

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
2 May 2008 (02.05.2008)

PCT

(10) International Publication Number  
**WO 2008/051491 A2**

(51) International Patent Classification:  
**C12N 1/00** (2006.01)

(21) International Application Number:  
PCT/US2007/022372

(22) International Filing Date: 19 October 2007 (19.10.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/853,227 20 October 2006 (20.10.2006) US  
60/853,258 20 October 2006 (20.10.2006) US

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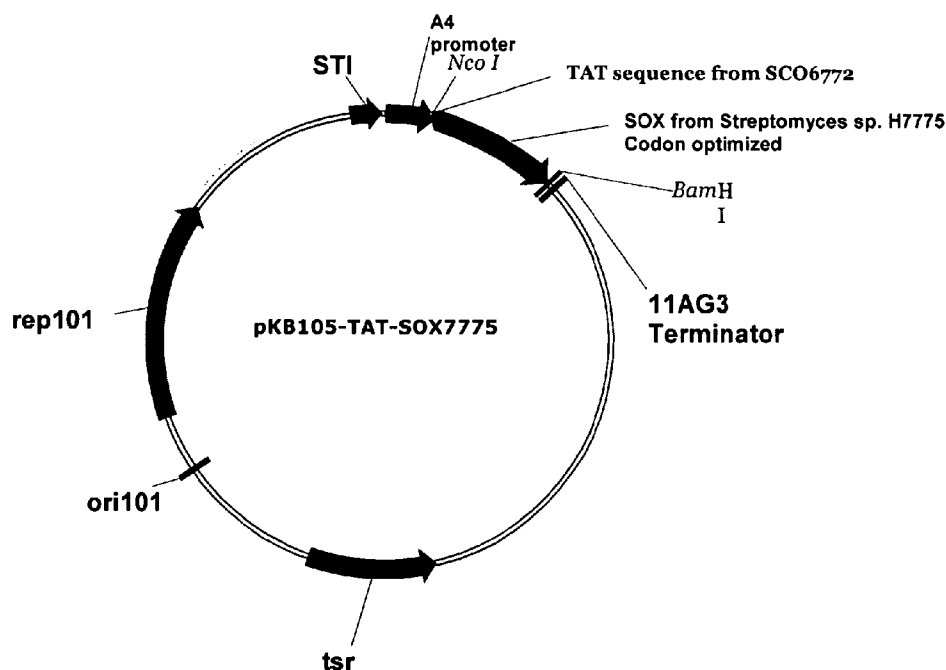
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(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH,  
CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG,  
ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL,  
IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK,  
LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW,  
MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL,  
PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY,  
TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA,  
ZM, ZW.

(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,  
PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: POLYOL OXIDASES



(57) Abstract: The present invention provides compositions and methods for producing a polyol oxidase in microorganisms, and the use of polyol oxidases in cleaning compositions. The invention includes cleaning compositions that contain combinations of two or more POx oxidases, and cleaning compositions that contain combinations of two or more POx oxidases and a perhydrolase. In particular, the invention provides methods for expressing polyol oxidases in bacterial hosts for use in detergent applications for cleaning, bleaching and disinfecting.

WO 2008/051491 A2



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**Published:**

- *without international search report and to be republished  
upon receipt of that report*

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**POLYOL OXIDASES****CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims priority to United States provisional application US  
10 60/853,227, filed October 20, 2006, United States provisional application US 60/853,258,  
filed on October 20, 2006, which are herein incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

15 [001] The present invention provides compositions and methods for producing a polyol  
oxidase in microorganisms, and the use of polyol oxidases in cleaning compositions.  
The invention includes cleaning compositions that contain combinations of two or more  
POx oxidases, and cleaning compositions that contain combinations of two or more POx  
oxidases and a perhydrolase. In particular, the invention provides methods for  
20 expressing polyol oxidases in bacterial hosts for use in detergent applications for  
cleaning, bleaching and disinfecting.

**BACKGROUND OF THE INVENTION**

[002] Oxidoreductases are enzymes that catalyze the transfer of electrons from one  
25 molecule (the reductant, also called the hydrogen acceptor or electron donor) to another  
(the oxidant, also called the hydrogen donor or electron acceptor). Oxidoreductases are  
classified as EC 1 in the EC number classification of enzymes. Oxidoreductases can be  
further classified into 22 subclasses, and the oxidoreductases belonging to EC class  
1.1.3. act on the CH-OH group of donors with oxygen as acceptor. The oxidoreductase  
30 enzymes of the EC1.1.3 class of enzymes are oxidases, and their ability to generate  
hydrogen peroxide has found use in improving the storage stability of food products  
including cheese, butter, meat, wine and fruit juice (See e.g., Hammer, Oxidoreductases:  
Enzymes in Food Processing, Nagodawithana and Reed (eds). Academic Press, NY,  
[1998]; pp.251-254; and Tiina and Sandholm, Int. J. Food Microbiol., 8:165-74 [1989]).  
35 Indeed, oxidases have found use as components of various compositions, including  
foods, personal care items, and detergents. However, there remains a need for methods

and compositions that facilitate the efficient and economical production of oxidases for use in any suitable setting.

## SUMMARY OF THE INVENTION

5 [003] The present invention provides compositions and methods for producing a polyol oxidase in micoroorganisms, and the use of polyol oxidases in cleaning compositions. The invention includes cleaning compositions that contain combinations of two or more POx oxidases, and cleaning compositions that contain combinations of two or more POx oxidases and a perhydrolase. In particular, the invention provides methods  
10 for expressing polyol oxidases in bacterial hosts for use in detergent applications for cleaning, bleaching and disinfecting.

[004] In one embodiment, the invention provides an isolated chimeric polynucleotide comprising a sequence encoding a mature polyol oxidase protein (POx) that is operably linked to a sequence encoding a secretory signal peptide, which is derived from a  
15 prokaryotic microorganism.

[005] In another embodiment, the invention provides an isolated chimeric polynucleotide that encodes a mature polyol oxidase protein (POx) that is operably linked to a sequence encoding a secretory signal peptide derived from a *Streptomyces sp.*, an *Acidothermus sp.* or an *Arthrobacter sp.*

20 [006] In another embodiment, the invention provides for an isolated chimeric polynucleotide that encodes a polypeptide selected form SEQ ID NO:35, 37, 38, and 40, wherein the chimeric polynucleotide comprises a sequence encoding a mature polyol oxidase protein (POx) operably linked to a sequence encoding a secretory signal peptide, which is derived from a prokaryotic microorganism.

25 [007] In another embodiment, the invention provides an isolated chimeric polynucleotide comprising a sequence encoding a mature polyol oxidase protein (POx) that is operably linked to a sequence encoding a secretory signal peptide, which is peptide is selected from the signal sequence encoded by the *S. coelicolor* gene SCO7637, the signal sequence encoding the *S. lividans* gene SCO 0624 and the signal  
30 sequence encoding the *B. subtilis* gene P43379.

[008] In another embodiment, the invention provides for a recombinant expression vector comprising an isolated chimeric polynucleotide that comprises a sequence that encodes a mature polyol oxidase protein (POx) that is operably linked to a sequence encoding a secretory signal peptide derived from a *Streptomyces sp.*, an *Acidothermus*  
35 *sp.* or an *Arthrobacter sp.*

[009] In another embodiment, the invention provides a host cell that comprises a recombinant expression vector that an comprises isolated chimeric polynucleotide that

comprises a sequence that encodes a mature polyol oxidase protein (POx) that is operably linked to a sequence encoding a secretory signal peptide derived from a *Streptomyces sp.*, an *Acidothermus sp.* or an *Arthrobacter sp.*

5 [0010] In another embodiment, the invention provides a host cell that comprises a polynucleotide that encodes a POx polypeptide that has a sequence selected from SEQ ID NO: 6, 35, 37, 38 and 40.

[0011] In another embodiment, the invention provides a host cell that comprises a polynucleotide that encodes a POx polypeptide that has sorbitol oxidase and/or xylitol oxidase activity, and that has a sequence selected from SEQ ID NO: 6, 35, 37, 38 and 10 40.

[0012] In another embodiment, the invention provides a host cell that comprises a polynucleotide that polynucleotide is present in the genome of said host cell or in a vector that autonomously replicates in said host cell, and that encodes a POx polypeptide that has a sequence selected from SEQ ID NO: 6, 35, 37, 38 and 40.

15 [0013] In another embodiment, the invention provides an *S. lividans*, a *B. subtilis* or an *Acidothermus cellulolyticus* host cell that comprises a polynucleotide that encodes a POx polypeptide that has a sequence selected from SEQ ID NO: 6, 35, 37, 38 and 40.

[0014] In another embodiment, the invention provides a method for producing a 20 polypeptide having POx activity that includes (a) transforming a host cell with a recombinant expression vector that comprises a polynucleotide sequence that encodes a POx polypeptide having a sequence selected from SEQ ID NO:6, 35, 37, 38 and 40, (b) growing the transformed host cell under conditions suitable for the expression of said POx polypeptide; and (c) recovering the POx polypeptide.

25 [0015] In another embodiment, the invention provides a method for producing a polypeptide having sorbitol and/or xylitol activity that includes (a) transforming a host cell with a recombinant expression vector that comprises a polynucleotide sequence that encodes a POx polypeptide having a sequence selected from SEQ ID NO:6, 35, 37, 38 and 40, (b) growing the transformed host cell under conditions suitable for the expression 30 of said POx polypeptide; and (c) recovering the POx polypeptide.

[0016] In another embodiment, the invention provides a method for producing a polypeptide having POx activity that includes (a) transforming a *Bacillus*, a *Streptomyces* or an *E. Coli* host cell with a recombinant expression vector that comprises a polynucleotide sequence that encodes a POx polypeptide having a sequence selected 35 from SEQ ID NO:6, 35, 37, 38 and 40, (b) growing the transformed host cell under conditions suitable for the expression of said POx polypeptide; and (c) recovering the POx polypeptide.

[0017] In another embodiment, the invention provides a method for producing a polypeptide having POx activity that includes (a) transforming a host cell with a recombinant expression vector that comprises a polynucleotide sequence that encodes a POx polypeptide having a sequence selected from SEQ ID NO:6, 35, 37, 38 and 40, (b) growing the transformed host cell under conditions suitable for the expression of said POx polypeptide; and (c) recovering the POx polypeptide, wherein the expression of the polypeptide is extracellular.

[0018] In another embodiment, the invention provides a method for producing a polypeptide having POx activity that includes (a) transforming a host cell with a recombinant expression vector that comprises a polynucleotide sequence that encodes a POx polypeptide having a sequence selected from SEQ ID NO:6, 35, 37, 38 and 40, (b) growing the transformed host cell under conditions suitable for the expression of said POx polypeptide; and (c) recovering the POx polypeptide, wherein the expression of the polypeptide is intracellular.

[0019] In another embodiment, the invention provides for a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

[0020] In another embodiment, the invention provides for a detergent composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

[0021] In another embodiment, the invention provides for a detergent composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14, at least one additional enzyme, and a bleach activator.

[0022] In another embodiment, the invention provides for a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO: 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14 and least one additional enzyme.

[0023] In another embodiment, the invention provides for a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO: 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14 and least one additional enzyme that is selected from hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases,

oxidoreductases, perhydrolases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases, and amylases, or mixtures thereof.

5     **[0024]**     In another embodiment, the invention provides for a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14 and least one additional enzyme that is a perhydrolase.

10     **[0025]**     In another embodiment, the invention provides for a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14 and least one additional enzyme that is a glucose oxidase.

15     **[0026]**     In another embodiment, the invention provides for a bleaching composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14 and least one additional enzyme that is a glucose oxidase.

20     **[0027]**     In another embodiment, the invention provides for a method of cleaning that includes contacting a hard surface and/or an article comprising a fabric with a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

25     **[0028]**     In another embodiment, the invention provides for a method of cleaning that includes contacting a hard surface and/or an article comprising a fabric with a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14, and rinsing said surface and/or article after contacting said surface or article with said cleaning composition.

30     **[0029]**     In another embodiment, the invention provides for a method of cleaning that includes contacting a hard surface and/or an article comprising a fabric stained with a substance containing at least one polyol, with a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

35     **[0030]**     In another embodiment, the invention provides for a method of cleaning that includes contacting a hard surface and/or an article comprising a fabric stained with a

substance containing at least one polyol selected from the group of D-sorbitol, D-xylitol, D-mannitol, D-ribitol, myo-inositol, glycerol, 1,3-propanediol and 1,2-propanediol, with a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

[0031] In another embodiment, the invention provides for a method of cleaning that includes contacting a hard surface and/or an article comprising a fabric, which is soiled with juice, wine and/or tea, with a cleaning composition that comprises an effective amount of an isolated POx that has an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and/or 14.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

[0032] Figure 1 provides a map of expression plasmid pKB105-TAT-SOx7775 (SEQ ID NO:29).

[0033] Figure 2 provides a map of expression plasmid pKB105-CelA-SOx-7775 (SEQ ID NO:30).

[0034] Figure 3 provides a map of expression plasmid pet24a-SOx (neutral codons).

[0035] Figure 4 provides a map of expression plasmid pSMM-SOx *S. lividans*.

[0036] Figure 5 provides a graph showing the results of a washing study to determine the ability of sorbitol oxidase to remove tea stains on cotton discs.

[0037] Figure 6 provides a graph showing the results of a SOx dose study for bleaching of tea stains on cotton discs.

[0038] Figure 7 provides a graph showing the results of a washing and bleaching performance study of sorbitol oxidase to remove of wine stains on cotton discs.

[0039] Figure 8 provides a graph showing the results of a bleaching performance study of the ability of sorbitol oxidase to remove blueberry stains on cotton discs.

[0040] Figure 9 (A-B) show the rate of H<sub>2</sub>O<sub>2</sub> production by a combination of a constant amount of a first polyol oxidase (SOx) with an increasing amount of a second polyol oxidase (HOx or GOx). The amount of GOx or HOx relative to the constant amount of SOx was increased between 1X and 3000X (A) or 1X and 100X (B), and the rate of H<sub>2</sub>O<sub>2</sub> production was measured over 5 minutes in a 300ul ABTS assay. The combination of SOx with HOx (♦) or SOx with GOx (■) resulted in a synergistic increase in the production of H<sub>2</sub>O<sub>2</sub> of between 250 and 300% of that seen with SOx alone (--) at 3000X (A).

## DESCRIPTION OF THE INVENTION

[0041] The present invention provides compositions and methods for producing a polyol  
5 oxidase in microorganisms, and the use of polyol oxidases in cleaning compositions.

The invention includes cleaning compositions that contain combinations of two or more  
POx oxidases, and cleaning compositions that contain combinations of two or more POx  
oxidases and a perhydrolase. In particular, the invention provides methods for

expressing polyol oxidases in bacterial hosts for use in detergent applications for  
10 cleaning, bleaching and disinfecting.

### Definitions

[0042] Unless otherwise indicated, the practice of the present invention involves  
15 conventional techniques commonly used in molecular biology, microbiology, protein  
purification, protein engineering, protein and DNA sequencing, recombinant DNA fields,  
and industrial enzyme use and development, all of which are within the skill of the art. All  
patents, patent applications, articles and publications mentioned herein, both supra and  
infra, are hereby expressly incorporated herein by reference in their entirety.

[0043] Furthermore, the headings provided herein are not limitations of the various  
20 aspects or embodiments of the invention, which can be had by reference to the  
specification as a whole. Accordingly, the terms defined immediately below are more fully  
defined by reference to the specification as a whole. Nonetheless, in order to facilitate  
understanding of the invention, definitions for a number of terms are provided below.

[0044] Unless defined otherwise herein, all technical and scientific terms used herein  
25 have the same meaning as commonly understood by one of ordinary skill in the art to  
which this invention pertains. For example, Singleton and Sainsbury, Dictionary of  
Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY (1994); and Hale  
and Margham, The Harper Collins Dictionary of Biology, Harper Perennial, NY (1991)  
30 provide those of skill in the art with a general dictionaries of many of the terms used in the  
invention. Although any methods and materials similar or equivalent to those described  
herein find use in the practice of the present invention, preferred methods and materials  
are described herein. Accordingly, the terms defined immediately below are more fully  
described by reference to the Specification as a whole. Also, as used herein, the singular  
35 terms "a," "an," and "the" include the plural reference unless the context clearly indicates  
otherwise. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3'  
orientation; amino acid sequences are written left to right in amino to carboxy orientation,

respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art.

[0045] It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[0046] As used herein, the term "oxidase" refers to enzymes that catalyze an oxidation/reduction reaction involving molecular oxygen ( $O_2$ ) as the electron acceptor. In these reactions, oxygen is reduced to water ( $H_2O$ ) or hydrogen peroxide ( $H_2O_2$ ). The oxidases are a subclass of the oxidoreductases.

[0047] As used herein, "polyol oxidase", "POx" or "POx polypeptide" refers to enzymes that catalyze the oxidation of various polyols (e.g., sorbitol, xylitol, arabitol, mannitol, ribitol, glycerol, propanediol, and propylene glycol). Polyol oxidase enzymes include oxidoreductase enzyme of the EC1.1.3 class of enzymes that includes sorbitol oxidase, xylitol oxidase, glucose oxidase, hexose oxidase, alcohol oxidase, choline oxidase, mannitol oxidase. As used herein, "polyol" refers to chemical compounds that contain multiple hydroxyl groups.

[0048] As used herein, the term "glucose oxidase" ("GOx") refers to the oxidase enzyme (EC 1.1.3.4), a dimeric protein which catalyzes the oxidation of beta-D-glucose into D-glucono-1,5-lactone, which then hydrolyzes to gluconic acid with concomitant reduction of molecular oxygen to hydrogen peroxide.

[0049] As used herein, the term "alcohol oxidase" ("AOx") refers to the oxidase enzyme (EC 1.1.3.13) that converts an alcohol to an aldehyde with concomitant reduction of molecular oxygen to hydrogen peroxide.

[0050] As used herein, the term "choline oxidase" ("COx") refers to an oxidase enzyme (EC 1.1.3. 17) that catalyzes the four-electron oxidation of choline to glycine betaine, with betaine aldehyde as an intermediate with concomitant reduction of two molecules of molecular oxygen to two molecules of hydrogen peroxide.

[0051] As used herein, the term "hexose oxidase" ("HOx") refers to an oxidase enzyme (EC 1.1.3.5) that is capable of the oxidation of mono- and disaccharides to their corresponding lactones, with concomitant reduction of molecular oxygen to hydrogen peroxide. Hexose oxidase is able to oxidize a variety of substrates including D-glucose,

D-galactose, maltose, cellobiose, and lactose, etc. It is not intended that the present invention be limited to any particular hexose.

[0052] As used herein, "glycerol oxidase" refers to an oxidase enzyme (EC 1.1.3.) that catalyzes the oxidation of glycerol to glyceraldehyde, with concomitant reduction of molecular oxygen to hydrogen peroxide.

[0053] As used herein, "sorbitol oxidase" or "SOx" refers to a polyol oxidase enzyme (EC 1.1.3.) that catalyzes the oxidation of a substrate (e.g., D-sorbitol) to D-glucose, with concomitant reduction of molecular oxygen to hydrogen peroxide. The definition further includes oxidase enzymes with a  $V_{max}/K_m$  value which is highest for sorbitol than for xylitol, galactitol, D-mannitol, glycerol and D-arabitol, and no significant activity towards xylose, D-glucose, galactose, mannose and arabinose.

[0054] As used herein, the term "xylitol oxidase" ("XOx") refers to an oxidase enzyme that catalyzes the oxidation of xylitol to xylose with concomitant reduction of molecular oxygen to hydrogen peroxide. The definition further includes oxidase enzymes with a  $V_{max}/K_m$  value which is highest for xylitol than for sorbitol, galactitol, D-mannitol, and D-arabitol, and no activity towards xylose, D-glucose, galactose, mannose and arabinose.

[0055] In some particularly preferred embodiments, the sorbitol oxidase of the present invention has a higher specific activity, (or  $V_{max}/K_m$  ratio) on sorbitol substrate, as compared to an alternative substrate under standard assay conditions (e.g. in the in vitro assay provided below) and/or using an in situ in an application media, conducted as known in the art. In some preferred alternative embodiments, the alternative substrate is xylitol.

[0056] In some preferred embodiments, the following in vitro assay finds use. An assay mixture containing 266  $\mu$ l substrate (e.g., sorbitol -Sigma P-5504 or xylitol) (0.055 M, in 0.1 M sodium phosphate buffer, pH 6.3), 12  $\mu$ l 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)(Sigma A-9941, 5 mg/ml aqueous solution), 12  $\mu$ l peroxidase (POD)(Sigma P-6782, 0.1 mg/ml in 0.1 M sodium phosphate buffer, pH 6.3) and 10  $\mu$ l enzyme (SOx or HOx) aqueous solution was prepared. The assay was performed at 25°C. The incubation was started by the addition of glucose to the assay mixture. The absorbance was monitored at 405 nm in an ELISA reader. A standard curve, based on varying concentrations of  $H_2O_2$ , was used for calculation of enzyme activity. In this assay, 1 polyol unit (POx), such as SOx or XOX units, corresponds to the amount of enzyme, which under the specified conditions results in the conversion of 1  $\mu$ mole of the specified polyol per minute, with resultant generation of 1  $\mu$ mole of hydrogen peroxide ( $H_2O_2$ ).

[0057] In some particularly preferred embodiments, the sorbitol oxidase of the present invention has no significant activity on the corresponding sugar product, such as glucose,

xylose, galactose, mannose or arabinose, preferably glucose as determined using the assay provided in the Examples.

[0058] In some alternative preferred embodiments, the xylitol oxidase of the present invention has a higher specific activity, (or  $V_{\max}/K_{\text{m}}$  ratio) on xylitol substrate, as compared to sorbitol under standard assay conditions (e.g., in the in vitro assay provided herein) and/or in an in situ in an application media, conducted as known in the art.

[0059] As used herein, "inhibitors" refers to chemical compounds that can reduce or stop the catalytic activity of an enzyme. In particularly preferred embodiments, the inhibitors reduce or stop the catalytic activity of at least one oxidase. Examples of oxidase inhibitors include acetate, silver salts, halide ions, sec- and tert-alcohols, isocyanate, isothiocyanate, glucose analogs, bisulfite, sulfite, thiosulfate, metabisulfite, zinc salts, diethyl dicarbamate, methyl methane sulfonate, acrylonitrile, 2-amino,2-methyl 1-propanol.

[0060] As used herein, "reversible enzyme inhibitor" refers to molecules that bind to an enzyme and decrease its rate of reaction. In some embodiments, reversible enzyme inhibitors are affected by varying the concentration of the enzyme's substrate in relation to the inhibitor. In some embodiments, reversible enzyme inhibitors bind to the enzyme using weak bonds that are similar to those used to bind to substrate. Thus, the reversible inhibitor does not permanently disable the enzyme, as removal of the inhibitor allows the enzyme to bind to and turnover its substrate. In some embodiments, reversible enzyme inhibitors are competitive inhibitors that interact non-covalently with the enzyme, and/or compete with the substrate for the enzyme's active site, and/or have structures that are similar to the substrate, products and/or transition state. In additional embodiments, the reversible inhibitor is a non-competitive enzyme inhibitor that binds at a site present on the enzyme other than the active site, and/or causes conformational changes in the enzyme that decrease, and/or stop catalytic activity. It is not intended that the term be limited to any particular mechanism or type of reversible enzyme inhibitor. It is only necessary that the effects of the enzyme inhibitor be reversible, such that the enzyme will function in the absence of the inhibitor and/or dilutions of the enzyme inhibitor mixture.

[0061] As used herein, the term "compatible," means that other composition materials (i.e., other materials in a given composition, in addition to an oxidase of the present invention) do not reduce the enzymatic activity of the oxidase enzyme(s) provided herein to such an extent that the oxidases(s) is/are not effective as desired during normal use situations. Specific composition materials are exemplified in detail hereinafter.

[0062] As used herein, "effective amount of enzyme" refers to the quantity of enzyme necessary to achieve the enzymatic activity required in the specific application. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based

on many factors, such as the particular enzyme variant used, the application, the specific contents of the composition, and whether a liquid or dry (e.g., granular) composition is required, and the like.

**[0063]** As used herein, "having improved properties" used in connection with "mutant oxidative enzymes," refers to oxidative enzymes with improved performance and/or improved stability with retained performance, relative to the corresponding wild-type oxidase. In some particularly preferred embodiments, the improved properties are selected from the group consisting of improved dishwash performance and improved stability, as well as the combination of improved dishwash performance and improved stability.

**[0064]** As used herein, the phrase "detergent stability" refers to the stability of a detergent composition. In some embodiments, the stability is assessed during the use of the detergent, while in other embodiments, the term refers to the stability of a detergent composition during storage.

**[0065]** The term "improved stability" is used to indicate better stability of oxidases combined with mutant protease(s) in compositions during storage and/or better stability during use (e.g., in the sud). In preferred embodiments, the mutant oxidases combined with mutant protease(s) exhibit improved stability in formulations during storage and/or improved stability during use, which includes stability against oxidizing agents, sequestering agents, autolysis, surfactants and high alkalinity, relative to the corresponding wild-type enzyme.

