

COMPOSITIONS AND METHODS FOR 2,5-FURAN DICARBOXYLIC ACID PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. provisional patent application Serial No. 62/988,841 filed March 12, 2020, and entitled "Compositions and Methods for 2,5-Furan Dicarboxylic Acid Production from 5-Hydroxymethylfurfural," which is hereby incorporated herein by reference in its entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

FIELD

[0003] The present disclosure relates to the production of high purity 2,5-furan dicarboxylic acid. More particularly, this disclosure relates to the chemoenzymatic synthesis of high purity 2,5-furan dicarboxylic acid under mild conditions.

BACKGROUND

[0004] 2,5-furan dicarboxylic acid (FDCA) is regarded by the US Department of Energy as one of the top 12 value-added chemicals derived from biomass. FDCA is used in the production of a wide array of compounds including succinic acid, isodecylfuran-2, 5-dicarboxylate, isononyl furan-2,5-dicarboxylate, dipentyl furan-2, 5-dicarboxylate, diheptyl furan-2,5-dicarboxylate, and poly(ethylene dodecanedioate-2,5-furandicarboxylate) (PEDF). FDCA is an important ingredient in the preparation of hexanoic acid, macrocyclic ligands, fungicides, corrosion inhibitors, and tiolene films. The compound can also be used as a precursor in the synthesis of monomers like dichloride-, dimethyl-, diethyl-, or bis(hydroxyethyl)-derivatives for the production of polyesters, polyamides, and plasticizers. FDCA has also been used in medicine as an anesthetic, antibiotic, and chelating agent for the removal of kidney stones. FDCA is particularly interesting as it is a precursor in the synthesis of polyethylene furanoate (PEF), an alternative polymer to petroleum-based polyethylene terephthalate (PET) and polybutylene terephthalate (PEB). The PEF polymer consists of furan-2,5-dicarboxylic acid (FDCA) monomers linked with monoethylene glycol (MEG), another renewable chemical. Structural similarities between

the PET monomer para-terephthalic acid (PTA) and FDCA allow for PEF polymerization using existing polyester infrastructure. In addition, PEF exhibits enhanced barrier, thermal, and mechanical properties when compared to PET.

[0005] FDCA can be produced from renewable sugars such as glucose and fructose. The conventional approaches to generate FDCA currently include two major competing routes: 1) oxidation and dehydration of fructose through an HMF intermediate, and 2) ketone formation at the C2 or C5 position of aldaric acids to promote furan formation.

SUMMARY

[0006] Disclosed herein is a chemoenzymatic process for the preparation of 2,5-furan dicarboxylic acid, the process comprising contacting D-glucose with (i) at least two enzymes selected from the group consisting essentially of galactose oxidase, pyranose 2-oxidase, glucarate dehydratase, catalase, and a combination thereof to produce an intermediate; and (ii) contacting the intermediate with a metal catalyst and acid catalyst to form 2,5-furan dicarboxylic acid.

[0007] Also disclosed herein is a chemoenzymatic process for the preparation of 2,5-furan dicarboxylic acid, the process comprising enzymatic oxidation of 5-hydroxymethylfurfural using an enzymatic oxidizing composition comprising one or more enzymes selected from the group consisting of Aryl-alcohol oxidase (AAO), chloroperoxidase (CPO), 5-hydroxymethylfurfural oxidase (HMFO), glyoxal oxidase (GLOX), periplasmic aldehyde oxidase (PaoABC), unspecific peroxygenase (UPO), horseradish peroxidase (HRP), galactose oxidase (GAO) with and without the activating enzyme horseradish peroxidase (HRP), lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO), thyroid peroxidase (TPO), ovoperoxidase, salivary peroxidase, vanadium haloperoxidase, non-mammalian vertebrate peroxidase (POX), peroxidase (Pxd), bacterial peroxidase (Pxc), invertebrate peroxidase (Pxt) and short peroxidase (PxDo), short peroxidase (Pxt), alpha-dioxygenase (aDox), dual oxidase (DuOx), prostaglandin H synthase or cyclooxygenase (PGHS/CyOx), linoleate diol synthase (LDS), functional variants thereof, and any combination thereof to form an intermediate; and oxidizing the intermediate using a metal catalyst to form 2,5-furan dicarboxylic acid.

BRIEF DESCRIPTION OF DRAWINGS

[0008] For a detailed description of the aspects of the disclosed processes and systems, reference will now be made to the accompanying drawings in which:

[0009] Figure 1 is a graph of the specific activities for glucose conversion for the samples from Example 1.

[0010] Figure 2 is a graph of the specific activities for glucose and gluconate conversion for the samples from Example 2.

[0011] Figure 3 is a graph of the activity of GAO-Mut47 and GAO-Mut107 on 0.5 and 2% glucose.

[0012] Figure 4 is a plot of the residual glucose concentration in a Parr reaction.

[0013] Figure 5 is a plot of the specific activity of oxidizing enzymes on glucose and oxidized derivatives.

[0014] Figure 6 is a compilation of HPLC-MS traces of pyranose 2-oxidase in glucodialdose or glucose reactions.

[0015] Figure 7 is an aspect of a process flow diagram or a chemoenzymatic process of the type disclosed herein.

DETAILED DESCRIPTION

[0016] To define more clearly the terms used herein, the following definitions are provided. Unless otherwise indicated, the following definitions are applicable to this disclosure. If a term is used in this disclosure but is not specifically defined herein, the definition from the IUPAC Compendium of Chemical Terminology, 2nd Ed (1997) can be applied, as long as that definition does not conflict with any other disclosure or definition applied herein, or render indefinite or non-enabled any claim to which that definition is applied.

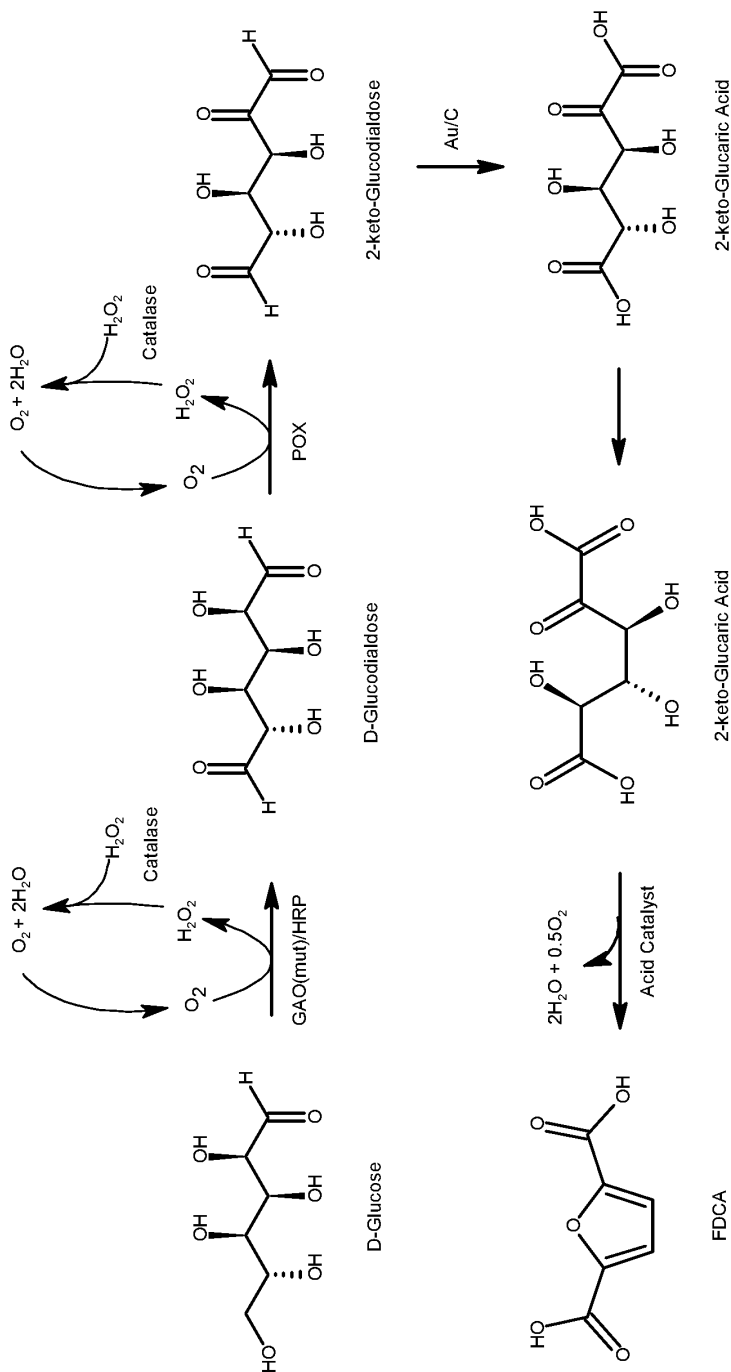
[0017] Groups of elements of the periodic table are indicated using the numbering scheme indicated in the version of the periodic table of elements published in Chemical and Engineering News, 63(5), 27, 1985. In some instances, a group of elements can be indicated using a common name assigned to the group; for example alkali metals for Group 1 elements, alkaline earth metals for Group 2 elements, transition metals for Group 3-12 elements, and halogens for Group 17 elements, among others.

[0018] Regarding claim transitional terms or phrases, the transitional term "comprising," which is synonymous with "including," "containing," "having," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. The transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. The phrase "consisting essentially of" occupies a middle ground between closed claims that are written in a "consisting of" format and fully open claims that are drafted in a "comprising" format. Absent an indication to the contrary, when describing a compound or composition "consisting essentially of" is not to be construed as "comprising," but is intended to describe the recited component that includes materials that do not significantly alter the composition or method to which the term is applied. While compositions and methods are described in terms of "comprising" various components or steps, the compositions and methods can also "consist essentially of" or "consist of" the various components or steps.

