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(54) **Title:** METHODS AND COMPOSITIONS FOR THE PRODUCTION OF DIACIDS AND GLYCOLIC ACID

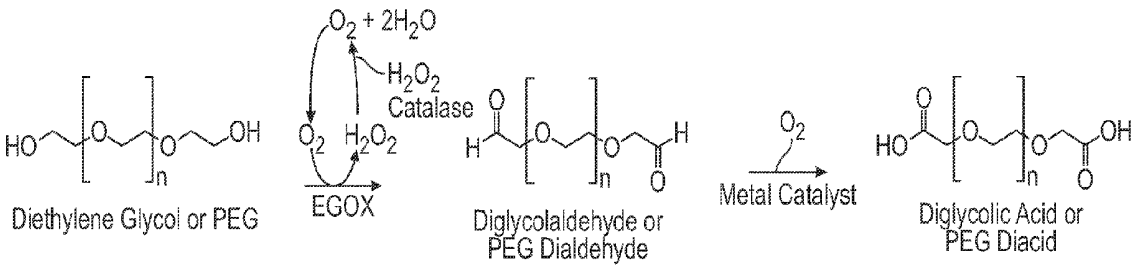

FIG. 1A

(57) **Abstract:** A method for preparing one or more acids comprising contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate; contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid and recovering the acid.

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METHODS AND COMPOSITIONS FOR THE PRODUCTION OF DIACIDS AND GLYCOLIC ACID

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 63/518,711 filed August 10, 2023 and entitled "ACID DEHYDRATION CATALYST," and to U.S. Provisional Application Serial No. 63/557,445 filed February 23, 2024 and entitled "METHODS AND COMPOSITIONS FOR THE PRODUCTION OF DIACIDS AND GLYCOLIC ACID," each of which is hereby incorporated herein by reference in its entirety for all purposes.

REFERENCE TO SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in XML file format and is hereby incorporated by reference in its entirety. Said XML file, created on February 23, 2023, is named "24PROC002-PRO_3416-18900 and is 21.3 kilobytes in size.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] Not applicable.

TECHNICAL FIELD

[0004] The present disclosure relates generally to compositions and methods for the production of value-added chemicals. More particularly, the present disclosure relates to chemoenzymatic methods for the production of diacids and glycolic acid.

BACKGROUND

[0005] Over the last 150 years, the synthesis of inexpensive chemicals from fossilized forms of carbon (e.g. oil, coal, natural gas) has dramatically altered society through their broad applications, ranging from cosmetics to plastics. This petroleum-based carbon feedstock generates a small collection of platform chemicals from which highly efficient chemical conversions lead to the manufacture of a large variety of chemical products. However, the current approach to producing these carbon-based chemicals is un-sustainable as feedstocks that required millions of years to form are being depleted. The

creation of a truly sustainable chemical industry will only occur when the timescale of the feedstock formation matches the timescale of its utilization to make chemicals.

[0006] Organic acids have conventionally been used in goods such as feed and food. The renaissance of bio-plastics has pushed bio-production of organic acids as bi-functional monomers. For example, acids with additional keto- or hydroxyl-groups may be employed as building blocks for polyesters, and di-carboxylic acids may be used for the production of polyamides.

[0007] With respect to glycolic acid, diesters of diglycolic acid with branched higher alcohols can be used as (i) softeners for polyvinyl chloride ((ii) as a diester component in homopolymeric and copolymeric polyester. The polymeric blends of diglycolic diesters find applications as tissue adhesives, cartilage substitutes, or implant materials.

[0008] Diglycolic acid is also used as a corrosion inhibitor, a chelating agent, and in herbicides and pesticides. Commercial production of diethylene glycol typically involves either oxidation with molecular oxygen and nitric acid or oxidation using a combintion of chloroacetic and sodium hydroxide.

[0009] Nonetheless, an ongoing need exists to find novel methods and compositions for the production of diacids that lack one or more of the aforementioned challenges.

SUMMARY

[0010] In various aspects disclosed here is a method for preparing one or more acids. The method may comprise contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate. The method may comprise also contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid. The method may also comprise recovering the acid.

BRIEF DESCRIPTION OF DRAWINGS

[0011] For a detailed description of various exemplary embodiments, reference will now be made to the accompanying drawings in which:

[0012] Figure 1A depicts oxidation of a polyglycol to the corresponding dialdehyde and oxidation of the dialdehyde to the corresponding diacid catalyzed by ethylene glycol oxidase and a metal oxidation catalyst, respectively.

[0013] Figure 1B depicts the ethylene glycol oxidase catalyzed oxidation of ethylene glycol to glyoxal.

[0014] Figure 1C depicts oxidation of ethylene glycol to the aldehyde and oxidation of the aldehyde to the corresponding acid catalyzed by ethylene glycol oxidase and galactose oxidase, respectively.

[0001] Figure 1D hydrolysis of a dicarboxylic acid to the corresponding monocarboxylic acids catalyzed by an acid dehydration catalyst.

[0002] Figure 1E depicts oxidation of a polyglycol to the corresponding diacid catalyzed a metal oxidation catalyst.

[0003] Figure 1F depicts oxidation of a polyglycol to the corresponding dialdehyde and oxidation of the dialdehyde to the corresponding diacid catalyzed by galactose oxidase and a metal oxidation catalyst, respectively.

[0004] Figure 2A is a scanning electron micrograph image of the acid dehydration catalyst ZSM-5.

[0005] Figure 2B depicts a surface area plot.

[0006] Figure 3 is a depiction of an exemplary reactor system for production of a diacid from a polyglycol.

[0007] Figure 4 is a bar graph depicting the catalytic activity of galactose oxidase mutants in a glucose oxidation reaction.

[0008] Figure 5 is a bar graph depicting the catalytic activity of galactose oxidase mutants in a gluconate oxidation reaction.

[0009] Figure 6A is a plot of the catalytic activity of the galactose oxidase mutant Mut47 in a Parr bomb reaction as a function of time.

[0010] Figure 6B is a plot of the specific activity of machine learning mutants compared with GAO-mut47 and GAO-Mut107 controls.

[0011] Figure 6C is a plot of the time to 50% inactivation of machine learning mutants compared with GAO-mut47 and GAO-Mut107.

[0012] Figure 7 is a plot of the specific activities of GAO mutants on the indicated substrates.

[0013] Figure 8 is a plot of the concentration glycol oxidation products as a functio of the indicated enzyme using ethylene glycol as a substrate.

[0014] Figure 9 is a plot of the activity of galactose oxidase mutant 142 using ethylene glycol as a substrate.

[0015] Figure 10A is schematic depiction of the proposed purpald reaction with either glycolaldehyde or glyoxal.

[0016] Figure 10B is an image of the colorimetric response obtained in the purpald reaction for ethylene glycol, glyoxal and glycolaldehyde over the indicated concentration range.

[0017] Figure 10C is a plot of the absorbance as function of wavelength for the indicated samples.

[0018] Figure 11 is bar graph of the glyoxal to glycolaldehyde ratio after oxidation reactions of ethylene glycol (EG) catalyzed by the indicated galactose oxidase mutants.

[0019] Figure 12A is a plot of the amount of glyoxal and glycolaldehyde produced in an ethylene glycol oxidation reaction catalyzed by lysates of a galactose mutant relative to that of GAO Mut321 wild type.

[0020] Figure 12B is a plot of the amount of glyoxal and glycolaldehyde produced in an ethylene glycol oxidation reaction catalyzed by lysates of a galactose mutant relative to that of GAO Mut321 wild type.

[0021] Figure 12C is a plot of the amount of glyoxal and glycolaldehyde produced in an ethylene glycol oxidation reaction catalyzed by lysates of a galactose mutant relative to that of GAO Mut321 wild type.

[0022] Figure 13 is a plot of the specific activity of different galactose oxidase mutants using diethylene glycol as a substrate.

[0023] Figure 14A is an image of Purpald assay results for the oxidation of diethylene glycol with the indicated galactose oxidase.

[0024] Figure 14B is a plot of the absorbance as a function of wavelength for the indicated samples.

[0025] Figure 14C is a bar graph of the percent conversion of diethylene glycol using the indicated galactose oxidase after 16 hours.

[0026] Figure 14D is a bar graph of the percent conversion of diethylene glycol using the indicated galactose oxidase after 16 hours.

DETAILED DESCRIPTION

[0027] Disclosed herein are chemoenzymatic methods of generating glycol derivatives. In one or more aspects, the method comprises oxidation of a glycol to the corresponding aldehyde and oxidation of the aldehyde to form glyoxal. Oxidation of ethylene glycol may be carried out using one or more biocatalysts, one or more transition-metal oxidation catalysts, or combinations thereof. In one or more aspects, the glycol is

ethylene glycol. In one or more aspects, the glycol is a polyglycol such as diethylene glycol, polyethylene glycol, or combinations thereof.

[0028] In one or more aspects, a method of the present disclosure comprises oxidation of ethylene glycol to glyoxal. This is reaction is depicted in Figure 1B. In some aspects, oxidation of ethylene glycol is carried out in the presence of a first biocatalyst. A first biocatalyst suitable for use in the present disclosure may comprise one or more oxidases, such as alcohol oxidases. Catalysts suitable for use in the present disclosure will be described in more detail later herein.

[0029] In an aspect, ethylene glycol is contacted with a first biocatalyst under conditions suitable for formation of the corresponding aldehyde, glycolaldehyde.

[0030] In one or more aspects, glycolaldehyde is recovered and used with or without further processing. In some aspects, at least a portion of the glycolaldehyde produced is subjected to further oxidation in the presence of a second biocatalyst under conditions suitable for the production of glyoxal.

[0031] In one or more aspects, the second biocatalyst comprises one or more oxidase enzymes. The oxidase enzymes in the second biocatalyst may be the same as the oxidase enzymes used in the first biocatalyst. In some aspects, the oxidase enzymes in the second biocatalyst differ from the oxidase enzymes used in the first biocatalyst. In an aspect, the second biocatalyst comprises a galactose oxidase and a peroxidase such as horseradish peroxidase. In some aspects, the galactose oxidase is a galactose oxidase which has one or more amino acids is mutated.

[0032] Oxidation of ethylene glycol to glyoxal facilitated by two biocatalysts is depicted in Figure 1C. The reaction product, glyoxal, may be used without further purification. In the alternative, glyoxal may be processed to meet one or more user and/or process goals. In one or more aspects, glyoxal is produced at a yield ranging from about 50 weight percent (wt.%) to about 90 wt.% or from about 60 wt.% to about 90 wt.% or from about 70 to about 90 wt.% based on the total weight of recovered product and a purity of from about 50% to about 80% or from about 60% to about 70% or from about 70% to about 90% based on the total weight of recovered product.

[0033] In one or more aspects, a method of the present disclosure comprises oxidation of a polyglycol molecule to form the corresponding dialdehyde which can be further oxidized to form the corresponding diacid. In some aspects, the polyglycol molecule is diethylene glycol. In other aspects, the polyglycol molecule is polyethylene glycol having

the polymerization of n monomer units where n ranges from 10 to 1000 or from 100 to 750 or from 100 to 400. Hereinafter the reactant will be referred to as the polyglycol molecule (PGM) which may be exemplified by diethylene glycol or polyethylene glycol (PEG).

[0034] In one or more aspects, PGM is contacted with a biocatalyst comprising one or more oxidase enzymes under conditions suitable for formation of the dialdehyde. In an aspect, the biocatalyst comprises an alcohol oxidase such as EGOX. In the alternative aspect, the biocatalyst comprises a galactose oxidase and a peroxidase such as horseradish peroxidase. In such aspects, the galactose oxidase is a galactose oxidase which has one or more amino acids mutated. In such aspects, a peroxidase is included to facilitate regeneration of galactose oxidase.

[0035] The reaction product, a dialdehyde, may be used without further purification. In the alternative, the dialdehyde may be processed to meet one or more user and/or process goals. In one or more aspects, the dialdehyde is produced at a yield ranging from about 70 weight percent (wt.%) to about 99 wt.% or from about 75 wt.% to about 90 wt.% or from about 80 wt.% to about 90 wt.% based on the total weight of recovered product and a purity of from about 75% to about 99% or from about 80% to about 99% or from about 85% to about 99%.

[0036] In some aspects, at least a portion of the dialdehyde produced is subjected to further oxidation in the presence of an oxidation catalyst under conditions suitable for the production of the corresponding diacid. In one or more aspects the oxidation catalyst comprises a transition metal or a supported transition metal. An oxidation catalyst suitable for use in the present disclosure will be described in more detail later herein.

[0037] Conditions suitable for the formation of the diacid may include one or more of the following reaction parameters: an oxidation catalyst present in an amount of from about 0.0001 wt.% to about 1 wt.% or from about 0.001 wt.% to about 0.5 wt.% or from about 0.01 wt.% to about 0.1 wt.% based on the total amount of the reaction mixture; a solvent comprising aqueous fluids; a temperature of from about 0 °C to about 300 °C, or from about 25 °C to about 150 °C or from about 50 °C to about 100; an oxygen pressure of from about 100 psi to about 500 psi or from about 50 psi to about 250 psi or from about 100 psi to about 200 psi; and a reaction time of from about 30 minutes to about 96 hours or from about 5 hours to about 72 hours or from about 12 hours to about 24 hours.

[0038] The reaction product, a diacid, may be used without further purification. In the alternative, the diacid may be processed to meet one or more user and/or process goals. In one or more aspects, the diacid is produced at a yield ranging from about 50 weight percent (wt.%) to about 90 wt.% or from about 60 wt.% to about 90 wt.% or from about 70 wt.% to about 90 wt.% based on the total weight of recovered product and a purity of from about 70% to about 99% or from about 80% to about 95% or from about 85% to about 95%.

[0039] The reaction of a PGM to the corresponding dialdehyde and then diacid using an alcohol oxidase and oxidation catalyst is depicted in Figure 1A. The reaction of a PGM to the corresponding dialdehyde and then diacid using a galactose oxidase and oxidation catalyst is depicted in Figure 1F.

[0040] In some aspects, the oxidation catalyst is used to catalyze both the oxidation of a PGM to the corresponding dialdehyde and oxidation of the dialdehyde to the corresponding diacid. In such aspects, reaction conditions suitable for conversion of the PGM to the corresponding diacid may include the following parameters: an oxidation catalyst present in an amount of from about 0.0001 wt.% to about 1 wt.% or from about 0.001 wt.% to about 0.5 wt.% or from about 0.01 wt.% to about 0.1 wt.% based on the total amount of the reaction mixture; a solvent comprising aqueous fluids; a temperature of from about 0 °C to about 300 °C, or from about 25 °C to about 150 °C or from about 50 °C to about 100; an oxygen pressure of from about 100 psi to about 500 psi or from about 50 psi to about 250 psi or from about 100 psi to about 200 psi; and a reaction time of from about 30 minutes to about 96 hours or from about 5 hours to about 72 hours or from about 12 hours to about 24 hours.

[0041] The reaction product, a diacid, may be used without further purification. In the alternative, the diacid may be processed to meet one or more user and/or process goals. In one or more aspects, the diacid is produced at a yield ranging from about 50 weight percent (wt.%) to about 90 wt.% or from about 60 wt.% to about 90 wt.% or from about 70 wt.% to about 90 wt.% based on the total weight of recovered product and a purity of from about 70% to about 99% or from about 80% to about 95% or from about 85% to about 95%.

[0042] The reaction of a PGM to the corresponding dialdehyde and then diacid using an acid dehydration catalyst is depicted in Figure 1E.

[0043] In one or more aspects, a diacid disclosed herein is hydrolyzed to produce two equivalents of the corresponding monoacid. In one or more aspects, hydrolysis of the diacid is facilitated by an acid catalyst or acid dehydration catalyst. Acid catalysts suitable for use include homogeneous acids and heterogeneous acids. Nonlimiting examples of acid catalysts suitable for use in the present disclosure include mineral acids such as HI, HBr, HCl, sulfuric acid (H_2SO_4), methanesulfonic acid, p-toluenesulfonic acid, camphorsulfonic acid, phosphoric acid (H_3PO_4), mixture of methanesulfonic acid and 1,5,7-triazabicyclo[4.4.0]dec-5-ene, zeolites such as H-ZSM-5, H-β zeolite, solid-supported acids such as AMBERLYST 15 and SILICABOND TOSIC AICD, silica-alumina, ion-exchange resins, sulfated zirconia, alumina, solid Lewis acids such as Al_2O_3, WO_3/γ-Al_2O_3, and combinations thereof.

[0044] Without wishing to be limited by theory, a diacid when contacted with an acid catalyst is protonated at the ether oxygen increasing its electrophilicity. Water, acting as a nucleophile, may then attack the carbon atom adjacent to the protonated ether oxygen, leading to the formation of an oxonium intermediate. The oxonium intermediate then undergoes deprotonation, cleaving the ether bond, forming two molecules of glycolic, acid, and regenerating the acid catalyst. The reaction of an acid catalyst with a diacid is depicted schematically in Figure 1D.