**[0066]** As used herein, "oxidative stability" refers to the ability of a protein to function under oxidative conditions. In particular, the term refers to the ability of a protein to function in the presence of various concentrations of H<sub>2</sub>O<sub>2</sub>, peracids and other oxidants.

Stability under various oxidative conditions can be measured either by standard procedures known to those in the art and/or by the methods described herein. A substantial change in oxidative stability is evidenced by at least about a 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the enzymatic activity, as compared to the enzymatic activity present in the absence of oxidative compounds.

**[0067]** As used herein, "pH stability" refers to the ability of a protein to function at a particular pH. In general, most enzymes have a finite pH range at which they will function. In addition to enzymes that function in mid-range pHs (i.e., around pH 7), there are enzymes that are capable of working under conditions with very high or very low pHs. Stability at various pHs can be measured either by standard procedures known to those in the art and/or by the methods described herein. A substantial change in pH stability is evidenced by at least about 5% or greater increase or decrease (in most embodiments, it

is preferably an increase) in the half-life of the enzymatic activity, as compared to the enzymatic activity at the enzyme's optimum pH. However, it is not intended that the present invention be limited to any pH stability level nor pH range.

**[0068]** As used herein, "thermal stability" refers to the ability of a protein to function at a particular temperature. In general, most enzymes have a finite range of temperatures at which they will function. In addition to enzymes that work in mid-range temperatures (e.g., room temperature), there are enzymes that are capable of working in very high or very low temperatures. Thermal stability can be measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the catalytic activity of a mutant when exposed to given temperature. However, it is not intended that the present invention be limited to any temperature stability level nor temperature range.

**[0069]** As used herein, the term "chemical stability" refers to the stability of a protein (e.g., an enzyme) towards chemicals that may adversely affect its activity. In some embodiments, such chemicals include, but are not limited to hydrogen peroxide, peracids, anionic detergents, cationic detergents, non-ionic detergents, chelants, etc. However, it is not intended that the present invention be limited to any particular chemical stability level nor range of chemical stability.

**[0070]** As used herein, the terms "purified" and "isolated" refer to the removal of contaminants from a sample. For example, polyol oxidases are purified by removal of contaminating proteins and other compounds within a solution or preparation that are not polyol oxidases. In some embodiments, recombinant polyol oxidases are expressed in bacterial or fungal host cells and these recombinant polyol oxidases are purified by the removal of other host cell constituents; the percent of recombinant polyol oxidase polypeptides is thereby increased in the sample. In particularly preferred embodiments, the polyol oxidases of the present invention are substantially purified to a level of at least about 99% of the protein component, as determined by SDS-PAGE or other standard methods known in the art.

**[0071]** As used herein, "protein of interest" and "polypeptide of interest" refer to a protein (e.g., an enzyme or "enzyme of interest") which is being analyzed, identified and/or modified. Naturally-occurring, as well as recombinant proteins find use in the present invention.

**[0072]** As used herein, "protein" refers to any composition comprised of amino acids and recognized as a protein by those of skill in the art. The terms "protein," "peptide" and polypeptide are used interchangeably herein. Wherein a peptide is a portion of a protein, those skilled in the art understand the use of the term in context.

[0073] The terms "mature form" and "mature region" refer to the final functional portion of the protein. To exemplify, a mature form of the POx of the present invention at least includes the amino acid sequence identical to SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6. In this context, the "mature form" is "processed from" a full-length POx, wherein  
5 the processing of the full-length carrageenase encompasses the removal of the signal peptide. Thus, for example, the mature form of the POx of SEQ ID NO:2 corresponds to amino acid residues 48 to 467 of SEQ ID NO:16; and the mature form of the POx of SEQ ID NO:6 corresponds to amino acid residues 28 to 480 of SEQ ID NO:37.

[0074] A "full-length" protein herein refers to a POx polypeptide that comprises a  
10 secretory signal peptide and a mature portion. The fusion POx enzymes of the invention are encoded by chimeric polynucleotides.

[0075] The term "chimeric polynucleotide" or "fusion polynucleotide" herein refer to a polynucleotide that comprises at least two separate and distinct regions that may or may not originate from the same gene. For example, a polynucleotide sequence encoding a  
15 signal peptide linked to the polynucleotide that encodes the mature form of the polypeptide would be termed a chimeric polynucleotide. In some embodiments, a chimeric POx polynucleotide encodes a POx fusion polypeptide.

[0076] As used herein, the terms "chimeric polypeptide" and "fusion polypeptide" are used interchangeably to refer to a protein that comprises at least two separate and  
20 distinct regions that may or may not originate from the same protein. For example, a signal peptide linked to the protein of interest wherein the signal peptide is not normally associated with the protein of interest would be termed a chimeric polypeptide or chimeric protein. A "POx fusion polypeptide" or "POx chimeric polypeptide" herein refers to a polypeptide comprising the sequence of the mature form of the POx enzyme operably  
25 linked to a POx signal peptide.

[0077] The terms "signal sequence," "signal peptide" and "secretory signal peptide" refer to any sequence of nucleotides and/or amino acids which may participate in the production of the mature or fusion forms of the protein. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-  
30 terminal portion of the protein gene, which participate in the effectuation of the secretion of the protein. A "POx signal peptide" herein refers to a signal peptide that is linked to the N-terminus of the mature form of the POx enzyme.

[0078] "Naturally-occurring" or "wild-type" refers to a POx protein or a polynucleotide encoding a POx protein having the unmodified amino acid sequence identical to that  
35 found in nature. Naturally occurring enzymes include native enzymes, such as those enzymes naturally expressed or found in the particular microorganism. A sequence that is wild-type or naturally-occurring refers to a sequence from which a variant, or a

synthetic sequence is derived. The wild-type sequence may encode either a homologous or heterologous protein.

**[0079]** As used herein the term "expression" refers to a process by which a polynucleotide is transcribed and the resulting transcript is translated to yield a polypeptide.

**[0080]** As used herein, functionally and/or structurally similar proteins are considered to be "related proteins." In some embodiments, these proteins are enzymatically active on polyols. In some embodiments, these proteins are derived from a different genus and/or species, including differences between classes of organisms (e.g., a bacterial protein and a fungal protein). In some embodiments, these proteins are derived from a different genus and/or species, including differences between classes of organisms (e.g., a bacterial enzyme and a fungal enzyme). In additional embodiments, related proteins are provided from the same species. Indeed, it is not intended that the present invention be limited to related proteins from any particular source(s). In addition, the term "related proteins" encompasses tertiary structural homologs and primary sequence homologs (e.g., the polyol oxidases of the present invention). In further embodiments, the term encompasses proteins that are immunologically cross-reactive.

**[0081]** As used herein, the term "derivative" refers to a protein which is derived from a protein by addition of one or more amino acids to either or both the C- and N-terminal end(s), substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, and/or deletion of one or more amino acids at either or both ends of the protein or at one or more sites in the amino acid sequence, and/or insertion of one or more amino acids at one or more sites in the amino acid sequence. The preparation of a protein derivative is preferably achieved by modifying a DNA sequence which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative protein.

**[0082]** Related (and derivative) proteins comprise "variant proteins." In some preferred embodiments, variant proteins differ from a parent protein and one another by a small number of amino acid residues. In some particularly preferred embodiments, related proteins and particularly variant proteins comprise at least about 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% amino acid sequence identity. Additionally, a related protein or a variant protein as used herein, refers to a protein that differs from another related protein or a parent protein in the number of prominent regions. For example, in some embodiments, variant proteins have 1, 2, 3, 4, 5, or 10 corresponding prominent regions that differ from the parent protein.

**[0083]** In some embodiments, amino acid and polynucleotide homology is determined using methods known in the art (e.g., the ClustalW algorithm, using standard settings,

with EMBOSS::water (local): Gap Open = 10.0, Gap extend = 0.5, using Blosum 62 (protein), or DNAsfull for nucleotide sequences.)

**[0084]** As used herein, the term "variant(s)" as used in context of a polypeptide sequence (e.g., SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40) refers to a polypeptide prepared from the original (i.e., parent) polypeptide, or by using the sequence information from the original (i.e., parent) polypeptide, by insertion, deletion and/or substitution of one or more amino acids in the original (i.e., parent) sequence. In some embodiments, at least one insertion, deletion, and/or substitution is made in the original (i.e., parent) sequence, while in other embodiments, preferably less than about 50 amino acids, less than about 40, less than about 30, less than about 20, or less than about 10 amino acids are modified by insertion, deletion and/or substitution of the original (i.e., parent) sequence in order to produce variant polypeptides. In some preferred embodiments, only one amino acid modification (i.e., insertion, deletion or substitution) is made, while in other preferred embodiments two amino acids are modified, and in still further embodiments, three amino acids are modified, and in yet additional embodiments, four amino acids are modified, and in still further embodiments, five amino acids are modified. It is not intended that the variants of the present invention be limited to any specific number nor type of amino acid modifications.

**[0085]** The terms "precursor" or "parent" polypeptide herein refer to the polypeptide that was modified to provide a variant polypeptide.

**[0086]** As used herein, the term "homologue(s)," when used herein in the context of a polypeptide sequence (e.g., SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40) refers to a polypeptide which is at least about 70% homologous, more preferably at least about 80% homologous, still more preferably at least about 85% homologous, further more preferably at least about 90% homologous, even more preferably at least about 95%, more preferably about 96%, still more preferably about 97%, even more preferably about 98%, or most preferably about 99% homologous to the polypeptide sequence of interest (e.g., a polyol oxidase provided by the present invention). In some embodiments, homology between two polypeptide sequences is determined using ClustalW alignment algorithm using standard settings, as referred to herein. However, it is not intended that the present invention be limited to any particular method for determining homology.

**[0087]** The term "heterologous" used in this context refers to a sequence which originates from a species or strain other than the species or strain from which the polypeptide, or the parent polypeptide from which the polypeptide is derived (i.e., a variant, homologue or fragment) is naturally found, for example a heterologous signal peptide encoding polynucleotide.

**[0088]** As used herein, the term "fragment(s)." as used herein in the context of a polypeptide sequence (e.g., SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40) refers to a polypeptide which consists of only a part of the original (i.e., parent) polypeptide sequence. In some embodiments, fragments comprise at least  
5 about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% of the original (i.e., parent) polypeptide sequence.

**[0089]** The variants, homologues and fragments provided by the present invention all retain at least some of the desired enzymatic activity of the parent enzyme, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least  
10 about 50%, at least about 60%, at least about 70%, at least about 80% at least about 90%, or all the enzyme activity of the parent enzyme. In some alternative embodiments, the variants and/or homologues have greater enzymatic activity than the original (i.e., parent) enzyme.

**[0090]** It is intended that although preferred enzymes for use in the vectors and methods  
15 of the invention are referred to herein by their specific SEQ ID NOS , the present invention encompasses enzymes which are derived from the nucleic acids which encode the corresponding amino acid SEQ ID NOS, when expressed, either in their native host species or a heterologous host species. Thus, the present invention encompasses embodiments in which the enzymes are co- or post- translationally processed.

**[0091]** Several methods are known in the art that are suitable for generating variants of  
20 the enzymes of the present invention, including but not limited to site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches.

**[0092]** Characterization of wild-type and mutant proteins is accomplished via any means  
25 suitable and is preferably based on the assessment of properties of interest. For example, pH and/or temperature, as well as detergent and /or oxidative stability is/are determined in some embodiments of the present invention. Indeed, it is contemplated that enzymes having various degrees of stability in one or more of these characteristics  
30 (pH, temperature, proteolytic stability, detergent stability, and/or oxidative stability) will find use.

**[0093]** As used herein, "expression vector" refers to a DNA construct containing a DNA  
sequence that is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to  
35 effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage

particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid," "expression plasmid," and "vector" are often used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors that serve equivalent functions and which are, or become, known in the art.

[0094] In some preferred embodiments, the POx gene is ligated into an appropriate expression plasmid. The cloned POx gene is then used to transform or transfect a host cell in order to express the sorbitol oxidase gene. This plasmid may replicate in hosts in the sense that it contains the well-known elements necessary for plasmid replication or the plasmid may be designed to integrate into the host chromosome. The necessary elements are provided for efficient gene expression (e.g., a promoter operably linked to the gene of interest). In some embodiments, these necessary elements are supplied as the gene's own homologous promoter if it is recognized, (i.e., transcribed, by the host), a transcription terminator (a polyadenylation region for eukaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the sorbitol oxidase gene. In some embodiments, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media is also included.

[0095] As used herein, "corresponding to," refers to a residue at the enumerated position in a protein or peptide, or a residue that is analogous, homologous, or equivalent to an enumerated residue in a protein or peptide.

[0096] As used herein, "corresponding region," generally refers to an analogous position along related proteins or a parent protein.

[0097] The terms "nucleic acid molecule encoding," "nucleic acid sequence encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

[0098] The term "POx polynucleotide" herein refers to the DNA sequence encoding a POx polypeptide.

[0099] As used herein, the term "analogous sequence" refers to a sequence within a protein that provides similar function, tertiary structure, and/or conserved residues as the protein of interest (i.e., typically the original protein of interest). For example, in epitope regions that contain an alpha helix or a beta sheet structure, the replacement amino acids in the analogous sequence preferably maintain the same specific structure. The term

also refers to nucleotide sequences, as well as amino acid sequences. In some embodiments, analogous sequences are developed such that the replacement amino acids result in a variant enzyme showing a similar or improved function. In some preferred embodiments, the tertiary structure and/or conserved residues of the amino acids in the protein of interest are located at or near the segment or fragment of interest. Thus, where the segment or fragment of interest contains, for example, an alpha-helix or a beta-sheet structure, the replacement amino acids preferably maintain that specific structure.

**[00100]** As used herein, "homologous protein" refers to a protein (e.g., polyol oxidase) that has similar action and/or structure, as a protein of interest (e.g., a polyol oxidase from another source). It is not intended that homologs be necessarily related evolutionarily. Thus, it is intended that the term encompass the same or similar enzyme(s) (i.e., in terms of structure and function) obtained from different species. In some preferred embodiments, it is desirable to identify a homolog that has a quaternary, tertiary and/or primary structure similar to the protein of interest, as replacement for the segment or fragment in the protein of interest with an analogous segment from the homolog will reduce the disruptiveness of the change. In some preferred embodiments, the homologs are selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13, while in other embodiments, the homolog is SEQ ID NO:14.

**[00101]** As used herein, "homologous genes" refers to at least a pair of genes from different species, which genes correspond to each other and which are identical or very similar to each other. The term encompasses genes that are separated by speciation (i.e., the development of new species) (e.g., orthologous genes), as well as genes that have been separated by genetic duplication (e.g., paralogous genes). These genes encode "homologous proteins."

**[00102]** As used herein, "ortholog" and "orthologous genes" refer to genes in different species that have evolved from a common ancestral gene (i.e., a homologous gene) by speciation. Typically, orthologs retain the same function during the course of evolution. Identification of orthologs finds use in the reliable prediction of gene function in newly sequenced genomes.

**[00103]** As used herein, "paralog" and "paralogous genes" refer to genes that are related by duplication within a genome. While orthologs retain the same function through the course of evolution, paralogs evolve new functions, even though some functions are often related to the original one.

**[00104]** As used herein, "wild-type" and "native" proteins are those found in nature. The terms "wild-type sequence," and "wild-type gene" are used interchangeably herein, to refer to a sequence that is native or naturally occurring in a host cell. In some

embodiments, the wild-type sequence refers to a sequence of interest (i.e., a sequence that is being analyzed, assessed, modified, etc.) that is the starting point of a protein engineering project. The genes encoding the naturally-occurring protein may be obtained in accord with the general methods known to those skilled in the art. The methods  
5 generally comprise synthesizing labeled probes having putative sequences encoding regions of the protein of interest, preparing genomic libraries from organisms expressing the protein, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

**[00105]** The term "recombinant DNA molecule" as used herein refers to a DNA

10 molecule that is comprised of segments of DNA joined together by means of molecular biological techniques.

**[00106]** The term "recombinant oligonucleotide" refers to an oligonucleotide created using molecular biological manipulations, including but not limited to, the ligation of two or more oligonucleotide sequences generated by restriction enzyme digestion of a

15 polynucleotide sequence, the synthesis of oligonucleotides (e.g., the synthesis of primers or oligonucleotides) and the like.

**[00107]** The degree of homology between sequences may be determined using any suitable method known in the art (See e.g., Smith and Waterman, *Adv. Appl. Math.*, 2:482 [1981]; Needleman and Wunsch, *J. Mol. Biol.*, 48:443 [1970]; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 [1988]; programs such as GAP, BESTFIT, FASTA, and  
20 TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI); and Devereux et al., *Nucl. Acid Res.*, 12:387-395 [1984]).

**[00108]** For example, PILEUP is a useful program to determine sequence homology levels. PILEUP creates a multiple sequence alignment from a group of related

25 sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, (Feng and Doolittle, *J. Mol. Evol.*, 35:351-360 [1987]). The method is similar to that described by Higgins and Sharp (Higgins and Sharp, *CABIOS* 5:151-153 [1989]). Useful PILEUP parameters including a  
30 default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

Another example of a useful algorithm is the BLAST algorithm, described by Altschul et al., (Altschul et al., *J. Mol. Biol.*, 215:403-410, [1990]; and Karlin et al., *Proc. Natl. Acad. Sci. USA* 90:5873-5787 [1993]). One particularly useful BLAST program is the WU-BLAST-2 program (See, Altschul et al., *Meth. Enzymol.*, 266:460-480 [1996]).

35 parameters "W," "T," and "X" determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix

(See, Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 [1989]) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

**[00109]** As used herein, "percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that is identical with the nucleotide residues of the sequence.

**[00110]** As used herein, the term "hybridization" refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing, as known in the art.

**[00111]** As used herein, the phrase "hybridization conditions" refers to the conditions under which hybridization reactions are conducted. These conditions are typically classified by degree of "stringency" of the conditions under which hybridization is measured. The degree of stringency can be based, for example, on the melting temperature ( $T_m$ ) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ$  below the  $T_m$  of the probe); "high stringency" at about  $5 - 10^\circ$  below the  $T_m$ ; "intermediate stringency" at about  $10 - 20^\circ$  below the  $T_m$  of the probe; and "low stringency" at about  $20 - 25^\circ$  below the  $T_m$ . Alternatively, or in addition, hybridization conditions can be based upon the salt or ionic strength conditions of hybridization and/or one or more stringency washes. For example, 6xSSC = very low stringency; 3xSSC = low to medium stringency; 1xSSC = medium stringency; and 0.5xSSC = high stringency. Functionally, maximum stringency conditions may be used to identify nucleic acid sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify nucleic acid sequences having about 80% or more sequence identity with the probe. For applications requiring high selectivity, it is typically desirable to use relatively stringent conditions to form the hybrids (e.g., relatively low salt and/or high temperature conditions are used).

**[00112]** The phrases "substantially similar" and "substantially identical" in the context of at least two nucleic acids or polypeptides typically means that a polynucleotide or polypeptide comprises a sequence that has at least about 40% identity, more preferable at least about 50% identity, yet more preferably at least about 60% identity, preferably at least about 75% identity, more preferably at least about 80% identity, yet more preferably at least about 90%, still more preferably about 95%, most preferably about 97% identity, sometimes as much as about 98% and about 99% sequence identity, compared to the reference (i.e., wild-type) sequence. Sequence identity may be determined using known programs such as BLAST, ALIGN, and CLUSTAL using standard parameters. (See e.g., Altschul, et al., J. Mol. Biol. 215:403-410 [1990]; Henikoff et al., Proc. Natl. Acad. Sci. USA 89:10915 [1989]; Karin et al., Proc. Natl. Acad. Sci. USA 90:5873 [1993]; and

Higgins et al., Gene 73:237 - 244 [1988]). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. Also, databases may be searched using FASTA (Pearson et al., Proc. Natl. Acad. Sci. USA 85:2444-2448 [1988]). One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions (e.g., within a range of medium to high stringency).

**[00113]** As used herein, "equivalent residues" refers to proteins that share particular amino acid residues. For example, equivalent residues may be identified by determining homology at the level of tertiary structure for a protein (e.g., polyol oxidase) whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the protein having putative equivalent residues and the protein of interest (N on N, CA on CA, C on C and O on O) are within 0.13 nm and preferably 0.1 nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the proteins analyzed. The preferred model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available, determined using methods known to those skilled in the art of crystallography and protein characterization/analysis.

**[00114]** As used herein, the terms "hybrid polyol oxidases" and "fusion polyol oxidases" refer to proteins that are engineered from at least two different or "parental" proteins. In preferred embodiments, these parental proteins are homologs of one another. For example, in some embodiments, a preferred hybrid sequence or fusion protein contains the N-terminus of a protein and the C-terminus of a homolog of the protein. In some preferred embodiment, the two terminal ends are combined to correspond to the full-length active protein.

**[00115]** The term "regulatory element" as used herein refers to a genetic element that controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Additional regulatory elements include splicing signals, polyadenylation signals and termination signals.

[00116] As used herein, "host cells" are generally prokaryotic or eukaryotic hosts which are transformed or transfected with vectors constructed using recombinant DNA techniques known in the art. Transformed host cells are capable of either replicating vectors encoding the protein variants or expressing the desired protein variant. In the case of vectors which encode the pre- or prepro-form of the protein variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

[00117] The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means transformation, transduction or transfection. Means of transformation include protoplast transformation, calcium chloride precipitation, electroporation, naked DNA and the like as known in the art. (See, Chang and Cohen, Mol. Gen. Genet., 168:111 - 115 [1979]; Smith et al., Appl. Env. Microbiol., 51:634 [1986]; and the review article by Ferrari et al., in Harwood, Bacillus, Plenum Publishing Corporation, pp. 57-72 [1989]).

[00118] The term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (for example, the long terminal repeats of retroviruses contain both promoter and enhancer functions). The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An endogenous enhancer/promoter is one which is naturally linked with a given gene in the genome. An exogenous (heterologous) enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques).

[00119] The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York [1989], pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

[00120] The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign or exogenous DNA into the genomic DNA of the transfected cell.

[00121] The terms "selectable marker" or "selectable gene product" as used herein refer to the use of a gene which encodes an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed.

[00122] As used herein, the terms "amplification" and "gene amplification" refer to a process by which specific DNA sequences are disproportionately replicated such that the amplified gene becomes present in a higher copy number than was initially present in the genome. In some embodiments, selection of cells by growth in the presence of a drug

(e.g., an inhibitor of an inhibitable enzyme) results in the amplification of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (i.e., input) sequences encoding this gene product, or both. Selection of cells by growth in the presence of a drug (e.g., an inhibitor of an inhibitable enzyme) may result in the amplification of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (i.e., input) sequences encoding this gene product, or both.

**[00123]** "Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (i.e., replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (i.e., synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid.

Amplification techniques have been designed primarily for this sorting out.

**[00124]** As used herein, the term "co-amplification" refers to the introduction into a single cell of an amplifiable marker in conjunction with other gene sequences (i.e., comprising one or more non-selectable genes such as those contained within an expression vector) and the application of appropriate selective pressure such that the cell amplifies both the amplifiable marker and the other, non-selectable gene sequences. The amplifiable marker may be physically linked to the other gene sequences or alternatively two separate pieces of DNA, one containing the amplifiable marker and the other containing the non-selectable marker, may be introduced into the same cell.

**[00125]** As used herein, the terms "amplifiable marker," "amplifiable gene," and "amplification vector" refer to a marker, gene or a vector encoding a gene which permits the amplification of that gene under appropriate growth conditions.

**[00126]** As used herein, the term "amplifiable nucleic acid" refers to nucleic acids which may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

**[00127]** As used herein, the term "sample template" refers to nucleic acid originating from a sample which is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

**[00128]** "Template specificity" is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q $\beta$  replicase, MDV-1 RNA is the specific  
5 template for the replicase (See e.g., Kacian et al., Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acids are not replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (See, Chamberlin et al., Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where  
10 there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (See, Wu and Wallace, Genomics 4:560 [1989]). Finally, Taq and Pfu polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer  
15 hybridization with the target sequences and not hybridization with non-target sequences.

**[00129]** As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid  
20 strand is induced, (i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an  
25 oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

**[00130]** As used herein, the term "probe" refers to an oligonucleotide (i.e., a sequence  
30 of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be  
35 labeled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical

assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

**[00131]** As used herein, the term "target," when used in reference to amplification methods (e.g., the polymerase chain reaction), refers to the region of nucleic acid  
5 bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

**[00132]** As used herein, the term "polymerase chain reaction" ("PCR") refers to the methods of U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, hereby incorporated  
10 by reference, which include methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase.  
15 The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase  
20 extension can be repeated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter.  
25 By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

**[00133]** As used herein, the term "amplification reagents" refers to those reagents  
30 (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

**[00134]** With PCR, it is possible to amplify a single copy of a specific target sequence  
35 in genomic DNA to a level detectable by several different methodologies (e.g., hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of <sup>32</sup>P-labeled deoxynucleotide

triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR

5 amplifications.