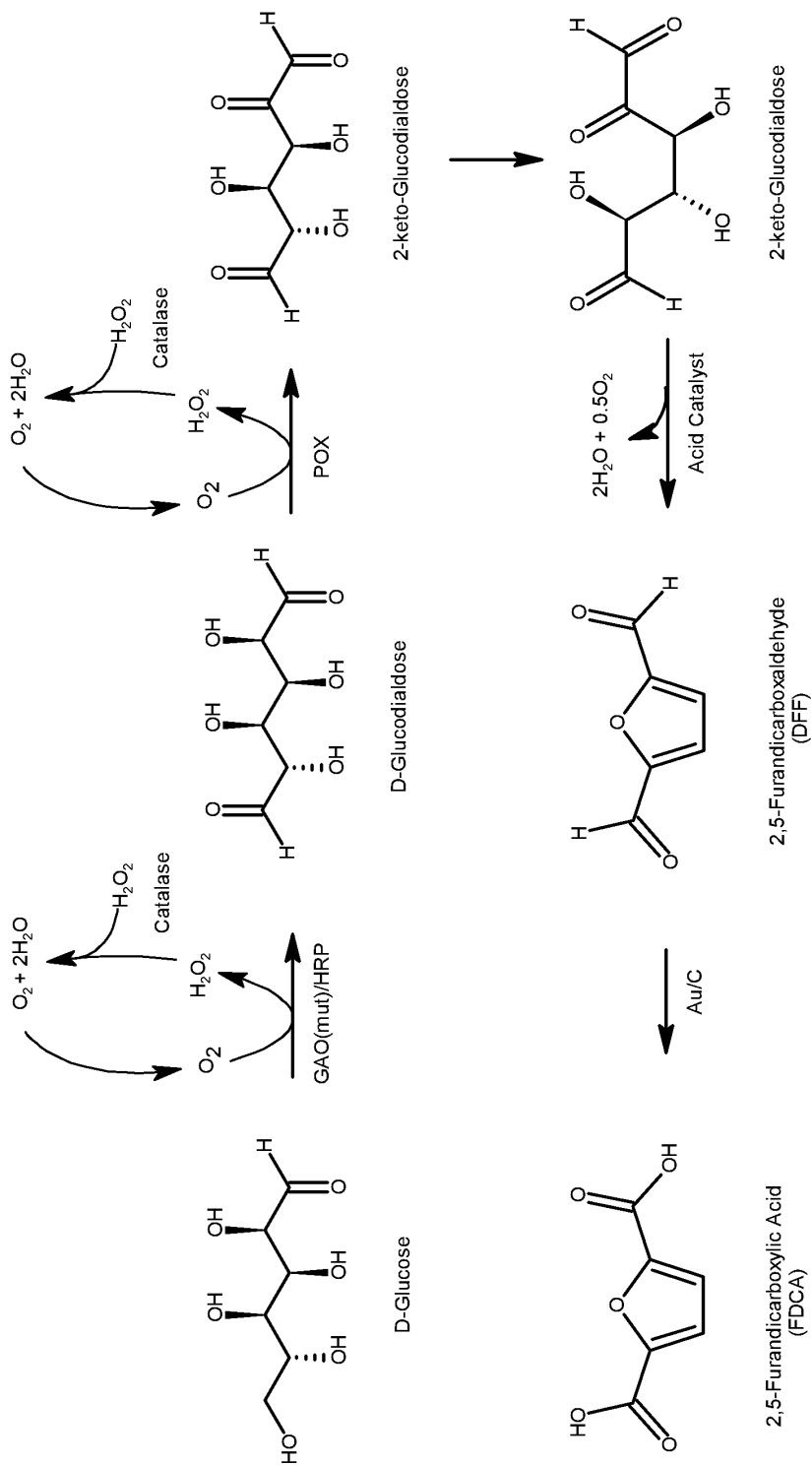
[0019] As previously described, the conventional approaches to generate FDCA currently include two major competing routes: 1) oxidation and dehydration of fructose through an HMF intermediate, and 2) ketone formation at the C2 or C5 position of aldaric acids to promote furan formation. To date, most routes towards FDCA proceed through an HMF intermediate. However, current chemical methods of producing FDCA require harsh reaction conditions (e.g., high temperatures) and are characterized by poor selectivity to the desired product. Accordingly, an ongoing need exists for novel, cost-effective methods of producing FDCA.

[0020] Disclosed herein are chemoenzymatic methods for the production of FDCA. In an aspect, a chemoenzymatic method of generating FDCA comprises contacting glucose with a multiple-enzyme system (MES), an acid catalyst, and a metal catalyst to generate the final diacid. In an aspect, the metal catalyst is heterogeneous, alternatively the metal catalyst is homogeneous. One aspect of this method is depicted in Scheme I. Referring to Scheme I, in this process, D-glucose is oxidized using a mutant galactose oxidase (GAO) to form D-glucodialdose. A pyranose-2-oxidase (POX) is used to convert D-glucodialdose to 2-keto-glucodialdose. This molecule is then converted to the diacid over a

heterogeneous noble metal catalyst and to the furan form using an acid catalyst. Efficient cyclization may require the diacid instead of the dialdehyde, meaning the heterogeneous catalyst oxidation proceeds before reacting over the acid catalyst. However, if this is not the case and 2-keto-glucodialdose is able to sample the correct furan form at a sufficiently high proportion to promote formation of 2,5-furandicarboxaldehyde (DFF), the acid catalyst step may be conducted before the metal oxidation as is depicted in Scheme II.