[0045] As will be understood by one of ordinary skill in the art with the benefit of this disclosure, the reaction conditions utilized for any particular biocatalyst composition disclosed herein will depend on a variety of factors. In one or more aspects, the reaction conditions disclosed herein contemplate the use of a biocatalyst in commercial scale reactions carried out in the presence of an oxygen source. In one or more aspects, the reactions disclosed herein are carried out in a bubble column reactor where oxygen is introduced under pressure to increase availability to the biocatalyst.

[0046] The biocatalyst reactions disclosed herein may be carried out using one or more of the following reaction parameters: a biocatalyst amount of about 5 milligrams/liter (mg/L) to about 1000 mg/L, or from about 10 mg/L to about 500 mg/L or from about 20 mg/L to about 200 mg/L; a suitable buffered media providing a pH ranging from about 5 to about 10, or from about 5.5 to about 9; or from about 6.5 to about 8.5, or rom about 6.5 to about 8.5 or from about 7 to about 8; an oxygen pressure of equal to or less than about 500 psi, alternatively from about 50 to about 250 psi or alternatively from about 70 to about 150 psi; a reaction temperature of from about 10 °C to about 50 °C or from

about 10 °C to about 40 °C or from about 10°C to about 25 °C; a reaction period of from about 1 hour to about 48 hours or from about 1 hour to about 36 hours or from about 5 hours to about 24 hours.

[0047] In one or more aspects, a biocatalyst suitable for use in the present disclosure comprises an immobilized biocatalyst. The term immobilized biocatalyst herein refers to a biocatalyst (e.g., enzyme) that is physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously. An immobilized enzyme may be associated with any suitable support such as a natural polymer (e.g., polysaccharide), a synthetic polymer (e.g., polystyrene), or an inorganic material (e.g., silica).

[0048] In one or more aspects, the biocatalyst comprises a copper radical oxidase (CRO). CROs are a class of non-flavoprotein alcohol oxidoreductases that employ molecular oxygen as a terminal electron acceptor to generate hydrogen peroxide.

[0049] GAO is a copper enzyme secreted by some fungal species, including *Fusarium graminearum* (also known as *Gibberella zeae*), to aid in degradation of extracellular carbohydrate food sources through catalyzing the oxidation of primary alcohols to aldehydes while generating hydrogen peroxide. Not intending to be bound by theory, the native function of GAO is the oxidation of D-galactose to D-galacto-hexodialdose. In an aspect, the CRO is galactose oxidase (GAO EC 1.1.3.9) and the reaction is carried out in the presence of a small molecule activator . The small molecule activator is a cofactor that functions to rescue the activity of the GAO and is itself activated by a biocatalyst such as horseradish peroxidase (HRP).

[0050] Nonlimiting examples of other small molecule activators suitable for use in the present disclosure include L-tryptophan, 2-mercaptobenzothiazole, L-histidine, methylchloroisothiazolinone, o-dianisidine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 4-aminoantipyrine, L-tyrosine, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl, chloromethylisothiazolinone, 4-thiazolecarboxylic acid, Sunset yellow FCF, tartrazine, p-benzoquinone, dicoumarol, phthalimide, saccharin, phthalic anhydride, erythrosine B, 2-aminobenzothiazole, thiabendazole, 2-hydroxybenzothiazole, phenothiazine, 6-aminobenzothiazole, indigo carmine, naphthalimide, 2-aminothiazole, thiazole, 2H-1,4-benzothiazin-3(4H)-one, 2-oxindole, beta-lapachone, menaquinone, thiamine, 4-methyl-5-thiazoleethanol, disodium 6-hydroxy-5-((2-methoxy-5-methyl-4-sulfophenyl)azo)-2-naphthalenesulfonate (Allura Red AC), menadione, p-cresol, ethyl-

[4-[[4-[ethyl-[(3-sulfophenyl)methyl]amino]phenyl]-(4-hydroxy-2-sulfophenyl)methylidene]-1-cyclohexa-2,5-dienylidene]-[(3-sulfophenyl)methyl]azanium (Fast green FCF), disodium α-(4-(N-ethyl-3-sulfonatobenzylamino) phenyl)-α-(4-N-ethyl-3-sulfonatobenzylamino) cyclohexa-2,5-dienylidene) toluene-2-sulfonate (Brilliant Blue FCF), methylisothiazolinone, caffeine, veratryl alcohol, fluorescein, and combinations thereof.

[0051] In an aspect, the biocatalyst comprises an alcohol oxidase (AOX, EC 1.1.3.13). In another aspect, an immobilized AOX with catalase is used as a biocatalyst in the present disclosure. Nonlimiting examples of sources of an AOX suitable for use in the biocatalyst include *Achatina achatina, Achatina fulica, Arion ater, Aspergillus ochraceus Aspergillus ochraceus AIU 031, Aspergillus nidulans, Aspergillus terreus Aspergillus terreus MTCC 6324, Basidiomycota Basidiomycota B191039, Byssochlamys spectabilis Byssochlamys spectabilis RI017 Paecilomyces variotii, Candida boidinii, Candida methanolica, Candida koshuensis, Candida olivarium, Candida ooitensis, Candida queretana, Candida silvicola Hanensula alcoolica, Kloeckera boidinii, Torulopsis enokii, Candida cariosilignicola, Candida guilliermondii, Pichia guilliermondii, Yamadazyma guilliermondii, Endomyces guilliermondii, Candida methanolovescense, Ogatea minuta, Candida methanosorbosa, Candida methanosorbosa M-2003, Candida sithepensis, Candida sonorensis, Torulopsis sonorensis, Candida sp. (in: Saccharomycetales), Candida sp. (in: Saccharomycetales) 25-A, Candida succiphila, Candida tropicalis, Comamonas sp., Comamonas sp. UVS, Gloeophyllum trabeum, Hansenula polymorpha , Ogataea polymorpha, Pichia angusta, Hanensula angusta, Ogataea angusta Ogataea angusta DL-1, Ogataea angusta NCYC 495, Helix aspersa, Kuraishia capsulata, Lachnellula arida, Lachnellula cervina, Lachnellula occidentalis, Lachnellula subtilissima, Lachnellula suecica, Lachnellula willkommii, Methylococcus capsulatus, Methylophilus methylotrophus, Ochrobactrum sp., Ochrobactrum sp. AIU 033, Ogataea glucozyma, Ogataea henricii, Ogataea methanolica, Pichia pinus, Ogataea minuta, Ogataea naganishii, Ogataea philodendri, Ogataea pignaliae, Ogataea pini, Ogataea siamensis, Ogataea trehalophil, Ogataea wickerhamii, Passalora fulva, Penicillium chrysogenum, Penicillium purpurascens, Penicillium purpurascens AIU 063, Phanerochaete chrysosporium, Phanerochaete chrysosporium DSMZ 1547,*

Phanerochaete chrysosporium K-3, Phlebiopsis gigantea, Pichia pastoris, Komagataella pastoris, Komagataella phaffii, Komagataella pseudopastoris, Endomyces pastoris, Petasospora pastoris, Zygosaccharomyces pastoris, Zygowillia pastoris, Zymopichia pastoris, Komagataella pastoris GS115, Komagataella pastoris IFP 206, Komagataella pastoris X33, Pichia putida, Polyporus obtusus, Poria contigua, Radulodon casearius, Thodotorula toruloides, Thermoascus aurantiacus, Thermoascus aurantiacus NBRC 31693, and *Trametes cinnabarina.* In one or more aspects, the *AOX* is sourced from *Phanerochaete chrysosporium.*

[0052] In one or more aspects, the biocatalyst comprises a glycerol oxidase (GlyOX, EC 1.1.3.9). For example, a GlyOX enzyme may have been endogenously expressed and characterized for activity on ethylene glycol. In one or more aspects, the GlyOX is an endogenous glycerol oxidase produced from *Aspergillus japonicus.* Additional glycerol oxidases suitable for use in the present disclosure may be sourced from *Neurospora* and *Penicillium.* Enzymes suitable for use in the present disclosure may

[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 may result in the production of byproducts (e.g., hydrogen peroxide) that can detrimentally impact other components of the reaction mixture. For example, hydrogen peroxide is a by-product of the GAO catalyzed oxidations. Hydrogen peroxide can degrade the biocatalyst resulting in a loss of catalytic activity which can mitigated by the introduction of a catalase (E.C. 1.11.1.61). In some aspects, the effect of hydrogen peroxide on the catalyst in the biocatalyst is mitigated. For example, mitigation of the effects of hydrogen peroxide on the biocatalyst may be through using any suitable mechanism such as the introduction of a catalase to the reaction media, through the use of a hydrogen peroxide-resistant biocatalyst, or through recovery of the hydrogen peroxide.

[0054] In an aspect, a biocatalyst is reacted in the presence of one or more cofactors. Herein a cofactor refers to non-protein chemical compound that modulates the biological activity of the biocatalyst. Nonlimiting examples of 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 biocatalyst and/or be added at various points during the reaction. In some aspects, cofactors included with the

biocatalyst preparation may be readily regenerated with oxygen and/or may remain stable throughout the lifetime of the enzyme(s). A biocatalyst suitable for use in the present disclosure may have any of SEQ ID NO:1 through SEQ ID NO:14.

[0055] In one or more aspects, the biocatalyst is a wild type enzyme, a functional fragment thereof or a functional variant thereof. “Fragment” as used herein is meant to include any amino acid sequence shorter than the full-length biocatalyst (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 biocatalyst sequence. Alternatively, the fragment may have or include multiple (e.g., several) different shorter segments where each segment is identical in amino acid sequence to a different portion of the amino acid sequence of the biocatalyst but linked via amino acids differing in sequence from the biocatalyst. Herein, a "functional variant" of the biocatalyst refers to a polypeptide which 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.

[0056] In the alternative or in combination with the aforementioned mutations, the biocatalyst 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 glycolaldehyde and/or hydrogen peroxide, increase protein yield, or combinations thereof.

[0057] Herein, reference has been made to “sources” of biocatalysts. It is to be understood this refers to the biomolecule as expressed by the named organism. It is contemplated the biocatalyst may be obtained from the organism or a version of said biocatalyst (wildtype or recombinant) provided as a suitable construct to an appropriate expression system.

[0058] In an aspect, any biocatalyst 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.* As used herein, "vector" refers to 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 AOX 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.

[0059] In an aspect, the gene of a biocatalyst 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.

[0060] The term "expression control sequence" or "regulatory sequences" are used interchangeably and as used herein refer to polynucleotide sequences that 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 may differ depending upon the host organism; for example, 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.

[0061] 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.

[0062] In one or more aspects of the present disclosure, gold-based catalysts are used as oxidation catalysts.

[0063] In one or more aspects, the oxidation catalyst is a bimetallic catalyst. A bimetallic catalyst may provide advantages such as improved throughput and catalyst stability. In one or more aspects, an oxidation catalyst for use in the present disclosure comprises equimolar concentrations of gold (Au) and platinum (Pt).

[0064] Nonlimiting examples of metallic catalysts for use in the present disclosure include (i) monometallic formulations that contain have a single transition metal species such as gold, platinum, palladium (Pd), nickel (Ni), iron (Fe), manganese (Mn), copper (Cu) or cobalt (Co) on a support material such as activated carbon (ii) bifunctional formulations: For example, the oxidation catalyst may comprise formulations of Au with a second transition metal such as platinum, pallidum, silver, copper or cobalt where a specific ratio of metals is achieved and(iii) doped alloy formulations. In doped alloy formulations the dopant is characterized by being present in minor amounts and is an atom not originally part of the crystal structure of the material present in major amounts. Dopants, including Zn, Sr, Bi, and most rare earth metals may enhance the catalyst stability against deactivation and leaching in aerobic oxidations in aqueous media. The introduction of dopants can alter the electronic density around the active sites of the major materials (e.g., gold nanoparticles) resulting in increased catalytic selectivity and activity by facilitating the formation of reaction intermediates. For example, Bi or La dopants can help in the dispersion of gold nanoparticles on the support, reducing the tendency for sintering. Further, by anchoring the gold nanoparticles more firmly to the support or by forming stable bimetallic structures, the resistance to corrosive environments would be improved

[0065] In one or more aspects, an oxidation catalyst suitable for use in the present disclosure may be a nanoparticle having particle size distributions ranging from about 1 nm to about 99 nm, or from about 2 nm to about 80 nm or from about 10 nm to about 70 nm or from about 20 nm to about 60 nm or from about 25 nm to about 50 nm. In one

or more aspects, the total amount of metal present in the oxidation catalyst ranges from about 0.1 weight percent (wt.%) to about 10 wt.%, or from about 0.5 wt.% to about 7.5 wt.%, or from about 1 wt.% to about 5 wt.%, or from about 1 wt.% to about 5 wt.%, or from about 1 to about 3 wt.%, or from about 1 wt.% to about 2 wt.%, or from about 0.1 wt.% to about 2 wt.% based on the total weight of the catalyst.

[0066] In one or more aspects an acid dehydration catalyst suitable for use in the present disclosure comprises a zeolite. Zeolites refer herein to a group of crystalline hydrated aluminosilicates of monovalent or polyvalent bases which give up their water without changing their crystal structure and are capable of adsorbing other compounds in place of the water removed and which, moreover, are capable of exchanging bases. Zeolites are characterized by their crystal structure. Among the numerous known natural and synthetic zeolites, those of the type of zeolite A, faujasite, mordenite, chabasite, erionite and clinoptilolite. Each of these types has its own crystal structure which gives its own characteristic X-ray diffraction diagram by the method of Debye and Scherrer. In one or more aspects, the zeolite comprises Zeolite Socony Mobil–5 (framework type MFI from ZSM-5 (five)), which is an aluminosilicate zeolite belonging to the pentasil family of zeolites and has the chemical formula $Na_nAlnSi96–nO192 \cdot 16H_2O$ ($0<n<27$).

[0067] In one or more aspects, the acid dehydration catalyst comprises a metal, alternatively zinc. In one or more aspects, a, acid dehydration catalyst of the present disclosure is characterized by an increased number of Bronstead acid sites when compared to Lewis acid sites. In one or more aspects, the sites are located on the surface of the zeolite.

[0068] In one or more aspects, methods of the present disclosure may be carried out using any suitable reactor system. An exemplary reactor system is depicted in Figure 3. With reference to Figure 3, a reactor system for production of a diacid 100 may comprise an enzyme oxidation reactor (EOR) 120. The EOR 120 may use water as a solvent, and is fed reactant (diethylene glycol or PEG), enzymes (as catalyst), and oxygen. The EOR 120 can be a stirred sparged tank, a tubular reactor, a bubble column, an airlift reactor, or other gas-liquid reactor system and operate at elevated pressures if higher dissolved oxygen concentrations are required. The EOR and all subsequent components of the reactor system may be operated under conditions suitable for the unit to perform its intended function. In one or more aspects, a first product mixture is generated in the EOR 120 (e.g., dialdehyde). The first product mixture may be withdrawn from the EOR

120 via a tangential flow filter (TFF) loaded with molecular weight cutoff (MWCO) 130 ultrafiltration membranes. In one or more aspects, the MWCO 130 separates enzyme from the product mixture which generates an enzyme rich fraction and a second product mixture (e.g., containing dialdehyde intermediate and buffer) is conveyed to an electrodialysis membrane 140 (EDM) downstream. At least a portion of the enzyme rich fraction may be recycled to the EOR 120. Fresh buffer can be dosed as needed. The second product mixture is introduced to the EDM 140 in order to remove charged buffer compounds, such as phosphates. Makeup water may be added to the second product mixture within or prior to entering the EDM 140. The makeup water acts as a sink for charged compounds while uncharged dialdehydes are not removed via the EDM 140. The buffer components may be recycled back to the EOR 120. The material containing buffer free product, the third product mixture may then be fed into the Metal Oxidation Reactor 150 (MOR). The MOR 150 could be a packed bed reactor, a slurry reactor, a trickle bed reactor, or other solid-liquid-gas contacting reactor. Disposed within the MOR 150 is an oxidation catalyst of the type disclosed herein. In an exemplary aspect, within the MOR 150, the third product mixture containing one or more dialdehyde intermediates in water and high-pressure air or oxygen are contacted over the oxidation catalyst to form a crude di-acid product, along with side products. The crude diacid product may be conveyed from the MOR 150 to Separator 1 160. Separator 1 160 may be a single unit operation, or a mix of unit operations configured to obtain a user or process desired yield and /or purity. Nonlimiting examples of separators that could be concatenated include vacuum distillation, evaporation, pervaporation, nanofiltration, liquid-liquid extraction, adsorption, stripping, and chromatography. On-path impurities may be conveyed from Separator 1 160 back to the MOR 150 to form a reactor-separator train that maximizes integrated yield of dialdehyde intermediate to a final product. In one or more aspects, a reactor system of the type disclosed herein can be used to produce a diacid as the final product. In other aspects, the reactor system 100 can be used to product can be used to produce the monoacid as the final product. For example, a diacid that is separated in Separator 1 may be fed to a Hydrolysis Reactor, 170 having an acid disposed therein. The Hydrolysis Reactor 170 combines the diacid with a water molecule to split the diacid and form two mono-acids via hydrolysis. This mixture of mono-acid and di-acid may then fed to Separator 2 180 which to function to separate the product mixture and produce high purity mono-acid. This Separator 2 could

be a concatenation of liquid-liquid extraction and chromatography. Unreacted di-acid may be reintroduced to the Hydrolysis Reactor 170 feed, while purified mono-acid is withdrawn as product.