**[00135]** As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

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**[00136]** As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

**[00137]** "Recovering" a polypeptide from a culture medium refers to collecting the polypeptide in the culture medium into which it was secreted by the host cell. The polypeptide can also be recovered from a lysate prepared from the host cells and further purified. A secreted polypeptide may be recovered from the cell wall fraction prepared according to methods known in the art. One skilled in the art can readily follow known methods for isolating polypeptides and proteins in order to obtain one of the isolated polypeptides or proteins of the present invention. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography, and immuno-affinity chromatography. See, e.g., Scopes, Protein Purification: Principles and Practice, Springer-Verlag (1994); Sambrook, et al., in Molecular Cloning: A Laboratory Manual; Ausubel et al., Current Protocols in Molecular Biology. The protein produced by a recombinant host cell comprising a secretion factor of the present invention will be secreted into the culture media.

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**[00138]** As used herein, "compositions", "cleaning compositions" and "cleaning formulations" refer to compositions that find use in the removal of undesired compounds from items to be cleaned, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin (soaps and creams), teeth (mouthwashes, toothpastes) etc. The term encompasses any materials/compounds selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid, gel, granule, or spray composition), as long as the composition is compatible with the oxidase and other enzyme(s) used in the composition, and any reversible enzyme inhibitors in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use.

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**[00139]** The terms further refer to any composition that is suited for cleaning, bleaching, disinfecting, and/or sterilizing any object and/or surface. It is intended that the terms include, but are not limited to detergent compositions (e.g., liquid and/or solid laundry detergents and fine fabric detergents; hard surface cleaning formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; fabric fresheners; fabric softeners; and textile and laundry pre-spotters, as well as dish detergents).

**[00140]** Indeed, the term "cleaning composition" as used herein, includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels and foam baths and metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types.

**[00141]** As used herein, the terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some preferred embodiments, the term is used in reference to laundering fabrics and/or garments (e.g., "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (e.g., "dishwashing detergents"). It is not intended that the present invention be limited to any particular detergent formulation or composition. Indeed, it is intended that in addition to oxidase, the term encompasses detergents that contain surfactants, transferase(s), hydrolytic enzymes, oxido reductases, perhydrolases builders, bleaching agents, bleach activators, bluing agents and fluorescent dyes, caking inhibitors, masking agents, enzyme activators, enzyme inhibitors, antioxidants, and solubilizers. In some preferred embodiments, the detergent formulations include, but are not limited to those set forth in US Pat. Appln. Ser. Nos. 10/576,331 and 10/581,014, as well as WO 05/52161 and WO 05/056782 find use in the present invention. However, it is not intended that the present invention be limited to any particular detergent formulation(s), as any suitable detergent formulation finds use in the present invention.

**[00142]** As used herein, "textile materials" is a general term for fibers, yarn intermediates, yarn, fabrics, and products made from fabrics (e.g., garments and other articles).

[00143] As used herein, "fabric" encompasses any textile material. Thus, it is intended that the term encompass garments, as well as fabrics, yarns, fibers, non-woven materials, natural materials, synthetic materials, and any other textile material.

5 [00144] As used herein, "dishwashing composition" refers to all forms of compositions for cleaning dishware, including cutlery, including but not limited to granular and liquid forms. It is not intended that the present invention be limited to any particular type or dishware composition. Indeed, the present invention finds use in cleaning dishware (e.g., dishes, including, but not limited to plates, cups, glasses, bowls, etc.) and cutlery (e.g.,  
10 utensils, including but not limited to spoons, knives, forks, serving utensils, etc.) of any material, including but not limited to ceramics, plastics, metals, china, glass, acrylics, etc. The term "dishware" is used herein in reference to both dishes and cutlery.

[00145] As used herein, "wash performance" of an enzyme refers to the contribution of an enzyme to washing that provides additional cleaning performance to the detergent  
15 without the addition of the enzyme to the composition. Wash performance is compared under relevant washing conditions.

[00146] The term "relevant washing conditions" is used herein to indicate the conditions, particularly washing temperature, time, washing mechanics, sud concentration, type of detergent and water hardness, actually used in households in a  
20 detergent market segment.

[00147] The term "improved wash performance" is used to indicate that a better end result is obtained in stain removal from items washed (e.g., fabrics or dishware and/or cutlery) under relevant washing conditions, or that less enzyme, on weight basis, is needed to obtain the same end result relative to another enzyme.

25 [00148] The term "retained wash performance" is used to indicate that the wash performance of an enzyme, on weight basis, is at least 80% relative to another enzyme under relevant washing conditions.

[00149] Wash performance of enzymes is conveniently measured by their ability to remove certain representative stains under appropriate test conditions. In these test  
30 systems, other relevant factors, such as detergent composition, sud concentration, water hardness, washing mechanics, time, pH, and/or temperature, can be controlled in such a way that conditions typical for household application in a certain market segment are imitated.

[00150] As used herein, the term "bleaching" refers to the treatment of a material (e.g.,  
35 fabric, laundry, pulp, etc.) or surface for a sufficient length of time and under appropriate pH and temperature conditions to effect a brightening (i.e., whitening) and/or cleaning of

the material. Examples of chemicals suitable for bleaching include but are not limited to  $\text{ClO}_2$ ,  $\text{H}_2\text{O}_2$ , peracids,  $\text{NO}_2$ , etc.

**[00151]** As used herein, the term "disinfecting" refers to the removal of contaminants from the surfaces, as well as the inhibition or killing of microbes on the surfaces of items.

5 It is not intended that the present invention be limited to any particular surface, item, or contaminant(s) or microbes to be removed.

**[00152]** As used herein, the term "perhydrolase" refers to an enzyme that is capable of catalyzing a reaction that results in the formation of sufficiently high amounts of peracid suitable for applications such as cleaning, bleaching, and disinfecting. In some preferred  
10 embodiments, the perhydrolases encompassed by the present invention include the *M. smegmatis* perhydrolase, variants and/or homologs thereof as described in PCT/US05/056782. However, it is not intended that the present invention be limited to this specific *M. smegmatis* perhydrolase, specific variants of this perhydrolase, nor specific homologs of this perhydrolase.

## 15 **Polyol oxidases**

**[00153]** Polyols, or sugar alcohols, are polyhydric alcohols produced by hydrogenation or fermentation of different carbohydrates. Chemically, polyols are derived from mono- and disaccharides. Most polyols occur naturally in a variety of food products like vegetables, fruits and mushrooms. They are also regularly presented in fermented foods  
20 like wine or soy sauces. Polyols are therefore a normal constituent of the human diet. Polyols comprise a variety of sugar alcohols such as sorbitol, galactitol, lactitol, xylitol, and mannitol, or alcohols such as glycerol, propylene glycol and in addition to being constituents of human diet they are also commonly used in in personal care products, food applications, surfactants, vitamins, plastics and in enzyme product formulations.

25 Polyols are converted to their corresponding sugars and peroxide ( $\text{H}_2\text{O}_2$ ) by polyol oxidase enzymes. Thus, polyol oxidases are attractive biobleaching agents for use in detergents, personal care (e.g., toothpastes, cosmetics, etc.), and other products that incorporate polyol-containing enzyme product formulations.

**[00154]** The invention encompasses polyol oxidases that can be derived or isolated  
30 from *Streptomyces*, *Acidothrmus*, *Arthrobacter*, *Brevibacterium*, *Frankia*, *Nocardia*, *Janibacter*, *Marinobacter*, *Burkholderia*, *Paracoccus*, *Chromabacterium*, *Thermobifida*, *Xanthomonas*, *Pseudomonas*, *Corynebacterium* and *Bacillus*. In some embodiments, the invention encompasses polyol oxidases of the class E.E.1.1.3. In some preferred embodiments, the invention encompasses polyol oxidases including sorbitol oxidase  
35 ( $\text{SOx}$ ), glycerol oxidase ( $\text{GLOx}$ ), xylitol oxidase ( $\text{XOx}$ ), and alditol oxidase ( $\text{ALOX}$ ). In

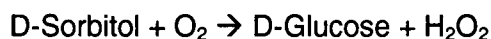
some preferred embodiments, the invention encompasses combining a polyol oxidase e.g. sorbitol oxidase with a second oxidase such as glucose oxidase, hexose oxidase, pyranose oxidase, glucooligosaccharide oxidase.

**[00155]** Sorbitol oxidase ("SOx") is an enzyme that catalyzes conversion of sorbitol to glucose and hydrogen peroxide. Sorbitol oxidases are known and used in various settings, including diagnostic methods (See e.g., Oda and Hiraga, Ann. NY Acad. Sci., 864:454-457 [1998]; and Yamashita et al J. Biosci. Bioengin., 89:350-360 [2000]).

Sorbitol (D-glucitol, C<sub>6</sub>H<sub>14</sub>O<sub>6</sub>, MW 182.2, CAS 50-70-4) is commonly used in personal care products, food applications, surfactants, vitamins, plastics and in enzyme product formulations. In preferred embodiments of the invention, SOx is used in cleaning, bleaching and/or disinfecting compositions.

**[00156]** In some particularly preferred embodiments, the sorbitol oxidase of the present invention has a higher specific activity, (or  $V_{max}/K_{km}$  ratio) on sorbitol substrate, as compared to an alternative substrate under standard assay conditions (e.g., in the in vitro assay provided below) and/or using an in situ in an application media, conducted as known in the art. In some preferred alternative embodiments, the alternative substrate is xylitol. In some particularly preferred embodiments, the sorbitol oxidase of the present invention has no significant activity on the corresponding sugar product, such as glucose, xylose, galactose (3.5%), mannose (1%) or arabinose.

**[00157]** In some preferred embodiments, the SOx is an oxidoreductase that uses covalently bound FAD as a cofactor for oxidation of sorbitol to glucose. This enzyme offers a unique opportunity for its potential use as a bio bleach agent on its own, as well as used in combination with other oxidases such as carbohydrate oxidases e.g. glucose oxidase and/or hexose oxidase (See e.g., WO 96/39851), pyranose oxidase (See e.g., WO04/100669), glucooligosaccharide oxidase and M. nivale carbohydrate oxidase (See e.g., WO99/31990). An advantage of the use of such combinations is due to the fact that SOx converts sorbitol to glucose, which can then be converted to gluconate by glucose oxidase and/or hexose oxidase and/or carbohydrate oxidases (e.g., *Michrodochium nivale* carbohydrate oxidase) and/or glucooligosaccharide oxidase (e.g., *Acremonium strictum* glucooligosaccharide oxidase), and/or pyranose oxidase thus generating two moles of hydrogen peroxide per mole of sorbitol, as illustrated below.

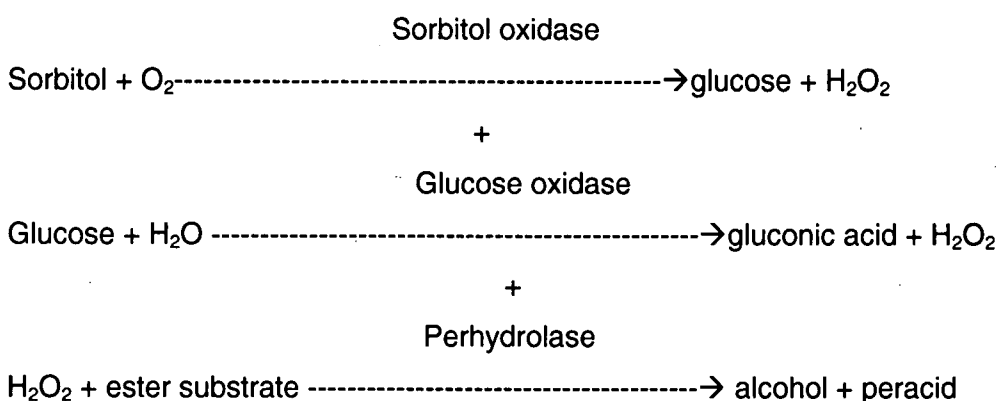


**[00158]** In addition, the preferred sorbitol oxidase provided by the present invention produces glucose, an aldehyde product that can be further oxidized to gluconic acid, a

carboxylic acid product using other oxidases such as hexose oxidase, releasing another molecule of hydrogen peroxide from starting substrate sorbitol. Similarly oxidation of polyols such as xylitol, arabitol, mannitol, ribitol, inositol, by sorbitol oxidase, xylitol oxidase, mannitol oxidase with the assistance of atmospheric oxygen with formation of, xylose, arabinose, mannose, ribose, respectively as secondary substrate for further oxidation by other relevant oxidases such as hexose oxidase, xylose oxidase, pyranose oxidase, arabinose oxidase, mannose oxidase, and ribose oxidase is feasible.

**[00159]** Thus, in some embodiments, a polyol oxidase e.g. SOx can be used alone, while in other embodiments, SOx can be used in combination with at least another oxidase that catalyzes the conversion of the product of the SOx reaction. For example, SOx can be used in combination with any one or multiple sugar oxidase enzymes of the class EC1.1.3. For example, SOx can be used in combination with at least one additional sugar oxidase including but not limited to GOx and /or HOx. In other embodiments, SOx can be used in combination with other oxidases that catalyze the conversion of polyol substrates that are not the products of the SOx reaction.

**[00160]** In yet other embodiments, SOx can be used alone, while in other embodiments, SOx can be used in combination with other oxidase enzymes and/or enzymes that convert the H<sub>2</sub>O<sub>2</sub> product of oxidase reactions to peracid. For example, SOx can be used in combination with a second oxidase e.g. glucose oxidase, and at least one perhydrolase enzyme. Examples of perhydrolase enzymes that can be used in combination with the POx enzymes described herein are described in PCT/05/056782. The following equation provides an example of a coupled system that finds use with the present invention.



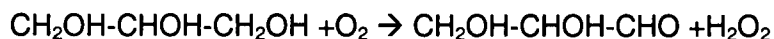
POx enzymes can also be used in combination with bleach activators such as TAED, NOBS etc., which can be activated by H<sub>2</sub>O<sub>2</sub>.

**[00161]** Other POx enzymes that find use in the present invention include xylitol oxidase, glycerol oxidase and alditol oxidase.

**[00162]** Xylitol oxidase ("XOx") is a monomeric oxidase containing one molecule of FAD per molecule of protein, and it can be derived from *Streptomyces* sp. IKD472. The enzyme catalyzes the conversion of xylitol to glucose and H<sub>2</sub>O<sub>2</sub>, and can also oxidize D-sorbitol (Yamashita *et al.*, J. Biosci. Bioeng. 89 (2000) 350-360). Thus, the invention encompasses cleaning, disinfecting and/or antimicrobial compositions that comprise XOx. In some embodiments, the compositions of the invention comprise XOx in combination with at least one other sugar oxidase. In other embodiments, compositions of the invention comprise XOx in combination with at least one other sugar oxidase and/or a perhydrolase.

**[00163]** In some embodiments, the present invention encompasses a xylitol oxidase that has a higher specific activity, (or  $V_{\max}/K_m$  ratio) on xylitol substrate, as compared to sorbitol under standard assay conditions (e.g., in the in vitro assay provided herein) and/or in an in situ in an application media, conducted as known in the art. In some preferred alternative embodiments, the alternative substrate is sorbitol.

**[00164]** Glycerol oxidase (GLOx) is an enzyme found in the genera *Penicillium* and *Botrytis* (See e.g., Lin *et al.* *Enz. Micro. Technol.*, 18:383-387 [1996]; and Uwajima *et al.*, *Agric. Biol. Chem.*, 44:399-406 [1989]). This enzyme catalyzes the conversion of glycerol and oxygen to glyceraldehyde and hydrogen peroxide as shown below.



**[00165]** Glycerol (glycerin, C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>, MW 92.09, CAS 56-81-5) is a commonly used in enzyme product formulations, soap and detergent formulations, food and beverages, pharmaceuticals and is widely used in cosmetics and personal care applications. Thus, glycerol oxidase provides an attractive biobleaching agent for use in detergents that incorporate these glycerol-containing enzyme product formulations. Thus, the invention encompasses cleaning, disinfecting and/or antimicrobial compositions that comprise GLOx. In some embodiments, the compositions of the invention comprise GLOx in combination with at least one other polyol oxidase. In other embodiments, compositions of the invention comprise GLOx in combination with at least one other polyol oxidase and/or a perhydrolase. In other embodiments, GLOx can be combined with a bleach activator.

**[00166]** In some embodiments, the invention encompasses hexose oxidases ("HOx") (Sullivan, *et al.*, *Biochim. Biophys. Acta* 309 (1973) 11-22; Bean *et al.*, *J. Biol. Chem.* 218 (1956); Bean *et al.*, *J. Biol. Chem.* 236 (1961)), which catalyze the conversion of D-glucose to D-glucono-1,5-lactone and H<sub>2</sub>O<sub>2</sub>. Hexose oxidase also utilizes other

substrates including D-galactose, D-mannose, maltose, lactose and cellobiose, Thus, the invention encompasses cleaning, disinfecting and/or antimicrobial compositions that comprise HOx. In some embodiments, the compositions of the invention comprise HOx in combination with at least one other polyol oxidase. In other embodiments,

5 compositions of the invention comprise HOx in combination with at least one other polyol oxidase and/or a perhydrolase.

**[00167]** In some embodiments, the compositions of the invention comprise glucose oxidase (GOx), which catalyzes the conversion of glucose to D-glucono-1,5-lactone + H<sub>2</sub>O<sub>2</sub>. Thus, GOx can utilize the product of polyol oxidase reactions as a substrate to  
10 convert glucose to the corresponding lactone and generate a second molecule of H<sub>2</sub>O<sub>2</sub>. Thus, the invention encompasses cleaning, disinfecting and/or antimicrobial compositions that comprise GOx. In some embodiments, the compositions of the invention comprise GOx in combination with at least one other polyol oxidase. In other embodiments, compositions of the invention comprise GOx in combination with at least one other polyol  
15 oxidase and/or a perhydrolase. In other embodiments, compositions of the invention comprise GOx in combination with at least one other POx and/or a bleach activator.

**[00168]** As indicated above, key components to peracid production by enzymatic perhydrolysis are enzyme, ester substrate, and hydrogen peroxide. In the present invention, hydrogen peroxide is generated in a coupled enzyme system that includes at  
20 least one POx to provide the H<sub>2</sub>O<sub>2</sub> substrate for the perhydrolase. In some embodiments, the POx oxidase that can be used in cleaning compositions in combination with a perhydrolase can be a polyol oxidases of the class E.E.1.1.3. For example, enzymes (e.g., sorbitol oxidase, glucose oxidase, hexose oxidase, xylitol oxidase, alcohol oxidase, ethylene glycol oxidase, glycerol oxidase, amino acid oxidase, etc.) that can generate  
25 hydrogen peroxide also find use with ester substrates in combination perhydrolase enzymes to generate peracids. In some preferred embodiments, the invention encompasses cleaning compositions comprising a combination of a perhydrolase with a sorbitol oxidase (SOx), glycerol oxidase, a glucose oxidase (GOx), a xylitol oxidase (XOx), and/or hexose oxidase (XOx). The invention includes cleaning compositions that  
30 include combinations of one or more perhydrolases with one/or more POx enzymes. In some embodiments, the invention cleaning compositions and methods that include a perhydrolase in combination with a sorbitol oxidase and a glucose oxidase. In other embodiments, the invention provides cleaning compositions and methods that encompass a perhydrolase in combination with a sorbitol oxidase and a hexose oxidase.  
35 In yet other embodiments, the invention provides cleaning compositions and methods that encompass a perhydrolase. In yet other embodiments, the invention provides compositions and methods that encompass a perhydrolase in combination with xylitol

oxidase and a pyranose oxidase. In some embodiments, the compositions of the invention comprise at least one POx enzyme and a bleach activator. The invention also provides for the use of additional sources of  $H_2O_2$  that can be used with POx alone or in combination with perhydrolase enzymes. For example, chemical sources of  $H_2O_2$  such as percarbonates and perborates, which spontaneously decompose to  $H_2O_2$ , which are included in some current washing powders find use in the present invention. One advantage of the methods of the present invention is that the generation of acid by a POx (e.g., gluconic acid in the above example) reduces the pH of a basic solution to the pH range in which the peracid is most effective in bleaching (i.e., at or below the pKa). In some preferred embodiments, the ester substrates are selected from one or more of the following acids: formic acid, acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, caprylic acid, nonanoic acid, decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid, and oleic acid. Importantly, the present invention provides means for effective cleaning, bleaching, and disinfecting over broad pH and temperature ranges. In some embodiments, the compositions of the invention provide effective cleaning, bleaching, and disinfecting at pH between 5.5 and 10.5. The preferred temperatures at which the composition of the invention provide effective cleaning, bleaching, and disinfecting range between 10 and 70 °C. Other POx enzymes that find use in the present invention are described in PCT Applications DK2006/000590 and DK2006/000591, which are herein incorporated by reference in their entirety.

**[00169]** The present invention encompasses compositions and methods for expressing polyol oxidases in microorganisms. Polyol oxidases encompassed by the invention include oxidases that can react with a sample containing at least one polyol selected from the group consisting of D-sorbitol, D-mannitol, D-xylitol, and D-arabitol to produce hydrogen peroxide and D-glucose, D-mannose, D-xylose, or D-arabinose, respectively. In some embodiments, the invention encompasses polyol oxidases that are sorbitol oxidases. In other embodiments, the invention encompasses polyol oxidases that are xylitol oxidases.

**[00170]** In some embodiments, the polyol enzymes of the invention have specificity for D-sorbitol and D-xylitol and thus possess both sorbitol oxidase and xylitol oxidase activity. It is understood that the polyol oxidases encompassed by the invention are not limited to oxidases that utilize any one particular substrate but can utilize any one or a combination of substrates including D-sorbitol, D-xylitol, D-mannitol, D-ribitol, myo-inositol, glycerol, 1,3 propanediol and 1,2 propanediol.

**[00171]** The polyol oxidases of the present invention can be isolated from a number of microorganisms including but not limited to *Streptomyces sp.*, *Bacillus sp.*, *Acidothermus sp.*, *Arthobacter sp.*, and marine *Acinobacterium sp.* In some preferred embodiments,

the *Streptomyces* sp. include *Streptomyces* strain H-7775, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces lividans* and *Streptomyces* species IKD472. In other preferred embodiments, the *Acidothermus* sp. is *Acidothermus cellulolyticus* 11B (Q2E2H5). In some embodiments, the *Arthobacter* sp. is *Arthobacter* sp. FB24 (Q4NJLO). In some embodiments, the marine *Actinobacterium* is *Actinobacterium* PHSC20C1.

[00172] In some embodiments, the invention provides for isolated recombinant polynucleotides that encode a POx. In some embodiments, the recombinant polynucleotides comprise a sequence encoding the mature form of the POx that is a wild-type sequence. In other embodiments, the recombinant polynucleotides comprise a sequence encoding the mature form of the POx that is a variant, homolog or fragment of the wild-type sequence. In some preferred embodiments, the polynucleotide sequence is the wild-type sequence of the SOx of *Streptomyces* H-7775 (e.g. SEQ ID NO:2). In other embodiments, the polynucleotide sequence encoding the POx is the wild-type sequence of the SOx from *Streptomyces lividans* (e.g. SEQ ID NO:4). In some embodiments, the polynucleotides encoding the POx enzymes of the invention are synthetic polynucleotides that encode the amino acid sequences of the polyol oxidases derived from the *Streptomyces* sp., *Bacillus* sp., *Acidothermus* sp., *Arthobacter* sp., and marine *Actinobacterium* sp. In some preferred embodiments, the synthetic polynucleotides encode the POx enzymes isolated from *Streptomyces* sp. including *Streptomyces* strain H-7775 (e.g. SEQ ID NO:2), *Streptomyces avermitilis* (e.g. SEQ ID NO:8), *Streptomyces coelicolor* (e.g. SEQ ID NO:9) and *Streptomyces* species IKD472 (e.g. SEQ ID NO:10). In other preferred embodiments, the synthetic polynucleotides encode the POx enzymes isolated from *Acidothermus cellulolyticus* 11B (Q2E2H5; SEQ ID NO:6). In some other embodiments, the synthetic polynucleotides encode the POx enzymes isolated from *Arthobacter* sp. FB24 (Q4NJLO; SEQ ID NO:12). In yet other embodiments, the synthetic polynucleotides encode the POx enzymes isolated from *Actinobacterium* PHSC20C1 (e.g. SEQ ID NO:13). Other POx sequences that find use in the present invention include but are not limited to polynucleotides that encode the POx of *Acidothermus cellulolyticus* (ZP\_01136416; e.g. SEQ ID NO:5), *Streptomyces avermitilis* (NP\_823266; e.g. SEQ ID NO:7), *Arthobacter* sp. FB24 (ZP\_00411614; e.g. SEQ ID NO:11), and *Streptomyces* sp. IKD472 (Q9KX73; e.g. SEQ ID NO:14). In some embodiments, the POx polynucleotides encode a POx enzyme that has greater SOx activity than XOx activity. In other embodiments, the POx polynucleotides encode a POx enzyme that has greater XOx activity than SOx activity when utilizing the same polyol substrate.