SCHEME 1

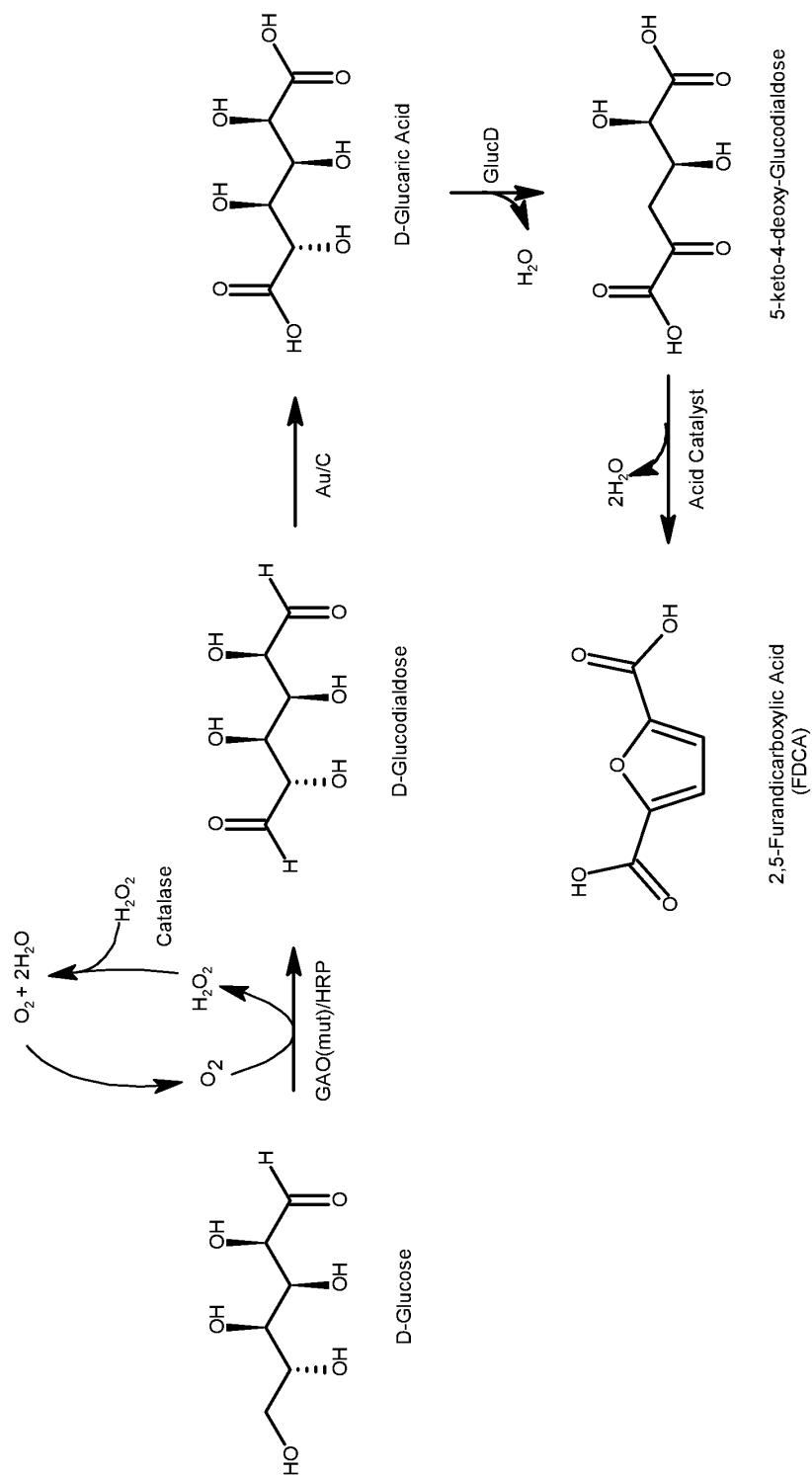


Scheme II

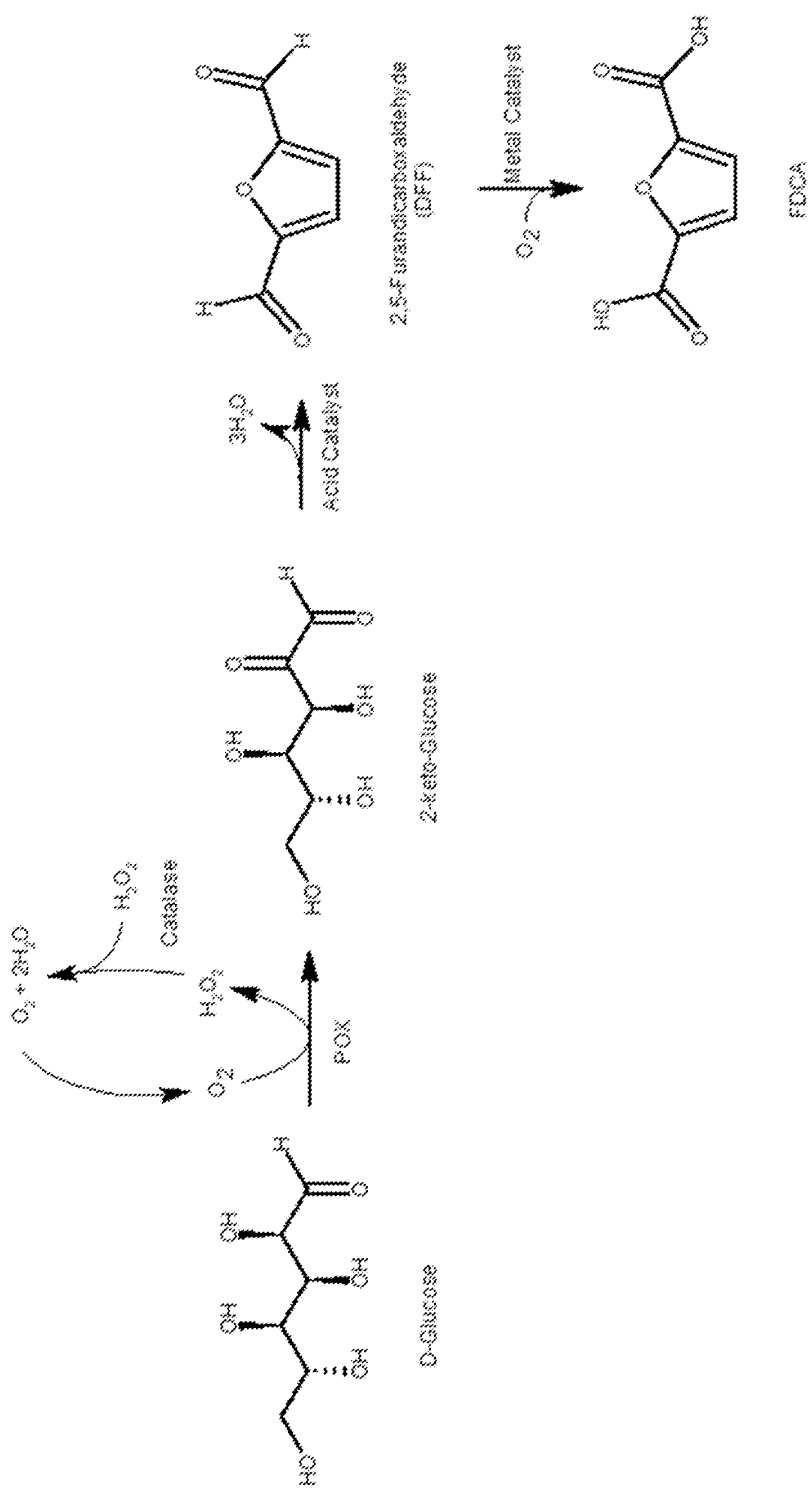
[0021] In an alternative aspect for production of FDCA, D-glucose is oxidized using a mutant galactose oxidase (GAO) to form D-glucodialdose. Subsequently, the keto group is formed by an enzyme called glucarate dehydratase (GlucD). This is depicted in Scheme III. This enzyme dehydrates glucodialdose, forming a 5-keto group while removing the 4-hydroxyl as a water. Similar to the reaction cycle depicted in Scheme II, cyclization and oxidation of the terminal aldehydes proceeds over an acid catalyst and heterogeneous noble metal catalyst. The order of the cyclization and terminal oxidation steps may be reversed.

[0022] In yet another aspect, D-glucose is oxidized by GAO to form glucodialdose. Glucodialdose may then be oxidized utilizing a metal catalyst to glucaric acid to form 5-keto-4-deoxy-glucodialdose which is subsequently cyclized to form FDCA. This is depicted in Scheme III.

[0023] In yet another aspect, D-glucose is oxidized by POX to form 2-ketoglucose, which is then cyclized to form DFF that can then be oxidized as described previously to form FDCA. This is depicted in Scheme IV.



SCHEME III



Scheme IV

[0024] The methods of the present disclosure are chemoenzymatic and utilize a combination of enzymes, one or more acid catalysts, and one or more metal catalysts. In an aspect, the enzymes comprise galactose oxidase, pyranose 2-oxidase, glucarate dehydratase, catalase, or a combination thereof. In aspect, the one or more acid catalysts, one or more metal catalysts or both are homogeneous. In an alternative aspect, the one or more acid catalysts, one or more metal catalysts or both are heterogeneous.

[0025] In an aspect, the MES comprises a member of the copper radical oxidase family. For example, and without limitation, a copper radical oxidase suitable for use in the present disclosure is galactose oxidase (GAO, EC 1.1. 3.9). GAO is one of the most extensively studied alcohol oxidases with respect to both mechanistic investigations and practical applications. Other members in the copper radical oxidase family may be suitably employed in the present disclosure.

[0026] GAO is secreted by some fungal species, particularly *Fusarium graminearum* (also known as *Gibberella zeae*), to aid in the degradation of extracellular carbohydrate food sources through catalyzing the oxidation of primary alcohols to aldehydes while generating hydrogen peroxide. The native function of GAO is the oxidation of D-galactose at the C6 position to generate D-galacto-hexodialdose. A small molecule (potassium ferricyanide) or auxiliary enzyme (i.e., horseradish peroxidase or HRP) is typically included to facilitate GAO activity. Typically, HRP is added to the reaction at a tenth of the weight percent (wt.%) of GAO. Catalase is also added to decompose hydrogen peroxide. Although the GAO is promiscuous, the native form is unable to bind glucose due to steric clashes with F464 and F194 in the active site and the equatorial C4 hydroxyl group on glucose. Efforts to engineer GAO to accept D-glucose as a substrate to form the C6 aldehyde have resulted in improved activity as shown in Table 1. The M-RQW variant (R330K, Q406T, W290F) shows a specific activity of 1.6 U mg⁻¹. Another variant, the Des3-2 (Q326E, Y329K, R330K) showed four times higher activity on glucose than the native enzyme. In addition, the mutation C383S was found to improve catalytic efficiency up to three times by reducing the K_M of the enzyme on non-native substrates guar gum and methylgalactose through improved binding of the catalytic copper ion. Tables 1 and 2

provide listings of some GAO mutants that may be useful in the methods of the present disclosure.

Table 1

Enzyme Name	Mutations	Benefits
M-RQW	R330K Q406T W290F	1.6 U/mg on glucose
Des3-2	Q326E Y329K R330K	4x activity on glucose vs WT
NA	C383S	Reduces K_M through improved copper binding
M1	S10P M70V P136 G195E V494A N535D	Improves E. coli expression and solubility
N6M1	A2A (GCC→GCA) S3S (TCA→TCT) I5I (ATC→ATT)	Silent N-term mutations for enhanced E. coli expression
NA	F194T C383E N245W/R	Improved specific activity of up to 3 U/mg with N245R and C383E in N6M1 background
NA	W290F R330K Q406T Y405F Q406E	1.6 μ M/min conversion on glucose

Table 2

Name	Starting Mut	Additional mutations
M1	wild-type	S10P M70V P136P G195E V494A N535D
M-RQW	M1	R330K Q406T W290F I463P
GAO	M-RQW	C383S
GAO-mut1	GAO	Y405F Q406E
GAO-mut2	GAO	F194T
GAO-mut3	GAO	C383E
GAO-mut4	GAO	N245R
GAO-mut5	GAO	Q326E Y329K

[0027] In an aspect, a GAO suitable for use in the present disclosure may have any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0028] In an aspect, the MES comprises a pyranose 2-oxidase (E.C. 1.1.3.10). Pyranose 2-oxidase (POX) is an flavin-dependent oxidoreductase, and a member of the glucose-methanol-choline (GMC) superfamily of oxidoreductases. POX oxidizes several monosaccharides including D-glucose, D-galactose, and D-xylose, while concurrently oxygen is reduced to hydrogen peroxide. For example, in lignocellulose-degrading fungi POX catalyzes the oxidation of α or β -D-glucose to 2-ketoglucose concomitantly with hydrogen peroxide formation during lignin solubilization.

[0029] In fungi, POX is extracellularly associated with membrane-bound vesicles or other membrane structures in the periplasmic space of hyphae. POX homologs are also found in actinobacteria, protobacteria, and bacilli species. POX enzymes from *Spongipellis unicolor* (aka *Polyporus obtusus*), *Phanerochaete chrysosporium* (PDB 4MIF), *Trametes multicolor* (aka *Trametes ochracea* PDB 1TT0), *Peniophora gigantea* (PDB 1TZL), *Aspergillus nidulans*, *A. oryzae*, *Irpex lacteus*, *Arthrobacter siccitolerans*, and *Kitasatospora aureofaciens* (aka *Streptomyces aureofaciens*) have been characterized. Although most POX enzymes exist as homotetramers with FAD covalently bound to a histidine, exceptions exist. POX is a monomer in solution and non-covalently binds FAD. KaPOX forms dimers in solution. In addition to this oxidase activity, POX shows pronounced activity with alternative electron acceptors that include various quinones or (complexed) metal ions. In an aspect, a POX suitable for use in the present disclosure may have any of SEQ ID NO.:7 to SEQ ID NO.:11.

[0030] In an aspect, the MES comprises a glucarate dehydratase (E.C. 4.2.1.40). GluD belongs to the mechanistically diverse enolase superfamily, specifically the glucarate dehydratase subgroup. GluD catalyzes the dehydration of both D-glucarate and L-idarate to form 5-keto-4-deoxyglucarate (KDG) as well as the epimerisation of the two substrates. In the first step, the His 339 residue acts as a general base towards the C5 atom of D-glucarate, while Lys 207 acts as a general base towards the related epimer L-idarate. Each residue is associated with a different stereo selective function; Lys 207 acts as an S specific base, while His 339 acts as an R specific base. The enolate anion intermediate is stabilized by hydrogen bonds to residues Lys 205 and Asn 237, as well as interaction with the catalytically essential divalent Mg cation.