[0069] Disclosed herein are chemoenzymatic methods that advantageously produces high purity materials such as diglycolaldehyde and PEG aldehyde intermediates using an *in-vitro* system; utilizes purified or partially pure enzymes as catalysts that eliminates or reduces the presence of additional compounds and materials thereby facilitating separation and purification of the product and leading to lower production costs. Additionally, the present disclosure contemplates in addition to the use of a biocatalyst, the use of a metal catalyst, either in a monofunctional formulation or bifunctional formulation, will promote selective oxidation of the enzyme-derived intermediates into the targeted diacid products.

[0070] The methods and compositions disclosed herein result in a cleaner production process because the use of enzymes and metal catalysts removes hazardous chemicals from the process such as nitric acid or chloracetic acid. Additionally, the presently disclosed processes may be greener or more environmentally-friendly because although the diethylene glycol feedstock is currently derived from petrochemical sources, the compound may also be derived from renewable resources (i.e., ethanol or polyethylene), providing a pathway to a greener process than the conventional methods.

ADDITIONAL DISCLOSURE

[0071] A 1st embodiment is a method for preparing one or more acids comprising contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate; contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid; and recovering the acid.

[0072] A 2nd embodiment is the method of the 1st embodiment wherein the glycol comprises ethylene glycol.

[0073] A 3rd embodiment is the method of the 2nd embodiment wherein the aldehyde intermediate comprises glycolaldehyde.

[0074] A 4th embodiment is the method of the 3rd embodiment wherein the acid comprises glyoxal.

[0075] A 5th embodiment is the method of one of the 1st—4th embodiments wherein the glycol is a polyglycol.

[0076] A 6th embodiment is the method of the 5th embodiment wherein the polyglycol comprises diethylene glycol,

[0077] A 7th embodiment is the method of the 6th embodiment wherein the aldehyde intermediate comprises diglycolaldehyde.

[0078] An 8th embodiment is the method of the 7th embodiment wherein the acid comprises diglycolic acid.

[0079] A 9th embodiment is the method of the 5th embodiment wherein the polyglycol is polyethylene glycol.

[0080] A 10th embodiment is the method of the 9th embodiment wherein the aldehyde intermediate is polyethylene glycol aldehyde.

[0081] An 11th embodiment is the method of the 10th embodiment wherein the acid is polyethylene glycol diacid.

[0082] A 12th embodiment is the method of one of the 1st –11th embodiments wherein the one or more oxidation catalysts comprise one or more oxidase enzymes.

[0083] A 13th embodiment is the method of the 12th embodiment wherein the oxidase is selected from the group consisting of a copper radical oxidase, an alcohol oxidase, a glycerol oxidase, a mutant thereof, and combinations thereof.

[0084] A 14th embodiment is the method of one of the 1st—13th embodiments wherein the oxidation catalyst comprises a metal oxidation catalyst selected from the group consisting of (i) monometallic formulations that contain have a supported single transition metal species (ii) bimetallic formulations: and(iii) doped alloy formulations.

[0085] A 15th embodiment is the method of the 14th embodiment wherein the oxidation catalyst comprises nanoparticle with a particle size of from about 1 nm to about 99 nm.

[0086] A 16th embodiment is the method of the 14th embodiment wherein the oxidation catalyst comprises gold.

[0087] A 17th embodiment is the method of the 7th embodiment further comprising contacting the diacid with an acid dehydration catalyst under conditions suitable for formation of the monoacid.

[0088] An 18th embodiment is the method of the 17th embodiment wherein the acid dehydration catalyst comprises a zeolite.

[0089] A 19th embodiment is the method of the 10th embodiment further comprising contacting the diacid with an acid dehydration catalyst under conditions suitable for formation of the monoacid.

[0090] A 20th embodiment is the method of the 10th embodiment wherein the acid dehydration catalyst comprises a zeolite.

EXAMPLES

[0091] The subject matter having been generally described, the following examples are given as particular aspects of the disclosure and are included to demonstrate the practice and advantages thereof, as well as aspects and features of the presently disclosed subject matter. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the present subject matter, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific aspects which are disclosed and still obtain a like or similar result without departing from the scope of the instant disclosure. It is understood that the examples are given by way of illustration and are not intended to limit the specification of the claims to follow in any manner.

EXAMPLE 2

[0092] An investigation of a GAO mutant capable of converting ethylene glycol to glyoxal was carried out. The GAO mutant was initially engineered to accept glucose as a substrate, but was found to additionally convert ethylene glycol to glyoxal.

Directed Evolution

[0093] 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 to introduce less than 1 U mg^{-1} activity on glucose to GAO, 2) C383S to lower the K_M of the enzyme on galactose, and 3) Y405F and Q406E to enhance activity on a D-N-acetyl glucosamine substrate. The new combination sequence was termed GAO-Mut1. The full sequence of the expressed construct is given as follows:

GAO-Mut1

[0094] MGHHHHHHSSGHIEGRHMASAPIGSAIPRNNWAVTCDSAQSGNECNKAIDG
NKDTFWHTFYGANGDPKPPHTYTIDMKTTQNVNGLSVLPRQDGNQNGWIGRHEVY
LSSDGTNWGSPVASGSWFADSTTKYSNFETRPARYVRLVAITEANGQPWTSIAEINV

FQASSYTAPQPGLGRWGPTIDLPIVPAAAAIEPTSGRVLMWSSYRNDAFEGSPGGIT LTSSWDPSTGIVSDRTVTVTKHDMFCPGISMDGNGQIVVTGGNDAKKTSLYDSSSD SWIPGPDMQVARGYQSSATMSDGRVFTIGGSFSGGVFEKNGEVYSPSSKTWTSLP NAKVNPMLTADKQGLYKSDNHAWLFGWKKGSVFQAGPSTAMNWYYTSGSGDVKS AGKRQSNRGVAPDAMSGNAVMYDAVKGKILTFGGSPDFEDSDATTNAHIITLGEPG TSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQD TFYKQNPNSIVRAYHSISLLLPDGRVFNGGGGLCGDCTTNHFDAQIFTPNYLYDSNG NLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTATHTVNTDQRRIPLTLTNNG GNSYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ and is present as SEQ ID No.1. Other mutations that were carried out are described in Table I.

Table I

Name	Staring Mut	Addition 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

[0095] 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 Luria 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. 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 ΔA405/min and taking the extinction coefficient of ABTS at 405 nm as 36.8 $mM^{-1}cm^{-1}$, dividing by two to account for the fact that one GAO oxidation event produces one molecule of H_2O_2 which oxidizes two molecules of ABTS. The results are presented in Figure 4.

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

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

EXAMPLE 2

[0096] Mutant lysates exhibiting a ΔA405/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 by from 5ml culture with auto-induction medium in 24 well plate. Harvested cells were lysed with Bacterial Protein Extraction Reagent (B-PER) and the lysate was spun down with 15,000 rcf for 30min at 4 °C. The lysate supernatant was subjected to 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 $CuSO_4$, and specific activity was measured using the ABTS assay outlined above for. T_{50} 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 enough 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 T_{50}, 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, and A172V, T50 and the specific activity of point and combination mutants identified via directed evolution (Table II). These mutations were combined into a single combination mutant named GAO-Mut47 which exhibited a specific activity of 19.68 U mg^{-1} and a T_{50} of 56.8 °C.

Table II

Name	Mutations	U/mg	T50 °C	Kcat S-1	Km 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 G461A I462R S383C	13.3	46.3	24.5	70.8
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 3

Rational Engineering

[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). Previously, we identified that GAO-M-RQW-S (the GAO-Mut1 sequence without the Y405F and Q406E mutations could accept both glucose and gluconate as substrate, Figure 5. As efforts were underway to produce a GAO active on both substrates, rational design was performed on the GAO-M-RQW-S sequence rather than GAO-Mut1. Structural methods employed included applying FoldX11 (40 predicted mutations) and PROSS12 (80 mutations) to a modified form of the PDB structure 2WQ8 to contain the GAO-M-RQW-S mutations. MSA-based predictions were made using previously described methods 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 previously 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 (Table III). 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 T_{50} of 60.56 °C. Additional mutations identified from machine learning algorithms were later incorporated to generate GAO-mut142 and GAO-mut164.

Table III

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-mut107a	N66S S306A S311F Q486L	Removed N28I, Y189W, S331R, A378D, R459Q, and V477D	34.96	1.20	60.56
GAO-mut142b	N66S S306A S311F Q486L H40C	H40C	37.53	1.29	58.76
GAO-mut164b	N66S S306A S311F Q486L H40C L71C	L71C	38.22	1.32	52.97

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.

One-Step Parr Bomb Reaction with GAO-Mut47 to Produce D-Glucodialdose

[00100] A 50ml reaction was conducted in a 200 mL vessel pressurized to 100 psi. The vessel was charged with 50mM sodium phosphate pH 8 buffer, 50 µM $CuSO_4$, 15 w/v% glucose, 0.005 w/v% catalase, 0.001% horseradish peroxidase, and 0.001 w/v% engineered GAO. The reaction was stirred at 500 rpm, 11 °C for 48 hours. Samples were taken at 0, 24, and 48 hours then assayed with high pressure liquid chromatography (HPLC) to measure residual glucose, the results are presented in Figure 6A. The activity and stability of GAO mutants containing mutations discovered through machine learning was assayed and the results are presented in Figures 6B and 6C, respectively.

EXAMPLE 4

[00101] GAO mutants described in Example 1 were discovered using an ABTS assay to oxidize ethylene glycol and related compounds to various degrees and the results are presented in Figure 7. Because ethylene glycol oxidation products were also found to be substrates for further oxidation by GAO, reactions were performed to mimic industrially-relevant conditions and product profile was determined by high pressure liquid chromatography refractive index (HPLC-RI).

[00102] Specifically, oxidation reactions of ethylene glycol by AOX and GAO mutants were performed in a Parr bomb pressurized to 100 psi O_2. Reactions were prepared in 20 mL scintillation vials equipped with stir bars, to which was added 10% w/v ethylene glycol in 50 mM KP, pH 7.5 (AOX) or 50 mM NaP, pH 8.0 GAO, 0.005% w/v Katalase-AK, then charged with the specified amount (%w/v) of the oxidase. Reactions were quenched as 50 µL aliquots into 950 µL 1% TFA in H_2O and analyzed by HPLC-RI with a Waters Acquity H-Class and a 15 cm Aminex HPX-87C column at 65°C operating at 0.6 mL/min eluting with 100% H_2O. The results are presented in Figure 8 where the values presented represent relative areas of integrated peaks.

[00103] For GAO-Mut107, glyoxal was found to be the major product and glycolaldehyde the only other detectable product. A similar product profile was discovered for GAO-Mut147. Specifically, oxidation reactions of ethylene glycol by GAO-mut142 were performed in a Parr bomb reactor pressurized to 100 psi O_2. Reactions were prepared in 20 mL scintillation vials equipped with stir bars, to which was added ethylene glycol at the given concentration (in %w/v) in 50 mM NaP, pH 8.0, 0.005% w/v Katalase-AK, then charged with the specified amount (%w/v) of the oxidase. Reactions were analyzed

by HPLC-RI with a Waters Acquity H-Class and a 15 cm Aminex HPX-87 °C column at 65 °C operating at 0.6 mL/min eluting with 100% H_2O. Values of concentration (mg/mL) were calculated using a standard curve generated with analytical standards. The results are presented in Figure 9.

EXAMPLE 5

Development of purpald assay for the quantification of glyoxal and glycolaldehyde in GAO catalyzed ethylene glycol (EG) oxidation reactions

[00104] Further GAO engineering was carried out to obtain higher ethylene glycol conversion, e.g., by high throughput screening of GAO mutation libraries. A rapid method to quantify the product was developed. A chromogenic method, the purpald assay, was developed to rapidly (greater than 600 samples/hour) and accurately quantify the amount of glyoxal and glycolaldehyde. The reaction of aldehyde with the purpald reagent is depicted in Figure 10A. Glycolaldehyde and glyoxal was reacted with 100 mM purpald reagent (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) in 1 M NaOH solution via their aldehyde group(s) and formed purple and yellow color products after oxidation by dioxygen (O_2) in the ambient air. However, since ethylene glycol does not contain any aldehyde group, it did not react with purpald reagent. These results are shown in Figure 10B.

[00105] Wavelength scan analysis showed that the products of glycolaldehyde and glyoxal have maximum absorption at 550 nm and 400 nm, respectively. The results are depicted in Figure 10C. With reference to Figure 10C, the absorbance values were found to correlate linearly with the concentrations of starting materials (20 – 200 μM). Further, the product of glyoxal did not have any absorbance at 550 nm though the product of glycolaldehyde has absorbance at 400 nm. Therefore, the A550 value can be used to quantify the glycolaldehyde in a mixture of ethylene glycol, glycolaldehyde and glyoxal such as reaction samples from the GAO and ethylene glycol. Nevertheless, the combination of A550 and A400 can be used to quantify glyoxal in the same mixture. This is validated by the quantification of glycolaldehyde and glyoxal in their mixtures with known concentrations and comparison of quantification data from purpald assay and those from HPLC-RI analysis.

EXAMPLE 6

Application of purpald assay in the quantification of glyoxal and glycolaldehyde in GAO catalyzed ethylene glycol oxidation reactions

[00106] A series of more GAO variants (e.g. Mut142, Mut290, Mut312 and Mut314) were generated that displayed increased glucose oxidation function. These variants were also tested for ethylene glycol oxidation function. Oxidation reactions of ethylene glycol with GAO mutants were performed in Parr bomb pressurized to 150 psi O_2. Reactions were prepared in 20 mL scintillation vials equipped with stir bars, to which was added 10% (v/v) ethylene glycol in 50 mM NaPi, pH 8.0, 0.02% antifoam, 0.01 mM $CuSO_4$, 0.05 mM $MnSO_4$, 100 mg/L Katalase-AK, 5 mg/L HRP_GoldBio and 20 mg/L GAO enzyme. Reaction samples were collected at 20 hours and diluted by 2000-fold in water. The purpald assay was used to quantify glyoxal and glycolaldehyde produced from ethylene glycol by GAO variants using commercial glyoxal and glycolaldehyde as standards. With reference to Figure 11, the GAO mutants that were obtained were found to have evolved for glucose oxidation with a concomitant increase in ethylene glycol oxidation activity.

EXAMPLE 7

Application of purpald assay in direct evolution of GAO enzyme for GAO-glyoxal reaction

[00107] Based on developed machine learning predictions, 210 residues of GAO Mut321 were individually mutated to all 20 amino acids using primers containing NNK codons to create site saturation mutagenesis (SSM) libraries. The plasmid DNA for the libraries was then transformed into *E. coli* BL21 (DE3) Gold competent cells. Around ~1800 GAO library colonies were picked and used to inoculate one well each in a 96-well deep well plate charged with LB alongside three wells reserved for GAO Mut321 wild type enzyme. The grown clones were then used to inoculate autoinduction media in a separate 96-well deep well plate for protein expression. Harvested cells were lysed with Bacterial Protein Extraction Reagent and appropriately diluted lysate was then screened for glyoxal production from 6% (v/v) EG in 96-well microtiter plates shaken at 11oC (ambient air) for 20 hours. Glyoxal and glycolaldehyde in the reaction samples were quantified by the purpald assay. Ethylene glycol conversion was calculated by dividing the concentration of total product ([Glyoxal] + [Glycolaldehyde]) with the starting concentration of ethylene glycol.

[00108] Clones that displayed a 10% increase in ethylene glycol conversion over the GAO Mut21 parent on the same plate were assigned as hits. In addition, clones showing a greater than 10% increase in glyoxal production over the GAO Mut321 parent on the

same plate were also assigned as hits. Overall, approximately 300 hits were obtained from primary screening of approximately 1800 library clones. Then, the hits obtained from the primary screening were retested from protein expression to enzyme reaction using the same conditions as primary screening except biological triplicate was set up for the retesting. 80 hits were confirmed with a greater than 10% increase in EG conversion or glyoxal production over the parent enzyme. The results are presented as a graph of catalytic activity as a function of the mutation in Figure 11. Notably, several single mutations (K330R (Mut323), H382Y(Mut324), E465K(Mut325) and F471L(Mut326)) increased glyoxal production >30% and H382Y mutation increased glyoxal production by 2.5-fold. These results are depicted in Figures 12A, 12B, and 12C respectively.

