D-erythrose to yield D-erythritol.
22. A method for producing carbohydrate from dihydroxyacetone comprising the steps of:
- a) subjecting said dihydroxyacetone to isomerase enzyme reaction to yield glyceraldehyde;
 - b) combining said dihydroxyacetone and said glyceraldehyde and subjecting the combined mixture to transferase enzyme reaction to yield a carbohydrate with five carbons and formaldehyde; and
 - C) subjecting reaction mixture from transferase enzymes mixture to a fractionation process to recover the carbohydrate with five carbons free of formaldehyde
23. The method of claim 20 and claim 22 further comprising a step of subjecting said D-xylulose to an epimerization reaction to yield D-ribulose.

24. The method of claim 20 and claim 22, further comprising an isomerization step wherein said carbohydrate with five carbons is subjected to an isomerization reaction using an isomerase enzyme selected from a group consisting of, D-arabinose isomerase, D-mannose isomerase, D-lyxose isomerase, xylose isomerase, L-fucose isomerase, ribose isomerase and any mutant derivatives thereof to yield a carbohydrate selected from a group consisting of D-xylose, D-lyxose, D-arabinose and D-ribose.
25. The method of claim 20 and claim 22 further comprising a step of subjecting said D-xylulose to an epimerization reaction to yield L-ribulose.
26. The method of claim 25, further comprising an isomerization step wherein said L-ribulose is subjected to an isomerization reaction using an isomerase enzyme selected from a group consisting of, L-arabinose isomerase, D-mannose isomerase, D-lyxose isomerase, L-ribose isomerase and any mutant derivatives thereof to yield a carbohydrate selected from a group consisting of L-arabinose and L-ribose.
27. A carbohydrate produced according to the method in claim 3.
28. A process for manufacturing dihydroxyacetone comprising the steps of:
 - a) preparing syngas from methane;
 - b) liquefying syngas to yield methanol
 - c) producing formaldehyde from methanol; and condensing formaldehyde to yield dihydroxyacetone.