[0031] In an aspect, an MES of the present disclosure comprises a catalase (E.C. 1.11.1.61). CAT is a tetrameric, heme-containing, antioxidant enzyme present in all aerobic organisms. Catalase catalyzes the decomposition of H₂O₂ into water and oxygen.

[0032] In an aspect, any of the enzymes present in the MES is a wild type enzyme, a functional fragment thereof, or a functional variant thereof. As used herein, "fragment" is meant to include any amino acid sequence shorter than the full-length enzyme (e.g., AOX), but where the fragment maintains a catalytic activity sufficient to meet some user or process goal. Fragments may include a single contiguous sequence identical to a

portion of the enzyme sequence. Alternatively, the fragment may have or include several different shorter segments where each segment is identical in amino acid sequence to a different portion of the amino acid sequence of the enzyme but linked via amino acids differing in sequence from the enzyme. Herein, a "functional variant" of the enzyme refers to a polypeptide that has at one or more positions of an amino acid insertion, deletion, or substitution, either conservative or non-conservative, and wherein each of these types of changes may occur alone, or in combination with one or more of the others, one or more times in a given sequence but retains catalytic activity.

[0033] In the alternative or in combination with the aforementioned mutations, an enzyme in the MES may be mutated to improve the catalytic activity. Mutations may be carried out in order to enhance the protein or a homolog activity, increase the protein stability in the presence of products and/or hydrogen peroxide, and increase protein yield.

[0034] Herein, reference is made to "sources" of enzymes. It is to be understood this refers to the biomolecule as expressed by the named organism. It is contemplated the enzyme may be obtained from the organism or a version of said enzyme (wildtype or recombinant) provided as a suitable construct to an appropriate expression system.

[0035] In an aspect, any enzyme of the type disclosed herein may be cloned into an appropriate expression vector and used to transform cells of an expression system such as *E. coli*, *Saccharomyces* sp., *Pichia* sp., *Aspergillus* sp., *Trichoderma* sp., or *Myceliophthora* sp. A "vector" is a replicon, such as plasmid, phage, viral construct, or cosmid, to which another DNA segment may be attached. Vectors are used to transduce and express a DNA segment in cells. As used herein, the terms "vector" and "construct" may include replicons such as plasmids, phage, viral constructs, cosmids, Bacterial Artificial Chromosomes (BACs), Yeast Artificial Chromosomes (YACs) Human Artificial Chromosomes (HACs), and the like into which one or more gene expression cassettes may be or are ligated. Herein, a cell has been "transformed" by an exogenous or heterologous nucleic acid or vector when such nucleic acid has been introduced inside the cell, for example, as a complex with transfection reagents or packaged in viral particles. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell.

[0036] In an aspect, the gene of an enzyme disclosed herein is provided as a recombinant sequence in a vector where the sequence is operatively linked to one or more control or regulatory sequences. "Operatively linked" expression control sequences refers to a linkage in which the expression control sequence is contiguous with the gene of interest to control the gene of interest, as well as expression control sequences that act in trans or at a distance to control the gene of interest.

[0037] The term "expression control sequence" or "regulatory sequences" are used interchangeably and refer to polynucleotide sequences, which are necessary to affect the expression of coding sequences to which they are operatively linked. Expression control sequences are sequences that control the transcription, post-transcriptional events, and translation of nucleic acid sequences. Expression control sequences include appropriate transcription initiation, termination, promoter, and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (e.g., ribosome binding sites); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0038] The term "recombinant host cell" ("expression host cell", "expression host system", "expression system" or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. A recombinant host cell may be an isolated cell or cell line grown in culture or may be a cell which resides in a living tissue or organism.

[0039] In an aspect, the enzymes suitable for use in an MES of the type disclosed herein may further include one or more purified cofactors. Herein, a “cofactor” refers to non-protein chemical compound that modulates the biological activity of the enzyme. Many enzymes require cofactors to function properly. Nonlimiting examples of purified enzyme cofactors suitable for use in the present disclosure include thiamine pyrophosphate, NAD⁺, NADP⁺, pyridoxal phosphate, methyl cobalamin, cobalamine, biotin, Coenzyme A, tetrahydrofolic acid, menaquinone, ascorbic acid, flavin mononucleotide, flavin adenine dinucleotide, and Coenzyme F420. Such cofactors may be included in the MES and/or be added at various points during a reaction. In some aspects, cofactors included with the MES may be readily regenerated with oxygen and/or may remain stable throughout the lifetime of the enzyme(s).

[0040] In one or more aspects, any enzyme component of the MES is present in an amount in the MES and/or reaction mixture sufficient to provide some user and/or process desired catalytic activity. In such aspects, any of the enzymes disclosed herein may be present in an amount ranging from about 0.0001 wt.% to about 1 wt.%, alternatively from about 0.0005 wt.% to about 0.1 wt.% or alternatively from about 0.001 wt.% to about 0.01 wt.% based on the total weight of the MES.

[0041] In the reactions depicted in Schemes 1 through V, the MES acts to initially oxidize glucose which is subsequently reacted to form an intermediate that is dehydrated. For example, methods of the present disclosure involve dehydration of the intermediates 5-keto-4-deoxy-glucarate, 5-keto-4-deoxy-glucodialdose, 2-keto-glucodialdose, 2-keto-glucaric acid, and/or 2-keto-glucose to form DFF. In an aspect, dehydration is carried out in the presence of an acid catalyst.

[0042] In an aspect, the acid catalyst that is employed to facilitate dehydration of the aforementioned intermediates is a Bronsted acid or contains strong Bronstead acid sites that are characterized by ability to provide a proton or hydronium ion in the reaction mixture. Bronstead acids suitable for use in the present disclosure include homogeneous acidic catalysts or heterogeneous acidic catalysts tested for furan formation in glucaric acid. In an aspect, the acid catalyst comprises ion exchange resins (such as DIAION series, Amberlyst-15), sulfonated silica, zeolites, niobium oxide, mineral acids such as HCl, or a combination thereof. In an aspect, the acid catalyst may be present in an

amount effective to catalyze conversion of the intermediate. In an aspect, the acid catalyst is present in a suitable solvent such as dimethyl formamide or dimethyl sulfoxide. For example, the acid catalyst may be present in an amount of from about 0,1 wt.% about 0,2 wt.% based on the total weight of the reaction mixture, alternatively from about 0,001 wt.% to about 2,0 wt.% or alternatively from about 0,001 wt.% to about 20 wt.%.

[0043] In one or more aspects of the present disclosure, a final oxidation step is carried out to convert an aldehyde into a carboxylic acid, such as depicted in Schemes I through IV. In an aspect of the present disclosure, the oxidation can be carried out using a metal catalyst, alternatively a supported metal catalyst. In an aspect, the metal catalyst comprises a supported metal catalyst such as a heterogeneous metal catalyst or a homogenous metal catalyst (HMC). In an aspect, the support comprises carbon, silica, alumina, titania (TiO_2), zirconia (ZrO_2), a zeolite, or any combination thereof, which contains less than about 1.0 weight percent (wt.%), alternatively less than about 0.1 wt.% or alternatively less than about 0.01 wt.% SiO_2 binders based on the total weight of the support.

[0044] Suitable support materials are predominantly mesoporous or macroporous, and substantially free from micropores. For example, the support may comprise less than about 20% micropores. In an aspect, the support of the HMC is a porous nanoparticle support. As used herein, the term "micropore" refers to pores with a diameter of <2 nm, as measured by nitrogen adsorption and mercury porosimetry methods and as defined by IUPAC. As used herein, the term "mesopore" refers to pores with a diameter of from ca. 2 nm to ca. 50 nm, as measured by nitrogen adsorption and mercury porosimetry methods and as defined by IUPAC. As used herein, the term "macropore" refers to pores with diameters larger than 50 nm, as measured by nitrogen adsorption and mercury porosimetry methods and as defined by IUPAC.

[0045] In an aspect, the HMC support comprises a mesoporous carbon extrudate having a mean pore diameter ranging from about 10 nm to about 100 nm, and a surface area greater than about $20 \text{ m}^2 \text{ g}^{-1}$ but less than about $300 \text{ m}^2 \text{ g}^{-1}$. Supports suitable for use in the present disclosure may have any suitable shape. For example, the support may be shaped into 0.8-3.0 mm trilobes, quadralobes, or pellet extrudates. Such shaped supports

enable the use of fixed trickle bed reactors to perform the final oxidation step under continuous flow.

[0046] In an aspect, the HMC comprises metals of main group IV, V, VI, alternatively the metal is from subgroup I, IV, V, VII, alternatively the HMC comprises gold, Au. In one or more aspects, the metal comprises a Group 8 metal (e.g., Re, Os, Ir, Pt, Ru, Rh, Pd, Ag), a 3d transition metal, an early transition metal, or a combination thereof. In an alternative aspect, a dehydration catalyst comprises hafnium, tantalum, zinc, or a combination thereof on a support such as a zeolite or a β -zeolite. In an aspect, a metal catalyst suitable for use in the present disclosure comprises a metal oxide, zirconia doped with alkaline-earth elements, rare earth orthophosphate catalyst, ruthenium, or a combination thereof.