EXAMPLE 8

[00109] The ability of an oxidation catalyst to catalyze the oxidation of diethylene glycol to diglycolic acid was investigated. Table IV lists the different oxidation catalysts investigated.

Table IV

Catalysts for DEG → DGA
K902 – Au/Pt/C (0.3/0.2 wt%)
K903 – Au/Pt/C (0.3/0.3 wt%)
10% Pt/C
Au/Pt/Sr/C (0.78/0.91/0.48 wt%)
Au/Pt/C (0.6/0.6 wt%)
Au/Pt/Bi/C (0.45/0.82/0.23 wt%)

[00110] Through the use of one or more of the catalysts in the table above, diglycolic acid was obtained, with some evidence that glycolic acid could also be obtained directly from diethylene glycol through the use of one or more of the catalysts in the table above. The reaction conditions are presented in Table V.

Table V

Catalyst	Reaction conditions	Diglycolic Acid Detection	Glycolic Acid Detection

K902	2 – 10wt% diethylene glycol in water 1 – 10% catalyst loading 80 – 150 oC 4 – 15 hours of reaction 500 – 1000 rpm 10 bar O2	Yes	Yes
K903		Yes	Yes
10%Pt/C		Yes	Yes

EXAMPLE 8

Hydrolysis of diglycolic acid

[00111] Both homogeneous (e.g., H_2SO_4, H_3PO_4) and heterogeneous (e.g., zeolites, AMBERLYST-15, Si-TsOH) acids were used to catalyze the hydrolysis of diglycolic acid. The cleavage of the C-O bond was carried out at elevated temperatures in the presence of high concentrations or loading of the acid catalysts. Reaction conditions, Table VI, overcame kinetic barriers without excessive side reactions and move the equilibrium conversion towards products. Utilizing solid supported acids (heterogeneous acids) facilitated the separation of the desired product, glycolic acid, as well as the recycling of the catalysts.

Table VI.

Catalyst	**Reaction condition**	**Glycolic Acid Detection**
HZSM-5	10-20 wt.% DGA in water 80-150 °C 2-4h, 2-50 wt.% catalyst loading	Yes
Si-TsOH		No
AMBERLYST-15		No
Camphorsulfonic acid (CSA)		No
p-Toluenesulfonic acid (*p*TsA)		Yes

HZSM-5	2-7 equiv. of H_2O 50-140 °C 2-4h 10-20 wt.% catalyst loading	Yes
Si-TsOH		Yes
Amberlyst-15 *(Reaction was run at 250 °C for 1h)*		Yes

Acid catalyst for dehydration of polyols:

Synthesis Methods:

[00112] Both ZSM-5 and $Zn(NO_3)_2$-$6H_2O$ was purchased from Sigma Aldrich. HZSM-5 was prepared by calcining ZSM-5 in a calcine oven at 600°C for 5 hours using a 10°C/min heat ramp. A 30 mL solution containing 1 wt.% of metal was prepared. Once the solution was fully dissolved, the solution was added dropwise over 8 g of HZSM-5. The catalyst was dried overnight at 92°C, and then calcined at 550°C using a 10/min heating ramp. The resulting Zn/ZSM-5 catalyst was then stored in a moisture sealed container. A scanning electron micrograph image of the catalyst is presented in Figure 2A while the surface area data is presented in Figure 2B. The catalyst was found to have a BET surface area of 281.135 m^2/g and a total pore volume 0.1448 cc/g.

Example 9

Use of GAO mutants for oxidation of DEG

[00113] GAO mutants isolated from previous enzyme evolution campion were discovered to oxidize diethylene glycol, Figure 13. Given the structure similarity of diethylene glycol with ethylene glycol, it was reasonable to propose the oxidized product from the enzymatic reaction is the compounds with either monoaldehyde (2',2'-hydroxyethoxyacetaldehyde) or dialdehyde (2,2'-oxybisacetaldehyde) or both.

[00114] The enzymatic oxidation reaction was performed under industrially relevant conditions. 2 mL of the reaction is setup with 50 mM Na phosphate, pH 7.5, 2 wt % diethylene glycol, 0.1 g/L galactose oxidase , 0.05 g/L catalase, 0.0025 g/L of HRP, 33 µM or 5 ppm small molecule activator, 50 µM $Mn(NO_3)_2$, 10 µM $Cu(NO_3)_2$, 0.05% antifoam. The reactor was pressured with pure O_2 at 140 psi and stirred for 16 hrs. The conversion % of diethylene glycol (DEG) was calculated based on the DEG consumption which was measured by HPLC-RI.

[00115] The observation of the clear color change on the Purpald assay demonstrated the production of both 2',2' -hydroxyethoxyacetaldehyde and 2,2'-oxybisacetaldehyde in the enzymatic reaction, Figure 14A. UV-Vis spectra of the reaction products are

presented in Figure 14B. The HPLC result showed the GAO variant Mut327 performed the best on oxidizing DEG in the enzymatic reaction (Figure 14C).

[00116] The reaction condition was further optimized by tuning the enzyme loading. The enzymatic reactions were set up with similar conditions as described above, but with different concentrations of GAO-mut327. The DEG conversion is clearly improved as increasing the enzyme GAO concentration in the reaction. With 0.5 g/L of Mut327 loaded in the reaction, the DEG conversion reached >80% after 66 hrs (Figure 14D).

[00117] The subject matter having 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.

[00118] 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.

CLAIMS

What is claimed is:

1. A method for preparing one or more acids, the method comprising:

contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate;

contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid and;

recovering the acid.

2. The method of claim 1, wherein the glycol comprises ethylene glycol.

3. The method of claim 2, wherein the aldehyde intermediate comprises glycolaldehyde.

4. The method of claim 3, wherein the acid comprises glyoxal.

5. The method of claim 1, wherein the glycol is a polyglycol.

6. The method of claim 5, wherein the polyglycol comprises diethylene glycol,

7. The method of claim 6, wherein the aldehyde intermediate comprises diglycolaldehyde.

8. The method of claim 7, wherein the acid comprises diglycolic acid.

9. The method of claim 5, wherein the polyglycol is polyethylene glycol.

10. The method of claim 9, wherein the aldehyde intermediate is polyethylene glycol aldehyde.

11. The method of claim 10, wherein the acid is polyethylene glycol diacid.

12. The method of claim 1, wherein the one or more oxidation catalysts comprise one or more oxidase enzymes.

13. The method of claim 12, wherein the oxidase is selected from the group consisting of a copper radical oxidase, an alcohol oxidase, a glycerol oxidase, a mutant thereof, and combinations thereof.

14. The method of claim 1, wherein the oxidation catalyst comprises a metal oxidation catalyst selected from the group consisting of (i) monometallic formulations that contain have a supported single transition metal species (ii) bimetallic formulations: and(iii) doped alloy formulations.

15. The method of claim 14, wherein the oxidation catalyst comprises nanoparticle with a particle size of from about 1 nm to about 99 nm.

16. The method of claim 14, wherein the oxidation catalyst comprises gold.

17. The method of claim 7, further comprising contacting the diacid with an acid dehydration catalyst under conditions suitable for formation of the monoacid.

18. The method of claim 17, wherein the acid dehydration catalyst comprises a zeolite.

19. The method of claim 10, further comprising contacting the diacid with an acid dehydration catalyst under conditions suitable for formation of the monoacid.

20. The method of claim 10, wherein the acid dehydration catalyst comprises a zeolite.

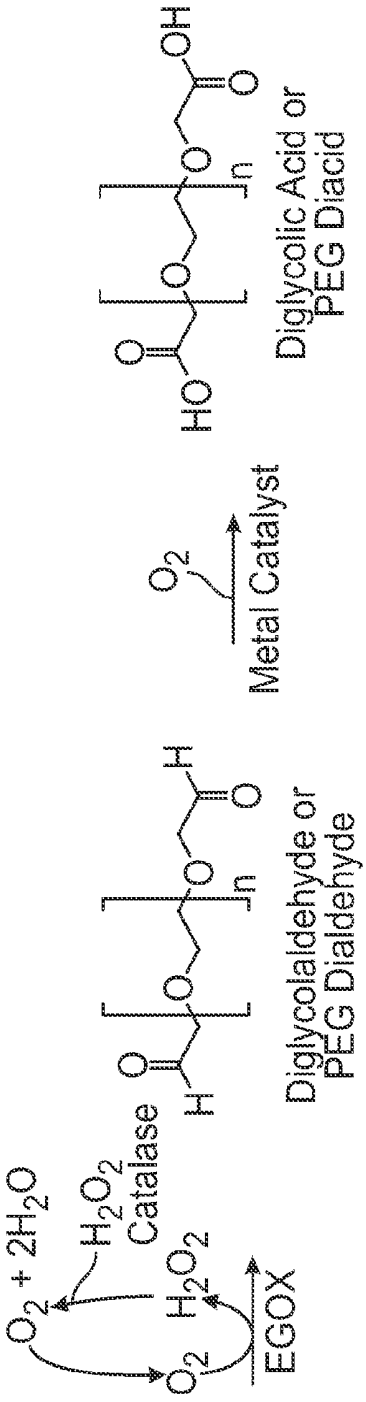
Diethylene Glycol or PEG
$O_2 + 2H_2O$
H_2O_2
Catalase
O_2 H_2O_2
EGOX
Diglycolaldehyde or PEG Dialdehyde
O_2
Metal Catalyst
Diglycolic Acid or PEG Diacid

FIG. 1A

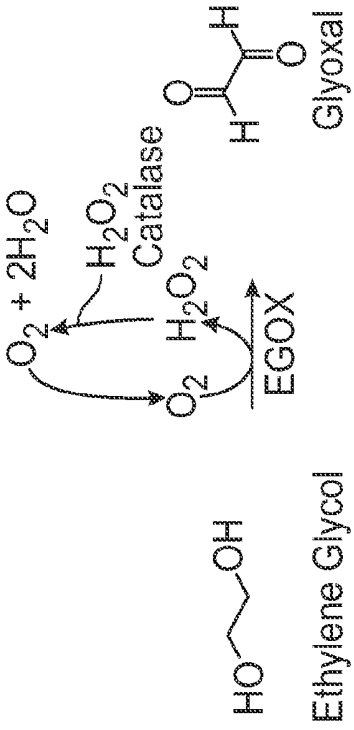
Ethylene Glycol
$O_2 + 2H_2O$
H_2O_2
Catalase
O_2 H_2O_2
EGOX
Glyoxal

FIG. 1B

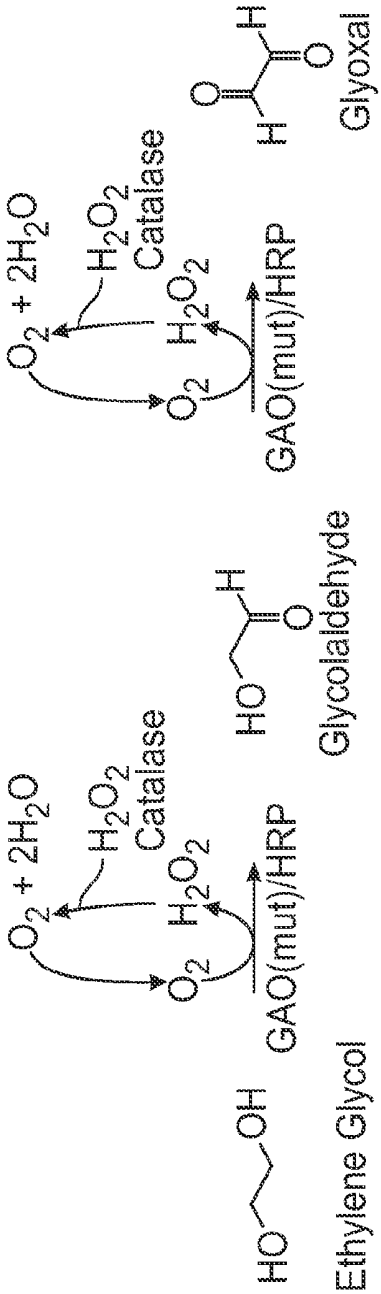
Ethylene Glycol
GAO(mut)/HRP
O_2
H_2O_2
Catalase
$O_2 + 2H_2O$
Glycolaldehyde
GAO(mut)/HRP
O_2
H_2O_2
Catalase
$O_2 + 2H_2O$
Glyoxal

FIG. 1C

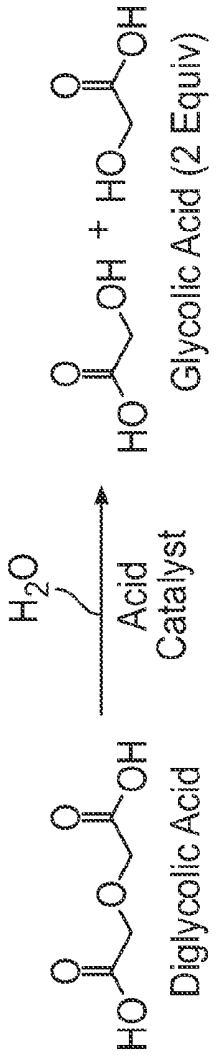
Diglycolic Acid
H_2O
Acid Catalyst
Glycolic Acid (2 Equiv)

FIG. 1D

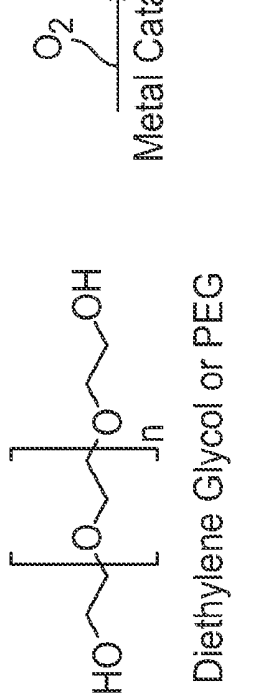
Diethylene Glycol or PEG
O_2
Metal Catalyst
Diglycolic Acid or
PEG Diacid

FIG. 1E

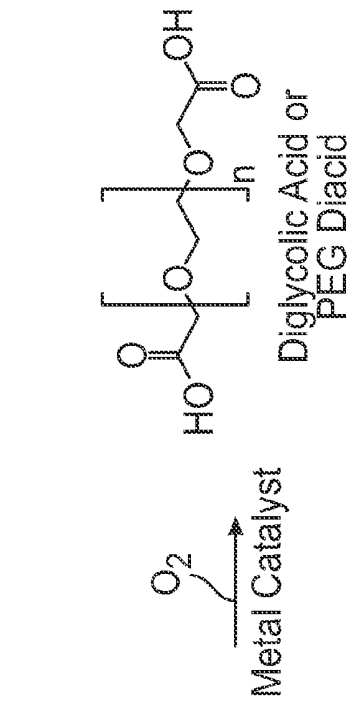
Diethylene Glycol
or PEG
GAO(mut)/SEO/SMA
$O_2 + 2H_2O$
H_2O_2
Catalase
O_2 H_2O_2
Diglycolaldehyde or
PEG Dialdehyde
O_2
Metal Catalyst
Diglycolic Acid or
PEG Diacid

FIG. 1F

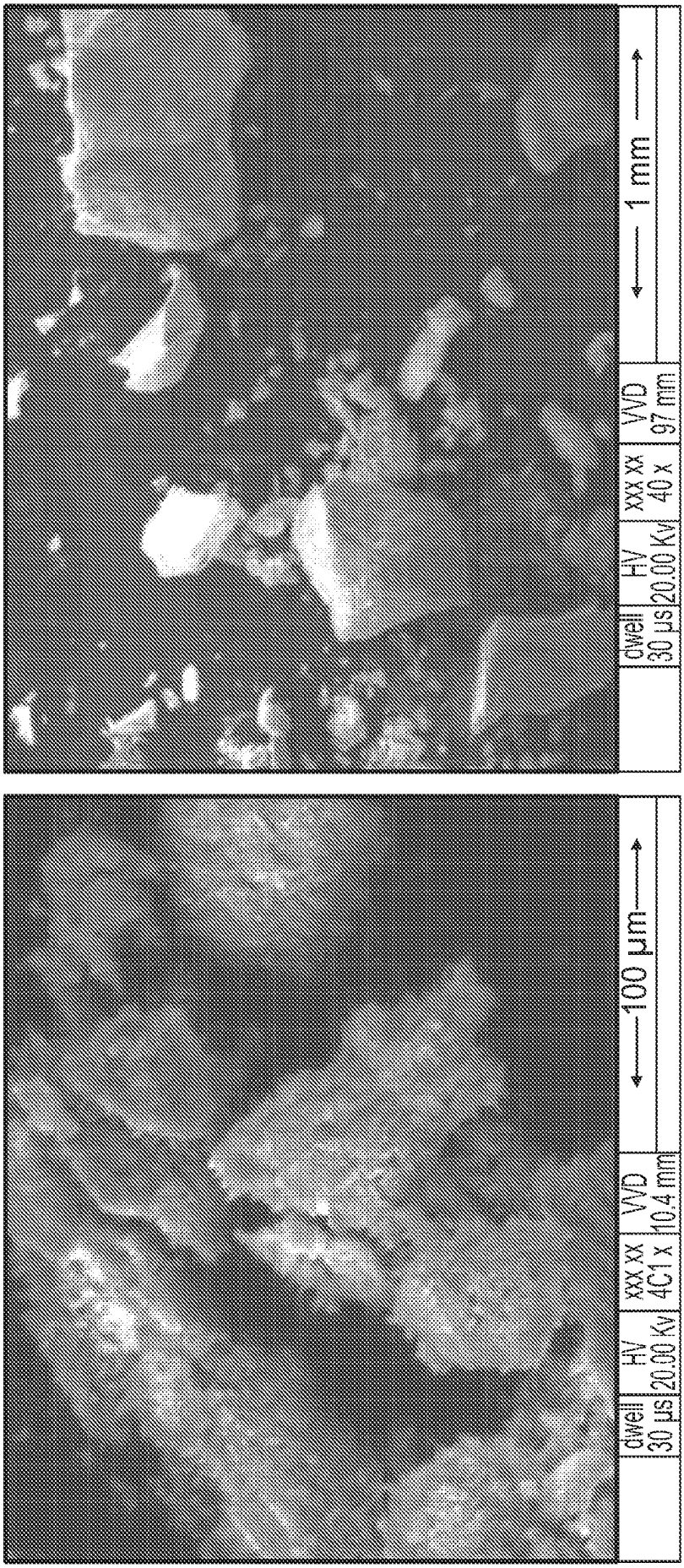
dwell
30 µs
HV
20.00 Kv
xxx xx
40 x
VVD
97 mm
1 mm
dwell
30 µs
HV
20.00 Kv
xxx xx
4C1 x
VVD
10.4 mm
100 µm