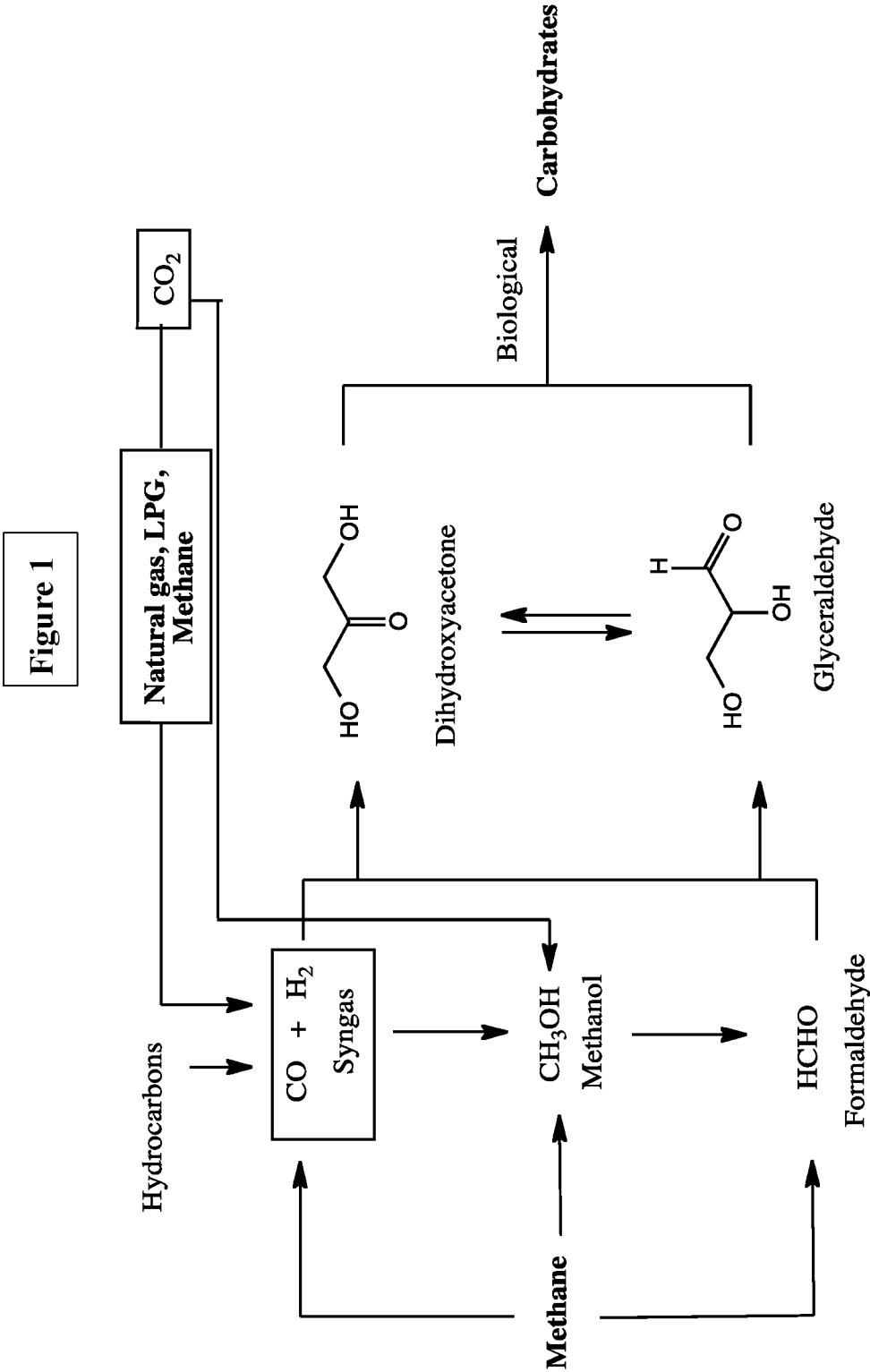


Figure 3