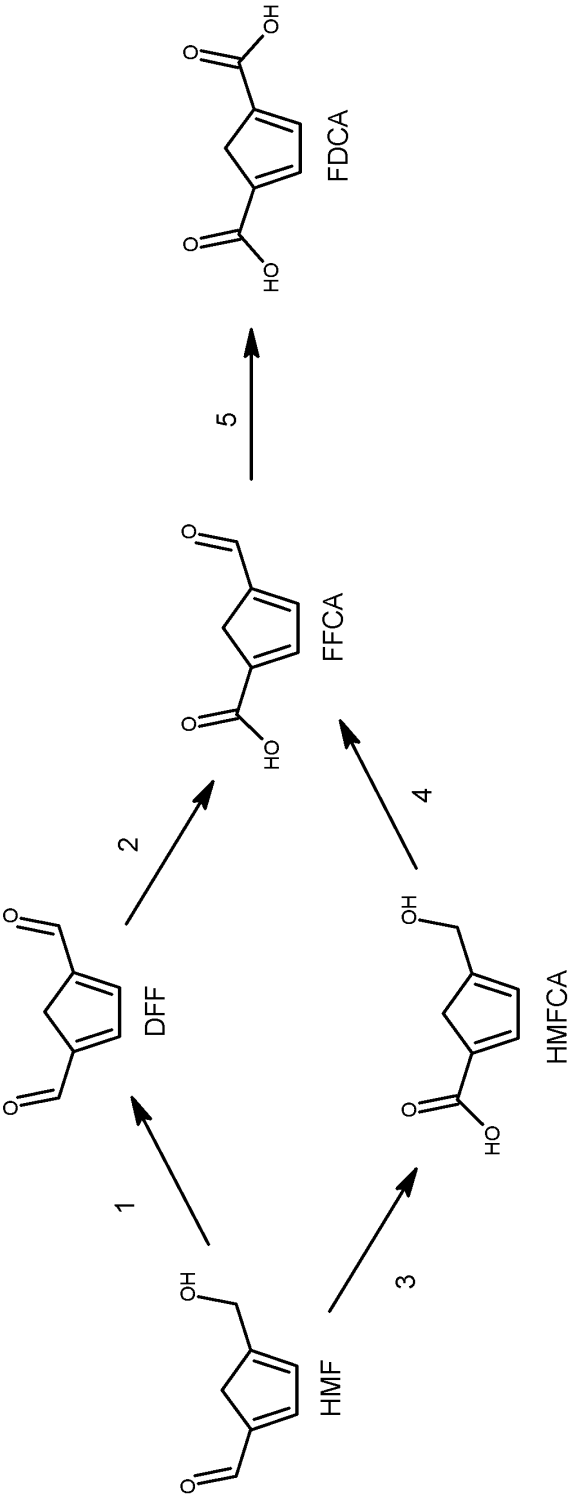
[0047] The HMC may be prepared using any suitable methodology. For example, the HMC may be prepared using gas phase reduction of the support (e.g., carbon) impregnated with metal salts in hydrogen at temperatures ranging from greater than about 200 °C to about 600 °C. In an alternative aspect, the HMC may be prepared using liquid phase reduction of the support impregnated with metal salts immersed in an aqueous oxygenate (e.g., formate, gluconate, citrate, ethylene glycol, etc.) solution at temperature between about 0 °C and about 100 °C. Alternatively, the impregnated support can be loaded into the hydrogenation reactor in a non-reduced form and reduced on stream by the reactants of the process during startup. Liquid Phase Reduction (LPR) is a synthetic method to obtain a core-shell dispersion of the active metallurgy over a surface annulus of the extrudate.

[0048] In an aspect, materials of the type disclosed herein are prepared via incipient wetness or bulk adsorption of a metal precursor salt solution onto the extrudate support followed by either Gas Phase Reduction (GPR) at temperatures between 100 °C and 500 °C under an H_2/N_2 atmosphere or followed by Liquid Phase Reduction (LPR) using an alkaline aqueous solution.

[0049] In one or more alternative aspects, a method of the present disclosure comprises enzymatic oxidation of HMF, under mild reaction conditions, to produce an intermediate. In an aspect, a method of the present disclosure further comprises oxidation of the

intermediate by a metal catalyst, alternatively a heterogeneous metal catalyst (HMC) to produce the FDCA. This scheme is generally depicted in Scheme V.

3416-00301
20ENZ006-PCT



Scheme V

[0050] Without wishing to be limited by theory, oxidases, which typically generate one molecule of hydrogen peroxide for each oxidation performed, can be combined with peroxygenases or peroxidases which use hydrogen peroxide as an oxidant to catalyze another oxidation. This not only removes highly reactive hydrogen peroxide from the solution, but also provides peroxide which is necessary for peroxygenase/peroxidase function.

[0051] In an aspect, a method of producing FDCA comprises enzymatic oxidation of HMF by an enzymatic oxidizing composition (EOC). In an aspect, the EOC comprises one or more enzymes selected from the group consisting of Aryl-alcohol oxidase (AAO), chloroperoxidase (CPO), 5-hydroxymethylfurfural oxidase (HMFO), glyoxal oxidase (GLOX), periplasmic aldehyde oxidase (PaoABC), unspecific peroxygenase (UPO), horseradish peroxidase (HRP), galactose oxidase (GAO) with and without the activating enzyme horseradish peroxidase (HRP), lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO), thyroid peroxidase (TPO), ovoperoxidase, salivary peroxidase, vanadium haloperoxidase, non-mammalian vertebrate peroxidase (POX), peroxidase (Pxd), bacterial peroxidase (Pxc), invertebrate peroxidase (Pxt) and short peroxidase (PxDo), short peroxidase (Pxt), alpha-dioxygenase (aDox), dual oxidase (DuOx), prostaglandin H synthase, cyclooxygenase (PGHS/CyOx), linoleate diol synthase (LDS), functional variants thereof and any combination thereof. Enzymes of the type disclosed herein may be isolated from any suitable source. Nonlimiting examples of enzymes suitable for use in the present disclosure along with their catalytic efficiencies (k_{cat}) are presented in Table 3 which also provides the step of Scheme 1 that may be catalyzed by the enzyme.

Table 3

Step	Enzyme	k_{cat} (s^{-1})
1	GAO (Novozymes)	0.7
1	Pleurotus ostreatus AAO	2.95
1	Pleurotus eryngii AAO	0.34, 3.65
1	Pleurotus eryngii AAO Bantha (F501W)	18.8
1	Agrocybe aegerita UPO	222.2 (split with step 3)
1	Methyloversus sp. strain MP688 HMFO	9.9
1	Methyloversus sp. strain MP688 7xHMFO	11.8
1	Pycnoporus cinnabarinus GLOX1	1.59
1	Pycnoporus cinnabarinus GLOX2	0.56
1	Pycnoporus cinnabarinus GLOX3	0.75
2	Pleurotus eryngii AAO	0.86

2	Pleurotus eryngii AAO Bantha (F501W)	ND
2	Agrocybe aegerita UPO	29.2
2	Methyloversus sp. strain MP688 HMFO	1.6
2	Pycnoporus cinnabarinus GLOX1	4.38
2	Pycnoporus cinnabarinus GLOX2	0.21
2	Pycnoporus cinnabarinus GLOX3	0.18
3	Agrocybe aegerita UPO	222.2 (split with step 1)
4	Methyloversus sp. strain MP688 HMFO	8.5
4	GAOM _{3.5} and HRP	ND
5	Aspergillus catalase	ND
5	Methyloversus sp. strain MP688 HMFO	ND
5	Agrocybe aegerita UPO	ND
5	Pleurotus eryngii AAO Bantha (F501W)	ND
5	Pycnoporus cinnabarinus GLOX1	0.03
5	Pycnoporus cinnabarinus GLOX2	2.02
5	Pycnoporus cinnabarinus GLOX3	0.04

[0052] Herein, the k_{cat} refers to the turnover rate, turnover frequency, or turnover number. This constant represents the number of substrate molecules that can be converted to product by a single enzyme molecule per unit time (usually per minute or per second).

[0053] As will be understood by one of ordinary skill in the art with the benefit of the present disclosure, reactions of the type disclosed herein (e.g., AAO oxidation of HMF) may result in the production of byproducts that can detrimentally impact other components of the reaction mixture. For example, hydrogen peroxide may degrade the enzymes of the EOC resulting in a reduced catalytic efficiency. In such aspects, the detrimental effects of hydrogen peroxide may be mitigated such as by introduction of a catalase (E.C. 1.11.1.61), which not only degrades the hydrogen peroxide but will also generate oxygen to drive oxidase function.

[0054] In an aspect, an EOC of the present disclosure comprises (i) an oxidase; (ii) a peroxidase; and (iii) a catalase. Alternatively, an EOC of the present disclosure comprises (i) an oxidase; (ii) a peroxygenase and (iii) a catalase; alternatively (i) an oxidase and (ii) a peroxidase; alternatively (i) an oxidase and (ii) a peroxygenase; alternatively an oxidase or alternatively a peroxidase. Each of these enzymes may be of the type disclosed herein.

[0055] In an aspect, one or more enzymes of an EOC of the present disclosure is characterized by a k_{cat} of equal to or greater than about 9 s^{-1} , alternatively equal to or greater than about 50 s^{-1} , or alternatively equal to or greater than about 100 s^{-1} .

[0056] In an aspect, an EOC of the type disclosed herein may be utilized to produce an intermediate in the conversion of HMF to FDCA. Nonlimiting examples of

intermediates that may be produced using an EOC of the type disclosed herein include diformyl furan (DFF), 5-hydroxymethyl-2-furoic acid (HMFCFA), and 5-formyl-2-furancarboxylic acid (FFCA). In an aspect, an EOC of the present disclosure when reacted with HMF forms one or more intermediates selected from the group consisting of diformyl furan (DFF), 5-hydroxymethyl-2-furoic acid (HMFCFA), and 5-formyl-2-furancarboxylic acid (FFCA). An intermediate produced by reacting an EOC with HMF (e.g., diformyl furan) may be further oxidized to produce FDCA using a transition metal catalyst.