**[00173]** The invention also encompasses polynucleotide sequences, whether wild-type or synthetic that are derived from genes homologous to those of *Streptomyces* H-7775 and/or *Streptomyces lividans*. For example, the invention provides for synthetic polynucleotide sequences e.g. 1, 3, 17, 20, 29, 30, 34, and 44 which comprise sequences  
5 encoding polyol oxidases. As indicated above, "homologous genes" are genes that correspond to each other and which are identical or very similar to each other, yet are obtained from different species. The term encompasses genes that are separated by speciation (*i.e.*, the development of new species) (*e.g.*, orthologous genes), as well as genes that have been separated by genetic duplication (*e.g.*, paralogous genes).

10 Homologous genes encode for homologous proteins.

**[00174]** During the development of the present invention, sorbitol oxidases were isolated from recombinant *Streptomyces lividans* strains expressing the putative sorbitol oxidase gene from *S. lividans* and also the known SOx gene from *Streptomyces* sp. H-7775 (See, Hiraga et al., *Biosci. Biotech. Biochem.*, 61:1699-1704 [1997]). The SOx  
15 gene from *S. lividans* was identified based on the sequence derived from the *Streptomyces coelicolor* SCO6147 annotated as a putative xylitol oxidase gene. Two different synthetic genes encoding the *Streptomyces* sp. H-7775 sorbitol oxidase gene were used for intracellular expression in *E. coli* strain BL21(DE3)pLysS and extracellular expression in *S. lividans* g3s3, which is a derivative of the TK23 strain in which the *cyc2*  
20 gene was deleted (Boremann et al., *J. Bacteriol* 178:1216-1218 (1996)). The prosthetic group is a covalently bound FAD (1 mol of FAD to 1 mol of SOx). Thus, it is a flavoprotein, with typical absorption maxima at 276, 358, and 455 nm. However, in other embodiments, the enzyme exhibits a second maximum, not at 358 but at 345, indicative of a histidine-flavin linkage. Thus, it is not intended that the present invention be limited  
25 to any particular mechanism and/or embodiment. Flavin is functionally involved in oxidation of sorbitol as observed by desired changes in UV-VIS spectra. FAD is very tightly bound with the protein and thus offers a stable enzyme for laundry and personal care (*e.g.*, oral care) applications.

**[00175]** The SOx gene from *Streptomyces* species H-7775 (SEQ ID NO:1) has been  
30 described (See, Genbank accession number AB000519). Applicants have identified several homologs of the SOx from *S. H-7775* using a BLAST search of the NCBI database. These include but are not limited to a xylitol oxidase from *Streptomyces avermitilis* (BAC69801; Q82LCO; SEQ ID NO:8), a xylitol oxidase from *Streptomyces coelicolor* (Q9ZBU1; SEQ ID NO:9), a xylitol oxidase from *Streptomyces* sp. IKD472 (Q9KX73; SEQ  
35 ID NO:10), a putative xylitol oxidase from *Acidothermus cellulolyticus* 11B (Q2E2H5; SEQ ID NO:6), and a FAD-linked oxidase from *Arthrobacter* sp FB24 (ZP00411614; SEQ ID NO:11), a FAD-linked oxidase from *Arthrobacter* sp FB24 (YP\_833485; Q4NJLO; SEQ

ID NO: 12), a putative xylitol oxidase from marine Actinobacterium PHSC20C1 (GenBank Accession no. ZP\_01129132) and a xylitol oxidase from Streptomyces sp. IKD472 (GenBank Accession no. Q9KX73; SEQ ID NO:14). As discussed above, in some embodiments, the invention encompasses polynucleotide sequences that encode POx that are homologous to that of S. H7775 (SEQ ID NO:2). Thus, the invention encompasses POx enzyme encodes by polynucleotide homologs of SEQ ID NO: 2.

**[00176]** The sorbitol oxidase gene from Streptomyces species H-7775 (Genbank accession number AB000519) comprises a 1260 bp open reading frame (ORF) encoding a protein having 420 amino acids with theoretical MW of 45,158 Daltons. The enzyme is stable for 24 hours at 30°C, between pH 7.5-10 with an optimum temperature of 50°C at pH 7.5. It is also heat stable up to 55°C.

**[00177]** The nearest homolog identified for this enzyme is xylitol oxidase (with 55% homology). SOx is an efficient enzyme for multiple applications, including detergents, fabric care, home care, oral care (e.g., dental whitening, antimicrobial and/or cleaning), personal care, textile processing, food processing and industrial cleaning. In addition, in some embodiments, SOx can catalyze other substrates. Thus, this enzyme uses wide spectrum of substrates, providing flexibility in substrate usage in various applications. It is noted that many of these substrates are present in typical detergent formulations, or can be added to them. The synthetic gene sequence (neutral codons) and the corresponding amino acid sequence of sorbitol oxidase from Streptomyces sp. H-7775 are known. These sequences are set forth in SEQ ID NOS:1 and 2, respectively, as shown below. It is noted that the amino acid sequence provided by P97011 (Streptomyces sp.) has been reported to be the same as that of Streptomyces sp. H-7775. SEQ ID NO. 1 is the synthetic gene cloned in an E. coli expression vector pET24a and expressed in E. coli strain BL21(DE3)pLysS.

**[00178]** Thus, in some embodiments, the invention provides for polynucleotides encoding a wild-type SOx derived from Streptomyces H-7775 (SEQ ID NO:1)

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GGTACCCATA TGACCCCTGC TGAAAAAAAC TGGGCCGGCA ATATCACTTT
CGGTGCAAAG AGACTTTGCG TTCCACGTTC TGTCAGAGAG CTGCGCGAAA
CAGTTGCTGC CAGTGGAGCA GTGAGACCTT TGGGAACGCG GCACTCCTTT
AACACTGTCTG CTGACACCTC AGGTGATCAT GTTCTTTTGG CCGGTCTCCC
GAGAGTCGTT GACATTGATG TGCCAGGCAG GGCTGTTAGC CTGTGGGCAG
GACTTAGATT CGGTGAGTTT GCTGCCGAAT TGCATGCTCG AGGTCTCGCC
CTGGCAAATC TGGGCTCACT TCCCCACATT TCTGTGCTG GGGCCGTGGC
AACCGGCACA CATGGAAGTG GAGTGGGTAA CCGTTCCTTG GCCGGTGCTG
TCAGAGCACT GAGCCTCGTT ACTGCTGATG GCGAGACACG CACCCTTAGG
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CGTACTGACG AAGATTTTGC CGGGGCTGTC GTGTCTCTGG GCGCATTGGG  
 AGTTGTGACG TCGCTTGAGT TGGACCTCGT TCCTGCCTTC GAAGTCAGAC  
 AGTGGGTGTA CGAGGATCTG CCAGAAGCTA CACTTGCCGC CAGATTTGAC  
 GAGGTTATGT CCGCTGCATA TAGCGTCAGT GTGTTACGG ATTGGAGACC  
 5 GGGTCCTGTT GGACAAGTCT GGCTCAAACA ACGAGTTGGC GACGAAGGGG  
 CTAGATCAGT ATGCCCGCA GAGTGGCTGG GTGCCAGATT GGCTGATGGA  
 CCACGTCACC CTGTTCCGGG ATGCCAGCC GGTAATTGTA CTGCACAGCA  
 AGGCGTTCCG GGCCCTTGGC ATGAAAGACT GCCCCACTTC CGCATGGAAT  
 TTACCCCATC CAACGGTGAC GAGTTGCAGT CGGAGTATTT TGTCGCTAGG  
 10 GCTGATGCCG TTGCCGCCTA CGAAGCTCTT GCACGCCTCC GCGACAGAAT  
 CGCACCTGTC CTGCAAGTGT CTGAGTTGCG TACAGTCGCT GCCGACGATC  
 TGTGGCTTTC ACCGGCTCAT GGAAGAGATA GCGTGGCCTT CCACTTTACC  
 TGGGTTCCAG ACGCTGCCGC AGTCGCTCCT GTGGCCGGTG CAATTGAGGA  
 AGCTCTCGCC CCCTTTGGCG CAAGACCGCA TGGGGGAAG GTTTTCTCTA  
 15 CTGCTCCCGA GGTCTGCGA ACGTTGTACC CACGCTATGC CGACTTTGAG  
 GAACTTGTGG GACGTCACGA TCCTGAAGGC ACCTTCAGGA ACGCCTTTCT  
 CGATCGCTAC TTCCGGCGTT AATAAGGATC CGAGCTC (SEQ ID NO:1)

MTPAEKNWAGNITFGAKRLCVPRSVRELRETVAASGAVRPLGTRHSFNTVADTSGDHV  
 20 SLAGLPRVVDIDVPGRAVSLSAGLRFGEFAAELHARGLALANLGSLPHISVAGAVATGTH  
 GSGVGNRSLAGAVRALSIVTADGETRTLRRDDEDFAGAVVSLGALGVVTSLELDLVPF  
 EVRQWVYEDLPEATLAARFDEVMSAAYSVSFVDWRPGPVGQVWLKQRVGDEGARSV  
 MPAEWLGARLADGPRHPVPGMPAGNCTAQQGVPGPWHERLPFRMEFTPSNGDELQ  
 SEYFVARADAVAAYEALARLRDRIAPVLQVSELRTVAADDLWLSPAHRDSDVAFHFTWV  
 25 PDAAAVAPVAGAIEEALAPFGARPHWGKVFSTAPEVLRTLYPRYADFEELVGRHDPEGT  
 FR NAFLDRYFRR (GENBANK ACCESSION NO. BAA19135; SEQ ID NO:2)

**[00179]** The nucleic and amino acid sequences of the putative sorbitol oxidases in  
*Streptomyces lividans* and *Streptomyces coelicolor* are provided below (SEQ ID NOS:3  
 30 and 4, respectively). The addition of the NcoI cloning site at the start methionine resulted  
 in amino acid change from serine to glycine of the second amino acid residue at the N-  
 terminus. This is the gene cloned and expressed as an intracellular protein in  
*Streptomyces lividans* g3s3 showing both sorbitol and xylitol oxidase activities, as  
 described herein.

35 GCCATGGGCG ACATCACGGT CACCAACTGG GCCGGCAACA TCACGTACAC  
 GGCGAAGGAA CTGCTGCGGC CGCACTCCCT GGACGCGCTG CGGGCCCTGG

TGGCGGACAG CGCCAGGGTG CGGGTGCTGG GCAGCGGGCA CTCCTTCAAC  
GAGATCGCCG AGCCGGGCGA CGGGGGTGTC CTGCTGTCGC TGGCGGGCCT  
GCCGTCCGTG GTGGACGTGG ACACGGCGGC CCGTACGGTG CGGGTCGGCG  
GCGGTGTGCG GTACGCGGAG CTGGCCCCGGG TGGTGACGC GCGGGGCCTG  
5 GCGCTGCCGA ACATGGCCTC GCTGCCGCAC ATCTCGGTG CCGGGTCGGT  
GGCCACCGGC ACCCACGGTT CGGGGGTGGG CAACGGTTCG CTGGCCTCGG  
TGGTGCGCGA GGTGGAGCTG GTCACCGCGG ACGGTTGAC CGTGGTGATC  
GCGCGGGGCG ACGAGCGGTT CGGCGGGGCG GTGACCTCGC TCGGCGCGCT  
GGGCGTGGTG ACGTCGCTCA CACTCGACCT GGAGCCGGCG TACGAGATGG  
10 AACAGCACGT CTTACCGAG CTGCCGCTGG CCGGGTTGGA CCCGGCGACG  
TTCGAGACGG TGATGGCGGC GCGGTACAGC GTGAGTCTGT TCACCGACTG  
GCGGGCGCCC GGTTCGGC AGGTGTGGCT GAAGCGGCGC ACCGACCGGC  
CGCTGGACGG TTTCCGTAC GCGGCCCGG CCGCCGAGAA GATGCATCCG  
GTGCCGGGCA TGCCCGCGGT GAACTGCACG GAGCAGTTCG GGGTGCCGGG  
15 GCCCTGGCAC GAGCGGCTGC CGCACTTCCG CGCGGAGTTC ACGCCCAGCA  
GCGGTGCCGA GTTGCAGTCG GAGTACCTGA TGCCCCGGA GCACGCCCTG  
GCCGCCCTGC ACGCGATGGA CGCGATACGG GAGACGCTCG CGCCGGTGCT  
CCAGACCTGC GAGATCCGCA CGGTCGCCGC CGACGCGCAG TGGCTGAGCC  
CGGCGTACGG GCGGGACACC GTGGCCGCGC ACTTCACCTG GGTCGAGGAC  
20 ACGGCGGCGG TGCTGCCGGT GGTGCGGCGG CTGGAGGAGG CGCTCGTCCC  
CTTCGCGGCC CGTCCGCACT GGGGGAAGGT GTTCACCGTC CCGGCGGGCG  
AGCTGCGTGC GCTGTACCCG CGGCTGGCCG ACTTCGGGGC GCTGGCCGGG  
GCGCTGGACC CGGCGGGGAA GTTCACCAAC GCGTTCGTGC GCGGGGTGCT  
CGCGGGCTGA GGATCCAT (SEQ ID NO:3)

25

MGDITVTNWA GNITYTAKEL LRP HSLDALR ALVADSARVR VLGSGHSFNE  
IAEPGDGGVL LSLAGLPSVV DVDTAARTVR VGGGVRYAEL ARVVHARGLA  
LPNMA SLPHI SVAGSVATGT HSGVGNGSL ASVVREVELV TADGSTVIA  
30 RGDERFGGAV TSLGALGVVT SLTLDLEPAY EMEQHVFTL PLAGLDPATF  
ETVMAAAYS SVLFTDWRAPG FRQVWLKRRT DRPLDGFPYA APAAEKMHPV  
PGMPAVNCTE QFGVPGPWHE RLP HFRAEFT PSSGAELQSE YLMPREHALA  
ALHAMDAIRE TLAPVLQTCE IRTVAADAQW LSPAYGRDTV AAHFTWVEDT  
AAVLPVVRRL EEALVPFAAR PHWGKVFTVP AGELRALYPR LADFGALAGA  
35 LDPAGKFTNA FVRGVLAG (SEQ ID NO:4)

[00180] The sequences of other sorbitol oxidases that are homologs of SEQ ID NO:2 or 4 are encompassed by the invention and include those set forth below. It is understood that any homolog of the SOx of SEQ ID NOs: 2 or 4 that retains sorbitol and/or xylitol oxidase activity is encompassed by the invention.

5

[00181] *Acidothermus cellulolyticus* (ZP\_01136416)

MDGGKRCRDG TPQPPAPSEQ VTPSAAASLR AAYDVEVSAP RLRNWAGNIA  
FRPRRYVQPRDLDELVEIIRVSDQVRVLGTGHSFNPIADTTGTLISLDHLPREVRVMPGR  
TAVSAGTRYGDLAFPLHEAG WALANVGSLP HISIAGACAT ATHGSGDRNG  
10 CLATAVAGMTGVDGTCRVFHLTAESPEFPGAVVHLGALGAVTEIELVTEPTFTVRQWVY  
EDAPLDNVFADLDDVTSAAYSVSIFTTWDPPTARQIWLKERVERAAGRDPDPA  
RRWGGRLAER DHNPVPGMPP ENCTPQLGRIGPWHERELPHF RLDVTPSAGD  
ELQSEYFVPRAAAVEAYRALRHIGSRIAPVLQISEIRTVADELWLSPAYHRPSVAFHFT  
WIADEEAVRPVVSEVERALAPLQPRPHWGKLFTMDPAVVRAAYPRFDDFV  
15 ALAERYDPEG KFQNDFLRRF FAG (SEQ ID NO:5)

[00182] *Acidothermus cellulolyticus* (11B Q2E2H5)

MDGGKRCRDG TPQPPAPSEQ VTPSAAASLR AAYDVEVSAP RLRNWAGNIA  
FRPRRYVQPR DLDELVEIIR VSDQVRVLGT GHSFNPIADT TGTLSLDHL  
20 PREVRVMPGR TAVSAGTRYG DLAFPLHEAG WALANVGSLP HISIAGACAT  
ATHGSGDRNG CLATAVAGMT GVDGTCRVFH LTAESPEFPG AVVHLGALGA  
VTEIELVTEP TFTVRQWVYE DAPLDNVFAD LDDVTSAAYS VSIFTTWDP  
TARQIWLKER VAAGRDPDPA RRWGGRLAER DHNPVPGMPP ENCTPQLGRI  
GPWHERELPHF RLDVTPSAGD ELQSEYFVPR AAVEAYRAL RHIGSRIAPV  
25 LQISEIRTVA ADELWLSPAY HRPSVAFHFT WIADEEAVRP VVSEVERALA  
PLQPRPHWGK LFTMDPAVVR AAYPRFDDFV ALAERYDPEG KFQNDFLRRF FAG  
(SEQ ID NO:6)

[00183] *Streptomyces avermitilis* (NP\_823266)

MTDAGTALTN WAGNITYSAK ELHRPQSLDA LRALVADSAK VRVLGSGHSF  
NEIAEPGADGVLLSLTALPP SVEVDTAART VRVAGGVRYA ELARVVHGHG  
LALPNMASLP HISVAGSVATGTHGSGVTNG SLASAVREVE LVTADGSAVR  
IGRGDDRFDG AVTALGALGV VTALTDLEPDYRVAQQVFT ELPLAGLDFD  
AVAASAYSVS LFTGWRTSGF AQVWLKRRTD RPSADFPWAAPATEAMHPVP  
35 GMPAVNCTQQ FGVPGPWHERE LPHFRAEFTP SSGAELQSEY LLPRPYALDA

LHALDAVRET VAPVLQICEV RTVAADAQWL SPAYGRDTVA LHFTWVEDLA  
 AVLPPVRRVEEALDPFDPRP HWGKVFAVPA RVLGRYPRL GDFRALVDSL  
 DPGGKFTNAF VREVLGSGDRPS (SEQ ID NO:7)

5 [00184] *Streptomyces avermitilis* (Q82LCO)

MTDAGTALTN WAGNITYSAK ELHRPQSLDA LRALVADSAK VRVLGSGHSF  
 NEIAEPGADG VLLSLTALPP SVEVDTAART VRVAGGVRYA ELARVVHGHG  
 LALPNMASLP HISVAGSVATGTHGSGVTNG SLASAVREVE LVTADGSAVR  
 IGRGDDRFDG AVTALGALGV VTALTLDLEP DYRVAQQVFT ELPLAGLDFD  
 10 AVAASAYSVS LFTGWRTSGF AQVWLKRRTD RPSADFPWAA PATEAMHPVP  
 GMPAVNCTQQ FGVPGPWHER LPHFRAEFTP SSGAELQSEY LLPRPYALDA  
 LHALDAVRET VAPVLQICEV RTVAADAQWL SPAYGRDTVA LHFTWVEDLA  
 AVLPPVRRVE EALDPFDPRP HWGKVFAVPA RVLGRYPRL GDFRALVDSL  
 DPGGKFTNAF VREVLGSGDR PS (SEQ ID NO:8)

15

[00185] *Streptomyces coelicolor* (Q9ZBU1)

MSDITVTNWA GNITYTAKEL LRPHSLDALR ALVADSARVR VLGSGHSFNE  
 IAEPGDGGVLLSLAGLPSVV DVDTAARTVR VGGGVRYAEL ARVVHARGLA  
 LPNMASLPHI SVAGSVATGTHGSGVNGSL ASVVREVELV TADGSTVIA  
 20 RGDERFGGAV TSLGALGVVT SLTLDLEPAYEMEQHVTEL PLAGLDPATF  
 ETVMAAAYS SVSLFTDWRAPG FRQVWLKRRT DRPLDGFPYAAPAAEKMHPV  
 PGMPAVNCTE QFGVPGPWHE RLPHFRAEFT PSSGAELQSE  
 YLMPREHALAALHAMDAIRE TLAPVLQTCE IRTVAADAQW LSPAYGRDTV  
 AAHFTWVEDT AAVLPVRRLEEALVPFAAR PHWGKVFTVP AGELRALYPR  
 25 LADFGALAGA LDPAGKFTNA FVRGVLAG (SEQ ID NO:9)

[00186] *Streptomyces* sp. IKD472/FERM P-14339 (Q9KX73)

MSTAVTNWAG NITYTAKEVH RPATAEELAD VVARSAWGAC AGAAGHSFNE  
 IADPGPDGVLLRLDALPAETDVDTTARTVRVGGGVRYAELARVVHAHGLALPNMASLPH  
 30 ISVAGSVATGTHGSGVTNGPLAAPVREVELVTADGSQVRIAPGERRFGGAVTSLGALGV  
 VTALTLDLEPAFEVGQHLFTLPLRGLDFETVAAAGYSVSLFTDWREPGRQVWLKRRT  
 DQELPDFPWARPATVALHPVPGMPAENCTQQFGVPGPWHERLP HFRAEFTPSS  
 GAELQSEYLL PRAHALDALDAVDRIKRTVA PVLQTCEVRT VAPDEQWLGP  
 SHGRDTVALH FTWVKDTEAV LPVVRREEALDAFDPRPHW GKVFTTSA  
 35 LRARYPRLAD FRALARELDP SGKFTNTFLR DLLDG (SEQ ID NO:10)

[00187] *Arthrobacter* sp. FB24 (ZP\_00411614)

MRTVSELPGL SGSTGAGSSA PELNWAGNYR YTAASIHRPR  
 TLEEVQEVVAGASKIRALGSRHSFNIAIDS PGSLVSLEDL DPGIRIDAAT  
 RTVTVSGGTR YGTLAEQLES AGFALSNLASLPHISVAGAI ATATHGSGDA  
 NGNLATSVAA LELVAADGTV HRLNRGSSPG FDGAVVGLGALGVVTKVTLT  
 5 IEPTFTVRQD VFEALPWDTV LGNFDAVTSS AYSVSLFTDW SGDDVAQAWL  
 KSRLSGSAAS SDAGSTLAGE AFAAGTFFGG TRAGVARHPL PGVSAENCTE  
 QLGVPGSWSERLAHFRMAFT PSSGEELQSE FFVRREHAVA AIGELRALSD  
 RITPLLLVSE IRTVAADKLWLSTAYGQDSV GFHFTWKQRQ DEVEKVLPVM  
 EEALAPFNAR PHWGKLFHAG ADAVAELYPRFSDFKDLAER MDPEQKFRNE  
 10 FLARKVFGN (SEQ ID NO:11)

[00188] *Arthrobacter* sp. FB 24 (Q4NJLO; GenBank Accession no. YP\_833485)  
 MRTVSELPGL SGSTGAGSSA PELNWAGNYR YTAASIHRPR TLEEVQEVVA  
 GASKIRALGS RHSFNIAIDS PGSLVSLEDL DPGIRIDAAT RTVTVSGGTR  
 15 YGTLAEQLES AGFALSNLAS LPHISVAGAI ATATHGSGDA NGNLATSVAA  
 LELVAADGTV HRLNRGSSPG FDGAVVGLGA LGVVTKVTLT IEPTFTVRQD  
 VFEALPWDTV LGNFDAVTSS AYSVSLFTDW SGDDVAQAWL KSRLSGSAAS  
 SDAGSTLAGE AFAAGTFFGG TRAGVARHPL PGVSAENCTE QLGVPGSWSE  
 RLAHFRMAFT PSSGEELQSE FFVRREHAVA AIGELRALSD RITPLLLVSE  
 20 IRTVAADKLW LSTAYGQDSV GFHFTWKQRQ DEVEKVLPVM EEALAPFNAR  
 PHWGKLFHAG ADAVAELYPR FSDFKDLAER MDPEQKFRNE FLARKVFGN (SEQ ID  
 NO:12)

[00189] Marine *Actinobacterium* PHSC20C1 (ZP\_01129132)  
 25 MLTNQTNWAG NLTYNAKAIM QPTNVDELQE LVARLPRVRA LGTRHSFTDI  
 ADTPGTLMSLANMPPNIHID TTAMTASVTG GTSYGLLMSE LQSNGFALHN  
 TGSLPHISVA GATATATHGSGDGNGILSTA IAALDVVTAD GSLVTVDRAS  
 DHLPALAVGL GAFGVIARVT LDIEPTYRVRQDVYRFAPWE TVLEQLDDIM  
 ASAYSVSLLA DFGSPTVAQI WLKTRLGVGD DPEVAPTLFGGIWYDDSDDEL  
 30 APQNVNQRRAS IPGPWSERMP HFRLDGEPSN GGDELQSEYY  
 VRREHGVQALEALRGLGAQI SPHLLISEIR TAAADSLWMS PAYGQDVLCI  
 GFTWAKHPAE VTALLPEIEATLAPFAPRQH WGKLFSSFRD IIAERFPRVA  
 DFTEL RDQYD PQRKFWNPFL ERTL GAP (SEQ ID NO:13)

35 [00190] In additional embodiments, xylitol oxidases find use in the present  
 invention. The following sequence is a xylitol oxidase.