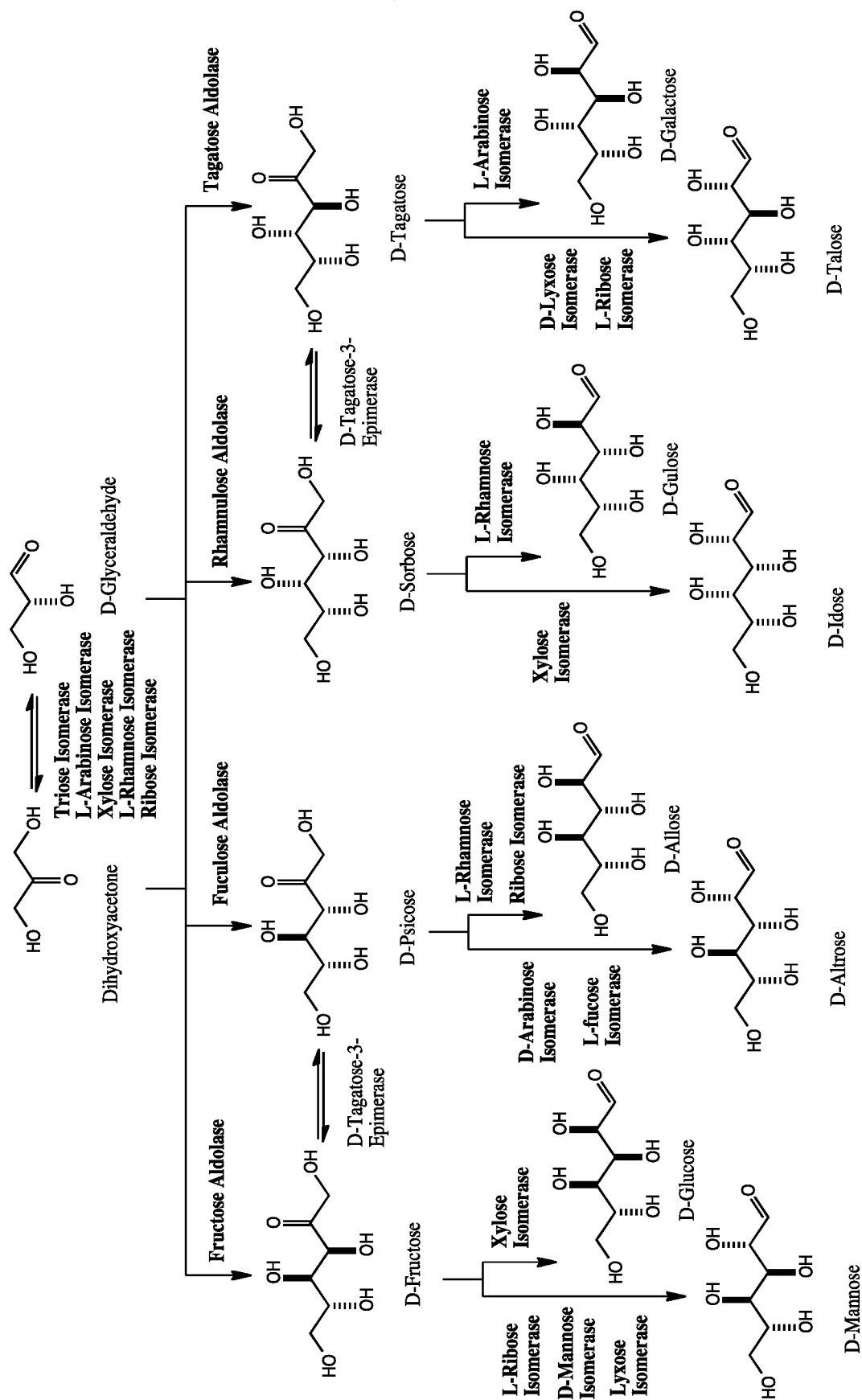
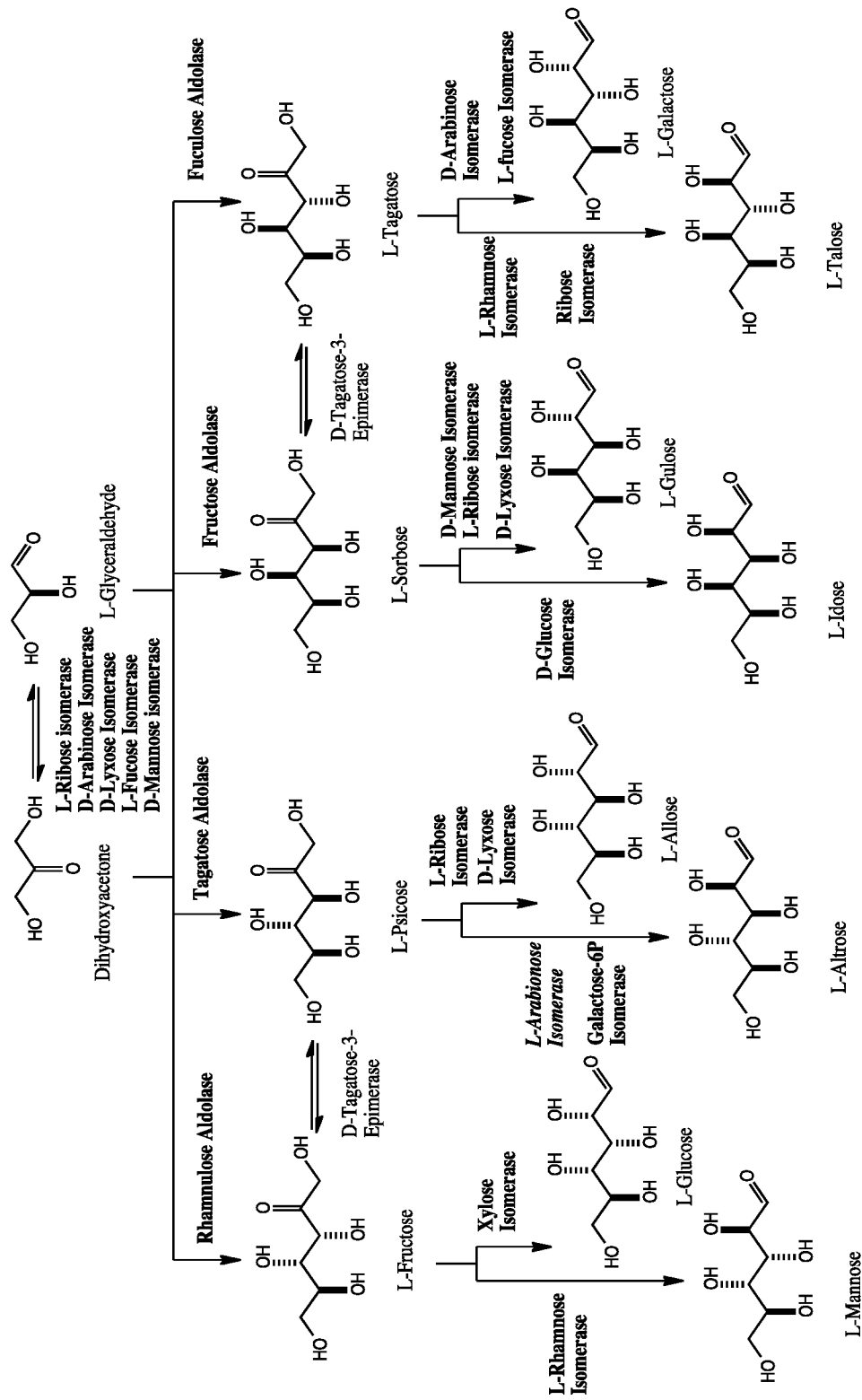