FIG. 2A

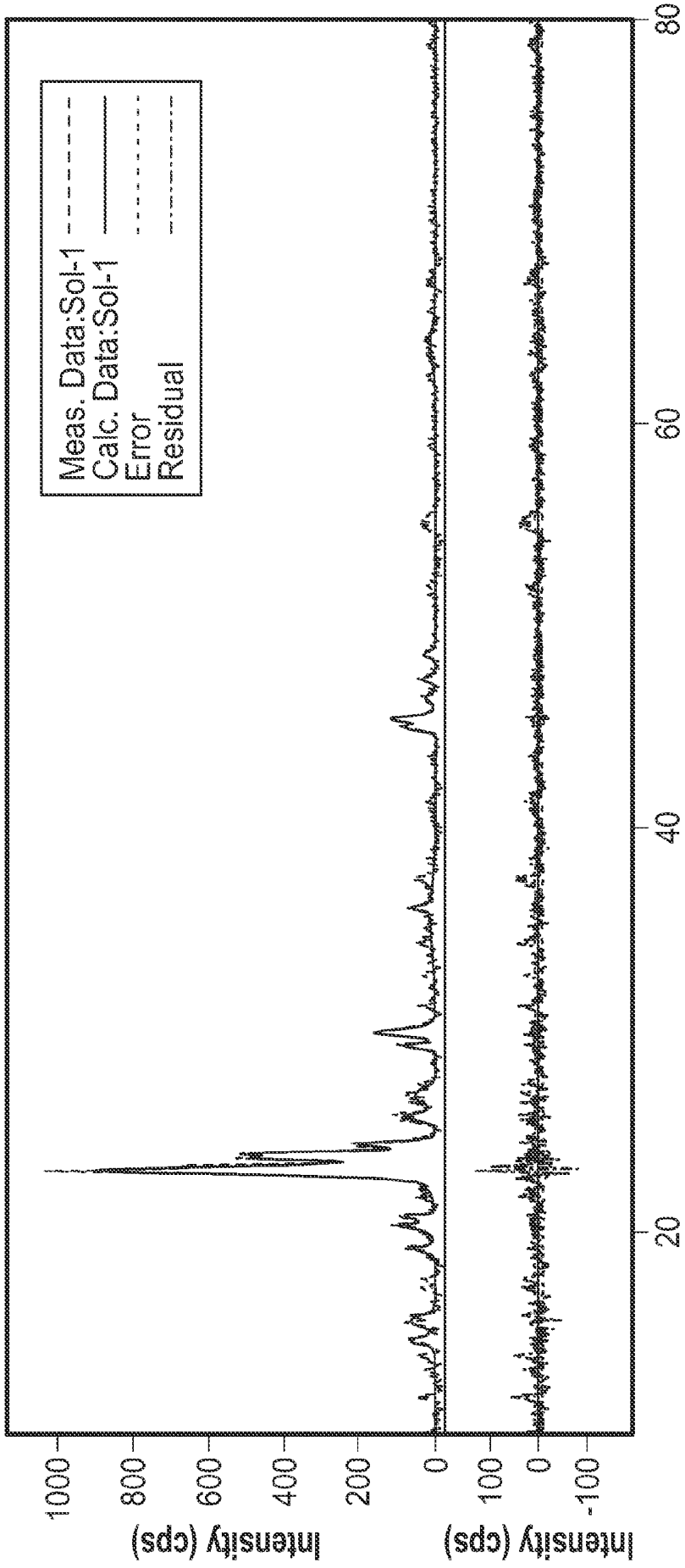
Meas. Data:Sol-1
Calc. Data:Sol-1
Error
Residual
Intensity (cps)
1000
800
600
400
200
0
100
0
-100
20
40
60
80
2-theta (Deg)

FIG. 2B

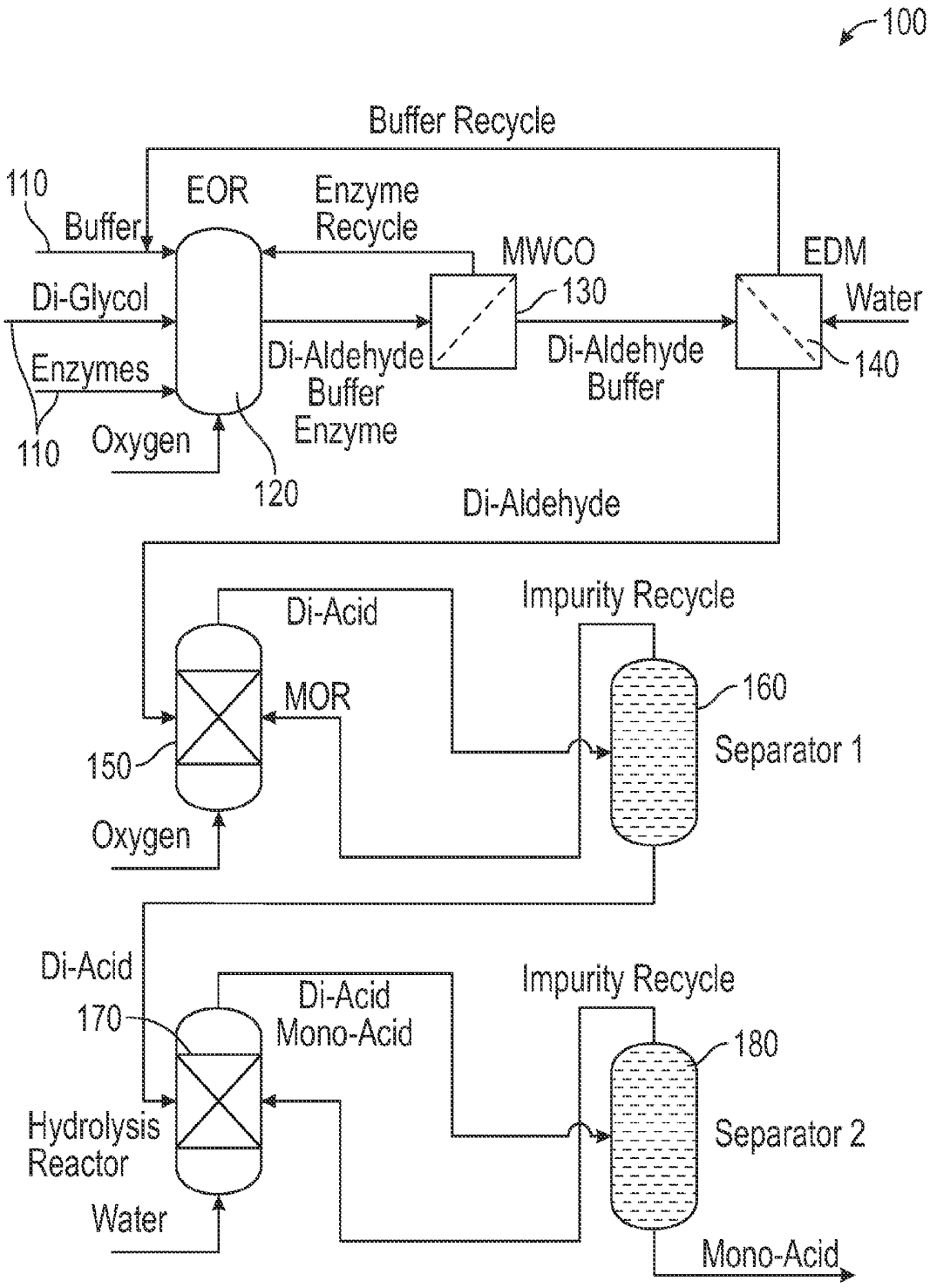
100
Buffer Recycle
110
Buffer
EOR
Enzyme Recycle
MWCO
130
EDM
Water
Di-Glycol
Enzymes
110
Oxygen
120
Di-Aldehyde Buffer Enzyme
Di-Aldehyde Buffer
140
Di-Aldehyde
Di-Acid
Impurity Recycle
MOR
150
160
Separator 1
Oxygen
Di-Acid
170
Di-Acid Mono-Acid
Impurity Recycle
180
Hydrolysis Reactor
Separator 2
Water
Mono-Acid

FIG. 3

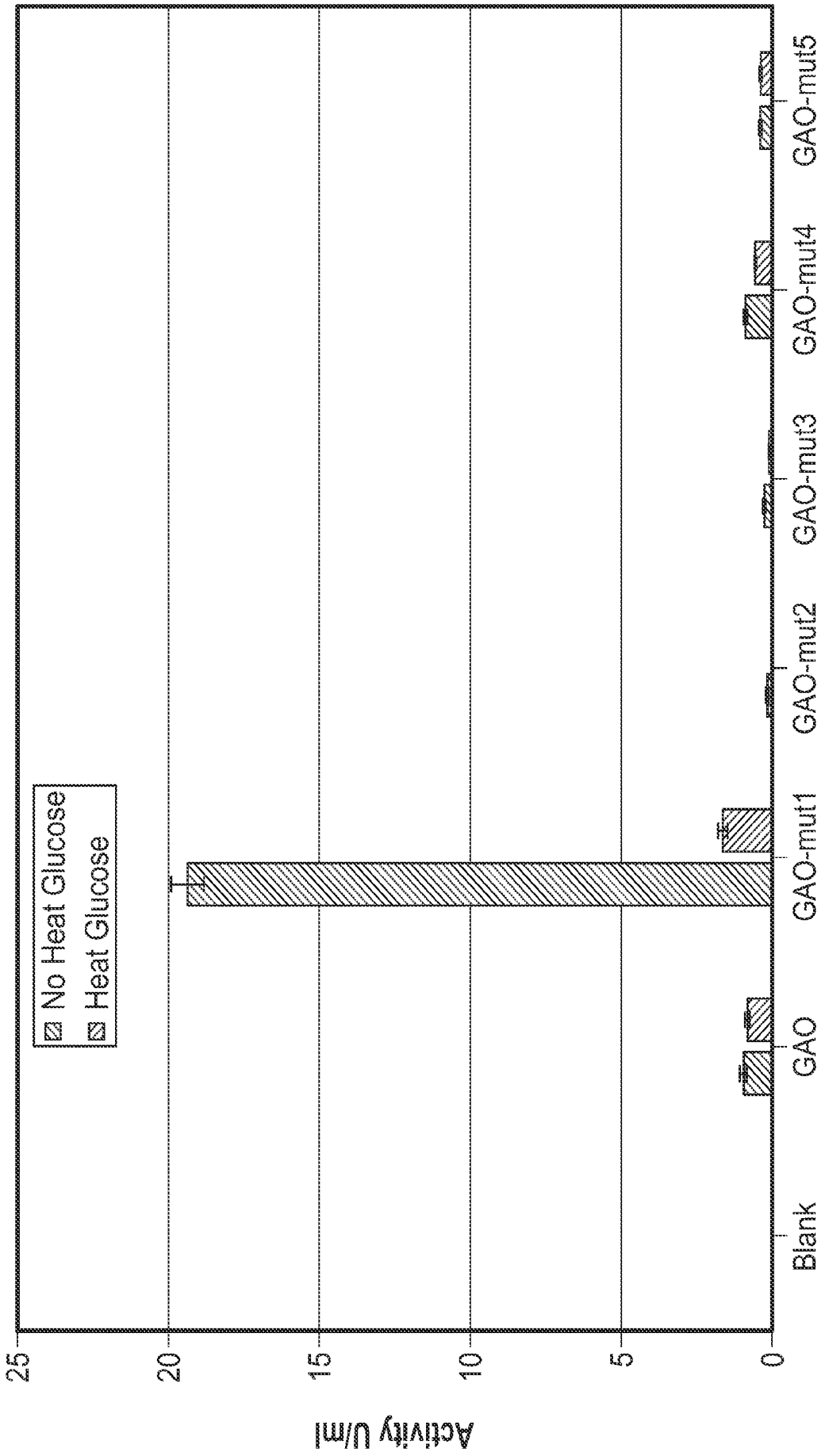
No Heat Glucose
Heat Glucose
Activity U/ml
0
5
10
15
20
25
Blank
GAO
GAO-mut1
GAO-mut2
GAO-mut3
GAO-mut4
GAO-mut5

FIG. 4

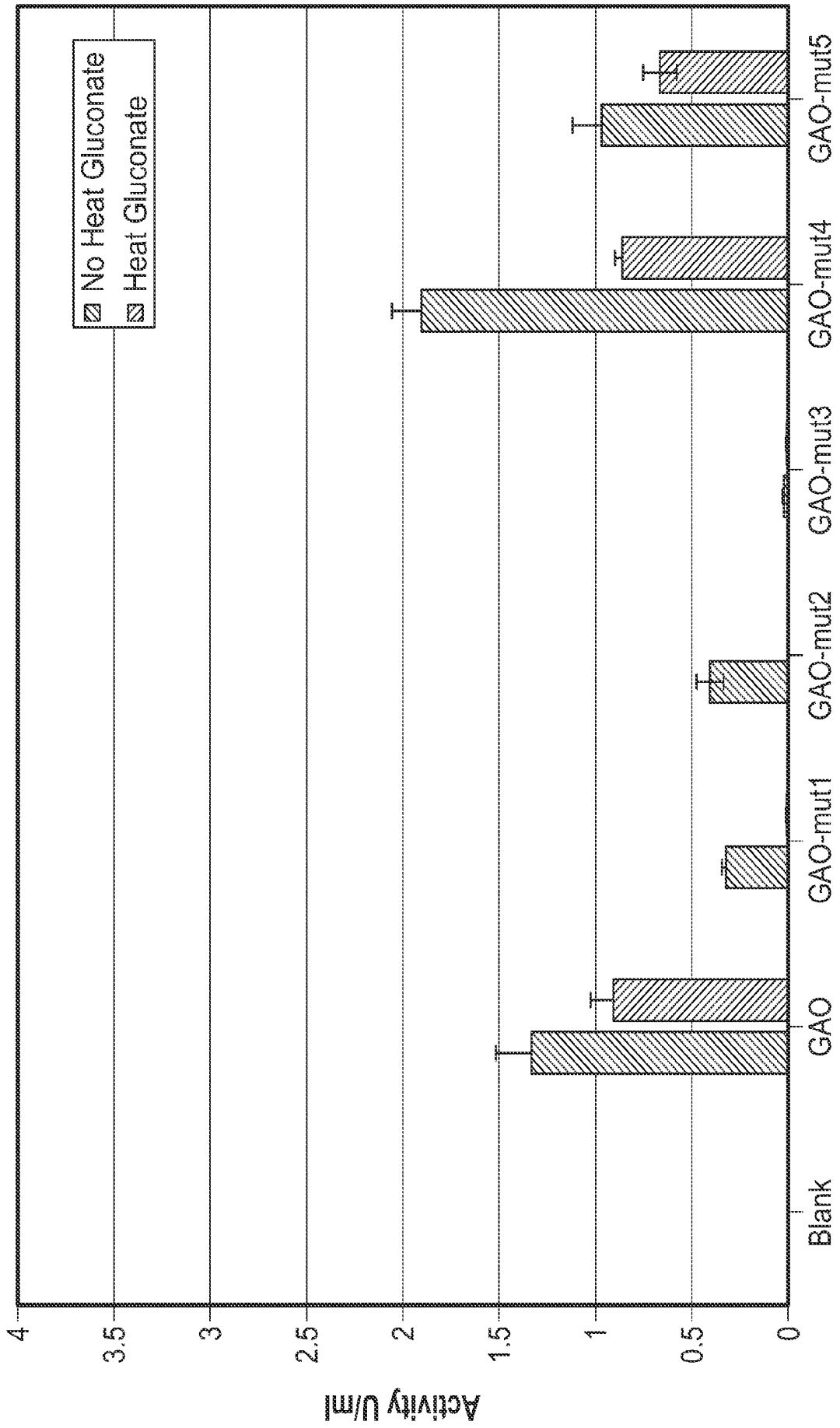
No Heat Gluconate
Heat Gluconate
Activity U/ml
0
0.5
1
1.5
2
2.5
3
3.5
4
Blank
GAO
GAO-mut1
GAO-mut2
GAO-mut3
GAO-mut4
GAO-mut5