[00191] *Streptomyces* sp.IKD472 (Q9KX73)

MSTAVTNWAG NITYTAKEVH RPATAEELAD VVARSAWGAC AGAAGHSFNE  
 IADPGPDGVL LRLDALPAET DVDTTARTVR VGGGVRYAEL ARVVHAHGLA  
 LPNMA SLPHI SVAGSVATGT HGSGVTNGPL AAPVREVELV TADGSQVRIA  
 5 PGERRFGGAV TSLGALGVVT ALTDLEPAF EVGQH LFTTEL PLRGLDFETV  
 AAAGYSVSLF TDWREPGFRQ VWLKRRTDQE LPDFPWARPA TVALHPVPGM  
 PAENCTQQFG VPGPWHERLP HFRAEFTPSS GAELQSEYLL PRAHALDALD  
 AVDRIRDVA PVLQTCEVRT VAPDEQWLGP SHGRDTVALH FTWVKDTEAV  
 LPVVRRL EEA LDAFDPRPHW GKVFTTSAAA LRARYPRLAD FRALARELDP  
 10 SGKFTNTFLR DLLDG (SEQ ID NO:14)

[00192] In some embodiments, the POx enzymes of the invention are encoded by chimeric polynucleotides that comprise a polynucleotide that encodes the mature form of the POx enzyme that is operably linked to a polynucleotide that encodes a signal  
 15 peptide. In some embodiments, the chimeric polynucleotide is a synthetic chimeric polynucleotide. In other embodiments, the chimeric polynucleotide comprises a wild-type sequence that encodes the signal peptide and a synthetic sequence that encodes the mature form of the POx enzyme.

[00193] In prokaryotes two pathways for protein translocation across the  
 20 cytoplasmic membrane have been recognized. In most bacteria the general secretory (Sec) pathway is the best-characterized route for protein export. Proteins exported by this pathway are translocated across the membrane in an unfolded state through a membrane-embedded translocon to which they are targeted by cleavable N-terminal signal peptides (Mori et al., (2001) *Trends in Microbiology* 9:494 – 500). More recently a  
 25 second general export pathway has been described, which is designated the twin-arginine translocation (Tat) pathway and reference is made to US 2002/0110860; WO 03/079007; Berks, B. C. (1996) *Mol. Microbiol.* 22:393-404 and Tjalsma et al., (2000) *Microbiol. & Molecul. Bio. Reviews* 64:515 - 547.

[00194] The choice of signal sequence largely depends on the host cell used. As  
 30 noted above, in certain embodiments, a *Streptomyces* and/or a *Bacillus* host cell is employed, the signal sequence may be any sequence of amino acids that is capable of directing the fusion protein into the TAT or SEC pathway of the *Streptomyces* and/or *Bacillus* host cell.

[00195] In some embodiments, signal sequences that may be employed include  
 35 the signal sequences of proteins that are secreted from wild-type *Bacillus* cells. Such signal sequences include the signal sequences encoded by  $\alpha$ -amylase, protease, (e.g., aprE or subtilisin E), or  $\beta$ -lactamase genes. Exemplary signal sequences include, but are

not limited to, the signal sequences encoded by an  $\alpha$ -amylase gene, an subtilisin gene, a  $\beta$ -lactamase gene, a neutral protease gene (e.g., *nprT*, *nprS*, *nprM*), or a *prsA* gene from any suitable *Bacillus* species, including, but not limited to *B. stearothermophilus*, *B. licheniformis*, *B. lentus*, *B. subtilis*, and *B. amyloliquefaciens*. In some embodiments, the signal sequence is encoded by the *aprE* gene of *B. subtilis* (See e.g., Appl. Microbiol. Biotechnol., 62:369-73 [2003]). Further signal peptides find use in the present invention (See e.g., Simonen and Palva, Micro. Rev., 57:109-137 [1993]; etc.). A preferred signal peptide encompassed by the invention is the signal peptide of the *Bacillus circulans* cyclomaltodextrin glucanotransferase (*cgt*) precursor (Accession no. P43379; SEQ ID NO:36).

**[00196]** In other embodiments, the signal sequence that may be employed includes is a signal sequences of a protein that is secreted from the wild-type *Streptomyces* host cells. Such signal sequences include the signal sequence encoded by the *S. coelicolor* SCO6772 gene (SEQ ID NO:15), the signal sequence encoded by the *S. coelicolor* *celA* gene (EMBL accession no. AL939132.1; SEQ ID NO: 27); the signal sequence encoded by the *S. coelicolor* secreted endoglucanase gene SCO7363 (SEQ ID NO:31), or the signal sequence encoded by the *S. coelicolor* possible secreted protein gene SCO624 (SEQ ID NO: 39). Other signal peptides encoded by the *Streptomyces* *sp.* genes are described in WO2007/071996 and in Widdick et al. (Science 103:17927-17932 (2006)).

**[00197]** In some embodiments, the POx polynucleotides encode mature POx polypeptides and/or POx fusion polypeptides that share at least about 65% amino acid sequence identity, preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, still more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, even more preferably at least about 90% amino acid sequence identity, more preferably at least about 92% amino acid sequence identity, yet more preferably at least about 95% amino acid sequence identity, more preferably at least about 97% amino acid sequence identity, still more preferably at least about 98% amino acid sequence identity, and most preferably up to about 99% amino acid sequence identity with the amino acid sequence of the precursor POx protein and have comparable or enhanced production activity, as compared to the precursor polypeptide.

**[00198]** As will be understood by the skilled artisan, due to the degeneracy of the genetic code, a variety of POx polynucleotides encode fusion and mature POx proteins. In some other embodiments of the present invention, polynucleotides comprising a nucleotide sequence having at least about 70% sequence identity, at least about 75% sequence identity, at least about 80% sequence identity, at least about 85% sequence

identity, at least about 90% sequence identity, at least about 92% sequence identity, at least about 95% sequence identity, at least about 97% sequence identity, at least about 98% sequence identity and at least about 99% sequence identity to the polynucleotide sequence of SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40, are provided.

**[00199]** In some embodiments, the percent identity shared by polynucleotide sequences is determined by direct comparison of the sequence information between the molecules by aligning the sequences and determining the identity by methods known in the art. In some embodiments, the percent identity (*e.g.*, amino acid sequence, nucleic acid sequence, and/or gene sequence) is determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs find use in these analyses including those described above. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above.

**[00200]** An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al.*, J. Mol. Biol., 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (See, Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M<sup>+</sup>5, N<sup>-</sup>4, and a comparison of both strands.

[00201] The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*See e.g.*, Karlin and Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 [1993]). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ( $P(N)$ ), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a serine protease nucleic acid of this invention if the smallest sum probability in a comparison of the test nucleic acid to a serine protease nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid encodes a serine protease polypeptide, it is considered similar to a specified serine protease nucleic acid if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

[00202] In some embodiments of the present invention, sequences were analyzed by BLAST and protein translation sequence tools. In some experiments, the preferred version was BLAST (Basic BLAST version 2.0). The program chosen was "BlastX", and the database chosen was "nr." Standard/default parameter values were employed.

[00203] Several methods are known in the art that are suitable for generating variant polynucleotide sequences of the POx enzymes of the present invention, include but are not limited to site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches.

[00204] This invention further provides expression vectors comprising at least a fragment of the polynucleotides set forth above and host cells or organisms transformed with these expression vectors. Useful vectors include plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art. Accordingly, the invention also provides a vector including a polynucleotide of the invention and a host cell containing the polynucleotide. In general, the vector contains an origin of replication functional in at least one organism, convenient restriction endonuclease sites, and a selectable marker for the host cell.

[00205] Preferably, a polynucleotide in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The control sequences may be modified, for example, by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. The control sequences may in particular comprise promoters.

[00206] In some embodiments, in the vector, the nucleic acid sequence encoding for the POx signal peptide or the POx signal peptide fusion polypeptide is operably

combined with a suitable promoter sequence. The promoter can be any DNA sequence having transcription activity in the host organism of choice and can be derived from genes that are homologous or heterologous to the host organism. Examples of suitable promoters for directing the transcription of the modified nucleotide sequence, such as modified enzyme nucleic acids, in a bacterial host include the promoter of the *Streptomyces coelicolor* agarase gene *dagA* promoters, GI and A4 promoters, the promoters of the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *aprE* promoter of *Bacillus subtilis*, the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes and a promoter derived from a *Lactococcus* sp.-derived promoter including the P170 promoter.

[00207] A mature POx or a POx fusion polypeptide of the invention can be expressed by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, e.g., by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the POx polynucleotides can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., Ausubel, et al. (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, 1992). A POx signal peptide-encoding nucleic acid can be cloned into an expression vector such that the fusion moiety e.g. polypeptide of interest, is linked in-frame to the POx signal peptide. An expression vector comprising a polynucleotide encoding a POx signal peptide can be any vector capable of expressing the polynucleotide encoding the mature form of the POx polypeptide or the POx signal peptide fused to the mature portion of the POx polypeptide in a selected host organism, and the choice of vector will depend on the host cell into which the expression vector is introduced. Thus, in some embodiments, the invention provides expression vectors that comprise a nucleotide sequence encoding a POx signal peptide, as recited herein, operably linked to a nucleotide sequence encoding a heterologous POx polypeptide. In another embodiment, the vectors of the invention comprise a polynucleotide sequence that encodes a POx polypeptide that lacks a signal peptide.

**[00208]** In some preferred embodiments, the POx polynucleotide is ligated into an appropriate expression plasmid. The cloned POx gene is then used to transform or transfect a host cell in order to express the POx gene. This plasmid may replicate in hosts in the sense that it contains the well-known elements necessary for plasmid replication or the plasmid may be designed to integrate into the host chromosome. The necessary elements are provided for efficient gene expression (e.g., a promoter operably linked to the gene of interest). In some embodiments, these necessary elements are supplied as the gene's own homologous promoter if it is recognized, (i.e., transcribed, by the host), a transcription terminator (a polyadenylation region for eukaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the POx gene. In some embodiments, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media is also included.

**[00209]** The mature POx polypeptides or POx fusion polypeptides may, in addition, can comprise a tag sequence that is fused to the C-terminus of the POx fusion polypeptide to generate a tagged POx fusion polypeptide. Such tag sequences can be used to identify transformants and/or to facilitate the purification of recombinant Tat fusion polypeptides. For example, the POx fusion polypeptide it may be expressed to contain a tag such as those of maltose binding protein (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX), or as a His tag. Kits for expression and purification of such fusion proteins are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and Invitrogen, respectively. The POx fusion polypeptide can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("FLAG.RTM.") is commercially available from Kodak (New Haven, Conn.). Another tag that can be used in the invention is the c-myc tag, as is described in the examples.

**[00210]** It is intended that although preferred enzymes for use in the vectors and methods of the invention are referred to herein by their specific SEQ ID NOS, the present invention encompasses enzymes which are derived from the nucleic acids which encode the corresponding amino acid SEQ ID NOS, when expressed, either in their native host species or a heterologous host species. Thus, the present invention encompasses embodiments in which the enzymes are co- or post- translationally processed.

**[00211]** The chimeric polynucleotides of the invention encode POx fusion proteins that are directed to a secretory pathway which leads to the secretion of the mature forms of the POx enzymes. In some embodiments, the fusion polypeptide comprises a Tat signal peptide that is the secretory leader sequence of polypeptides that are naturally

expressed by *Streptomyces* and/or *Bacillus* that is operably linked to a mature form of a POx polypeptide. In some embodiments, the signal peptide is a TAT signal peptide. In other embodiments, the signal peptide is a SEC signal peptide. In some embodiments, the fusion proteins of the invention comprise a signal peptide encoded by the *S. coelicolor* SCO6772 gene (SEQ ID NO:15), the signal sequence encoded by the *S. coelicolor* celA gene (EMBL accession no. AL939132.1; SEQ ID NO: 27); the signal sequence encoded by the *S. coelicolor* secreted endoglucanase gene SCO7363 (SEQ ID NO:31), or the signal sequence encoded by the *S. coelicolor* possible secreted protein gene SCO0624 (SEQ ID NO: 39). In yet other embodiments, the fusion proteins of the invention comprise SEC signal peptide of the *Bacillus circulans* cyclomaltodextrin glucanotransferase (cgt) precursor (Accession no. P43379; SEQ ID NO:36). It is understood that any signal peptide capable of directing the fusion protein of the invention to a secretory pathway finds use in the present invention.

**[00212]** The mature forms of the POx enzymes of the invention can be the POx enzymes identified from a number of microorganisms including but not limited to *Streptomyces sp.*, *Bacillus sp.*, *Acidothermus sp.*, *Arthobacter sp.*, and *marine Acinobacterium sp.* In some preferred embodiments, the *Streptomyces sp.* include *Streptomyces* strain H-7775, *Streptomyces lividans*, *Streptomyces avermitilis*, *Streptomyces coelicolor* and *Streptomyces* species IKD472. In other preferred embodiments, the *Acidothermus sp.* is *Acidothermus cellulolyticus 11B* (Q2E2H5). In some embodiments, the *Arthobacter sp.* is *Arthobacter sp. FB24* (Q4NJLO). In some embodiments, the marine *Actinobacterium* is *Actinobacterium PHSC20C1*.

**[00213]** POx enzymes encompassed by the invention include homologous or heterologous POx proteins. POx enzymes of interest include full-length POx polypeptides that are naturally synthesized with a signal peptide, the mature form of the full-length POx polypeptides, and POx polypeptides that naturally lack a signal peptide.

**[00214]** The mature POx polypeptides and/or the POx fusion polypeptides may, in addition, comprise a tag sequence that is fused to the C-terminus of the mature POx polypeptide and the POx fusion polypeptide to generate a tagged mature POx polypeptide of POx fusion polypeptide. Such tag sequences can be used to identify transformants and/or to facilitate the purification of recombinant POx fusion polypeptides.

**[00215]** For example, the POx fusion polypeptide it may be expressed to contain a tag such as those of maltose binding protein (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX), or as a His tag. Kits for expression and purification of such fusion proteins are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and Invitrogen, respectively. The Tat fusion polypeptide

can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("FLAG.RTM.) is commercially available from Kodak (New Haven, Conn.). Another tag that can be used in the invention is the c-myc tag.

5 **[00216]** Some preferred embodiments of fusion proteins include the fusion protein of SEQ ID NO:16 which comprises the mature form of *Streptomyces* species H-7775 (SEQ ID NO:2) operably linked (fused) to the signal peptide of *Streptomyces coelicolor* gene SCO6772 (SEQ ID NO:15); the fusion protein of SEQ ID NO:28 which comprises the mature form of *Streptomyces* species H-7775 (SEQ ID NO:2) operably linked (fused) to the signal peptide of *Streptomyces coelicolor* celA gene (EMBL Accession No. AL939132.1; SEQ ID NO:27). Other preferred embodiments of fusion proteins include the fusion protein of SEQ ID NO:38, which comprises the signal peptide of the Bacillus cgt precursor (SEQ ID NO:36) fused to the mature form of the POx protein of *Streptomyces* species H-7775 (SEQ ID NO:2); the fusion protein of SEQ ID NO:35, 15 which comprises the signal peptide of the secreted endoglucanase from *Streptomyces coelicolor* SCO 7363 (SEQ ID NO:31) fused to the mature form of the POx protein of *Acidothermus cellulolyticus* (SEQ IDNO:6); the fusion protein of SEQ ID NO:37, which comprises the signal peptide of the signal peptide of the Bacillus cgt precursor (SEQ ID NO:36) fused to the mature form of the POx protein of *Acidothermus cellulolyticus* (SEQ ID NO:6); the fusion protein of SEQ ID NO:40, which comprises the signal peptide of the secreted protein *Streptomyces coelicolor* SCO0624 (SEQ ID NO:39) fused to the mature form of the POx protein of *Arthrobacter* sp. gene FB24 (Q4NJLO; SEQ IDNO:11).

20 **[00217]** In some embodiments, the POx enzyme of the invention is expressed without a signal peptide. Any POx expressed in the absence of a signal peptide will not be translocated to the outside of the host cell, but will remain confined to the intracellular milieu. Examples of POx enzymes that are expressed intracellularly include the POx enzyme from *Streptomyces* sp. H-7775 (SEQ ID NO:2), the POx enzyme of *Streptomyces lividans* (SEQ IDNO:4) and the POx enzyme of *Acidothermus* sp. 11B (SEQ IDNO:6).

25 **[00218]** The invention encompasses POx proteins that are related to the POx polypeptide of SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40. In some embodiments, the invention encompasses variant proteins of SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40. In some preferred embodiments, variant proteins differ from a parent protein and one another by a small number of amino acid residues. The number of differing amino acid residues 30 may be one or more, preferably 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, or more amino acid residues. In some preferred embodiments, the number of different amino acids between variants is between 1 and 10. In some particularly preferred embodiments, related

proteins and particularly variant proteins share at least about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, or about 99% amino acid sequence identity with the amino acid sequence of the precursor POx and have comparable or enhanced POx activity of the precursor polypeptide.

5 **[00219]** In preferred embodiments, the invention encompasses variants of POx proteins of SEQ ID NO: 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35, 37, 38, and/or 40. As used herein, the term "variant(s)" as used in context of a polypeptide sequence refers to a polypeptide prepared from the original (i.e., parent) polypeptide, or by using the sequence information from the original (i.e., parent) polypeptide, by  
10 insertion, deletion and/or substitution of one or more amino acids in the original (i.e., parent) sequence. In some embodiments, at least one insertion, deletion, and/or substitution is made in the original (i.e., parent) sequence, while in other embodiments, preferably less than about 50 amino acids, less than about 40, less than about 30, less than about 20, or less than about 10 amino acids are modified by insertion, deletion  
15 and/or substitution of the original (i.e., parent) sequence in order to produce variant polypeptides. In some preferred embodiments, only one amino acid modification (i.e., insertion, deletion or substitution) is made, while in other preferred embodiments two amino acids are modified, and in still further embodiments, three amino acids are modified, and in yet additional embodiments, four amino acids are modified, and in still  
20 further embodiments, five amino acids are modified. It is not intended that the variants of the present invention be limited to any specific number nor type of amino acid modifications.

**[00220]** In other preferred embodiments, the invention encompasses homologues of the POx polypeptides of SEQ IDNO: 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 28, 33, 35,  
25 37, 38, and/or 40. The homologs of the POx polypeptides have are at least about 70% homologous, more preferably at least about 80% homologous, still more preferably at least about 85% homologous, further more preferably at least about 90% homologous, even more preferably at least about 95%, more preferably about 96%, still more preferably about 97%, even more preferably about 98%, or most preferably about 99%  
30 homologous to the polypeptide sequence of interest (e.g., a sorbitol oxidase provided by the present invention). In some embodiments, homology between two polypeptide sequences is determined using ClustalW alignment algorithm using standard settings, as referred to herein. However, it is not intended that the present invention be limited to any particular method for determining homology.

35 **[00221]** The variants, homologues and fragments provided by the present invention all retain at least some of the desired enzymatic activity of the parent enzyme, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least

about 50%, at least about 60%, at least about 70%, at least about 80% at least about 90%, or all the enzyme activity of the parent enzyme. In some alternative embodiments, the variants and/or homologues have greater enzymatic activity than the original (i.e., parent) enzyme.

5 [00222] The POx enzymes of the present invention were found to be thermally stable and stable over a wide pH range. For example the POx of SEQ ID NO:4 was found to have SOx activity at pH ranging from pH3.5 to 5.5, from pH 5.5-6.7, from pH 6-8, and from pH 7.6-9.0 at a temperature of 25°C. In preferred embodiments, the POx enzyme of the invention exhibits maximal activity at pH 5.5, pH 6.0 and pH 8.0. The POx of the  
10 invention retain at least 60% activity, more preferable at least 70% activity, more preferably at least 80% activity, more preferably at least 90% activity, more preferably at least 95% activity. Indeed, the pH profiles of the sorbitol oxidases of the present invention are compatible with the pHs necessarily used in industry, as well as detergents and other cleaning agents.

15 [00223] It is contemplated that the oxidases of the present invention will find use in numerous applications, including but not limited to cleaning compositions (e.g., laundry and dish detergents, etc.), personal care (e.g., oral care, skin care, etc.), textile processing, diagnostics (e.g., medical diagnostic methods), biosensors, and other suitable applications.

20 [00224] Suitable host cells for use in the present invention are members of those genera capable of being utilized for industrial biosynthetic production of desired POx enzymes. Accordingly, host cells can include prokaryotes belonging to the genera *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Acidothermus*, *Arthrobacter*, *Bacillus*,  
25 *Pseudomonas*, *Streptomyces*, *Staphylococcus*, or *Serratia*. Eukaryotic host cells can also be utilized, with yeasts of the genus *Saccharomyces* or *Schizosaccharomyces* being included.

[00225] More specifically, prokaryotic host cells suitable for use in the present invention include, but are not limited to, *Escherichia coli*, *Bacillus brevis*, *Bacillus cereus*,  
30 *Bacillus mesentericus*, *B. licheniformis*, *B. lentus*, *B. subtilis*, *B. amyloliquefaciens*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. coagulans*, *B. circulans*, *B. pumilus*, *B. thuringiensis*, *B. clausii*, *B. megaterium*, *S. lividans*, *Streptomyces aureofaciens*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, *Streptomyces kasugensis*, *Streptomyces murinus*, *S. rubiginosus*, and *S. griseus*. In  
35 preferred embodiments, the host cells for use in the present invention include *Streptomyces sp. H-7775*, *S. lividans*, *E. coli*, *Acidothermus* and *B. subtilis*. In some preferred embodiments, the host cells are *Streptomyces*, in other embodiments,

the host cells are *E. coli*, in yet other embodiments, the host cells are *Acidothermus*, and in still other embodiments, the host cells are *Bacillus*. Indeed, it is not intended that the present invention be limited to any particular species of host cells, as various organisms find use as host cells of the present invention.

5 [00226] The present invention provides host cells comprising a recombinant expression vector comprising at least one polynucleotide sequence encoding at least one polypeptide selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues, fragments or variants thereof. In alternative  
10 embodiments, the host cells comprise at least one polynucleotide sequence encoding at least one polypeptide selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues, fragments or variants thereof. In some embodiments, polynucleotide sequence encoding at least one polypeptide selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues, fragments or variants thereof is present in the  
15 genome of the host cell. In other embodiments, polynucleotide sequence encoding at least one polypeptide selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues, fragments or variants thereof is present in a vector that replicates autonomously in the host cell.

[00227] In some embodiments, the invention provides *E. coli* host cells comprising a  
20 polynucleotide that encodes a POx polypeptide of SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40. In preferred embodiments, the *E. coli* host cell comprises a POx polypeptide that encodes a POx protein of SEQ ID NO:2 or 4. In other embodiments, host cells of the invention are *S. lividans* cells that comprise a polynucleotide that encodes a POx polypeptide of SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11,  
25 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40. In preferred embodiments, the *S. lividans* host cell comprises a POx polypeptide that encodes a POx protein of SEQ ID NO:2, 4, 6 and/or 11, 16, 28, 35 and/or 40. In yet other embodiments, host cells of the invention are *B. subtilis* cells that comprise a polynucleotide that encodes a POx polypeptide of SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37,  
30 38, and/or 40. In preferred embodiments, the *B. subtilis* host cell comprises a POx polypeptide that encodes a POx protein of SEQ ID NO:2, 6 and/or 38.

[00228] In some preferred embodiments, the expression of the at least one polypeptide having oxidase activity is extracellular, while in other embodiments, the expression is intracellular.

35 [00229] Host cells transformed with POx polynucleotide sequences encoding heterologous or homologous protein may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture.

**[00230]** The present invention provides methods of using the above-described cells for the production of at least one recombinant POx polypeptide. In some embodiments, the methods include culturing a host cell of the invention to produce a POx protein. In some embodiments, the methods include providing a recombinant expression vector that  
5 contains a POx polynucleotide that encodes a POx enzyme; transforming the host cell with the expression vector to produce a transformed host cell; and growing the transformed host cell under conditions suitable for the expression of the at least one polypeptide; and recovering the at least one polypeptide expressed by the transformed host cell. In some additional embodiments and as discussed above, the protein is  
10 secreted into the culture medium. In yet further embodiments, the methods comprise the step of recovering the protein from the culture medium. Alternatively, the methods comprise the step of recovering the protein from within the host cell. Preferred embodiments provide for the production of at least one recombinant polypeptide, wherein said at least one polypeptide exhibits oxidase activity, wherein the polypeptide comprises  
15 SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues, fragments or variants thereof, comprising: (a) providing: a recombinant expression vector, wherein said expression vector comprises at least one polynucleotide sequence encoding at least one of said polypeptides selected from SEQ ID NOS:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 or homologues,  
20 fragments or variants thereof; and a host cell; (b) transforming the host cell with the expression vector to produce a transformed host cell; (c) growing the transformed host cell under conditions suitable for the expression of at least one polypeptide; and (d) recovering at least one polypeptide expressed by the transformed host cell. In some preferred embodiments, the oxidase activity is polyol oxidase activity. In some  
25 particularly preferred embodiments, the oxidase activity is sorbitol oxidase activity, while in some alternative particularly preferred embodiments, the oxidase activity is xylitol oxidase activity.