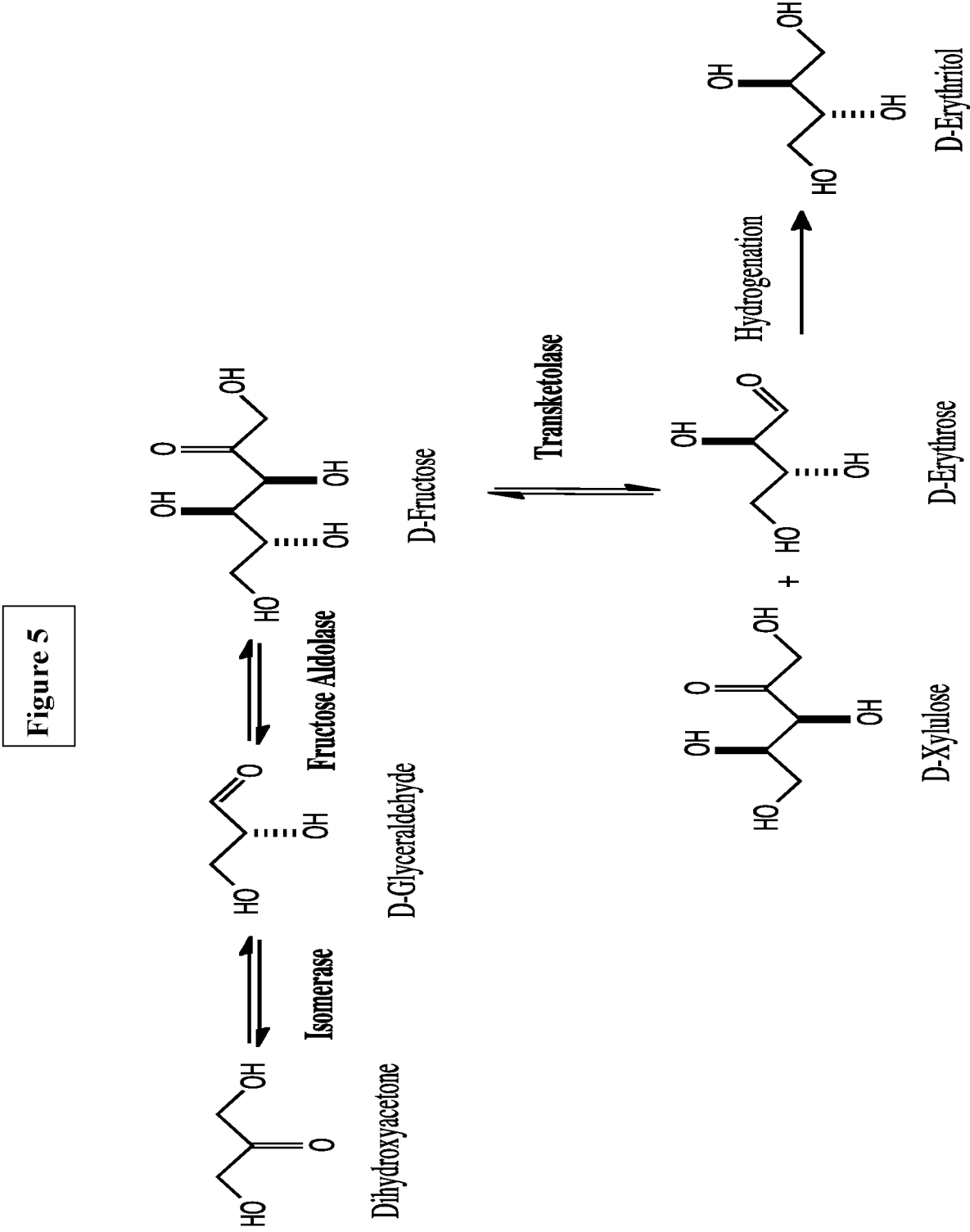