FIG. 5

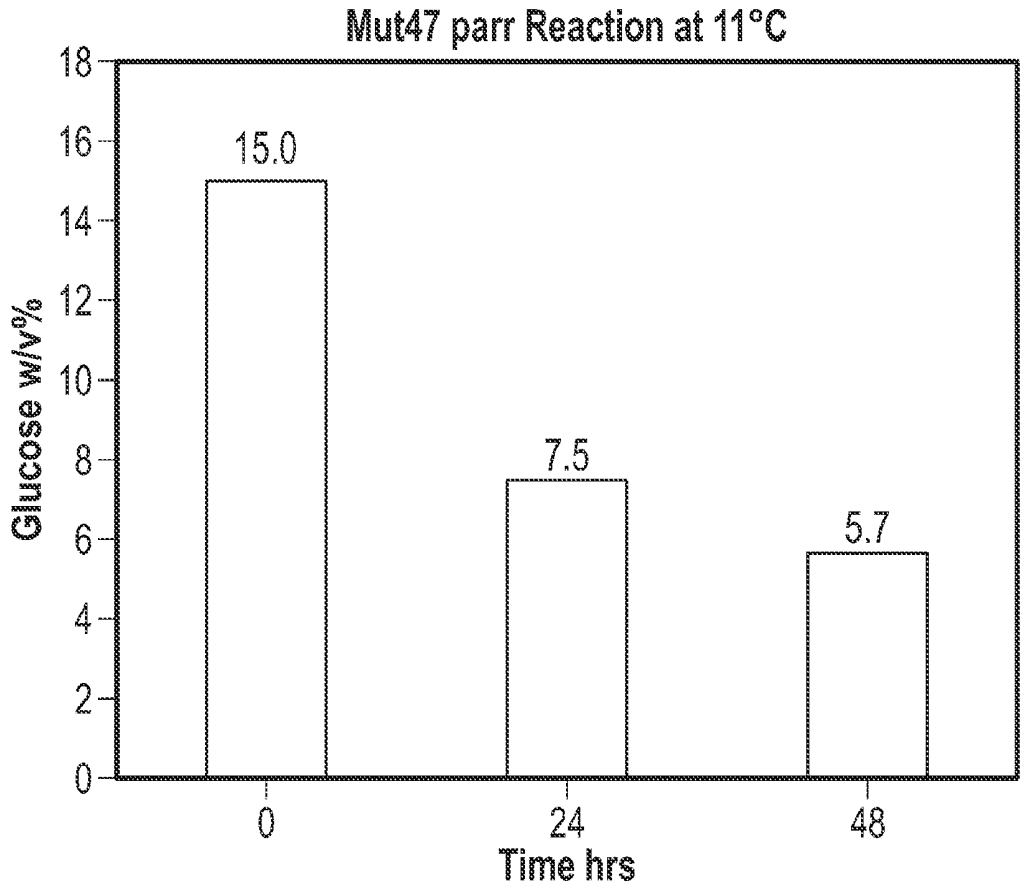
Mut47 parr Reaction at 11°C
Glucose w/v%
18
16
14
12
10
8
6
4
2
0
15.0
7.5
5.7
0
24
48
Time hrs

FIG. 6A

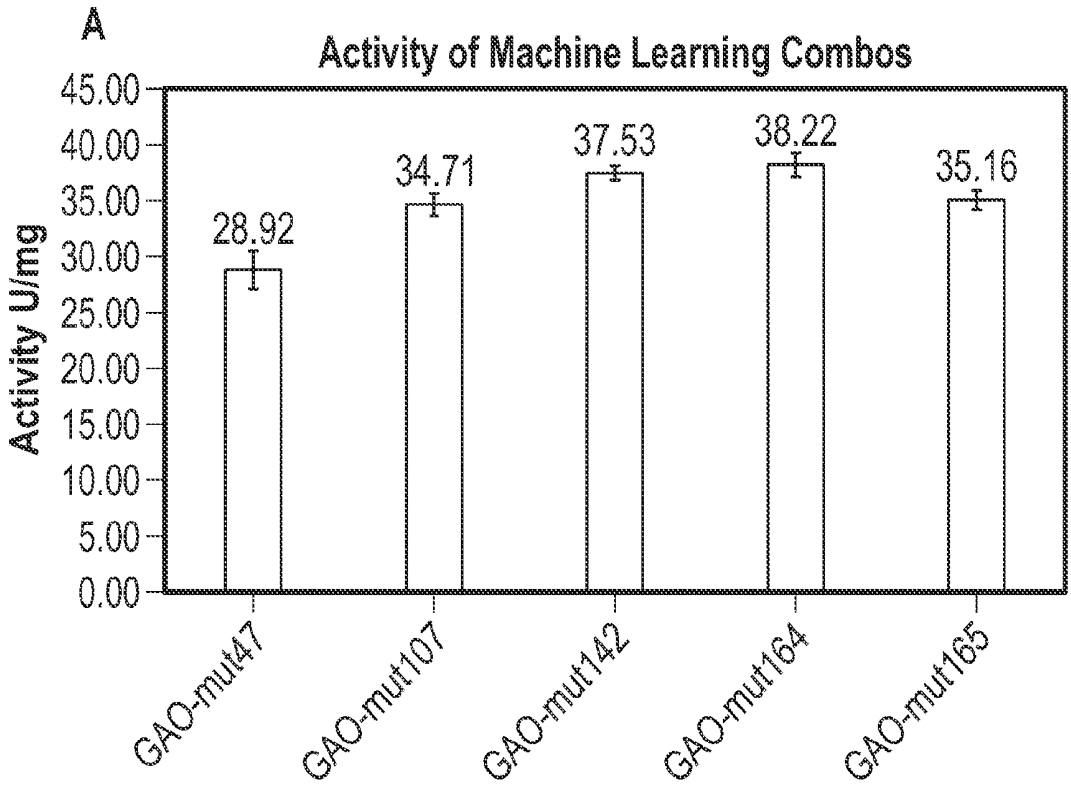
A
Activity of Machine Learning Combos
Activity U/mg
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
28.92
34.71
37.53
38.22
35.16
GAO-mut47
GAO-mut107
GAO-mut142
GAO-mut164
GAO-mut165

FIG. 6B

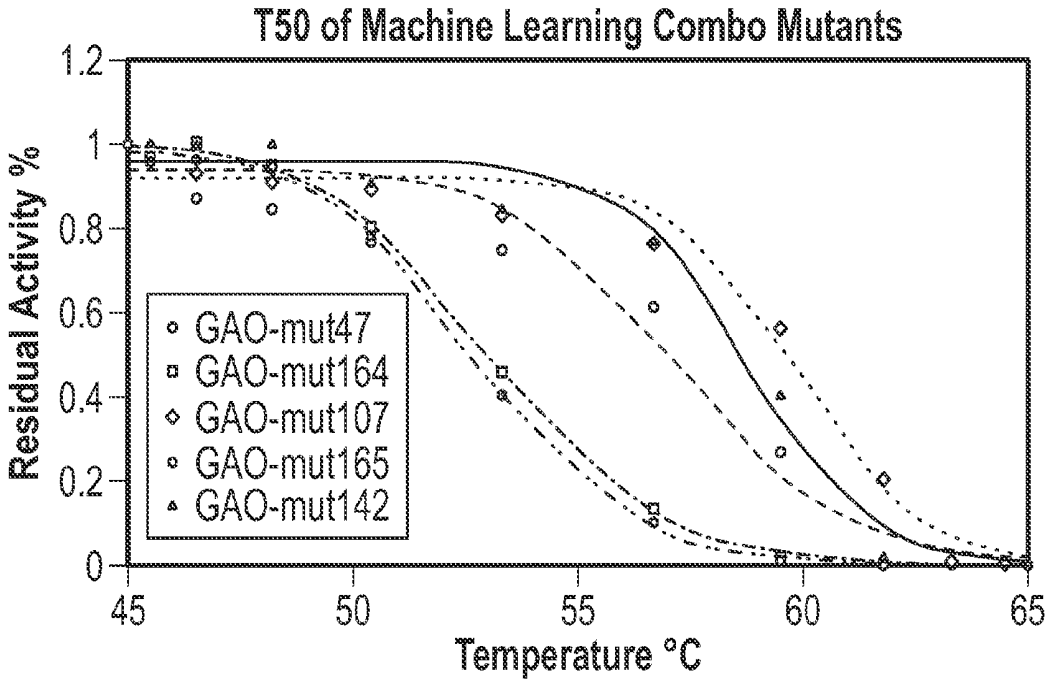
T50 of Machine Learning Combo Mutants
Residual Activity %
1.2
1
0.8
0.6
0.4
0.2
0
45
50
55
60
65
Temperature °C
GAO-mut47
GAO-mut164
GAO-mut107
GAO-mut165
GAO-mut142

FIG. 6C

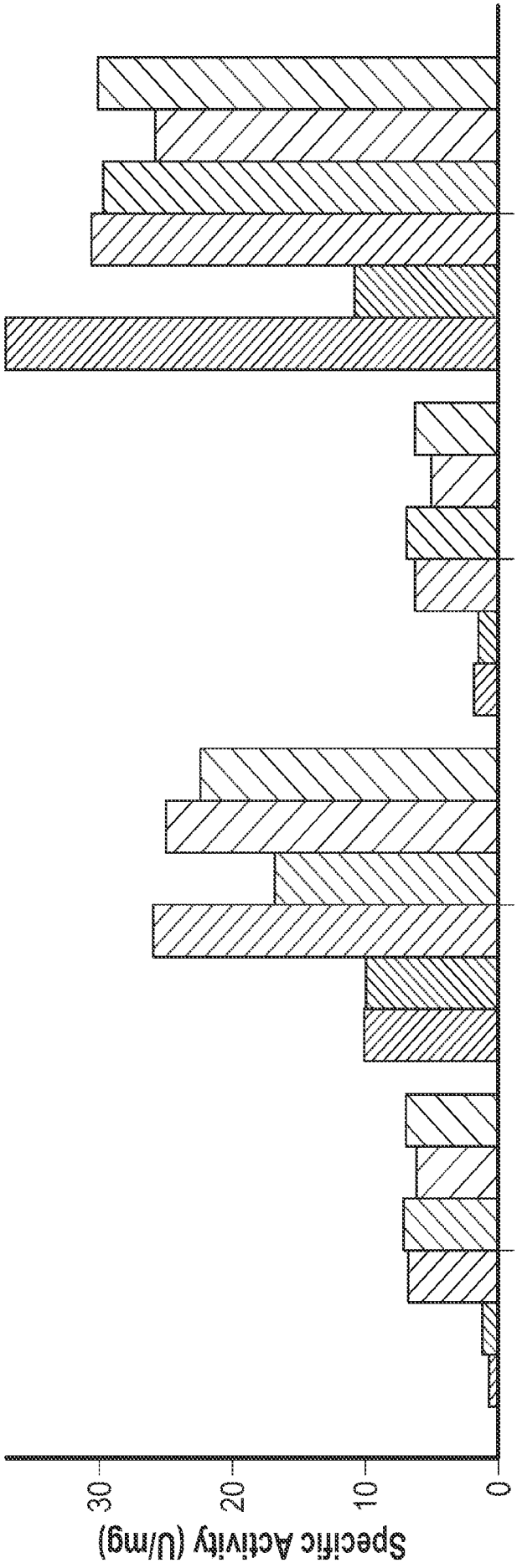
Specific Activity (U/mg)
0
10
20
30
Ethylene Glycol
Glycolaldehyde Dimer
Glyoxal
Glycolic Acid
Substrate

FIG. 7

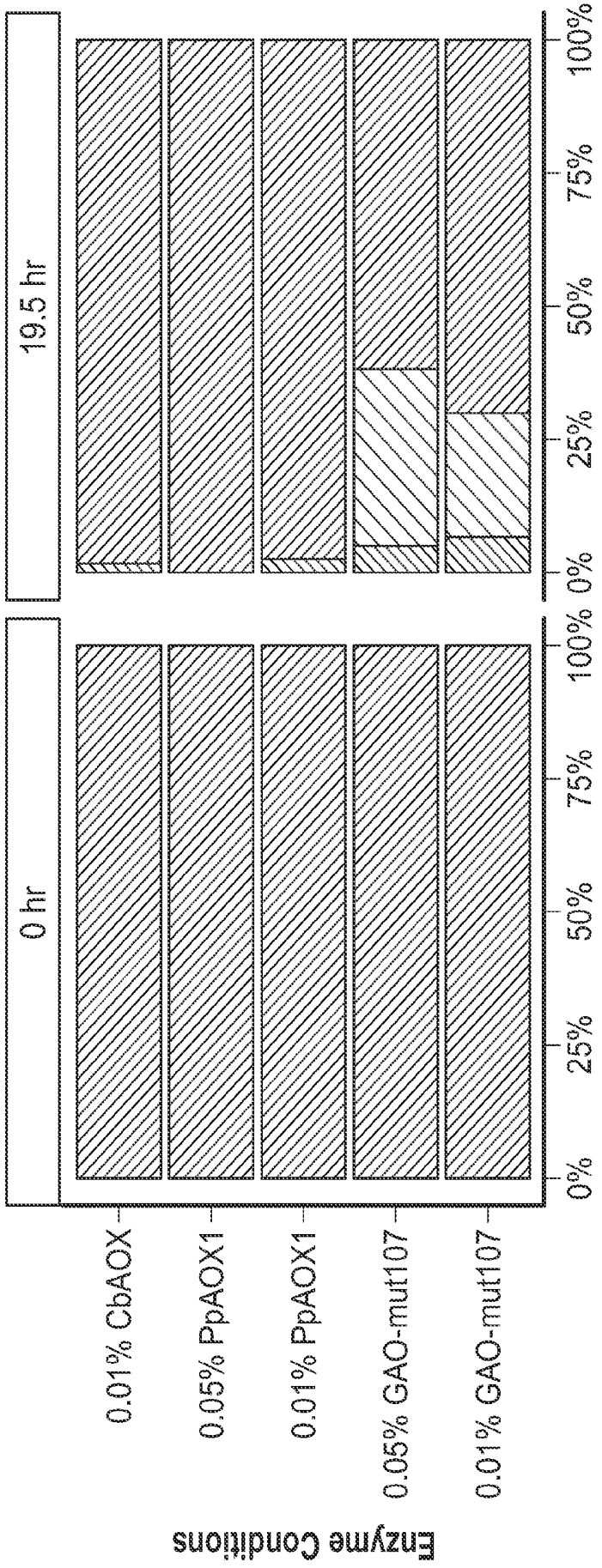
0 hr
19.5 hr
0.01% CbAOX
0.05% PpAOX1
0.01% PpAOX1
0.05% GAO-mut107
0.01% GAO-mut107
0%
25%
50%
75%
100%
Percent RI Peak Area
Enzyme Conditions