**[00231]** In some preferred embodiments, the POx protein expressed by the host cells is recovered by the removal of other host cell constituents in the growth media using any  
30 convenient method (e.g., by precipitation, centrifugation, affinity, filtration) or any other suitable method known in the art. For example, affinity chromatography (Tilbeurgh *et al.*, FEBS Lett., 16:215 [1984]); ion-exchange chromatographic methods (Goyal *et al.*, Biores. Technol., 36:37 [1991]; Fliess *et al.*, Eur. J. Appl. Microbiol. Biotechnol. 17:314 [1983]; Bhikhabhai *et al.*, J. Appl. Biochem., 6:336 [1984]; and Ellouz *et al.*, Chromatography  
35 396:307 [1987]), including ion-exchange using materials with high resolution power (Medve *et al.*, J. Chromatography A 808:153 [1998]; hydrophobic interaction chromatography (Tomaz and Queiroz, J. Chromatography A 865:123 [1999]; two-phase

partitioning (Brumbauer *et al.*, Bioseparation 7:287 [1999]); ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration (*e.g.*, using SEPHADEX G-75), find use in the present invention. In particularly preferred  
5 embodiments, the sorbitol oxidases of the present invention are substantially purified to a level of at least about 99% of the protein component, as determined by SDS-PAGE or other standard methods known in the art.

**[00232]** The invention also encompasses POx fusion polypeptides that comprise a signal peptide and a heterologous peptide and a polypeptide domain that will facilitate  
10 purification of POx enzymes (Kroll DJ *et al* (1993) DNA Cell Biol 12:441-53). Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3:263-281), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity  
15 purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and the heterologous protein can be used to facilitate purification. In some particular embodiments, the sorbitol oxidase is used without purification from the other components in the culture medium. In some embodiments, the components of the  
20 culture medium are simply concentrated and then used without further purification of the POx protein from the other components of the growth medium in order to produce a cleaning and/or other composition.

**[00233]** In some embodiments, recombinant POx are expressed in bacterial or fungal host cells and these recombinant POx are purified by the removal of other host cell  
25 constituents; the percent of recombinant POx polypeptides is thereby increased in the sample. In particularly preferred embodiments, the POx of the present invention are substantially purified to a level of at least about 99% of the protein component, as determined by SDS-PAGE or other standard methods known in the art.

**[00234]** Means for determining the levels of secretion of a heterologous or  
30 homologous protein in a gram-positive host cell and detecting secreted proteins include, using either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, a Laboratory Manual, APS  
35 Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 158:1211).

**[00235]** A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for

producing labeled hybridization or PCR probes for detecting specific polynucleotide sequences include oligo labeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the nucleotide sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

**[00236]** A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. The enzymatic activity of a secreted SOx-fusion polypeptide can be determined by contacting the secreted polypeptide with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate. In addition, verifying that the correct enzyme or other reporter molecule is secreted can be accomplished by performing mass spectroscopy of the secreted protein.

**[00237]** In some embodiments, the host cells are cultured under batch, fed-batch or continuous fermentation conditions. Classical batch fermentation methods use a closed system, wherein the culture medium is made prior to the beginning of the fermentation run, the medium is inoculated with the desired organism(s), and fermentation occurs without the subsequent addition of any components to the medium. In certain cases, the pH and oxygen content, but not the carbon source content, of the growth medium are altered during batch methods. The metabolites and cell biomass of the batch system change constantly up to the time the fermentation is stopped. In a batch system, cells usually progress through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general terms, the cells in log phase produce most protein.

**[00238]** A variation on the standard batch system is the "fed-batch fermentation" system. In this system, nutrients (*e.g.*, a carbon source, nitrogen source, O<sub>2</sub>, or other nutrient) are only added when their concentration in culture falls below a threshold. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of nutrients in the medium. Measurement of the actual nutrient concentration in fed-batch systems is estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the

partial pressure of waste gases such as CO<sub>2</sub>. Batch and fed-batch fermentations are common and known in the art.

5 **[00239]** Continuous fermentation is an open system where a defined culture medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

10 **[00240]** Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in some embodiments, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth are altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off  
15 may be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are known to those of skill in the art and find use in the production of the POx enzymes of the present invention.

20 **[00241]** The POx protein produced using the above described methods finds use in any product containing a POx, including, but not limited to cleaning compositions, (*e.g.*, fabric cleaning compositions, such as laundry detergents, surface cleaning compositions, dish cleaning compositions and automatic dishwasher detergent compositions; *See e.g.*, WO0001826, which is incorporated by reference herein), bleaching and disinfecting compositions.

25 **[00242]** The cleaning compositions and cleaning additives of the present invention require an effective amount of the POx enzymes provided by the present invention. The required level of enzyme may be achieved by the addition of one or more POx enzymes, variants, homologues, and/or other enzymes or enzyme fragments having the activity of the POx enzymes of the present invention. Typically, the cleaning compositions of the  
30 present invention comprise at least 0.1 ppm to about 10 ppm, from about 0.5 to about 2 ppm, or from about 1 to about 5 ppm. In some preferred embodiments, the cleaning compositions of the present invention comprise about 0.2 to about 1 ppm of POx enzymes. The cleaning compositions that comprise at least one POx enzyme in combination with a perhydrolase enzyme comprise an effective amount of POx enzyme  
35 as recited herein and an effective amount of perhydrolase that comprises about 0.1 to about 10ppm, about 0.5 to about 10 ppm or about 1 to about 5 ppm of perhydrolase. In preferred embodiments, the amount of perhydrolase contained in the cleaning

compositions is from about 0.2 to about 1 ppm. In some embodiments, the cleaning compositions comprise a perhydrolase substrate as recited above in amounts from about 0.2 to about 20 mM, or from about 0.5 to about 15 mM, from about 1 to about 10 mM. In preferred embodiments, the amount of perhydrolase substrate contained in the cleaning composition comprises from about 1 to about 5 mM of perhydrolase substrate.

**[00243]** In some particular embodiments, the POx protein is used in an POx-containing laundry detergent comprising from about 1% to about 80%, *e.g.*, about 5% to about 50% (by weight) of surfactant, which may be a non-ionic surfactant, cationic surfactant, an anionic surfactant or a zwitterionic surfactant, or any mixture thereof (*e.g.*, a mixture of anionic and nonionic surfactants). Exemplary surfactants include: alkyl benzene sulfonate (ABS), including linear alkyl benzene sulfonate and linear alkyl sodium sulfonate, alkyl phenoxy polyethoxy ethanol (*e.g.*, nonyl phenoxy ethoxylate or nonyl phenol), diethanolamine, triethanolamine and monoethanolamine. Exemplary surfactants that find use in laundry detergents are known in the art (*See e.g.*, U.S. Patent Nos. 3,664,961, 3,919,678, 4,222,905, and 4,239,659).

**[00244]** The laundry detergent may be in solid, liquid, gel or bar form, and may further contain a buffer such as sodium carbonate, sodium bicarbonate, or detergent builder, bleach, bleach activator, various enzymes, an enzyme stabilizing agent, suds booster, suppresser, anti-tarnish agent, anti-corrosion agent, soil suspending agent, soil release agent, germicide, pH adjusting agent, non-builder alkalinity source, chelating agent, organic or inorganic filler, solvent, hydrotrope, optical brightener, dye or perfumes. In some preferred embodiments, the laundry detergent comprises in addition to the POx enzymes of the present invention, at least one further enzyme (*e.g.*, hemicellulase, peroxidase, protease, cellulase, xylanase, lipase, phospholipase, esterase, cutinase, pectinase, keratinase, reductase, oxidase, phenoloxidase, lipoxxygenase, ligninase, pullulanase, tannase, pentosanase, mannanase,  $\beta$ -glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, and amylases, or mixtures thereof).

**[00245]** The POx protein of the present invention finds use in any suitable composition useful for cleaning a variety of surfaces in need of stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (*e.g.*, liquid, gel, bar and granular formation); detergent compositions for cleaning fabrics, unlimited in form (*e.g.*, granular, liquid, gel and bar formulations); dishwashing compositions (unlimited in form); oral cleaning compositions, unlimited in form (*e.g.*, dentifrice, toothpaste, gel and mouthwash formulations); denture cleaning compositions, unlimited in form (*e.g.*, liquid, gel or tablet); and contact lens cleaning compositions, unlimited in form (*e.g.*, liquid, tablet).

**[00246]** In some embodiments, the cleaning compositions of the present invention comprise an effective amount of a POx, alone or in combination with other POx enzymes and/or a perhydrolase enzyme as recited above. In one embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40. In one embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and/or 16. In one preferred embodiment, the cleaning composition comprises a POx of SEQ IDNO:2, 4, 6, and/or 11.

**[00247]** In yet other embodiments, the cleaning composition comprises a POx of SEQ ID NO:2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 in

combination with at least one additional enzyme including enzymes that are hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, oxidoreductases, perhydrolases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidases,

chondroitinases, laccases, and amylases. In yet other embodiments, the cleaning composition comprises a POx of SEQ ID NO:2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and/or 16 in combination with at least one additional enzyme including enzymes that are hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, oxidoreductases, perhydrolases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases, and amylases. In another preferred embodiments, the cleaning composition comprises a POx of SEQ IDNO:2, 4, 6, and/or 11 in combination

with at least one additional enzyme including enzymes that are hemicellulases,

peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, oxidoreductases, perhydrolases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases, and amylases. In another embodiment, the cleaning

composition comprises a POx of SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 in combination with a perhydrolase. In another embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and/or 16 in combination with a perhydrolase. In a preferred embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4, 6, and/or 11 in combination

with a perhydrolase. In another embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 28, 33, 35, 37, 38, and/or 40 in combination with a bleach activator. In another embodiment, the cleaning composition

comprises a POx of SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and/or 16 in combination with a bleach activator. In a preferred embodiment, the cleaning composition comprises a POx of SEQ ID NO:2, 4, 6, and/or 11 in combination with a bleach activator.

**[00248]** In some embodiments, the cleaning compositions of the present invention  
5 comprise one or more detergent enzymes which provide cleaning, bleaching and/or  
disinfecting benefits. Examples of suitable enzymes include, but are not limited to,  
hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases,  
esterases, cutinases, pectinases, keratinases, reductases, carrageenases,  
phenoloxidasases, lipoxxygenases, ligninases, pullulanases, tannases, pentosanases,  
10 mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and  
amylases, or mixtures thereof. In some embodiments, a combination of enzymes is used  
(*i.e.*, a "cocktail") comprising conventional applicable enzymes like protease, lipase,  
cutinase and/or cellulase in conjunction with the POx or POx-perhydrolase combination in  
the presence or absence of a bleach activator.

**[00249]** In some embodiments, the cleaning compositions also comprise, in  
15 addition to the proteins described herein, one or more cleaning composition materials  
compatible with the POx protein. As described herein, the term "cleaning composition  
material," refers any liquid, solid or gaseous material selected for the particular type of  
cleaning composition desired and the form of the product (*e.g.*, liquid, granule, bar, spray,  
20 stick, paste, gel), which materials are also compatible with the POx or POx-perhydrolase  
combinations used in the composition either in the presence or absence of a bleach  
activator. The specific selection of cleaning composition materials are readily made by  
considering the surface material to be cleaned, the desired form of the composition for  
the cleaning condition during use (*e.g.*, through the wash detergent use). As used herein,  
25 "non-fabric cleaning compositions" include hard surface cleaning compositions,  
dishwashing compositions, oral cleaning compositions, denture cleaning compositions  
and contact lens cleaning compositions.

**[00250]** The POx protein finds use with various conventional ingredients to provide  
fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering  
30 compositions, and the like. Such compositions find use in the form of liquids, granules,  
bars and the like. In some embodiments, such compositions are formulated as modern  
"concentrated" detergents which contain as much as about 30% to about 60% by weight  
of surfactants.

**[00251]** In some embodiments, the cleaning compositions of the present invention  
35 comprise various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are  
typically present at levels of from about 5% to about 35% of the compositions.  
Surfactants include include nonionic, anionic, cationic, anionic or zwitterionic detergents

(See e.g., U.S. Patent Nos 4,404,128 and 4,261,868). A suitable detergent formulation is that described in U.S. Patent No. 5,204,015 (previously incorporated by reference).

Those in the art are familiar with the different formulations which find use as cleaning compositions. As indicated above, in some preferred embodiments, the detergent

5 compositions of the present invention employ a surface active agent (*i.e.*, surfactant) including anionic, non-ionic and ampholytic surfactants well known for their use in detergent compositions. Some surfactants suitable for use in the present invention are described in British Patent Application No. 2 094 826 A, incorporated herein by reference. In some embodiments, mixtures surfactants are used in the present invention.

10 **[00252]** Suitable anionic surfactants for use in the detergent composition of the present invention include linear or branched alkylbenzene sulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefin sulfonates; alkane sulfonates and the like. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth  
15 metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3.

**[00253]** Ampholytic surfactants that find use in the present invention include quaternary ammonium salt sulfonates, betaine-type ampholytic surfactants, and the like. Such ampholytic surfactants have both the positive and negative charged groups in the  
20 same molecule.

**[00254]** Nonionic surfactants that find use in the present invention generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, fatty acid glycerine monoesters, and the like.

**[00255]** In some preferred embodiments, the surfactant or surfactant mixture  
25 included in the detergent compositions of the present invention is provided in an amount from about 1 weight percent to about 95 weight percent of the total detergent composition and preferably from about 5 weight percent to about 45 weight percent of the total detergent composition. In various embodiments, numerous other components are included in the compositions of the present invention. Many of these are described  
30 below. It is not intended that the present invention be limited to these specific examples. Indeed, it is contemplated that additional compounds will find use in the present invention. The descriptions below merely illustrate some optional components.

**[00256]** In some embodiments of the present invention, the composition contains  
35 from about 0 to about 50 weight percent of one or more builder components selected from the group consisting of alkali metal salts and alkanolamine salts of the following compounds: phosphates, phosphonates, phosphonocarboxylates, salts of amino acids, aminopolyacetates high molecular electrolytes, non-dissociating polymers, salts of

dicarboxylic acids, and aluminosilicate salts. Examples of suitable divalent sequestering agents are disclosed in British Patent Application No. 2 094 826 A, the disclosure of which is incorporated herein by reference.

**[00257]** In additional embodiments, compositions of the present invention contain from about 1 to about 50 weight percent, preferably from about 5 to about 30 weight percent, based on the composition of one or more alkali metal salts of the following compounds as the alkalis or inorganic electrolytes: silicates, carbonates and sulfates as well as organic alkalis such as triethanolamine, diethanolamine, monoethanolamine and triisopropanolamine.

**[00258]** The cleaning compositions herein may contain a chelating agent. Suitable chelating agents include copper, iron and/or manganese chelating agents and mixtures thereof. When a chelating agent is used, the cleaning composition may comprise from about 0.1% to about 15% or even from about 3.0% to about 10% chelating agent by weight of the subject cleaning composition.

**[00259]** In yet additional embodiments of the present invention, the compositions contain from about 0.1 to about 5 weight percent of one or more of the following compounds as antiredeposition agents: polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone and carboxymethylcellulose. In some preferred embodiments, a combination of carboxymethyl-cellulose and/or polyethylene glycol are utilized with the composition of the present invention as useful dirt removing compositions.

**[00260]** The use of the POx enzymes of the present invention are used in combination with additional bleaching agent(s) such as sodium percarbonate, sodium perborate, sodium sulfate/hydrogen peroxide adduct and sodium chloride/hydrogen peroxide adduct and/or a photo-sensitive bleaching dye such as zinc or aluminum salt of sulfonated phthalocyanine further improves the detergent effects. In additional embodiments, the POx enzymes of the present invention are used in combination with bleach boosters (e.g., TAED and/or NOBS).

**[00261]** The cleaning compositions can comprise one or more detergent enzymes which provide cleaning performance and/or fabric care benefits. Examples of suitable enzymes include, but are not limited to, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, phenoloxidases, lipoxxygenases, ligninases, pullulanases, tannases, pentosanases, malanases,  $\beta$ -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and amylases, or mixtures thereof.

**[00262]** In addition to the ingredients described above, perfumes, buffers, preservatives, dyes and the like also find use with the present invention. Other ingredients useful in detergent cleaning compositions also find use in the compositions

herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C<sub>10</sub> -C<sub>16</sub> alkylamides also find use in the compositions, typically at about 1% to about 10% levels.

5 [00263] In some embodiments, the detergent compositions comprise water and/or other solvents as carriers. For example, in some embodiments, low molecular weight primary or secondary alcohols (e.g., methanol, ethanol, propanol, and isopropanol) are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6  
10 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) also find use. In such embodiments, the compositions typically contain from about 5% to about 90%, or typically from about 10% to about 50% of such carriers.

[00264] In some embodiments, the detergent compositions provided herein are formulated such that during use in aqueous cleaning operations, the wash water will have  
15 a pH between about 5.5 and about 10.5. In some preferred embodiments, the pH of the wash water is between about pH 5 and pH 7. In other preferred embodiments the pH of the wash water is between about pH 7 and pH 10.5. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the  
20 art.

[00265] Various bleaching compounds, such as the percarbonates, perborates and the like, also find use in such compositions, typically at levels from about 1% to about 15% by weight. In some embodiments, such compositions also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which  
25 are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

[00266] Various soil release agents, especially of the anionic oligoester type, various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated  
30 tetraethylene pentamine, various dispersing agents, especially polyacrylates and polyaspartates, various brighteners, especially anionic brighteners, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays, and the like, all find use in various embodiments of the present compositions, at levels ranging from about 1% to about 35% by weight. Standard  
35 formularies and published patents contain multiple, detailed descriptions of such conventional materials.

[00267] Enzyme stabilizers also find use in the cleaning compositions of the present invention. Such stabilizers include, but are not limited to propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%), and calcium formate (preferably from about 0.1% to about 1%).

5 [00268] The cleaning compositions of the present invention are formulated into any suitable form and prepared by any suitable process chosen by the formulator, (*See e.g.*, U.S. 5,879,584, U.S. 5,691,297, U.S. 5,574,005, U.S. 5,569,645, U.S. 5,565,422, U.S. 5,516,448, U.S. 5,489,392, U.S. 5,486,303, U.S. 4,515,705, U.S. 4,537,706, U.S. 4,515,707, U.S. 4,550,862, U.S. 4,561,998, U.S. 4,597,898, U.S. 4,968,451, U.S. 10 5,565,145, U.S. 5,929,022, U.S. 6,294,514, and U.S. 6,376,445, all of which are incorporated herein by reference for some non-limiting examples). When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates 15 such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

[00269] Other optional ingredients include chelating agents, clay soil removal/anti-redeposition agents, polymeric dispersing agents, bleaches, brighteners, suds suppressors, solvents and aesthetic agents.

20 [00270] In some preferred embodiments, the cleaning compositions of the present invention find use in cleaning surfaces and/or fabrics. In some embodiments, at least a portion of the surface and/or fabric is contacted with at least one embodiment of the cleaning compositions of the present invention, in neat form or diluted in a wash liquor, and then the surface and/or fabric is optionally washed and/or rinsed. For purposes of 25 the present invention, "washing" includes, but is not limited to, scrubbing, and mechanical agitation. In some embodiments, the fabric comprises any fabric capable of being laundered in normal consumer use conditions. In some preferred embodiments, the cleaning compositions of the present invention are used at concentrations of from about 500 ppm to about 15,000 ppm in solution. In some embodiments in which the wash 30 solvent is water, the water temperature typically ranges from about 5 °C to about 70 °C. In some preferred embodiments for fabric cleaning, the water to fabric mass ratio is typically from about 1:1 to about 30:1.

[00271] In order to further illustrate the present invention and advantages thereof, the following specific Examples are given with the understanding that they are being 35 offered to illustrate the present invention and should not be construed in any way as limiting its scope.

**EXPERIMENTAL**

**[00233]** The following Examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

- 5 **[00234]** In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); H<sub>2</sub>O (water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg and ug (micrograms); mg (milligrams); ng (nanograms); µl and ul (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm and um (micrometer); M (molar); mM (millimolar); µM and uM (micromolar); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl<sub>2</sub> (magnesium chloride); NaCl (sodium chloride); OD<sub>280</sub> (optical density at 280 nm); OD<sub>600</sub> (optical density at 600 nm); EFT ("effective fermentation time"); HDL (Heavy Duty Detergent Liquid); EtOH (ethanol); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH
- 10 7.2]); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); TAED (N,N,N',N'-tetraacetythylenediamine); w/v (weight to volume); v/v (volume to volume); GOx and Gox (glucose oxidase); AOX and Aox (alcohol oxidase); COX and Cox (choline oxidase); HOx and Hox (hexose oxidase); SOx and Sox (sorbitol oxidase); PMS (phenazine methosulfate); NCBI (National Center for Biotechnology Information);
- 20 ATCC (American Type Culture Collection, Manassas, VA); Geneart (Geneart, Regensburg, Germany); Invitrogen (Invitrogen, Inc., Carlsbad, CA); Stratagene (Stratagene, Inc., San Diego, CA); Biospringer (Biospringer, Inc., Cedex, France); BASF (BASF, Mt. Olive, NJ); and Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO).

- [00235]** In the following Examples various growth media were used. In these
- 25 Examples, the TS medium was composed of 16 g Difco tryptone, 4 g Difco soytone, 20 g caseine (hydrolysate) (Sigma), and 5g K<sub>2</sub>HPO<sub>4</sub> brought to 1 liter. After autoclaving, 50% filtered sterilized glucose was added to a final concentration of 1.5%. The "Production Medium" was composed of 2.4 g citric acid\*H<sub>2</sub>O; 8.3 g yeast extract (Biospringer); 2.4 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 72.4 g MgSO<sub>4</sub>\*7H<sub>2</sub>O; 0.1 g CaCl<sub>2</sub>\*2H<sub>2</sub>O; 0.3 ml Mazu DF204 (BASF); 5 ml
- 30 *Streptomyces* modified trace elements (1 liter stock solution contains: 250 g citric acid \* H<sub>2</sub>O; 3.25 g FeSO<sub>4</sub>\*7H<sub>2</sub>O; 5 g ZnSO<sub>4</sub>\*7H<sub>2</sub>O; 5 g MnSO<sub>4</sub>\* H<sub>2</sub>O; 0.25 g H<sub>3</sub>BO<sub>3</sub>); 10 g glucose, adjust volume to 1 liter and the pH was adjusted to 6.9, with NaOH. The R5 medium used was the standard R5 medium commonly used to grow *Streptomyces*.

**EXAMPLE 1****Construction of Strains Expressing Sorbitol Oxidase of *Streptomyces* sp. H-7775 in *Streptomyces lividans***

5 [00236] In this Example, methods used to construct the strain that produced the sorbitol oxidase used in the development of the present invention are described. The protein sequence (SEQ ID NO:2) of the sorbitol oxidase was obtained from the published amino acid sequence (See e.g., Hiraga et al., Biosci. Biotechnol. Biochem., 62: 4347-353

10 [1998]). The signal sequence of the twin-arginine pathway of the *Streptomyces coelicolor* SCO6772 gene (SEQ ID NO:15) was obtained from complete genome sequence of *Streptomyces coelicolor*.

MTEVSRRKLMKGAAVSGGALALPALGAPPATAAPAAGPEDLPGPAAA (SEQ ID NO:15)

15 [00237] The sorbitol oxidase was expressed in *Streptomyces* as a fusion protein of the signal sequence of the SCO6772 protein (SEQ ID NO:15) and sorbitol oxidase (SEQ ID NO:2). A restriction site for *NcoI* was introduced at the 5' end of DNA for cloning purposes, which resulted addition of an amino acid glycine residue at position 2 (See,

20 SEQ ID NO:16).

MGTEVSRRKLMKGAAVSGGALALPALGAPPATAAPAAGPEDLPGPAAAMTPAEKNWA  
GNITFGAKRLCVRPSVRELRETVAASGAVRPLGTRHSFNTVADTSGDHVSLAGLPRVVD  
IDVPGRAVSLSAGLRFGEFAAELHARGLALANLGSLPHISVAGAVATGTHGSGVGNRSL  
25 AGAVRALSLVTADGETRTLRRRTDEDFAGAVVSLGALGVVTSLELDLVPFAFEVRQWVYED  
LPEATLAARFDEVMSAAYSVSFVDWRPQVGVQVWLKQRVGDEGARSVMPEAWLGAR  
LADGPRHPVPGMPAGNCTAQQGVPGPWHERELPFRMEFTPSNGDELQSEYFVARADA  
VAAYEALARLRDRIAPVLQVSELRTVAADDLWLSPAHRDVSFAHFTWVPDAAAVAPVA  
GAIEEALAPFGARPHWGKVFSTAPEVLRTLYPRYADFEELVGRHDPEGTFRNAFLDRYF  
30 RR (SEQ ID NO:16)

[00238] A restriction site for *Bam*HI was also introduced at the 3' end of DNA for cloning purposes. The codons of the fusion gene were optimized for expression in *Streptomyces lividans*. DNA was synthesized by Geneart. The DNA fragment spanning

35 the two restriction sites (*i.e.*, from *NcoI* to *Bam*HI (SEQ ID NO:17)) was cloned into *Streptomyces* expression plasmid pKB105 (See, U.S. Pat. Appln. Ser. No. 11/303,650,

filed December 16, 2005, incorporated by reference in its entirety) which was cut with *Bam*HI completely and *Nco*I partially.