Figure 4





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Figure 6

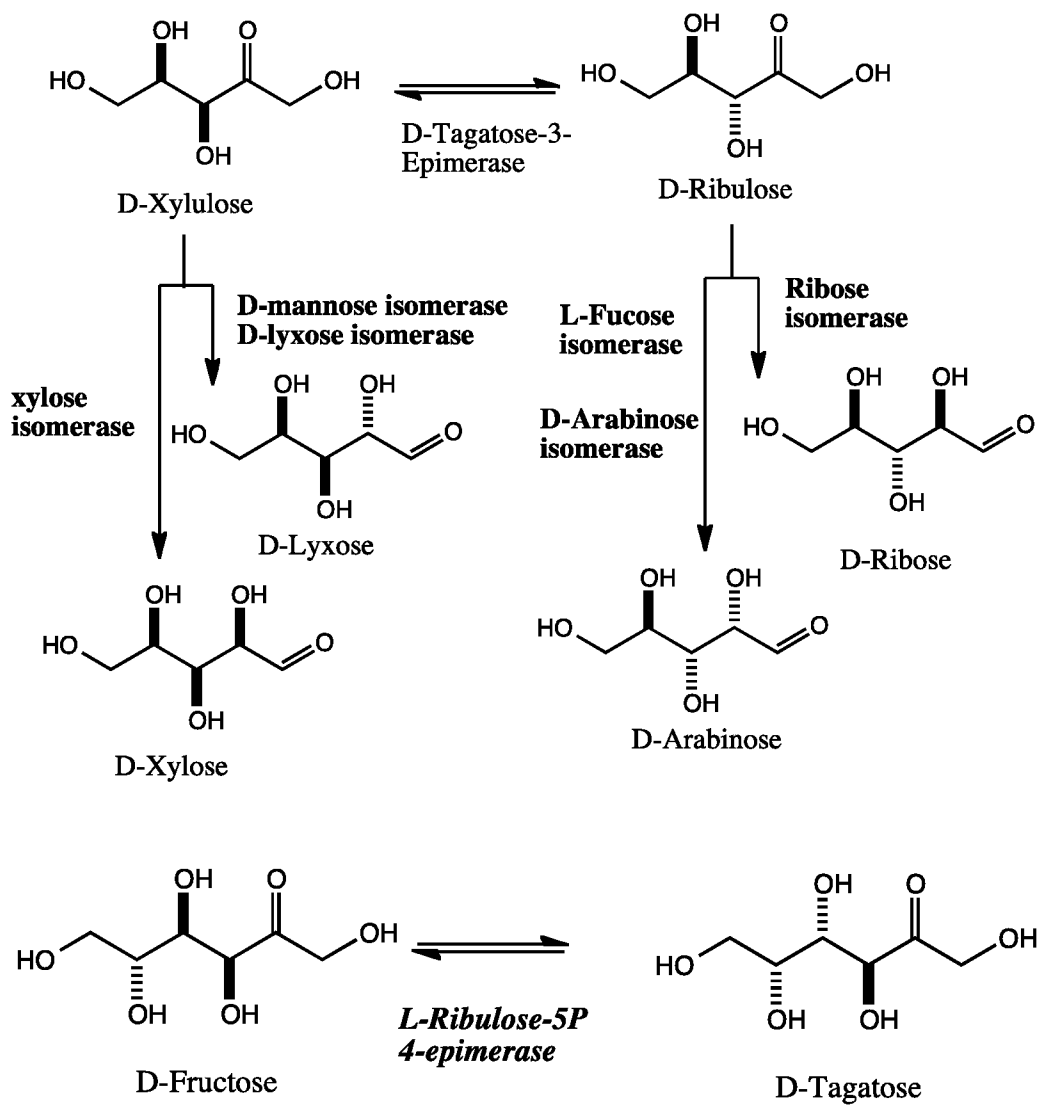


Figure 7

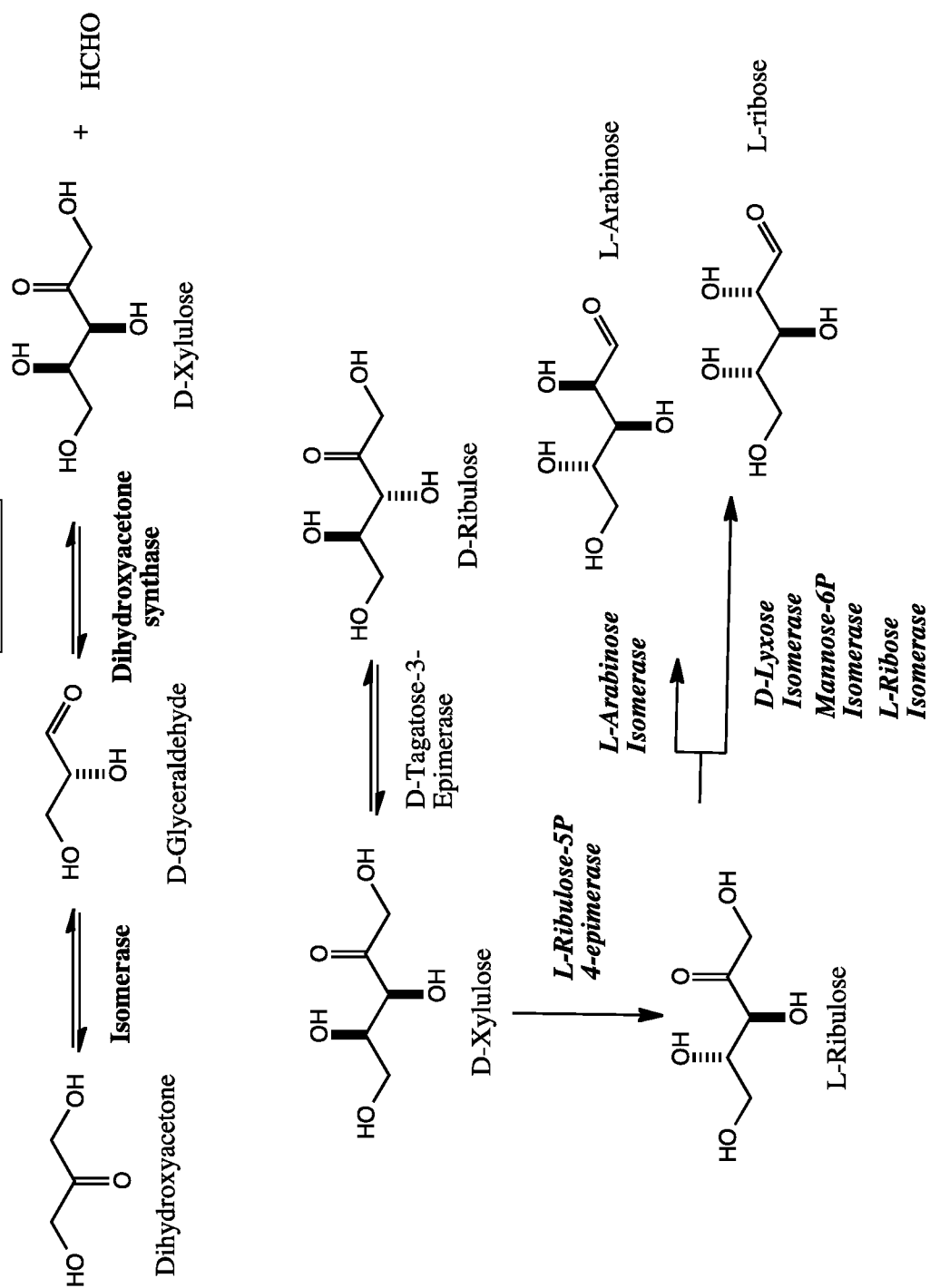