FIG. 8

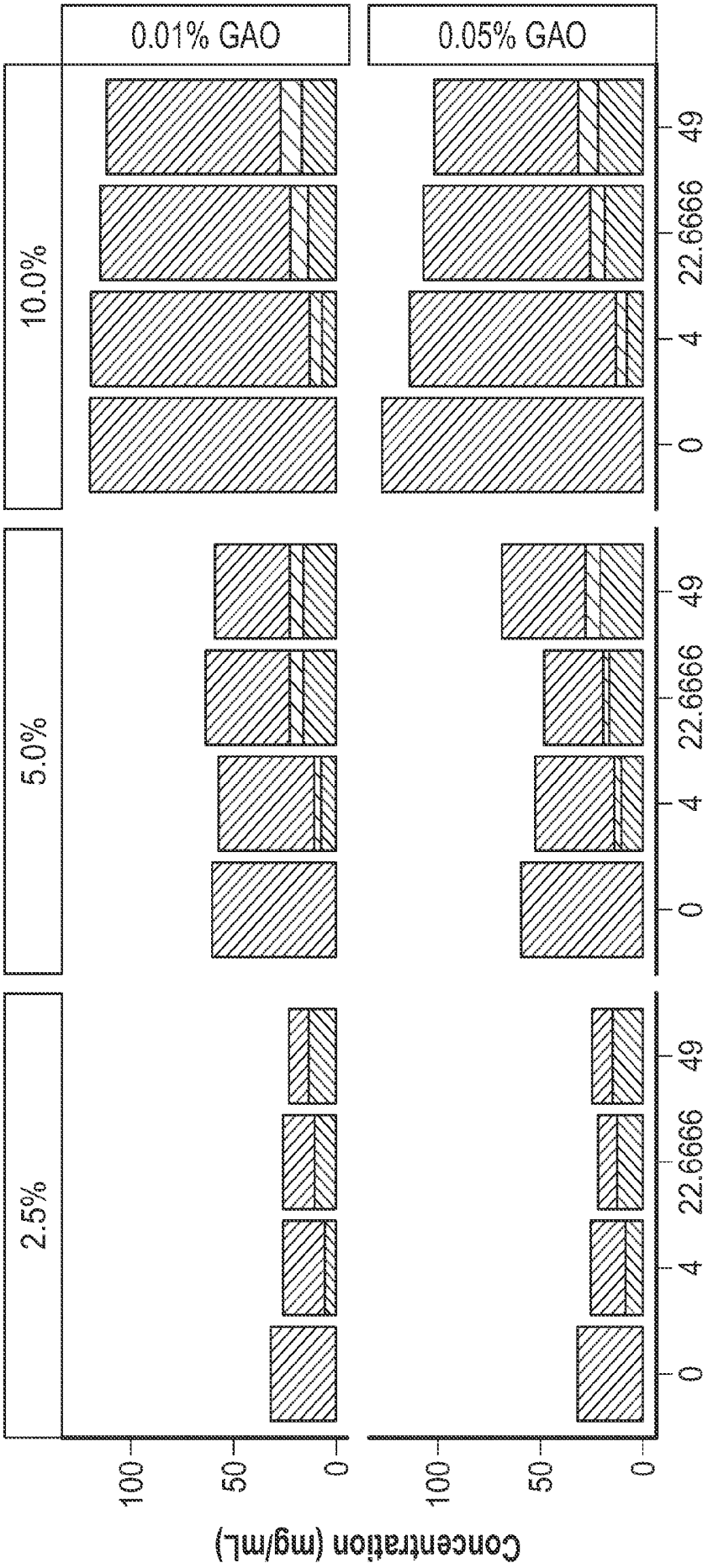
0.01% GAO
0.05% GAO
10.0%
5.0%
2.5%
100
50
0
0
4
22.6666
49
Concentration (mg/mL)
Reaction Time (hr)

FIG. 9

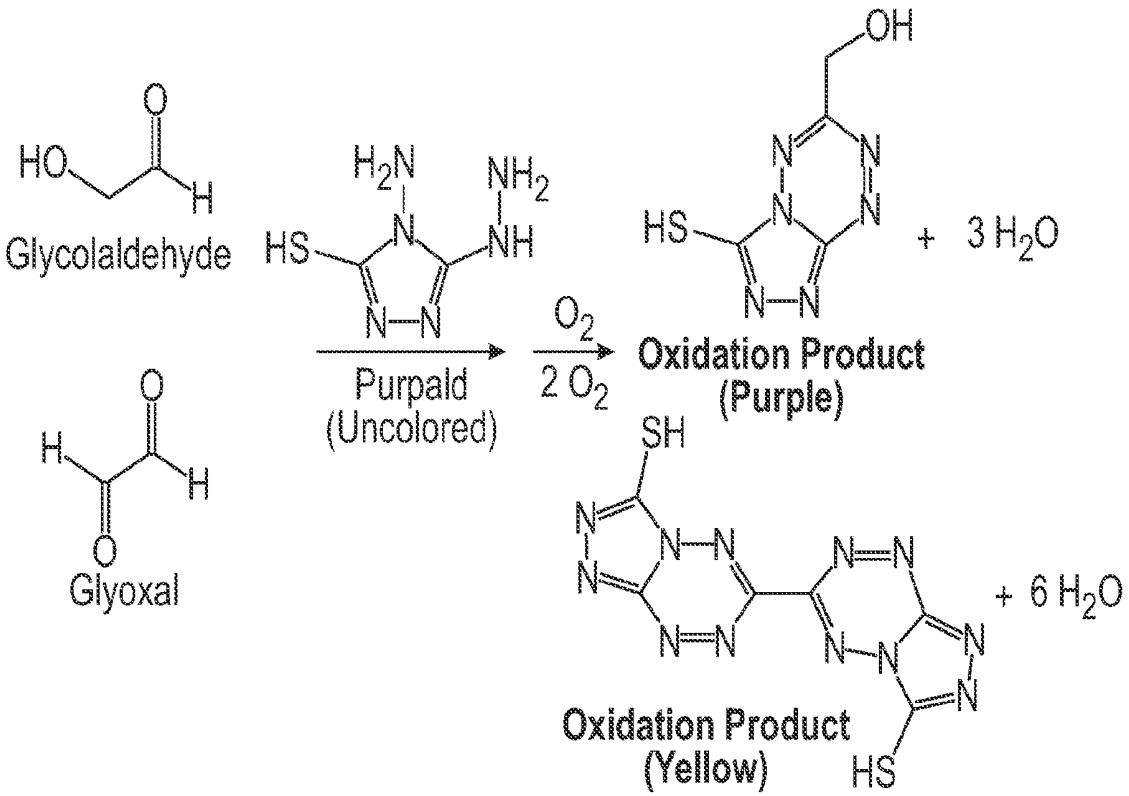

FIG. 10A

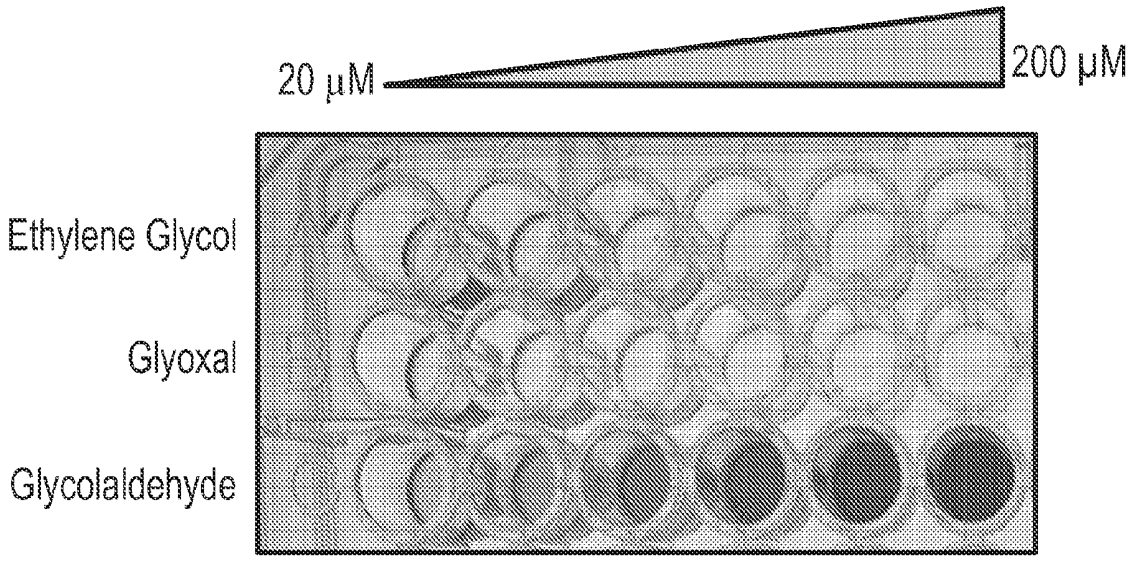

FIG. 10B

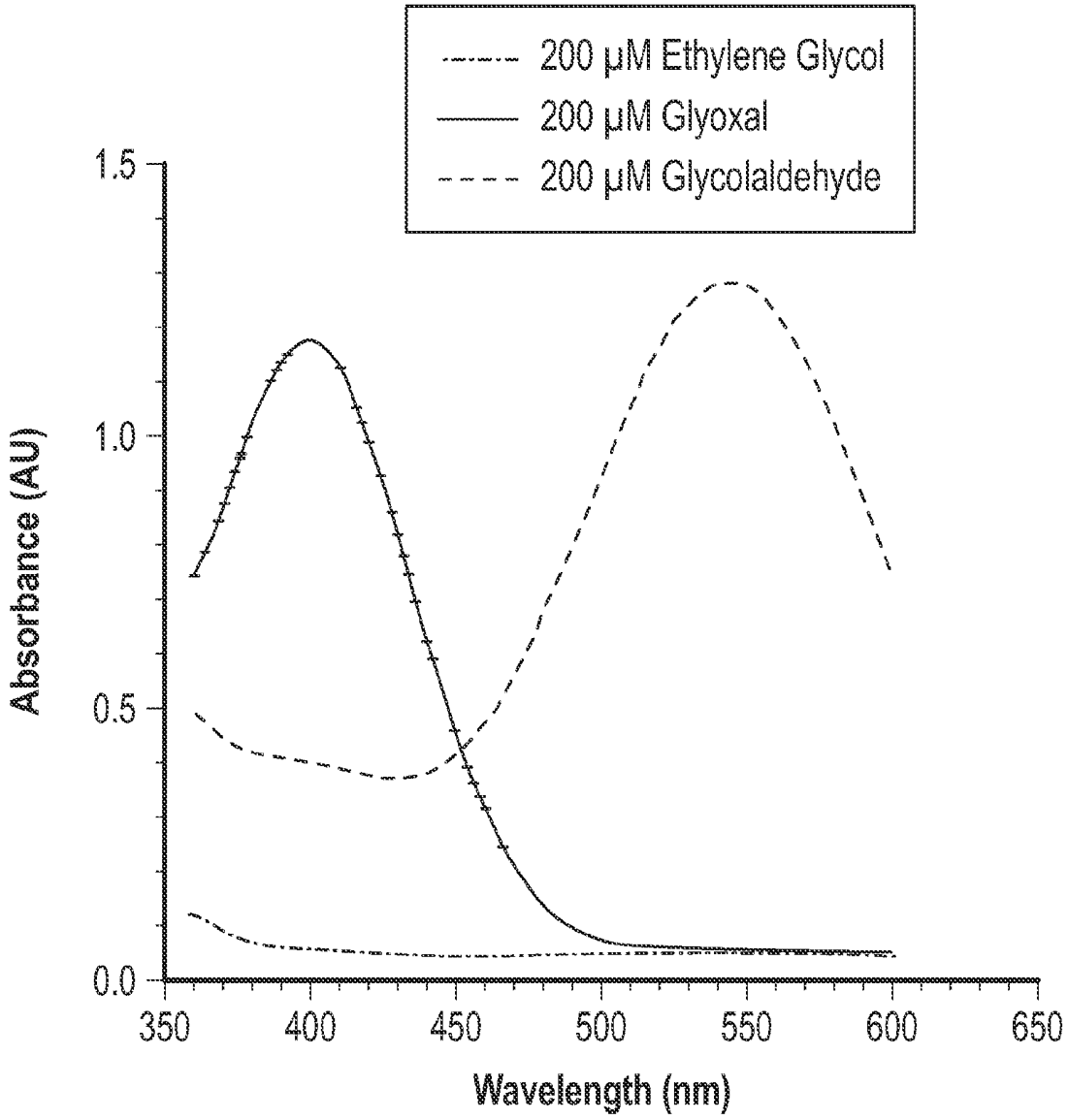
200 µM Ethylene Glycol
200 µM Glyoxal
200 µM Glycolaldehyde
Absorbance (AU)
1.5
1.0
0.5
0.0
350
400
450
500
550
600
650
Wavelength (nm)

FIG. 10C

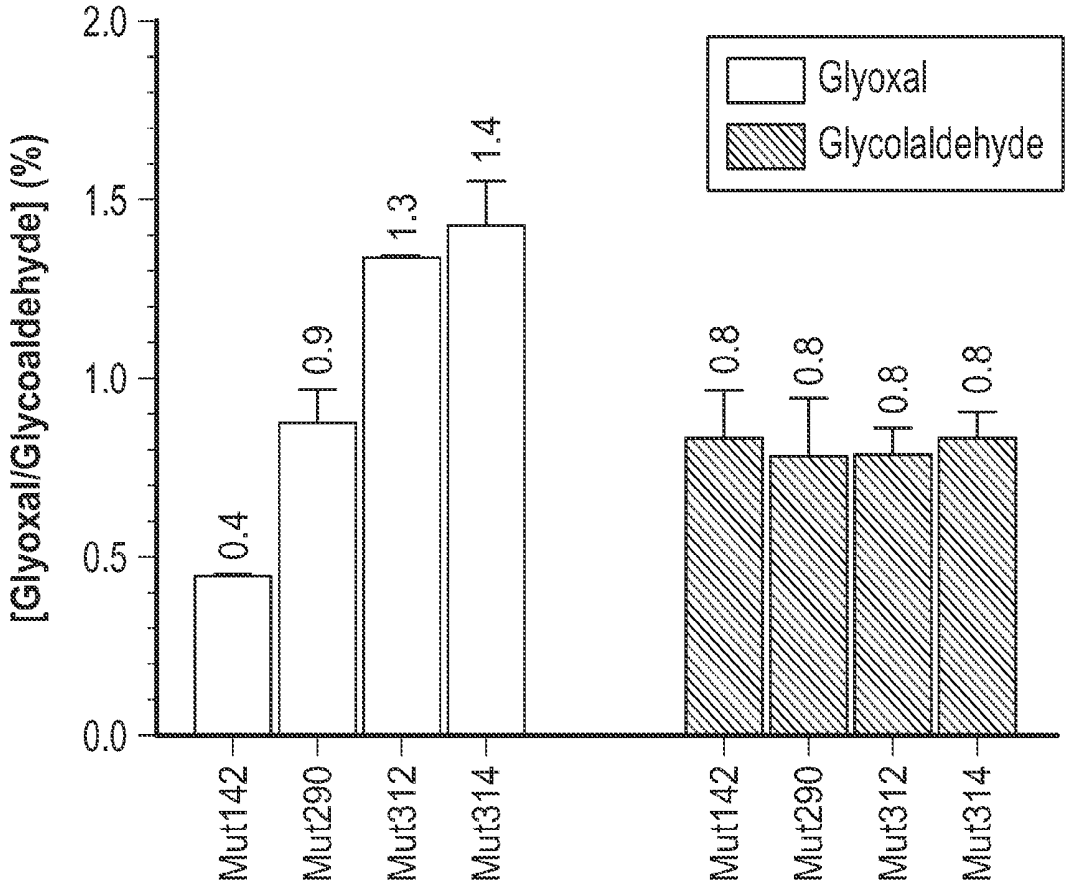
[Glyoxal/Glycoaldehyde] (%)
2.0
1.5
1.0
0.5
0.0
Glyoxal
Glycolaldehyde
0.4
0.9
1.3
1.4
0.8
0.8
0.8
0.8
Mut142
Mut290
Mut312
Mut314
Mut142
Mut290
Mut312
Mut314

FIG. 11

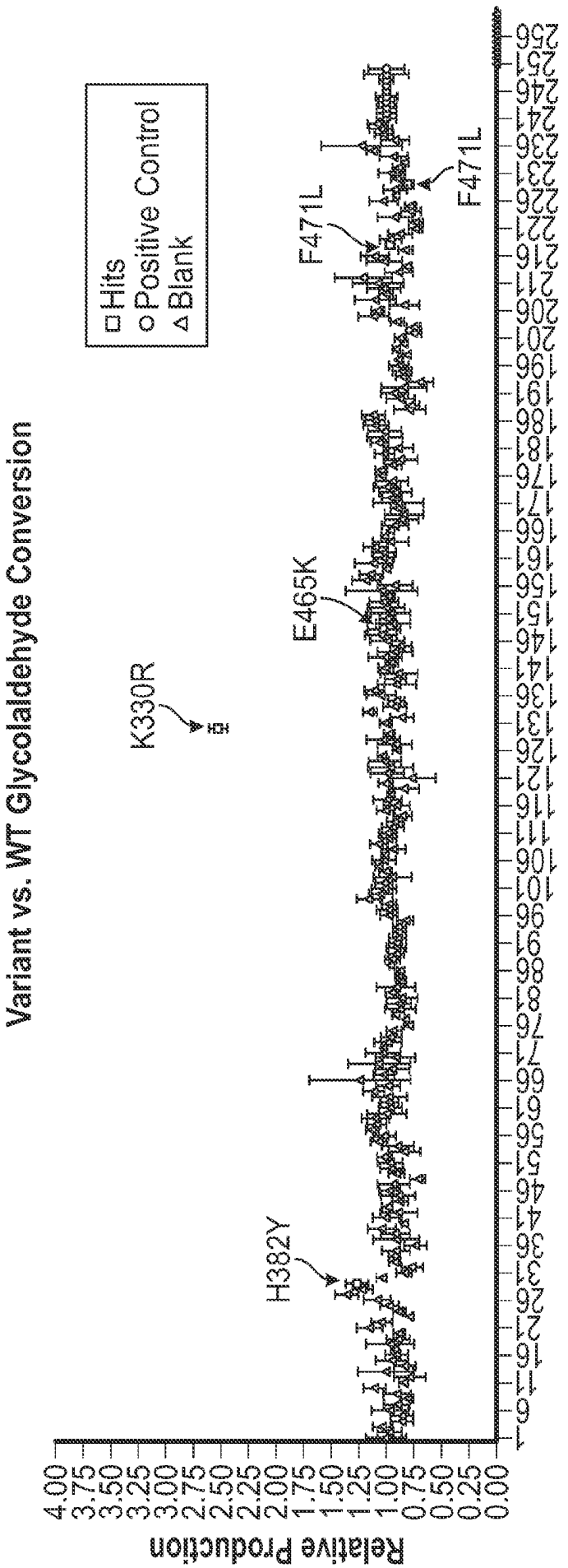
Variant vs. WT Glycolaldehyde Conversion
Hits
Positive Control
Blank
Relative Production
Hits
H382Y
K330R
E465K
F471L
F471L