CCATGGGCACCGAGGTCTCCCGCCGCAAGCTGATGAAGGGCGCGGCGGTGTCCG  
 5 GCGGCGCGCTGGCGCTGCCGGCCCTCGGCGCCCCGCCCCGCCACCGCGGCGCCG  
 GCCGCGGCCCCGAGGACCTCCCGGGCCCCGCGCGCCGCGCATGACCCCGGCGCGAG  
 AAGAACTGGGCGCGCAACATCACCTTCGGCGCCAAGCGCCTGTGCGTCCCGCGCT  
 CCGTCCGCGAGCTGCGCGAGACCGTGCCGCGCTCCGGCGCGGTGCGCCCGCTGG  
 GCACCCGCCACTCGTTCAACACCGTCGCCGACACCTCCGGCGACCACGTGTGCGT  
 10 GGCCGGCCTGCCGCGCGTCTGTCGACATCGACGTCCCGGGCCGGGCGGTGTCCCT  
 GTCCGCGCGCCTGCGCTTCGGCGAGTTCGCCGCGGAGCTGCACGCCCGCGGCCT  
 GGCCCTGGCCAACCTGGGCTCCCTGCCGCACATCTCCGTGGCGGGCGCGGTGCC  
 ACCGGCACCCACGGCTCCGGCGTCCGGCAACCGCTCCCTGGCGGGCGCCGTCCGC  
 GCCCTGTCCCTGGTCACCGCCGACGGCGAGACCCGCACCCTGCGCCGCACCGACG  
 15 AGGACTTCGCCGGCGCCGTCGTGTCCCTGGGCGCCCTGGGCGTCGTACCTCCCT  
 GGAGCTGGACCTGGTCCCGGCCCTTCGAGGTCCGCCAGTGGGTCTACGAGGACCTG  
 CCCGAGGCCACCCTGGCCGCCCCGCTTCGACGAGGTCATGTCCGCCGCCTACTCCG  
 TGTCCGTGTTACCGACTGGCGCCCCGGGCCCGGTCCGCCAGGTCTGGCTGAAGCA  
 GCGCGTCGGCGACGAGGGCGCCCGCTCCGTTCATGCCGGCCGAGTGGCTGGGCGC  
 20 CCGCCTGGCCGACGGCCCGCGCCACCCGGTCCCCGGCATGCCCGCCGGCAACTG  
 CACCGCCCAGCAGGGCGTCCCGGGCCCCGTGGCACGAGCGCCTGCCGCACTTCGG  
 CATGGAGTTCACCCCGTCCAACGGCGACGAGCTGCAGTCCGAGTACTTCGTGCCCC  
 GCGCGGACGCCGTCCGGCCCTACGAGGCGCTGGCCCGCCTGCGCGACCGCATCG  
 CCCCGGTCTTCAGGTCTCCGAGCTGCGCACCGTCGCCGCGGACGACCTGTGGCT  
 25 GTCCCCGGCCACGGCCGCGACTCCGTGCGCTTCCACTTCACCTGGGTCCCGGAC  
 GCCGCCGCGTCCGCCCGGTCCGCCGGCGCCATCGAGGAGGCCCTGGCCCCGTTC  
 GGCGCCCGCCCGCACTGGGGCAAGGTGTTCTCCACCGCCCCCGAGGTCCTGCGCA  
 CCCTGTACCCGCGCTACGCCGACTTCGAGGAGCTGGTCGGCCGCCACGACCCCGA  
 GGGCACCTTCGCAACGCCTTCCTCGACCGCTACTTCGCCCGCTGAGGATCC (SEQ  
 30 ID NO:17)

[00239] The expression plasmid (pKB105-TAT-SOx7775; Figure 1; SEQ ID NO:29) was transformed into *Streptomyces lividans* strain g3s3 (See, U.S. Pat. Appln. Ser. No. 11/305,650, supra) and three transformants were selected and grown in TS medium for  
 35 2- 3 days in the presence of 50 ug/ml thiostrepton at 30°C. Cells were then transferred to a production medium free of antimicrobials and growth was continued for another three days. Then, 1 ml of the culture was transferred to each of two culture tubes and the cells

were removed by centrifugation under conditions sufficient to separate the cells from the supernatants.. The supernatants obtained from these two culture tubes were tested in enzyme activity assays.

pKB105 TAT-SOx7775 (9495bps):

5 ctagagatcgaactcatgttcgagttctgttcacgtagaagccggagatgtgagaggtgatctggaactgctaccctcgttg  
gtggtgacctggaggtaaagcaagtgaccttctggcggagggtgtaaggaacgggggtccacggggagagagagatgg  
ccttgacggtcttggaaggggagcttcggcgcgggggaggatggtcttgagagagggggagctagtaatgtcgtacttg  
acagggagtgctccttctccgacgcatcagccacctcagcggagatggcatcgtgcagagacagacccccggaggtaac  
catgggcaccgaggtgtcccgccggaactcatgaaaggcgccggtgtccggcgcgctgtggcgctgcccgcgctc  
10 ggcgacccgcccggcgacggccgccccggcgaggccccgaggacctccccggccccgcagcggcgatgacccccg  
ccgagaagaactgggcccgaacatcaccttcggcgccaagcgctgtgctgccgctccgtccgagctgcgcga  
gacctgtggccgctccggcgccgtgcgcccgtgggcacccgccactcgttcaacaccgtcgccgacacctccggcgac  
cacgtgtcgtggccggcctgcgcgctgtgcacatcgactcccgggccggcgtgtccctgtccggcgccgtgcgc  
ttcggcgagttcgccgagctgcacgcccggcctggccctggccaacctgggctccctgccgacatctccgtggcg  
15 ggcgcggtcgccaccggcaccacggctccggcgctcggaacctgctccctggcgggcgccgtccgcgcccgtgctccgtgt  
caccgcccagcggcgagacccgcacctgcgcgcacccgacgaggacttcgcccggcgccgtcgtgtccctggcgcccgt  
ggcgctgcacctccctggagctggacctggtccggccttcgaggtccgccagtgggtctacgaggacctgcccaggcc  
acctggccgcccgttcgacgaggtcatgtccgcccctactccgtgtccgtgttcaccgactggcgcccggggccggtcg  
gccaggtctggtgaagcagcgctcgcgacgagggcgcccgtccgtcatgccggccgagtggtggcgcccgcct  
20 ggccgacggcccgcgccacccgggtccccggcatgccggccggcaactgcaccgcccagcagggcgctccggggcccgt  
ggcacgagcgctgccgacttccgcatggagttcaccctccaacggcgacgagctgcagtcaggtacttctgcgcc  
gcgcggacgcccgtcgccgctacgaggcgctggccgctgcgcgaccgcatgccccggctcgtcaggtctccgagct  
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ggacgcccgcgcccgtcgccccggtcgcccggccatcgaggaggccctggccccgttcggcgcccggccgactgggg  
25 caagggtgtctccaccgccccgaggtctgcgcacctgtaccgcgctacgccgacttcgaggagctggtcgccgccca  
cgaccccgagggcaccttcgcaacgccttctcgaccgctacttccggcgtgaggatccgcgagcggatcggctgacc  
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agtatcagcgccatgtccggcatatcccagccctccgcatgcaagcttggcactggccgtcgtttacaacgtcgtgactggg  
aaaacctggcggttacccaacttaatcgcttcgagcacatcccccttcgccagctggcgtaatagcgaagagggccgca  
30 ccgatcgcccttccaacagttgcgcagcctgaatggcgaatggcgccgtgatgcggtattttctccttacgcatctgtcggtatt  
tcacaccgcatatggtgcaactctcagtacaatctgctctgatgccgcatagttaagccagccccgacacccgccaacacccg  
ctgacgcgcccgtgacgggtgtgtctccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtaa  
gaggtttcaccgtcatcaccgaaacgcgcgagacgaaagggcctcgtgatacgccctattttataggttaatgtcatgataat  
aatgggtttcttagacgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgttttttaataacattcaaatatgt  
35 atccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcg  
cccttattcccttttttcgggcattttgccttctgttttctcaccagaaacgctggtgaaagtaaaagatgctgaagatcagttg  
ggtgcacgagtggttacatcgaactggaatctcaacagcggtgaagatccttgagagtttcgccccgaagaacgttttcaat

gatgagcacttttaaagtctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggctgccgcataca  
 ctattctcagaatgacttggtgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgca  
 gtgctgccataacatgagtataactgcccgaacttactctgacaacgatcggaggaccgaaggagctaaccgcttt  
 ttgcacaacatgggggatcatgtaactgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcg  
 5 tgacaccacgatgcctgtagcaatggcaacaacgttgcgcaactattaactggcgaacttacttacttagcttcccggcaa  
 caattaatagactggatggaggcggataaagtgcaggaccacttctgcgtcggcccttccggctgggttattgtgat  
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 ctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggctcactgattaagcattg  
 gtaactgtcagaccaagttactcataatatacttttagattgatttaaaacttcattttaatttaaaaggatctaggtgaagatcctttt  
 10 gataatctcatgaccaaatacccttaacgtgagtttctgctccactgagcgtcagaccccgtagaaaagatcaaaggatcttctt  
 gagatccttttttctgcgcgtaactgctgcttgcgaacaaaaaaaccaccgctaccagcgggtggttggttgccggatcaaga  
 gctaccaactcttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagttagccgtagttaggc  
 caccactcaagaactctgtagcaccgcctacatacctcgctctgctaactctgttaccagtggctgctgccagtggcgataag  
 tcgtgtcttaccgggttgactcaagacgatagttaccggataaggcgcagcggctcgggtgaacgggggggtcgtgcaca  
 15 cagcccagcttgagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccg  
 aaggagaaaaggcggacaggtatccggttaagcggcagggtcggaacaggagagcgcacgaggggagcttccagggg  
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 agcctatggaaaaacgccagcaacgcggccttttacgggtcctggccttttctggccttttctcacatgttcttctgcgttctc  
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 25 tcgaggtcgaggaaccgagcgtccgaggaacacaggcgttatcggttggccgcgagattcctgtcgatcctctcgtgcag  
 cgcatcgcaggggaaacggaaacgttgagagactcggctcgtcatatggggatggaaaccgagggcgggaagacgc  
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 30 tgtcgaccaggatgatccccgacgctccgagcgcgagcgcagcgtgcgtactatcgcgccgatgttcccgcagatcttcccc  
 gtcgagaacgacgacgtccccacgcccgtcgcgatatcgccgaacctggccgggaggggacgcccggcgatgccga  
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 acagatccagcaactcagatggaaaaggactgctgtcgtgccgttagacctcgatgaactccaccccgccgcatgctgt  
 gcatgaggggctcgacgtcctcgatcaacgttgtcttattgttgatcgcgacggcttggtagacatcgatgatccgctgcaccg  
 35 cgggatcggacggatttgcgatggtgtccaactcagtcattggtcgtcctaccggctgctgtgttcagtacgcgattcctgggt  
 gtgacaccctacgcgacgatggcggtggctgccctgaccggcaatcaccaacgcaagggggaagtcgtcgtctctgga  
 aagctccccgctcttcccgtccgggacccgcgcggtcgatccccgcataatgaagtattcgccttgatcagtcgggtggacg

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ccccaccaccaggagaccgacctgaccgacttcgacggacgcctgaccgaggggaccgtgaacctgggtccaggacc  
5 ccaacggcggtgggtgggtccgcccactgcgctgagcccgttgcgactgggcccgacttcgccggaccgctcggttcagg  
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ccctccaagtacaccagccccaaagggcgctgggagtgccggagggaacctctggcccgttggtgccaggattcca  
ccagaccaaaagagcaacgggcccggacttcgcacctccgacctccgctcccagactcgcgcccccttagccgggag  
acaggaacgttgctcgtgccagagtacggagcgtgcccaggcattgccagatcgccccgcccggccccgctgccact  
10 gcgggaccgcaattgccacacaccgggcaaacggccgctatctactgctcagaccgctgccggatggcagcgaagc  
gggcatcgcgctgtgacgcgagatgccgcccaggcaaaagcgaacacctgggaaagaacaacagagttcccg  
caccctccgacctgcggttctccggacggggtggatggggagagcccagagggcgacgcctctgggaagtaggaag  
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15 gaacgtccgtggtcctgggctgatgcggtgcgggcccgatctggctctgcccggtctgcgcccacgatccggcacaagc  
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cgaccgcgagggcatccgagaccggatcgggtacgtcgcatgatccgcgcgaccgaagtcaccgtggggcagatcaa  
20 cggctggcacccgcacatccacgcgatcgtcctggctggcgggccggaccgagggggagcgggtccgcgaagcagatcgt  
cgccacctcgagccgaccggcgccgctcgacgagtgccaggggacactggcggtccgtgtggaccgccgccctgcgc  
aaggtaacccccgccttcacgcccagcaccggcacggcgtcgactcaagcggctggagaccgagcgcgacgccaac  
gacctcgccgagtacatcgccaagaccaggacgggaaggcgcccgcctcgaactcgcccgccgacctcaagac  
ggcgaccggcggaacgtgccccgttcgaactcctcgacggatcggggacctgaccggcgcatgaccgaggacga  
25 cgccgcccgggtcggtcgtggagtggaaacctctcgcgctggcacgagtacgagcgggcaacccggggacgcccggc  
catcgaatggaccgctacctgcggcagatgctcgggctcgacggcgggcagaccgaggccgacgacctcgatctgctc  
ctggcgccgacgcccagcggggagctcgggccggggtcgccgtgaccgaggacggatggcacggtcaccgg  
ccgcccctcgacctcgaggcgacccgggcccgcgaaggcaaggacggcaacgaggattcgccggccgtggcgaa  
cgggtcgggaggtcctggcgtggccgacgcccgcacacagtgggtggtcgtcacggcgggggaggtggccgaggcg  
30 tacgccgacatgctcgccgccctcgccagcgccgaggaagcaactgcacgccgacggcgagagcaggacgacg  
accaggacgacgacgcccagcaccgcccaggagcgggcccggccgcacatcgccggctcgcaagtgggcccacttcg  
cactaactcgtcccccccgccgtacgtcatccgggtacgtacggcgggggtcggtgacgtacgcgcgacggcgccg  
gggtcgaagccgcccggagtaatcctgggattactcgccgggggtcgggcccgccggcacttcgtgaggcggtacctgc  
gcccgactcgctcgctacgagacgtgccgctacggctcgtcgccatgagcaccaccaccccgaggacgcccgacgg  
35 cgcaagctctgcgctggtgcggctcgagatcaagcaatccggcgtcgccggagccgggactactcgccgctcct  
gccgccagcgggctacgaggcccggcgccagcgagggcgtcgtccgcccgtggcgtcggcagtcgtcgccgag  
atacgtcacgtgacgaaatgcagcagccttcattccgtcacgtgacgaaactcgggccgcaggtcagagcacggtccgc

ccgctccggccctgccggacccccggctgcagctcgccggccgcccgtccccctgccgtccggcccgctccagaggca  
 gcgtcggcggctcctgcctccccgcccggcgccgaccgggacccgcaaacccttgatccgctgtcggggggatcact  
 acggtgggtgccgaagtgatcacggggaggactgatgcaccaccaggaccgggaccaggaccaggcgtagcggcag  
 tgctggccgactgctcctggcggggacgctgatcgtcggggagctcctgggctgtggcccgccgtggcggtcggc  
 5 ggcgcccgcctcgccctctacggaggccccgcccggcccgccgatatgccgtcgcggtcgaggtccgcccgttccgc  
 cggcatcttggccaccacgatcgggcagccgatgaccggccacgacggagccgcacggctgaccagctcgacggccg  
 ccacctcatcgcggcagcaggtgctccccagcaaccacgacggggctcagggtcgcctcacgcggtcagcaccg  
 acggcgggggtacggcgctccgggaggctgacaggcgctcagacggccgctagggccgcgagtccccaccctccc  
 cgctgccctgtcgggcagcacaacggcgatcccgcagtcggcgaggcaggcgccacgtaaaccgcccaccgatccg  
 10 ccccgctcgtgtgcgcgggcccgtcggcggccgggagcgggggaagacaggagcgtcgccggggccgtgggc  
 cgggcccgcggcccgcctcgcgggccgcttgatgacgtagggaagtgtaccgcaaaaaacgcagcctgaactagt  
 gcgatcct (SEQ ID NO:29)

[00240] Two oligos (SEQ ID NO:18 and SEQ ID NO:19) were obtained from  
 15 Invitrogen.  
 GCGCTAGCCGGCCCCCGGCACAGGCCATGACCCGGCCGAGAAGAAGAACTGGG  
 (SEQ ID NO:18)  
 CAGGAAACAGCTATGAC (SEQ ID NO:19)

[00241] The primers were used in PCR to amplify sorbitol oxidase gene and to fuse  
 20 the sorbitol oxidase gene to the *ce/A* signal sequence. The PCR reaction mixture  
 containing DNA, dNTPs, primer and 4% DMSO in 1x buffer was heated to 98°C for 4  
 minutes to denature the DNA templates. Herculanase® II enzyme (Stratagene) was added  
 to the tube and PCR reaction was performed in 30 cycles of 98°C for 30 seconds, 62°C  
 for 30 seconds and 72°C for 1 minute and 8 seconds. The final extension at 72°C was  
 25 done for 5 minutes and the reaction was chilled to 4°C

[00242] The resulting PCR fragment contained a portion of the *ce/A* signal sequence,  
 the sorbitol oxidase gene, and a portion of vector sequence containing two restriction  
 enzyme sites (SEQ ID NO:20).

30 GCGCTAGCCGGCCCCCGGCACAGGCCATGACCCGGCCGAGAAGAAGAACTGGGCC  
 GGCAACATCACCTTCGGCGCCAAGCGCCTGTGCGTCCCGCGCTCCGTCCGCGAGC  
 TGC GCGAGACCGTG GCGCCTCCGGCGCCGTGCGCCCGCTGGGCACCCGCCACT  
 CGTTCAACACCGTCGCCGACACCTCCGGCGACCACGTGTCGCTGGCCGGCCTGCC  
 GCGCGTCGTCGACATCGACGTCCCGGGCCGGGCGGTGTCCCTGTCCGCCGGCCTG  
 35 CGCTTCGGCGAGTTCGCCGCCGAGCTGCACGCCCGCGGCCTGGCCCTGGCCAACC  
 TGGGCTCCCTGCCGCACATCTCCGTGGCGGGCGCGGTGCGCACCGGGACCCACGG  
 CTCGGCGTGGCAACCGCTCCCTGGCGGGCGCCGTCCGCGCCCTGTCCCTGGTC

ACCGCCGACGGCGAGACCCGCACCCTGCGCCGCACCGACGAGGACTTCGCCGGC  
 GCCGTCGTGTCCCTGGGCGCCCTGGGCGTCGTACCTCCCTGGAGCTGGACCTGG  
 TCCCGGCCTTCGAGGTCCGCCAGTGGGTCTACGAGGACCTGCCCGAGGCCACCCT  
 GGCCGCCCGCTTCGACGAGGTCATGTCCGCCGCCTACTCCGTGTCCGTGTTACCG  
 5 ACTGGCGCCCCGGGCCCGGTCCGCCAGGTCTGGCTGAAGCAGCGCGTCGGCGACG  
 AGGGCGCCCGCTCCGTCATGCCGGCCGAGTGGCTGGGCGCCCGCCTGGCCGACG  
 GCCCGCGCCACCCGGTCCCCGGCATGCCCGCCGGCAACTGCACCGCCCAGCAGG  
 GCGTCCCGGGCCCGTGGCACGAGCGCCTGCCGCACTTCCGCATGGAGTTCACCCC  
 GTCCAACGGCGACGAGCTGCAGTCCGAGTACTTCGTGCGCCCGCGCGGACGCCGT  
 10 GCGGCCTACGAGGCGCTGGCCCGCCTGCGCGACCGCATCGCCCCGGTCCTGCAG  
 GTCTCCGAGCTGCGCACCGTCGCCGCCGACGACCTGTGGCTGTCCCCGGCCACG  
 GCCGCGACTCCGTGCGCTTCCACTTCACCTGGGTCCCGGACGCCGCCGCCGTGCG  
 CCCGGTCGCCGGCGCCATCGAGGAGGCCCTGGCCCCGTTCCGGCGCCCGCCCGCA  
 CTGGGGCAAGGTGTTCTCCACCGCCCCCGAGGTCCTGCGCACCTGTACCCGCGC  
 15 TACGCCGACTTCGAGGAGCTGGTCGGCCGCCACGACCCCGAGGGCACCTTCCGCA  
 ACGCCTTCCTCGACCGCTACTTCCGCCGCTGAGGATCCGAGCTCCAGCTTTTGTT  
 CCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG  
 (SEQ ID NO:20)

20 **[00243]** The PCR fragment was digested with restriction enzymes *NheI* and *BamHI* to  
 remove the vector sequence portion. The resulting fragment was then cloned to  
 expression vector pKB105 to generate the plasmid "pKB105-CelA-SOx7775" (See, Figure  
 2; SEQ ID NO: 30). The expression plasmid was transformed into *Streptomyces lividans*  
 strain g3s3 and three transformants were selected and grown in TS medium for 2- 3 days  
 25 in the presence of 50 ug/ml thiostrepton at 30°C. Cells were then transferred to a  
 production medium free of antibiotics and growth was continued for another three days.  
 Then, 1 ml sample was transferred to each of two new culture tubes and cells were  
 removed and the enzyme was purified as described in Example 5.

pKB105-celA-SOx7775 (9489bps):

30 ctagagatcgaacttcattgttcgagttctgttcacgtagaagccggagatgtgagaggtgatctggaactgctcacctcgttg  
 gtggtgacctggaggtaaagcaagtgaccttctggcggagggtgtaaggaacgggggtccacggggagagagagatgg  
 ccttgacggtcttgggaaggggagcttcggcgcgggggaggatggtcttgagagagggggagctagtaatgtcgtacttgg  
 acagggagtgctccttccgacgcacagccacctcagcggagatggcatcgtgcagagacagacccccggaggtaac  
 catgggcttgggagcgctcccatcgctgtgtccgcttcgcacgaggaggaacgcttgaaacgcctttggccctgctcgc  
 35 gaccggcgtgtgatcgctcggcctgactgcgctagccggccccccggcacaggccatgaccccgccgagaagaactg  
 ggccggcaacatcaccttcggcgccaagcgctgtgcgtcccgcgctccgtccgagctgcgcgagacctggccgcct  
 ccggcgccgtgcgcccgtgggcacccgccactcgttaacaccgtgcgcgacacctccggcgaccacgtgtcgtggcc

ggcctgccgcgctgctgacatcgacgtcccggggccggggccgtgtccctgtccgcccgtgcttggcgagttgcc  
gccgagctgcacgcccggcctggccctggccaacctgggtccctgccgcacatctccgtggcggggcgggcgccac  
cggcaccacgggtccggcgtcggaaccgctccctggcggggcgccgtccgcgccctgtccctgtcaccgcccagggc  
gagacccgcaccctgcccgcaccgacgaggacttcgcccggcgccgtcgtgtccctgggcgccctgggcgtcgtcacctc  
5 cctggagctggacctggtcccggccttcgaggtccgccagtgggtctacgaggacctgcccaggccacctggccgccc  
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cgccaccgggtcccggcatgcccggcgcaactgcaccgcccagcagggcgctccggggcccggtggcacgagcgccgtg  
ccgcaactccgcatggagttcaccccgctcaacggcgacgagctgcagtcgagtagtctcgtcgcccgcgggacgcccgtc  
10 gcggcctacgaggcgctggcccgcctgcgcgaccgcatgccccggctcgtcaggtctccgagctgcgcaccgtcgccgc  
cgacgacctgtggctgtccccggcccacggccgcgactccgtgccttccacttcacctgggtcccggacggccgcccgt  
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gcccccgaggtcctgcgcaccctgtacccgcgctacggcgacttcgaggagctggtcgccgcccacgacccccgagggca  
ccttcgcaacgccttctcgaccgctactccgcccgtgaggatccgcgagcggatcggtgacgggagcggggaggag  
15 gacggggcgccggcggaagtcgcccgtccgctgaatcgctccccgggcacggacgtggcagtatcagcgccatgtc  
cggcataatccagccctccgcatgcaagcttgccactggccgtcgtttacaacgtcgtgactgggaaaacctggcgttacc  
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25 atcgaactggatctcaacagcggtaagatccttgagagtttcgccccgaagaacgtttccaatgatgagcacttttaaagttc  
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35 ccttaacgtgagtttctgtccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttctgcgcgta  
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gcaccgcctacatacctcgctctgctaactctgttaccagtggtgctgctgccagtgggcgataagtctgtcttaccgggttgact  
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5 cctgtcgggtttcgccacctctgacttgagcgtcgattttgtgatgctcgtcagggggcgagcctatggaaaaacgccagc  
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25 gtccgggacccgcgcggtcgatccccgcataatgaagtattgccttgatcagtcgggtggacgcgcccagcggcccgcg  
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35 ccacacaccgggcaaacggccgctatctactgctcagaccgctgccggatggcagcgaagcgggagatcgcgcggtg  
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5 cggctcgcggacctcatggacgccctccagggcaccgggaagacgccggacagccccggcggccgggcccctacca  
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15 gacggcggggagctgcgggcccggggtcgcctgaccgaggacggatggcacgcggtcacccgccgcgcctcgacct  
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gccgacgaccgccaggagcgggcccggccgacatcgcccggtcgcaagtgggcccacttcgactaactcgtcccc  
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25 aaatgcagcagccttccattccgtcacgtgacgaaactcgggcccgcaggtcagagcacgggtccgcccgtccggccctgc  
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cctggtcggcgggacgctgatcgtgcgggagctcctgggcctgtggcccgcgtggcggtcggcattggcgcccgccctcg  
30 ccctctacggaggcccgcccgccgcccggatagccgtcgcggtcgaggtccgccgggtccgccggcatcttgcacac  
cacgatcgggcagccggatgaccggccacgacggagccgcacggctgaccagctcgacggccgccacctcatcgcg  
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35 gcgggcccgtcggcggccgggcccggagcggggcgaagacaggagcgtcggccgggcccgtgggcccggccgcgcgg  
cccgctcgcgggcccgttgatgacgtagggaagtgtaccgcaaaaaacgcagcctgaactagttgcgatcct (SEQ  
ID NO:30)

**EXAMPLE 2****Identification of Genes with Sequence Identity/Similarity to the  
*Streptomyces* sp. H7775 SOx Gene**

[00244] In this Example, experiments conducted to identify genes with sequence identity/similarity with the *Streptomyces* sp. H-7775 sorbitol oxidase gene are described. In these experiments, homologues encoding polyol oxidases (e.g., sorbitol and xylitol oxidase) were identified using the primary sequences of two functionally characterized polyol oxidases, namely *Streptomyces* sp. H-7775 sorbitol oxidase described herein and the *Streptomyces* sp. IKD472/FERM P-14339 xylitol oxidase as queries in BLAST analyses on the non-redundant (nr) protein database of the National Center for Biotechnology Information. Putative polyol oxidases were identified from different species of *Streptomyces*, *Acidothermus*, *Arthrobacter*, *Brevibacterium*, *Frankia*, *Nocardia*, *Janibacter*, *Marinobacter*, *Burkholderia*, *Paracoccus*, *Chromabacterium*, *Thermobifida*, *Xanthomonas*, *Pseudomonas*, *Corynebacterium* and *Bacillus*. In addition, the UniProtKB/TrEMBL/Swiss-Prot databases were also searched for genes which function as sorbitol or xylitol oxidases. In addition to the *Streptomyces* sp. IKD472/FERM P-14339 xylitol oxidase, the following were identified as xylitol oxidases in these searches: XYOA\_STRCO (Q9ZBU1) from *Streptomyces coelicolor*, XYOA\_STRSI (Q9KX73) from *Streptomyces* sp. strain IKD472 / FERM P-14339; Q2E2H5\_ACICE from *Acidothermus cellulolyticus* 11B; and Q82LC0\_STRAW from *Streptomyces avermitilis*.