[0057] In an aspect, chemoenzymatic processes of the type disclosed herein may be carried out in any suitable reactor. An aspect of a suitable reactor is depicted in Figure 7. Referring to Figure 7, a first enzymatic reactor 40 could be a sparged bubble column, an air lift column, a stirred sparged bioreactor, or a falling film high pressure oxidation vessel. The reactants, glucose, and a MES, can be introduced to the reactor from storage containers 10 and 20 via conduits 5 and 7 respectively. In an aspect, the enzyme reactor 40 may operate at temperatures of less than about 100 °C, alternatively at temperatures ranging from about 20 °C to about 60 °C and at pressures ranging from about 1 bar to about 15 bar. In an aspect, in the enzyme reactor 40 glucose is converted enzymatically by GAO and HRP to D-glucodialdose, with catalase present to degrade hydrogen peroxide for enzyme stability. Further, the enzyme reactor 40 may be sparged with both compressed air (for molecular oxygen) which may be supplied by an air compressor 30 via conduit 9. While not shown, pH can be controlled by the addition of strong acids, bases, or buffers. Effluent from the enzyme reactor 40 may be sent via conduit 13 to a tangential flow filter (TFF) 45 in order to preserve enzymes in the enzyme reactor and recycled as retentate via conduit 11, with D-glucodialdose permeate flowing further down the process to the second enzymatic reactor 60 via conduit 17.

[0058] The second enzymatic reactor 60 could be a sparged bubble column, an air lift column, a stirred sparged bioreactor, or a falling film high pressure oxidation vessel. In an aspect, the second enzyme reactor 60 may operate at temperatures of less than about 100 °C, alternatively at temperatures ranging from about 20 °C to about 60 °C and at pressures ranging from about 1 bar to about 15 bar. In the second enzyme reactor 60, D-glucodialdose is converted enzymatically by POX to 2-keto-glucodialdose, with catalase present to degrade hydrogen peroxide for enzyme stability. In the alternative as depicted in Scheme III GlucD replaces POX. The second

enzyme reactor 60 may be sparged with compressed air (for molecular oxygen). While not shown, pH can be controlled by the addition of strong acids, bases, or buffers. Effluent from the second enzyme reactor 60 may be sent to a TFF 55 via conduit 21 to preserve enzymes in the enzyme reactor as recycled retentate via conduit 19, with 2-keto-glucodialdose permeate flowing further down the process via conduit 23 to the metal oxidation reactor 65 and dehydration reactor 70.

[0059] In an aspect, permeate from the second enzyme reactor 60 is fed downstream to the metal oxidation reactor 65, where 2-keto-glucodialdose is converted to 2-keto-glucaric acid. In one or more aspects, the oxidation reactor 65 is operated as a trickle-bed reactor, utilizing metal catalysts of the type disclosed herein. The oxidation reactor 65 may be fed 2-ketoglucodialdose from the top and fed with high pressure air (to provide molecular oxygen) from the bottom, to ensure proper bed wetting and mass transfer. The oxidation reactor 65 may be operated at pressures ranging from about 100 °C to about 200 °C and pressures ranging from about 10 to about 100 bar. In an aspect, the reactor product is removed from the bottom and passed on to the dehydration reactor 70.

[0060] 2-keto-glucaric acid leaving the metal oxidation reactor 65 may be converted to FDCA via dehydration in the dehydration reactor 70. In an aspect, the dehydration reactor 70 is operated in either upstream or downstream configuration. The dehydration reactor 70 may be charged with an immobilized strong acid exchange catalyst, as described previously herein. The dehydration reactor 70 may operate at temperatures ranging from about 160 °C to about 200 °. For example, the dehydration reaction may take place with liquid water at elevated pressures.

[0061] In an aspect, the dehydration reactor product includes a mixture of water and FDCA, along with side product impurities. The dehydration reactor product stream may be transferred via conduit 37 to a purification train consisting of a water crystallization unit 75, a solvent crystallization unit 80, and a Nutsche Filter 85. As FDCA solubility in water is a strong function of temperature, the water crystallization unit 75 may be a cooling crystallizer or a cooling and vacuum crystallizer. In an aspect, FDCA crystals are then separated via filtration and sent to a second, organic solvent crystallizer.

[0062] In the organic solvent crystallizer 80, the solvent may be 1-butanol, isobutanol, methanol, or another suitable organic solvent. Without wishing to be limited by theory, by switching between water and an organic solvent, different impurities can be

removed in the mother liquor. FDCA is then crystallized out either by cooling or vacuum crystallization, with crystals removed and passed to a Nutsche filter 85. Organic solvent can be removed and regenerated via distillation to remove non-volatile impurities.

[0063] FDCA crystals are then washed in a Nutsche Filter 85 to remove any residual impurities. A polar, aprotic solvent like acetonitrile may be utilized, as (1) this would solvate impurities not previously picked up in water, a polar protic solvent, or 1-butanol, a non-polar protic solvent, and (2) FDCA is only sparingly soluble in acetonitrile. Acetonitrile leaving the Nutsche filter 85 could then be regenerated via distillation to remove non-volatile impurities. Highly pure FDCA crystals are then removed from the Nutsche Filter 85 as the final product.

[0064] Although the above drawing is at PFD level of detail, not all process interconnections are shown such as spillbacks, block and bleeds, recycle lines, control valves, cooling/heating elements, pumps, intermediate tankage, antifoam, etc.

[0065] In an aspect, the methods disclosed herein result in the preparation of high purity FDCA. The FDCA may have a purity of greater than about 80%, alternatively greater than about 85%, alternatively greater than about 95%, alternatively from about 80% to about 99%, alternatively from about 85% to about 99%, or alternatively from about 90% to about 99%.

ADDITIONAL DISCLOSURE

[0066] The following enumerated aspects of the present disclosures are provided as non-limiting examples.

[0067] A first aspect which is a chemoenzymatic process for the preparation of 2,5-furan dicarboxylic acid, the process comprising contacting D-glucose with (i) at least two enzymes selected from the group consisting essentially of galactose oxidase, pyranose 2-oxidase, glucarate dehydratase, catalase, and a combination thereof to produce an intermediate; and (ii) contacting the intermediate with a metal catalyst and acid catalyst to form 2,5-furan dicarboxylic acid.

[0068] A second aspect which is the chemoenzymatic process of the first aspect wherein D-glucose is contacted with galactose oxidase and catalase to form D-glucodialdose; and wherein the process further comprises contacting D-glucodialdose with pyranose-2-oxidase and catalase under conditions suitable for the formation of 2-keto-glucodialdose; contacting 2-keto-glucodialdose with a heterogeneous metal

catalyst to form 2-keto-glucaric acid; and dehydrating 2-ketoglucaric acid in the the presence of an acid catalyst to form 2,5-furan dicarboxylic acid.

[0069] A third aspect which is the chemoenzymatic process of any of the first through second aspects wherein D-glucose is contacted with galactose oxidase and catalase to form D-glucodialdose; and wherein the process further comprises contacting D-glucodialdose with pyranose-2-oxidase and catalase under condtions suitable for the formation of 2-keto-glucodialdose; dehydrating 2-keto-glucodialdose with an acid catalyst to form 2,5-furandicaboxaldehyde; and oxidizing 2,5-furandicaboxaldehyde in the the presence of a heterogeneous metal catalyst to form 2,5-furan dicarboxylic acid.

[0070] A fourth aspect which is the chemoenzymatic process of any of the first through third aspects, wherein D-glucose is contacted with galactose oxidase and catalase to form D-glucodialdose; and wherein the process further comprises contacting D-glucodialdose with a metal catalyst to form D-glucaric acid; dehydrating D-glucaric acid with glucarate dehydratase to form 5-keto-4-deoxy glucodialdose; and cyclizing 5-keto-4-deoxy glucodialdose in the presence of an acid catalyst to form 2,5-furan dicarboxylic acid.

[0071] A fifth aspect which is the chemoenzymatic process of any of the first through fourth aspects wherein D-glucose is contacted with pyranose-2-oxidase and catalase to form 2-keto-glucose; and wherein the process further comprises dehydrating 2-keto-glucose with an acid catalyst under condtions suitable for the formation of 2,5-furandicaboxaldehyde;

dehydrating 5-keto-4-deoxyglucodialdose with an acid catalyst to form 2,5-furandicaboxaldehyde; and oxidizing 2,5-furandicaboxaldehyde in the the presence of a heterogeneous metal catalyst to form 2,5-furan dicarboxylic acid.

[0072] A sixth aspect which is the chemoenzymatic process of any of the first through fifth aspects. wherein the galactose oxidase has any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0073] A seventh aspect which is the chemoenzymatic process of the second aspect wherein the galactose oxidase has any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0074] An eighth aspect which is the chemoenzymtic process of the third aspect wherein the galactose oxidase has any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0075] A ninth aspect which is the chemoenzymatic process of the fourth aspect wherein the galactose oxidase has any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0076] A tenth aspect which is the chemoenzymatic process of the fifth aspect, wherein the galactose oxidase has any of SEQ ID NO.:1 to SEQ ID NO.:6.

[0077] An eleventh aspect which is the chemoenzymatic process of any of the first through fifth aspects wherein the galactose oxidase has SEQ ID NO.:1.

[0078] A twelfth aspect which is the chemoenzymatic process of any of the first through eleventh aspects wherein the pyruvate-2-oxidase has any of SEQ ID NO.:7 to SEQ ID NO.:11.

[0079] A thirteenth aspect which is the chemoenzymatic process of any of the first through twelfth aspects carried out at a temperature of less than about 100 °C.

[0080] A fourteenth aspect which is the chemoenzymatic process of any of the first through thirteenth aspects wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0081] A fifteenth aspect which is the chemoenzymatic process of the the second aspect wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0082] A sixteenth aspect which is the chemoenzymatic process of the third aspect wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0083] A seventeenth aspect which is the chemoenzymatic process of the fourth aspect wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0084] An eighteenth aspect which is the chemoenzymatic process of the fifth aspect wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0085] A nineteenth aspect which is the chemoenzymatic process of any of the first through eighteenth aspects wherein the heterogeneous metal catalyst comprises a support comprising carbon, silica, alumina, titania (TiO₂), zirconia (ZrO₂), zeolite, or any combination thereof.