FIG. 12A

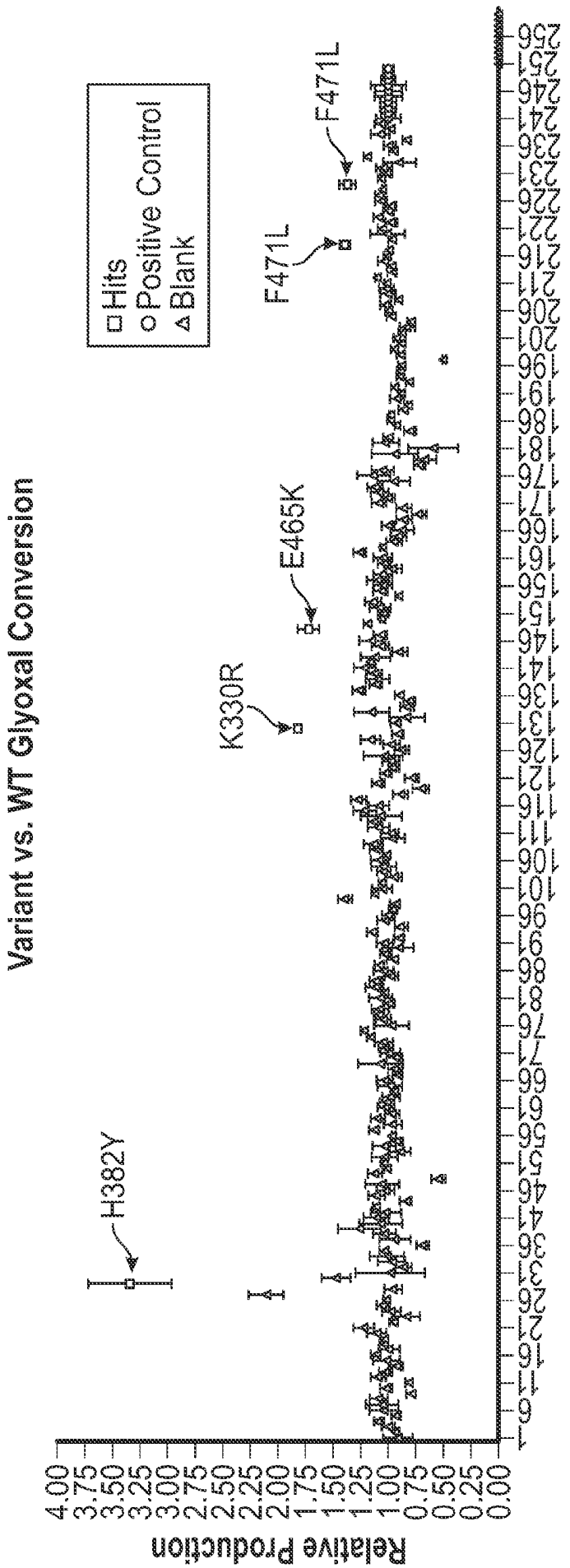
Variant vs. WT Glyoxal Conversion
Hits
Positive Control
Blank
H382Y
K330R
E465K
F471L
F471L
Relative Production
Hits

FIG. 12B

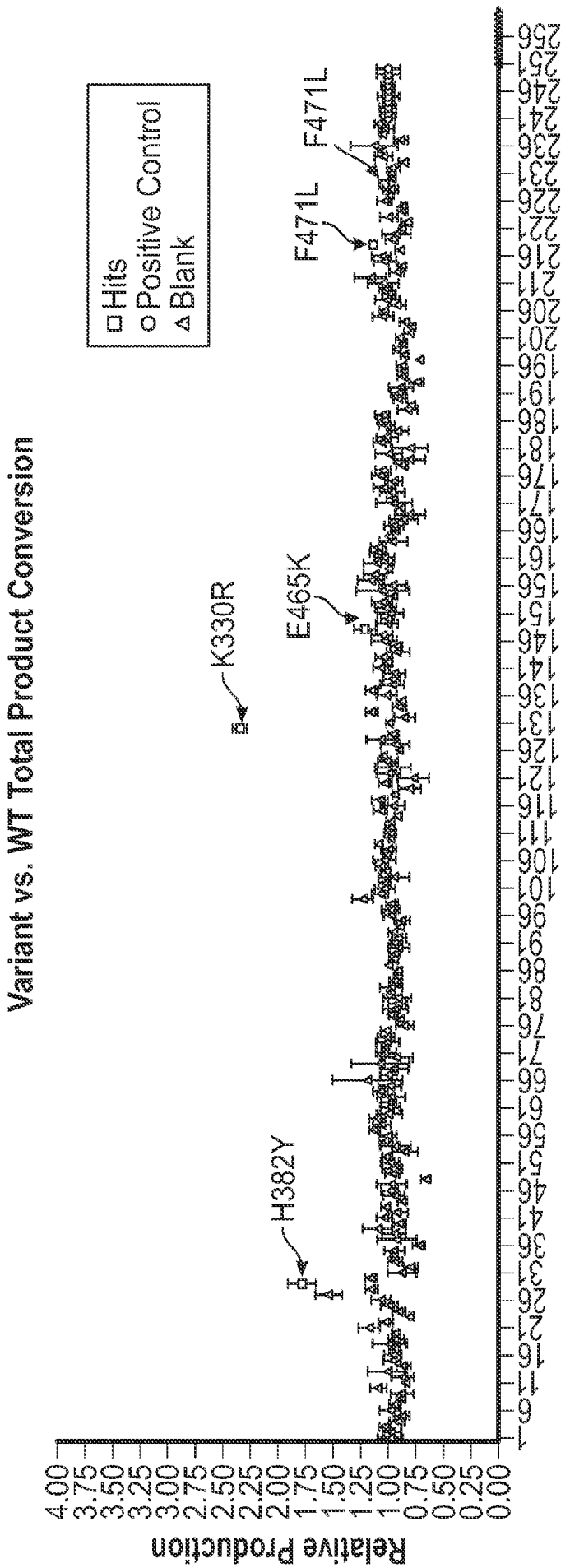
Variant vs. WT Total Product Conversion
Hits
Positive Control
Blank
H382Y
K330R
E465K
F471L
F471L
Relative Production
Hits

FIG. 12C

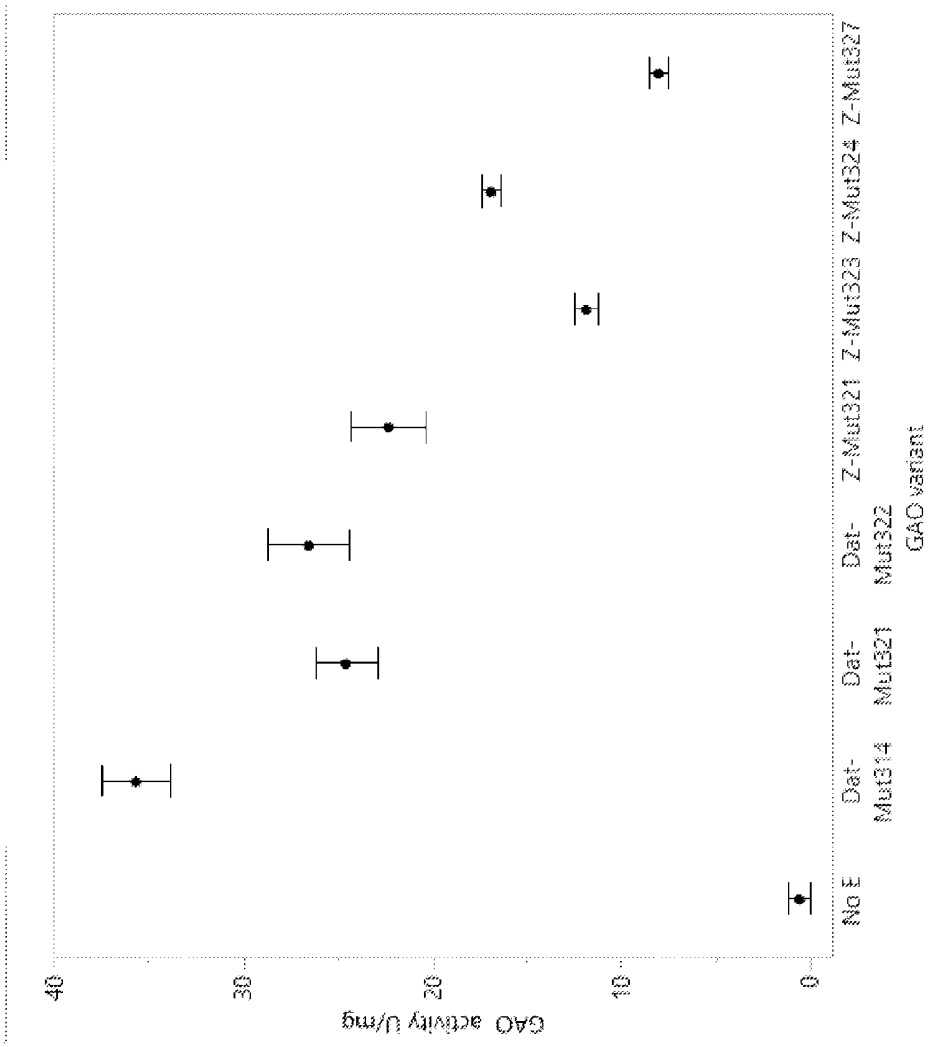
GAO activity U/mg
0
10
20
30
40
No E
Dat-Mut314
Dat-Mut321
Dat-Mut322
Z-Mut321
Z-Mut323
Z-Mut324
Z-Mut327
GAO variant

Figure 13

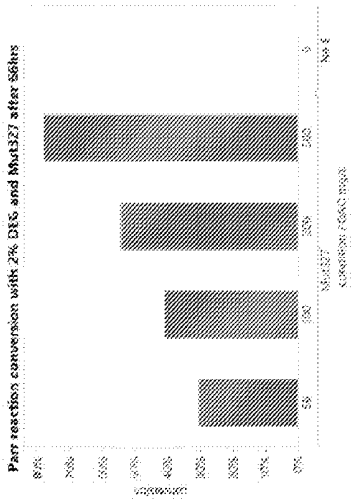

Figure 14D

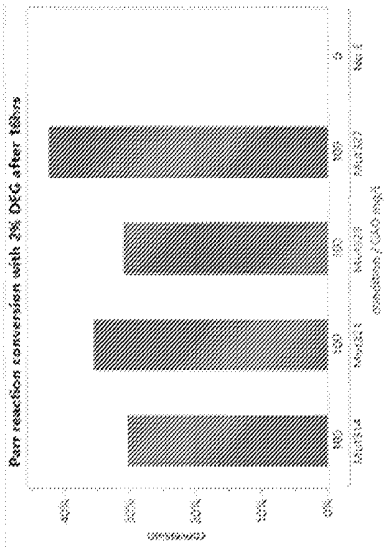

Figure 14C

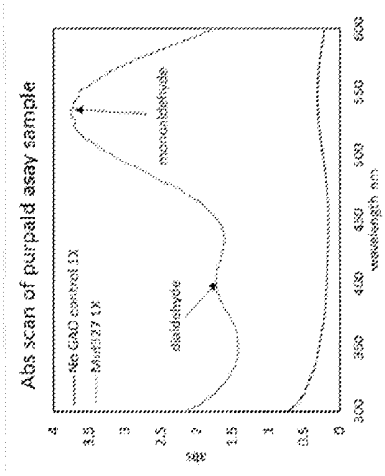

Figure 14B

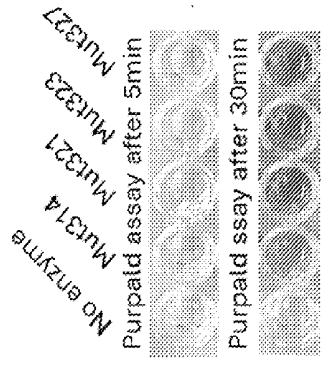

Figure 14A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/041995

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07C 51/235*** (2024.01); ***C12N 9/02*** (2024.01); ***C12N 9/04*** (2024.01); ***C07C 31/20*** (2024.01); ***C07C 47/127*** (2024.01)

CPC: ***C07C 51/235; C07C 31/202; C07C 47/127; C12N 9/0006; C12N 9/0008***

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011/0071262 A1 (PARK et al.) 24 March 2011 (24.03.2011) entire document	1-4, 12, 13
A	US 4,511,739 A (SAUER et al.) 16 April 1985 (16.04.1985) entire document	1-4, 12, 13
A	US 4,066,691 A (SCHROEDER) 03 January 1978 (03.01.1978) entire document	1-4, 12, 13
X	ISOBE et al. Enzymatic Production of Glyoxal from Ethylene Glycol Using Alcohol Oxidase from Methanol Yeast, Biosci Biotechnol Biochem, Vol. 58, No. 1, 31 January 1994 [Retrieved on 08 October 2024]. Retrieved from the Internet: <URL: https://www.jstage.jst.go.jp/article/bbb1992/58/1/58_1_170/_article>. Pgs. 170-173	1-4, 12, 13

☐ 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
"D" document cited by the applicant in the international application
"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	Date of mailing of the international search report
10 October 2024 (10.10.2024)	**11 December 2024 (11.12.2024)**

Name and mailing address of the ISA/US	Authorized officer
COMMISSIONER FOR PATENTS **MAIL STOP PCT, ATTN: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450** **UNITED STATES OF AMERICA**	**TAINA MATOS**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/041995

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/041995

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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-14 are drawn to methods for preparing one or more acids.

The first invention of Group I+ is restricted to a glycol selected to be ethylene glycol and one oxidation catalyst selected to be an oxidase enzyme, and methods for preparing one or more acids comprising the same. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on the first glycol and the first oxidation catalyst presented in the claims (see claims 2 and 12). It is believed that claims 1-4, 12, and 13 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the above embodiment.

Applicant is invited to elect additional glycols and/or oxidation catalysts to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be a glycol selected to be polyglycol and one oxidation catalyst selected to a metal oxidation catalyst, and methods for preparing one or more acids comprising the same. Additional glycols and/or oxidation catalysts will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/ examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

Groups I+ glycols and oxidation catalysts do not share a significant structural element, requiring the selection of alternatives for the glycols and oxidation catalysts where "the glycol comprises ethylene glycol", where "the glycol is a polyglycol", where "the one or more oxidation catalysts comprise one or more oxidase enzymes", and where "the oxidation catalyst comprises a metal oxidation catalyst selected from the group consisting of (i) monometallic formulations that contain have a supported single transition metal species (ii) bimetallic formulations: and (iii) doped alloy formulations".

Additionally, even if Group I+ were considered to share the technical features of a method for preparing one or more acids, the method comprising: contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate; contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid and; recovering the acid, these shared technical features do not represent a contribution over the prior art as disclosed by "Enzymatic Production of Glyoxal from Ethylene Glycol Using Alcohol Oxidase from Methanol Yeast" to Isobe et al. (hereinafter, "Isobe").

Isobe discloses a method for preparing one or more acids (Abstract - oxidized ethylene glycol to glyoxal), the method comprising: contacting a glycol with one or more oxidation catalysts under conditions suitable for formation of an aldehyde intermediate (pg 171 col 2 para 2 alcohol oxidases oxidized ethylene glycol to glyoxal via glycolaldehyde according to Fig. 4; see Fig 4 that shows the first step of oxidizing glycol to glycolaldehyde intermediate); contacting the aldehyde intermediate with one or more oxidation catalysts under conditions suitable for the formation of an acid (pg 171 col 2 para 2 alcohol oxidases oxidized ethylene glycol to glyoxal via glycolaldehyde according to Fig. 4; see Fig 4 that shows the second step of oxidizing the glycolaldehyde to glyoxal) and; recovering the acid (pg 170 col 2 para 4 The concentrations of glyoxal and glycolaldehyde were determined; therefore the glyoxal product was recovered in order to perform these tests).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/041995

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

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 additional fees, this Authority did not invite payment of 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.: **1-4, 12, 13**

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.