Figure 8

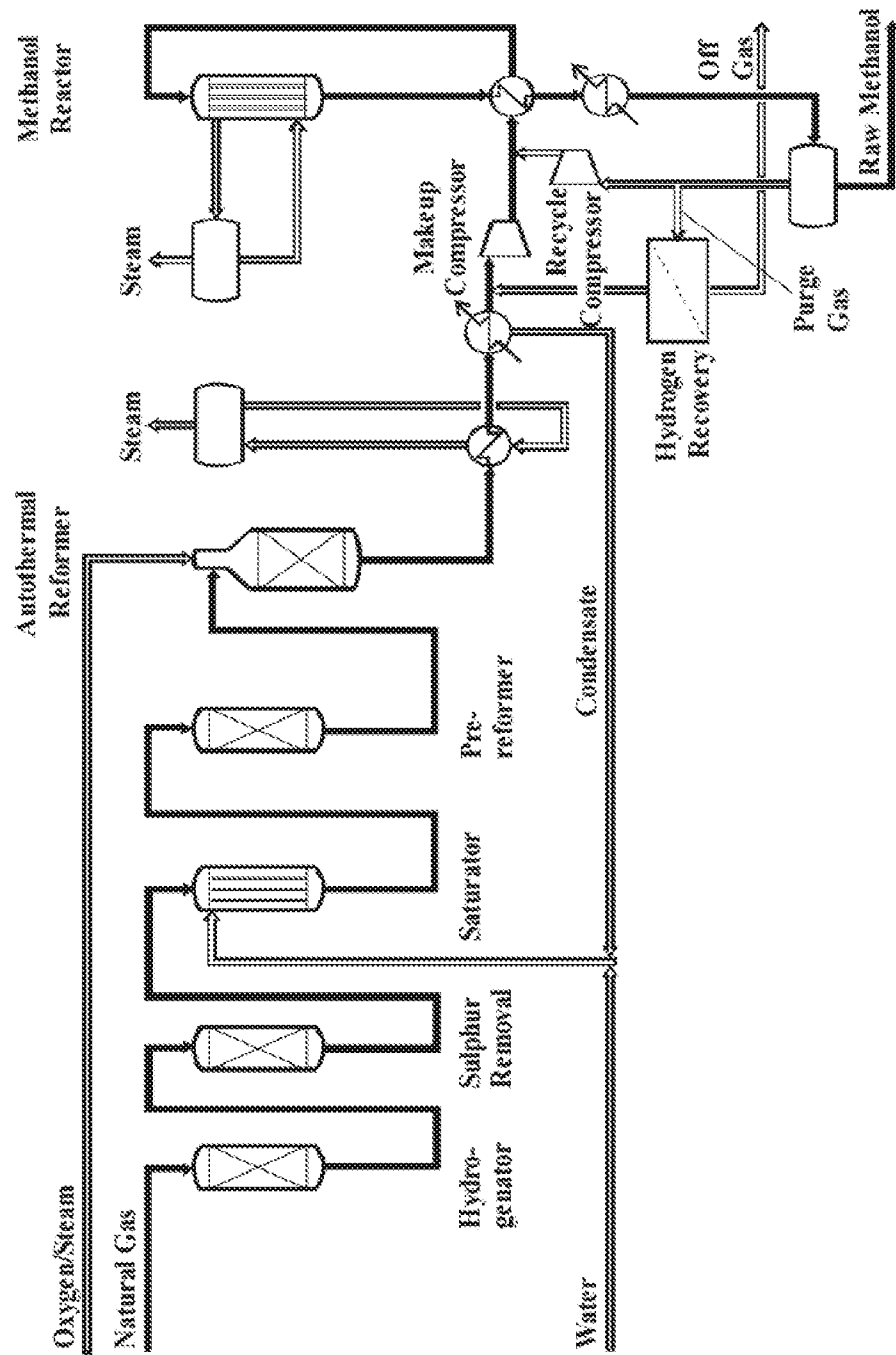
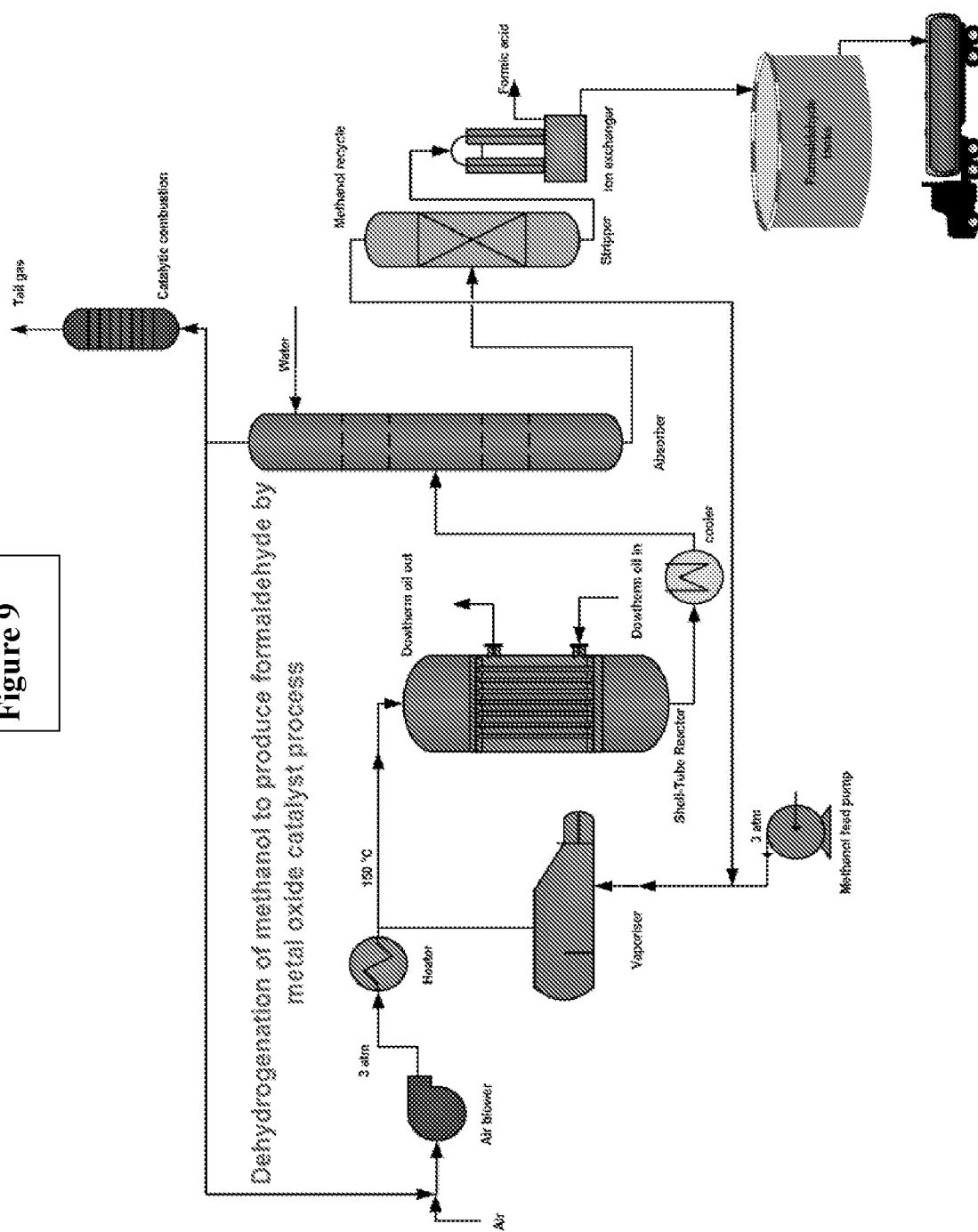