[0086] A twentieth aspect which is the chemoenzymatic process of any of the first through nineteenth aspects wherein the acid catalyst, the metal catalyst or both are heterogeneous.

[0087] A twenty-first aspect which is the chemoenzymatic process of any of the first through twentieth aspects wherein the acid catalyst, the metal catalyst or both are homogeneous.

[0088] A twenty-second aspect which is a chemoenzymatic process for the preparation of 2,5-furan dicarboxylic acid, the process comprising enzymatic oxidation of 5-hydroxymethylfurfural using an enzymatic oxidizing composition comprising one or more enzymes selected from the group consisting of Aryl-alcohol oxidase (AAO)

chloroperoxidase (CPO), 5-hydroxymethylfurfural oxidase (HMFO), glyoxal oxidase (GLOX), periplasmic aldehyde oxidase (PaoABC), unspecific peroxygenase (UPO), horseradish peroxidase (HRP), galactose oxidase (GAO) with and without the activating enzyme horseradish peroxidase (HRP), lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO), thyroid peroxidase (TPO), ovoperoxidase, salivary peroxidase, vanadium haloperoxidase, non-mammalian vertebrate peroxidase (POX), peroxidase (Pxd), bacterial peroxidase (Pxc), invertebrate peroxidase (Pxt) and short peroxidase (PxDo), short peroxidase (Pxt), alpha-dioxygenase (aDox), dual oxidase (DuOx), prostaglandin H synthase or cyclooxygenase (PGHS/CyOx), linoleate diol synthase (LDS), functional variants thereof, and any combination thereof to form an intermediate; and oxidizing the intermediate using a metal catalyst to form 2,5-furan dicarboxylic acid.

[0089] A twenty-third aspect which is the chemoenzymatic process of the twenty-second aspect wherein the enzymatic oxidation is carried out at a temperature of less than about 100 °C.

[0090] A twenty-fourth aspect which is the chemoenzymatic process of any of the twenty-second through twenty-third aspects wherein the 2,5-furan dicarboxylic acid has a purity of greater than about 80%.

[0091] A twenty-fifth aspect which is the chemoenzymatic process of any of the first through twenty-fourth aspects further comprising subjecting the 2,5-furan dicarboxylic to water crystallization, solvent crystallization, and Nutsche filtration.

EXAMPLES

[0092] The presently disclosed subject matter having been generally described, the following examples are given as particular aspects of the subject matter and to demonstrate the practice and advantages thereof. It is understood that the examples are given by way of illustration and are not intended to limit the specification or the claims in any manner.

Example 1

[0093] The specific activity of mutants from Table 1 on glucose were assessed and the results are presented in Figure 1. Another GAO mutant capable of converting glucose to glucodialdose was engineered. Following directed evolution and rational enzyme engineering, the improved GAO mutant exhibits a specific activity of 35 U mg⁻¹ on glucose. Directed evolution of thirty sites within 10 Å of the catalytic copper was performed on a parent sequence containing the following added mutations: 1) R330,

Q406T, W290F discovered by 2) C383S, and 3) Y405F and Q406E. Other mutations described in Table 4 were found to have neutral or deleterious effects on glucodialdose-generating activity. We named the new combination sequence GAO-Mut1. The full sequence of the expressed construct is given as SEQ ID No. 1, which contains a “MGHHHHHHSSGHIEGRHM” N-terminal his-tag and linker for expression and purification in *E. coli*.

[0094] Selected positions in GAO-Mut1 were mutated via the QUIKCHANGE method to all 20 amino acids using primers containing NNS codons. The constructs were then screened in the following manner: Colonies were picked and used to inoculate one well each in a 96-well deepwell plate charged with lysogeny broth (LB). The grown clones were then used to inoculate autoinduction media in a separate 96-well deepwell plate for protein expression. Harvested cells were lysed with Bacterial Protein Extraction Reagent (B-PER) and the lysate was then screened for oxidase activity using a colorimetric ABTS assay which detects hydrogen peroxide.

[0095] In short, lysate was assayed for activity with and without exposure to heat. To assay activity in the absence of a heat challenge, lysate was diluted 50 times. A volume of 5 μL of the diluted lysate was combined with ABTS assay solution (final concentration of 2% w/v glucose, 0.0125 mg/ml horseradish peroxidase, 50 mM sodium phosphate buffer at pH 8, 0.05% ABTS) to a final volume of 200 μL and the change in absorbance at 405 nm was monitored until the reaction was complete. To assay residual activity after a heat challenge, 50 μL lysate was incubated for ten minutes at 50 °C and 20 μL of the heat-treated lysate was added to the ABTS solution before monitoring change in absorbance at 405 nm. Specific activity was calculated from the formulas below using the linear portion of the curve to measure $\Delta A_{405}/\text{min}$ and taking the extinction coefficient of ABTS at 405 nm as $36.8 \text{ mM}^{-1}\text{cm}^{-1}$.

$$\text{Units } \text{mg}^{-1} = \frac{\Delta A_{405} \text{ min}^{-1}}{36.8 \times (\text{pathlength in cm}) \times (\text{mg enzyme}) / (\text{ml reaction mixture})}$$

$$\text{Units } \text{ml}^{-1} = \frac{\Delta A_{405} \text{ min}^{-1}}{36.8 \times (\text{pathlength in cm}) \times (\text{ml enzyme}) / (\text{ml reaction mixture})}$$

[0096] Mutant lysates exhibiting a $\Delta A_{405}/\text{min}$ greater than GAO-Mut1 were chosen for further characterization. Following identification of the mutation by DNA sequencing, hits were expressed, purified, and assayed for specific activity and thermostability as assessed by the temperature at which one half maximal activity was observed (T_{50}). Mutants were purified from 5 ml culture with auto-induction medium in a 24 well plate.

Harvested cells were lysed with B-PER and the lysate was spun down with 15,000 relative centrifugal force (rcf) for 30 min at 4 °C. The lysate supernatant was used for protein purification with HisPur™ Ni-NTA Spin Plates. The eluted protein sample was diluted with 100 mM potassium phosphate buffer pH 7.5 with 0.5 mM CuSO₄, and specific activity was measured using the ABTS assay. T₅₀ was measured by heating protein in the absence of substrate, cooling, and then measuring residual activity using the ABTS assay. Heating was accomplished by diluting the protein to a concentration of 2.5 mg/L in a volume of 100 mM phosphate buffer at pH 7.5, aliquoting 50 µL into a row of a 96-well PCR plate, and incubating over a temperature gradient sufficient to capture maximal and minimal enzyme performance for ten minutes. Promptly after heating, the mixture was cooled on ice and the ΔA405/min of 20 µL of enzyme solution in 200 µL of final volume of ABTS solution was measured as described above.

[0097] Hits were purified, tested for activity and T₅₀, and recombined to generate a final best mutant from the directed evolution step. Promising point mutants that could beneficially be combined in the Mut1 background included A193R D404H F441Y A172V are listed in Table 4. These mutations were combined into a single combination mutant named GAO-Mut47 which exhibited a specific activity of 27.3 U mg⁻¹ and a T₅₀ of 56.8 °C. Table 3 provides a list of point mutations carried out and their characteristics.

Table 4

Name	Mutations	U/mg	T ₅₀ °C	K _{cat} S ⁻¹	K _m mM
M-RQW-S		1.1	56.8	31.4	2168.3
GAO-mut1	Y405F Q406E	14.0	51.8	30.2	93.1
GAO-mut6	Y405F Q406E S383C	6.1	41.5	36.6	412.0
GAO-mut7	Y405F Q406E F441Y	16.9	53.6	27.3	42.7
GAO-mut8	Y405F Q406E D404H	15.0	53.7	30.7	83.9
GAO-mut9	Y405F Q406E G461A	13.4	53.2	27.8	83.7
GAO-mut10	Y405F Q406E I462R	12.1	53.2	31.6	130.7
GAO-mut11	Y405F Q406E A172V	21.2	48.5	39.5	72.6
GAO-mut12	Y405F Q406E A193R	15.4	56.3	28.0	64.8
GAO-mut13	Y405F Q406E A193T	14.6	53.8	30.4	75.5
GAO-mut14	Y405F Q406E D404H F441Y	18.8	55.0	26.5	29.7
GAO-mut15	Y405F Q406E G461A I462R	12.2	54.0	24.8	79.1
GAO-mut17	Y405F Q406E D404H F441Y G461A I462R	18.2	55.3	23.6	25.5
GAO-mut18	Y405F Q406E A193T D404H F441Y G461A I462R	18.1	56.6	24.1	28.0
GAO-mut19	Y405F Q406E A193T D404H F441Y	13.3	46.3	24.5	70.8

	G461A I462R S383C				
GAO-mut20	Y405F Q406E A193T D404H F441Y G461A I462R S383C A172V	21.4	37.9	35.6	58.2
GAO-mut21	Y405F Q406E F441Y G461A I462R	18.3	53.8	24.2	29.6
GAO-mut22	Y405F Q406E A193T D404H F441Y G461A I462R A172V	23.6	51.5	29.5	26.4
GAO-mut23	Y405F Q406E A193R D404H F441Y G461A I462R A172V	21.1	57.5	27.2	26.8
GAO-mut47	Y405F Q406E A193R D404H F441Y A172V	27.3	56.8	35.0	25.2
GAO-mut58	Y405F Q406E D404H F441Y A172V	27.1	52.9	35.4	26.6

EXAMPLE 2

[0098] Rational engineering of GAO to further accept a glucose substrate and identify stabilizing mutations was accomplished with a combination of computational methods based on structural and multiple sequence alignment data (MSA). It was identified that GAO-M-RQW-S could accept both glucose and gluconate as substrate, the results are displayed in Figure 2. Rational design was performed on the GAO-M-RQW-S sequence rather than GAO-Mut1. Structural methods employed included applying FoldX55 (40 predicted mutations) and PROSS56 (80 mutations) to a modified form of the Protein Database (PDB) structure 2WQ8 to contain the GAO-M-RQW-S mutations. MSA-based predictions were applied to a 185-member MSA. This MSA was generated from an initial set of 1000 sequences curated with JALVIEW to remove sequences with 98% redundancy and retain only sequences experimentally verified as carbohydrate oxidases. 30 mutations identified in designing a GAO for synthesizing an intermediate of the HIV drug Islatravir were also added to the panel.

[0099] In total, 202-point mutants were screened using the same methods described above for screening the directed evolution clones. Thirty-nine hits were identified from an initial screen and sixteen were reidentified from a second round of screening. Upon generation of combo mutants in the best combination mutant from the directed evolution step (GAO-Mut47), the mutations N66S, S306A, S311F, and Q486L were identified as complementary and beneficial while N28I, Y189W, S331R, A378D, and R459Q were deemed detrimental in this background. The results are summarized in Table 5. The final GAO-Mut107 construct containing the Mut47 mutations and N66S, S306A, S311F, and Q486L exhibits a specific activity of 34.96 U mg⁻¹ on 2% glucose and a T₅₀ of 60.56 °C as shown in Figure 3. Additional mutations identified from

machine learning algorithms were later incorporated to generate GAO-mut142 and GAO-mut164.

Table 5

Clone	Mutations from Mut47	New Mutations	U/mg	Fold Improvement	T50 °C
Mut47			31.11	1.00	57.64
GAO-mut68	N28I	N28I	30.84	0.99	56.76
GAO-mut69	N28I N66S	N66S	33.68	1.08	59.00
GAO-mut70	N28I N66S Y189W	Y189W	31.80	1.02	59.91
GAO-mut71	N28I N66S Y189W S306A	S306A	32.66	1.05	59.48
GAO-mut72	N28I N66S Y189W S306A S311F	S311F	33.87	1.09	60.81
GAO-mut73	N28I N66S Y189W S306A S311F S331R	S331R	27.56	0.89	59.87
GAO-mut74	N28I N66S Y189W S306A S311F S331R A378D	A378D	25.57	0.82	58.94
GAO-mut75	N28I N66S Y189W S306A S311F S331R A378D R459Q	R459Q	23.51	0.76	58.49
GAO-mut76	N28I N66S Y189W S306A S311F S331R A378D R459Q V477D	V477D	19.22	0.62	59.17
GAO-mut77	N28I N66S Y189W S306A S311F S331R A378D R459Q V477D Q486L	Q486L	24.57	0.79	59.88
GAO-mut107 ^a	N66S S306A S311F Q486L	Removed N28I, Y189W, S331R, A378D, R459Q, and V477D	34.96	1.20	60.56
GAO-mut142 ^b	N66S S306A S311F Q486L H40C	H40C	37.53	1.29	58.76
GAO-mut164 ^b	N66S S306A S311F Q486L H40C L71C	L71C	38.22	1.32	52.97

Bolded mutations are beneficial in a Mut47 background A193R D404H F441Y A172V

^aData collected in a separate experiment from other data. Fold improvement is calculated compared to an internal Mut47 control.

^bData collected in a separate experiment from other data. Fold improvement is calculated compared to an internal Mut47 control.

EXAMPLE 3

[00100] A 50 ml reaction was conducted in a 200 mL vessel pressurized to 100 psi. The vessel was charged with 50 mM sodium phosphate pH 8 buffer, 50 μM CuSO₄, 15 w/v% glucose, 0.005 w/v% catalase, 0.001% horseradish peroxidase, and 0.001 w/v% engineered GAO. The reaction was stirred 500 rpm, 11 °C for 48 hours. Samples were taken at 0, 24, and 48 hours then assayed with HPLC to measure residual glucose

and the results are presented in Figure 4. The amount of starting material declined over 48 hours from 15 to 5.7% (w/v).

EXAMPLE 4

Production of 2-keto-Glucodialdose from Glucodialdose via POX

[00101] To determine how POX might transform either D-glucose or D-glucodialdose to a product useful in the context of Scheme III, commercially available POX enzyme was obtained and used in a colorimetric 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reaction with either D-glucose, D-glucodialdose, 2-keto-D-gluconate, 2-keto-D-glucose, or 5-keto-D-glucose at a concentration of 20 mM in 50 mM pH 6 potassium phosphate buffer, at room temperature. Glucose oxidase (GOX) which is known to oxidize glucose at the C1 position to gluconolactone and GAO mutants were included in the assay as controls. In short, a 4x stock solution was prepared and diluted to a final reaction concentration of 20 mM substrate, 0.025 mg/ml horseradish peroxidase (HRP), 50 mM potassium phosphate buffer, pH 6, and 0.1% (w/v) ABTS. This solution was combined with enzyme to a final concentration of 0.8 µg/mL to begin the reaction and incubated for 25 minutes while monitoring A405 in a microtiter plate reader. Specific activity was calculated as:

$$\text{Units } \text{mg}^{-1} = \frac{\Delta A_{405} \text{ min}^{-1}}{36.8 \times (\text{pathlength in cm}) \times (\text{mg enzyme}) / (\text{ml reaction mixture})}$$

$$\text{Units } \text{ml}^{-1} = \frac{\Delta A_{405} \text{ min}^{-1}}{36.8 \times (\text{pathlength in cm}) \times (\text{ml enzyme}) / (\text{ml reaction mixture})}$$

[00102] The results of the activity screen demonstrate the potential of using POX to generate 2-keto-glucodialdose from glucodialdose. POX exhibits 8.8 U/mg specific activity on glucodialdose (77% of performance on the native glucose substrate). This specific activity could be improved upon through engineering. The results are shown in Table 6 and Figure 5. 2-ketoglucose, 2-ketogluconate, and 5-ketogluconate were not substrates for POX. None of the GAO enzymes were active on 2-keto-glucose. Given this data, production of 2-keto-glucodialdose should be possible in a dual reactor system where substrate passes through a first reactor charged with GAO to produce glucodialdose, then through a second reactor charged with POX to generate 2-keto-glucodialdose. Production via a single enzyme oxidation reactor will require engineering a GAO capable of accepting 2-keto-glucodialdose as a substrate. As expected, GOX exhibited high activity on the native substrate glucose.

Table 6

Enzyme	Glucose (U mg ⁻¹)	Glucodialdose (U mg ⁻¹)	2- ketoglucose (U mg ⁻¹)	2- ketogluconate (U mg ⁻¹)	5- ketogluconate (U mg ⁻¹)
GAO-mut1	5.7	0.2	0.0	0.2	0.0
GAO-mut47	46.9	n.d.	0.0	0.1	0.0
GAO-mut62	8.3	0.4	0.0	0.1	1.3
M-RQW-S	n.d.	n.d.	0.0	0.3	0.0
GOX	62.2	2.5	0.0	0.0	0.0
POX	11.4	8.8	0.0	0.0	0.0

[00103] The product profile was probed through HPLC-MS. A 50 μ L reaction in a 96-well microtiter plate containing 0.1% (w/v) POX, 10% (w/v) substrate, 0.005% (w/v) catalase, and 80 mM potassium phosphate buffer at pH 6.0 was analyzed using the following method: Mobile Phase A: 50 mM Ammonium Formate + 1% Formic Acid in Water

Mobile Phase B: 1% Formic Acid in Acetonitrile Column Temp: 50 C Column: Torus 2-Pic 1.7 μ m, 3.0 mm x 150 mm, Selected Ion Monitoring: 173, 175, 177, 179, 193, and 195 was done, Cone Voltages: Default values

[00104] The timed method is presented in Table 7.

Table 7

Time	Flow (mL/min)	%A	%B
0	0.8	30	70
3	0.8	80	20
4	0.8	80	20
5	0.8	30	70
5.1	0.8	30	70
7	0.8	30	70

[00105] A peak in the 175 m/z channel corresponding to the expected 2-keto-glucodialdose product was detected as early as 54 minutes and continued to grow in accordance with disappearance of glucodialdose (177 m/z). These results are presented in Figure 6. Mass channels in negative ion mode from top to bottom correspond to 173, 175, 177, 179, 193, 195 m/z. The 173 m/z channel is not shown for the glucose 0.9, 1.6, or 2.8 hr reactions

[00106] Glucodialdose typically exhibits three peaks in the 177 m/z channel potentially corresponding to different cyclized forms of the compound. It is curious that the glucodialdose peak with the longest retention time disappears more slowly than the others suggesting POX may prefer some configurations of the substrate over others.

Some peaks appear in the 193 m/z channel which could correspond to glucuronic or glucuronic acid.

[00107] While aspects of the presently disclosed subject matter have been shown and described, modifications thereof can be made by one skilled in the art without departing from the spirit and teachings of the subject matter. The aspects described herein are exemplary only, and are not intended to be limiting. Many variations and modifications of the subject matter disclosed herein are possible and are within the scope of the disclosed subject matter. Where numerical ranges or limitations are expressly stated, such express ranges or limitations should be understood to include iterative ranges or limitations of like magnitude falling within the expressly stated ranges or limitations (e.g., from about 1 to about 10 includes 2, 3, 4, etc.; greater than 0.10 includes 0.11, 0.12, 0.13, etc.). Use of the term "optionally" with respect to any element of a claim is intended to mean that the subject element is required, or alternatively, is not required. Both alternatives are intended to be within the scope of the claim. Use of broader terms such as comprises, includes, having, etc. should be understood to provide support for narrower terms such as consisting of, consisting essentially of, comprised substantially of, etc.

[00108] Accordingly, the scope of protection is not limited by the description set out above but is only limited by the claims which follow, that scope including all equivalents of the subject matter of the claims. Each and every claim is incorporated into the specification as an aspect of the present disclosure. Thus, the claims are a further description and are an addition to the aspects of the present invention. The discussion of a reference herein is not an admission that it is prior art to the presently disclosed subject matter, especially any reference that may have a publication date after the priority date of this application. The disclosures of all patents, patent applications, and publications cited herein are hereby incorporated by reference, to the extent that they provide exemplary, procedural or other details supplementary to those set forth herein.