Figure 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/036708**A. CLASSIFICATION OF SUBJECT MATTER****C12P 19/02(2006.01)i, C12P 19/24(2006.01)i, C07C 45/27(2006.01)i, C07C 49/17(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P 19/02; C07C 45/38; C07H 1/08; C07H 1/06; C07C 45/00; C12P 19/24; C07C 45/27; C07C 49/17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: dihydroxyacetone, glyceraldehyde, isomerase, aldolase, carbohydrate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FUSHINOBU, SHINYA et al., 'Structural basis for the bifunctionality of fructose-1,6-bisphosphate aldolase/phosphatase', Nature, 09 October 2011, Vol. 478, No. 7370, pp. 538-541 See abstract.	1-22, 27-28
X	US 8802843 B2 (OROSKAR, ANIL R. et al.) 12 August 2014 See claim 1; column 3, lines 27-28.	27
A		1-22, 28
Y	US 5410089 A (GEHRER, EUGEN et al.) 25 April 1995 See claim 1.	28
Y	KHIRSARIYA, PRIYANK et al., 'Single Step Oxidation of Methane to Methanol -Towards Better Understanding', PROCEEDIA ENGINEERING, 2013, Vol. 51, pp. 409-415 See abstract.	28
Y	US 8513470 B2 (ESTENFELDER, MARVIN et al.) 20 August 2013 See claim 1.	28



Further documents are listed in the continuation of Box C.



See patent family annex.

* 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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 November 2016 (22.11.2016)

Date of mailing of the international search report

23 November 2016 (23.11.2016)

Name and mailing address of the ISA/KR

International Application Division

Korean Intellectual Property Office

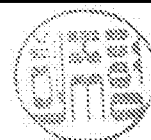
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

Facsimile No. +82-42-481-8578

Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/036708

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 26
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:
Claim 26 is unclear since it is referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 23-25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- Invention 1 (claims 1-26): A method for producing carbohydrate from dihydroxyacetone.
- Invention 2 (claim 27): A carbohydrate produced according to the method in claim 3.
- Invention 3 (claim 28): A process for manufacturing dihydroxyacetone.

The common technical feature among inventions 1-3 is dihydroxyacetone. However, the technical feature of invention 2 is disclosed in the following prior document: US 8802843 B2, 12 August 2014.

Thus, there is no technical relationship left over the prior art among the claimed inventions, leaving the claims without a single general inventive concept.

Therefore, the present application does not meet the requirements of unity of invention (Rule 13.1 and 13.2 PCT).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
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.:
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.:

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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/036708

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