[00245] In additional experiments, the genes encoding polyol oxidases (e.g., sorbitol oxidase/xylitol oxidase) were obtained by utilizing sequenced genomes to generate and screen genomic libraries of culturable organisms. In additional embodiments, novel polyol oxidase genes are obtained from microbes that are unculturable by generating and screening metagenomic libraries.

[00246] One example of such a metagenome is the complex microbial consortia derived from different soil sources. The microbiome metagenome database (JGI-DOE, USA) was searched for sequences with sequence identity to the SOx gene using the Function search: Genes in EC:1.1.3.41 - Xylitol oxidase, resulted in the identification of five FAD/FMN-containing dehydrogenases and one probable xylitol oxidase. The following table (Table 1) provides various polyol oxidases identified in searches in which the *Streptomyces* sp. H7775 SOx gene was used to query the Quick BLAST P.

| Table 1. Polyol Oxidases  |                                                    |                                           |
|---------------------------|----------------------------------------------------|-------------------------------------------|
| UniProt Accession Numbers | Protein Name                                       | Source Species Name                       |
| P97011                    | Sorbitol oxidase [SOx] SEQ ID NO:1                 | <i>Streptomyces sp</i>                    |
| Q9ZBU1                    | Probable xylitol oxidase (EC 1.1.3.41) SEQ ID NO:9 | <i>Streptomyces coelicolor</i>            |
| Q2E2H5                    | Putative xylitol oxidase SEQ ID NO:6               | <i>Acidothermus cellulolyticus</i> 11B    |
| Q82LC0                    | Putative xylitol oxidase SEQ ID NO:8               | <i>Streptomyces avermitilis</i>           |
| Q4NJL0                    | FAD linked oxidase N-terminal SEQ ID NO:12         | <i>Arthrobacter sp.</i> FB24              |
| Q9KX73                    | Xylitol oxidase (EC 1.1.3.41) SEQ ID NO: 10        | <i>Streptomyces sp.</i> IKD472            |
| Q412H8                    | FAD linked oxidase, N-terminal                     | <i>Kineococcus radiotolerans</i> SRS30216 |

### EXAMPLE 3

#### Expression of the *Streptomyces lividans* Sorbitol Oxidase Gene in *Streptomyces lividans* Strain g3s3

[00247] In this Example, experiments conducted to express the *S. lividans* sorbitol oxidase gene in *S. lividans* are described. Based on *in silico* analyses, two other *Streptomyces* species (*S. coelicolor* and *S. avermitilis*) were identified as having putative xylitol oxidase genes. The locus containing the *Streptomyces coelicolor* putative XOx gene (SCO6147) sequence was retrieved from the sequence database. The corresponding *S. lividans* gene was isolated using primers N2: 5'ctccagacgcgccggtaggtttc (SEQ ID NO: 23), Is-n2 5'ctgctgcgccgaccactgaccc (SEQ ID NO:24) and genomic DNA from *S. lividans* as template. The protocol suggested by the manufacturer for GC-rich templates was followed in a PCR reaction consisting of the Platinum Pfx DNA polymerase with the Enhancer solution (Invitrogen). Two parallel PCR reactions were carried out. The first PCR reaction (reaction #1) utilized primers designated as us-sco1 5'gcccatatgagcgacatcacgggtcacc (SEQ ID NO:21) and Is-sco1 5'ggatcctcagcccgcgagcacccc (SEQ ID NO:22). The previous PCR product was used as

the template. The PCR reaction product was a 1.269 kb PCR fragment. The PCR conditions used were: 30 cycles of denaturation at 94°C for 55 seconds, annealing at 55°C for 55 seconds, followed by extension for 1-2 minutes at 68°C. The polymerase used was Platinum Pfx DNA polymerase plus enhancer solution (Invitrogen). The resulting PCR product was cloned directly in an *E. coli* vector PCR Blunt TOPO (Invitrogen) and the sequence of the cloned SOx gene verified by DNA sequencing using primers provided in the kit.

[00248] The second PCR reaction (reaction #2) utilized primers us-s1

5'gccatgggcgacatcacggtcaccaac (SEQ ID NO:25) and ls-s1 5'

atggatcctcagcccgcgagcaccac (SEQ ID NO:26) were used in a PCR reaction using the previous PCR product as template and the same conditions as above.

[00249] The 1.268kb PCR product was digested with *Nco*I and *Bam*HI, and the resulting fragment was cloned directly into an *Nco*I (partial digest) and *Bam*HI digested *Streptomyces* vector pKB105. The final construct was the expression vector designated as pSMM-SOx (*S. lividans*) is shown in Figure 4. Five ul of plasmid DNA was used to transform *Streptomyces* g3s3 protoplasts using methods known in the art.

[00250] The transformation reaction product was plated on R5 plates and incubated at 32°C for 18 hours. A soft nutrient agar overlay containing thiostrepton at 50 ug/ml was then poured on the plates and the plates were incubated for an additional 3 days. Single colonies were used to inoculate a 250 ml flask with 20 ml TS-G media containing thiostrepton at 50 ug/ml. After 3 days of cultivation with shaking at 30°C, 2 ml aliquots were used to inoculate a 250 ml flask containing the *Streptomyces* Production Medium (See, above).

[00251] The cell pellets was collected by centrifugation, resuspended in buffer and disrupted, as described in Example 2. Table 2 shows the SOx activities present in the cell-free extracts derived from the different *Streptomyces* transformants. Twenty different transformants containing four DNAs encoding the SOx gene were analyzed for SOx activity, as described above. The results are provided in Table 2.

| Table 2. SOx Activities of Twenty Different <i>Streptomyces</i> Transformants |                         |          |          |          |
|-------------------------------------------------------------------------------|-------------------------|----------|----------|----------|
| Transformant                                                                  | SOx Activity (units/mg) |          |          |          |
|                                                                               | SOx DNA # 17            | DNA # 20 | DNA # 22 | DNA # 24 |
| 1                                                                             | 11.38                   | 8.03     | 10.89    | 13.60    |
| 2                                                                             | 6.77                    | 13.14    | 12.38    | 10.38    |
| 3                                                                             | 8.88                    | 16.81    | 8.55     | 17.03    |
| 4                                                                             | 9.37                    | 12.32    | 16.30    | 18.28    |

|   |      |       |      |       |
|---|------|-------|------|-------|
| 5 | 7.34 | 11.68 | 0.02 | 18.14 |
|---|------|-------|------|-------|

The activity of the SOx produced intracellularly by the *E. coli* host cells was greater than the activity of the SOx fusion protein produced extracellularly by other host cells as described in Examples

5

#### EXAMPLE 4

##### Expression of the *Streptomyces* sp. H-7775 in *E. coli*

- [00252] In this Example, methods for construction of an expression system for the expression of *Streptomyces* sp. H-7775 sorbitol oxidase (SOx) are described. A synthetic gene (with neutral codons) encoding the H-7775 SOx gene was used to express the sorbitol oxidase gene in *E. coli* strain BL21(DE3)pLysS. The expression vector pET 24a with the SOx gene was cloned as *Nde*I + *Bam*HI fragment. The resulting plasmid (See, Figure 3) was transformed into competent *E. coli* Top10 cells (Invitrogen) according to the manufacturer's protocol. Kanamycin resistant transformants containing the 1.2 kb SOx gene were identified by the direct colony PCR method using the TAQ Ready-to-go PCR beads (Amersham) using the PCR reaction conditions as suggested by the manufacturer. The following primers were used: us-sco1 5'gcccatatgagcgacatcacggtcacc (SEQ ID NO:21) and ls-sco1 5' ggatcctcagcccgcgagcacc (SEQ ID NO:22) in the PCR reaction.
- [00253] The SOx positive transformants were cultivated and plasmid DNA was isolated. Plasmid DNA containing the cloned SOx gene was then used to transform the commercially available BL21(DE3)pLysS competent cells (cat. # 200132; Stratagene) and following the protocol in the instruction manual.
- [00255] The entire transformation reaction was directly used to inoculate 250ml flasks containing 25ml LB, containing kanamycin (50ug/ml) and chloramphenicol (34ug/ml). The cultures were incubated overnight with shaking at 37°C. For each transformant, a 2 liter flask containing 500 mls of LB containing kanamycin (50ug/ml) and chloramphenicol (34ug/ml) was then inoculated with 25 mls of the overnight culture and was further allowed to grow for 2 hours to reach approximately OD<sub>600</sub> values of .0.4-0.6 (mid logarithmic phase). Then, IPTG at a final concentration of 1mM was added to the cultures. The cultures were further incubated for an additional 2 hours and then harvested by centrifugation. The resulting pellets were resuspended in phosphate buffer and the cells were passed through a French press for cell disruption/lysis. The SOx activity was determined using 10ul cell lysate and 990 ul assay reagent (100 mM potassium

phosphate, pH 7, 1% Sorbitol, 5mM ABTS, 10U/ml HRP) and incubated at 37°C for 3 minutes.

## EXAMPLE 5

### 5 Purification of Sorbitol Oxidase

[00256] In this Example, methods used to purify sorbitol oxidase produced by *S. lividans* (SEQ ID NO:4) are described. Sorbitol oxidase from *Streptomyces lividans* is localized in mycelia. Thus, the enzyme was isolated by cell lysis using a French press from cell-extract in 100mM Kpi buffer, potassium phosphate, pH 7.0. The cell extract was  
10 heated to 50°C for one hour, followed by centrifugation sufficient to remove the debris. The cell lysate supernatant was then mixed with ammonium sulfate to 32% saturation to precipitate the protein fraction containing sorbitol oxidase. The protein precipitate was kept at 4°C overnight and then was separated from mother liquor by centrifugation 10,000 RPM using Sorvall centrifuge and SLA-1500 Sorvall rotor.

15 [00257] The protein precipitate was then washed with 32% saturated ammonium sulfate solution. The washed protein precipitate was dissolved back in Kpi buffer and the insoluble material was discarded. The soluble fraction was dialyzed against 25 mM Kpi buffer, pH 7.0, overnight and then further purified using affinity chromatography on the reactive orange resin (Prometic). This partially purified sorbitol oxidase preparation and  
20 fermentation broth cell lysate (EFT of 108 hrs) were used as samples in experiments for biobleaching. The molecular weight of the enzyme was determined to be ~45,000 Da by SDS-PAGE gel electrophoresis. The prosthetic group is a covalently bound FAD (1mol of FAD to 1 mol of SOx).

## 25 EXAMPLE 6

### Stability and Bleaching Performance of SOx in HDL Laundry Wash Conditions

[00258] In this Example, methods to determine the stability and bleaching performance of SOx (produced as described above) in AATCC liquid detergent laundry wash conditions are described. In these experiments, AATCC standard  
30 detergent (American Association of Textile Chemists and Colorists Heavy Duty Liquid Detergent Version 2003 without brightener; key components include linear alkane sulfonate, alcohol ethoxylate, propanediol, citric acid, fatty acid, caustic soda and water; Testfabrics) was used.

[00259] Three bleachable cotton swatches with juice (STC CFT  
35 CS-15), wine (STC CFT CS-3), and tea (STC CFT BC-3) were used. The swatches were cut into 15mm circles with a textile punch (Model B equipped with a 5/8" die cutter; Model 93046; NAEF).

[00260]

Single swatch disks were placed into each well of a 24-well microplate (Costar 3526). One (1) ml of washing solution pH 8, containing per liter, 1.5 ml AATCC HDL detergent, 75-100 mM sorbitol, 6gpg hardness (diluted from stock 15000 gpg hardness solution containing 1.735 M calcium chloride and 0.67 M magnesium chloride), and 0.05% TAED (tetraacetythylenediamine, Fluka) was added to each well. Five to fifty (5-50 ul) microliters of partially purified sorbitol oxidase or sorbitol oxidase obtain from a late fermentation run (108 hr EFT "effective fermentation time") produced as described in Examples 1 and 2, were added with a positive displacement pipette to 3-8 wells in one column. The control wells contained no enzyme. The microplate was covered with a plastic lid and aluminum foil and incubated at 37°C with 100rpm gentle rotation for 14 hr. The plates were then removed from the shaker and tested for the presence of hydrogen peroxide with peroxide test strips (Baker).

[00261]

One hundred microliters (100 ul) of 0.1 mM sodium carbonate were added to each well to elevate the pH to 10. The microplates were incubated with rotation for another 90 min and the supernatants removed by aspiration. Each well was washed three (3) times with 1.5 ml Dulbecco's PBS, pH 7.3 and three (3) times with 1.5 ml distilled water. Each disk was removed from its well and dried overnight between sheets of paper towels and not exposed to direct light. The disks were inspected visually and then analyzed with a Reflectometer CR-200 (Minolta) calibrated on a standard white tile. The average L values were calculated as was the percent soil release ( $\% \text{ SR} = 100\% \times (\text{Final reflectance} - \text{Initial reflectance}) / (\text{Reflectance of a white standard} - \text{Initial reflectance})$ ). Graphs showing the results of these experiments are provided in Figures 5-8.

[00262]

The results confirmed that sorbitol oxidase is stable in a typical liquid detergent system and is able to produce effective concentration of hydrogen peroxide in presence of its substrate sorbitol mixed with detergent and available atmospheric oxygen. In addition, the sorbitol oxidase generated hydrogen peroxide in presence of a bleach booster (*i.e.*, TAED) was able to help bleach typical colored stains such as blueberry, tea and wine (See, Figures 5-8).

## EXAMPLE 7

### Substrate Range Study of Sorbitol Oxidase

[00263]

Sorbitol oxidase obtained using the methods described in Example 1, was tested for finding its activity with various polyol substrates. All substrates used in the assay were 50mM in 10 mM phosphate buffer pH 7.0. The relative activity using sorbitol as (+++++ = 100%) is shown below in Table 3. Although the activity was very high for

both substrates, it was noted that the SCO6147 SOx transformed in *S. lividans* exhibited 2.1 times more activity on D-sorbitol than on D-xylitol.

[00264] In addition to these substrates, it is contemplated that other substrates, including, but not limited to glycerol, will find use in the present invention.

5

**TABLE 3****Relative POx activities for corresponding substrates**

| Compound         | Relative Activity (+, 0) |
|------------------|--------------------------|
| D-Sorbitol       | ++++++                   |
| D-Xylitol        | ++++                     |
| D-Mannitol       | +++                      |
| D-Ribitol        | +                        |
| Myo-Inositol     | +                        |
| Glycerol         | +                        |
| 1,3-propanediol  | +/-2                     |
| 1,2-propanediol  | +/-2                     |
| Propylene glycol | 0                        |
| Ethylene glycol  | 0                        |

**Example 8**

**Combined enzyme system: Increased Hydrogen peroxide production using SOx + GOx (Glucose oxidase) and SOx + HOx (Hexose Oxidase)**

10

[00265] The activity of a combination of POx enzymes was tested. The enzymes that were tested in combination were SOx with GOx and SOx with HOx. The SOx used in the experiments was recombinant SOx purified from *S. lividans* strain transformant # 4 (SEQ ID NO:4), GOx is commercially available from Sigma (G7141) and HOx was prepared as described in WO 01/38544. HOx is available from Danisco A/S as DairyHOx™.

15

[00266] SOx, HOx and GOx have been discussed above, and can be summarized as follows.

20

[00267] Hexose oxidase (EC. 1.1.3.5) is a homo dimeric flavo enzyme containing 2 covalently bound FAD groups per molecule. The molecular weight is approx. 130 kDa. The enzyme catalyses the oxidation of D- hexose sugars including: glucose, galactose, lactose, maltose, malto-triose and cellobiose. The sugars are converted into their corresponding lactones, which undergo spontaneous hydrolysis to the corresponding

acids in aqueous environments. Upon re-oxidation of the reduced flavin group by molecular oxygen,  $H_2O_2$  is produced.

**[00268]** Sorbitol oxidase is a monomeric flavo enzyme containing one flavin group of unknown nature, bound covalently, probably to a histidine residue in the holoenzyme.

5 The molecular weight of the enzyme is approximately 45 kDa. The enzyme catalyses the oxidation of several polyols including: D-sorbitol, D-xylitol, D-glucose, D-mannitol, D-arabitol, glycerol, inositol, 1,2-propanediol, 1,3-butanediol, and 1,4-butanediol. Upon oxidation of the polyols the corresponding sugars are generated and upon contact with molecular oxygen the flavin is re-oxidized and  $H_2O_2$  is produced.

10 Glucose oxidase (EC, 1.1.3.4) is a dimeric protein with a molecular weight of about 160 kDa containing 1 non-covalently, but tightly associated flavin group per monomer. The enzyme catalyses the oxidations of D-glucose and to a lesser extent 2-deoxy-D-glucose, D-mannose and D-fructose. The sugars are converted into their corresponding lactones, which undergo spontaneous hydrolysis to the corresponding acids in aqueous  
15 environments. Upon re-oxidation of the reduced flavin group by molecular oxygen,  $H_2O_2$  is produced.

**[00269]** Activity assays of SOx, HOx or GOx activity were performed in microtiter plates (300  $\mu$ l).

**[00270]** The amount of enzyme in each combination was given as unit amount, wherein  
20 a unit of HOx is the amount of enzyme that produced 1  $\mu$ mol  $H_2O_2$ / min when substrates are in excess; a unit of GOx is the amount of enzyme that produced 1  $\mu$ mol  $H_2O_2$ / min when substrates are in excess; and a unit of SOx is the amount of enzyme that produced 1  $\mu$ mol  $H_2O_2$ / min when substrates are in excess. In the present example, only SOx had excess substrate. The generated sugar would be the limiting factor for the secondary  
25 enzyme. As noted above the activity is given as a percentage value of SOx catalysing D-sorbitol when both D-sorbitol and oxygen is in excess.

**[00271]** The commonly used horse radish peroxidase dye substrate ABTS was incorporated into an assay, measuring the production of  $H_2O_2$  produced by HOx or GOx respectively. ABTS serves as a chromogenic substrate for peroxidase. Peroxidase in  
30 combination with  $H_2O_2$  facilitates the electron transport from the chromogenic dye, which is oxidised to an intensely green/blue compound.

**[00272]** An assay mixture contained 266  $\mu$ l sorbitol (Sigma P-5504, 0.055 M in 0.1 M sodium phosphate buffer, pH 6.3), 11.6  $\mu$ l 2,2'-Azino-bis(3-ethylbenzothiazoline-6-Sulfonic acid)(ABTS)(Sigma A-9941, 5 mg/ml aqueous solution), 11.6  $\mu$ l peroxidase  
35 (POD)(Sigma P-6782, 0.1 mg/ml in 0.1 M sodium phosphate buffer, pH 6.3) and 10  $\mu$ l enzyme (SOx or HOx) aqueous solution.

[00273] The incubation was started by the addition of glucose at 25° C. The absorbance was monitored at 405 nm in an ELISA reader. A standard curve, based on varying concentrations of H<sub>2</sub>O<sub>2</sub>, was used for calculation of enzyme activity according to the definition above.

5 [00274] Initial velocities were measured over 5 minutes in 300 uL ABTS assay (as described previously). The production of rate of hydrogen peroxide production was extrapolated from a standard curve. The measured activity is given as a percentage value. 100 % is defined as the rate of hydrogen peroxide production by sorbitol oxidase alone, when the substrates D-sorbitol and oxygen is in excess (Linear velocity curves).

10 [00275] Figure 9 (A-B) shows the initial velocity of H<sub>2</sub>O<sub>2</sub> production using the compositions with between 1x and 3000x excess of the oxidoreductases (glucose oxidase & hexose oxidase) compared to the polyol oxidase (sorbitol oxidase) measured over 5 minutes in 300 ul ABTS assay. A dramatic synergy was seen with both glucose oxidase and hexose oxidase, with up to 250-300% increase in hydrogen peroxide  
15 production rate seen. Dosages greater than 1x, such as at least 2x of the oxidoreductase compared to the polyol oxidase resulted in increased hydrogen peroxide production.

### Example 9

#### 20 Construction of *Streptomyces lividans* & *Bacillus subtilis* Strains Expressing Polyol Oxidases (POx)

[00276] Table 4 shows the elements of polynucleotide constructs that were generated to express POx enzymes derived from *A. cellulolyticus*, *Arthrobacter* sp, and  
25 *Streptomyces* H-7775 in *Bacillus* and/or *Streptomyces* host cells.

TABLE 4

| Expression Constructs | Polyol Oxidase (POx) Genes (sorbitol/xylitol oxidases)                                          | N-terminal fusion to POx             | Expression Host              |
|-----------------------|-------------------------------------------------------------------------------------------------|--------------------------------------|------------------------------|
| pSMM-ES2              | <i>Acidothermus cellulolyticus</i> 11B Q2E2H5 (synthetic gene); amino acid sequence SEQ ID NO:6 | signal peptide SCO7637 SEQ ID NO: 31 | <i>Streptomyces lividans</i> |
| pSMM-POx(intra)       | <i>Acidothermus cellulolyticus</i> 11B Q2E2H5 (synthetic gene) amino acid sequence SEQ ID NO:6  | none                                 | <i>Streptomyces lividans</i> |

|                           |                                                                                                         |                                             |                                  |
|---------------------------|---------------------------------------------------------------------------------------------------------|---------------------------------------------|----------------------------------|
| pSM-ES3                   | <i>Arthrobacter sp.</i> FB 24<br>(Q4NJLO) (synthetic gene)<br>amino acid sequence SEQ ID<br>NO:12       | Signal peptide<br>SCO0624<br>SEQ ID NO:39   | <i>Streptomyces<br/>lividans</i> |
| pSM CG-SOx                | <i>Streptomyces</i> H-7775<br>(synthetic gene)<br>amino acid sequence SEQ ID<br>NO:2                    | Cgt signal<br>sequence.<br>SEQ ID NO:36     | <i>Bacillus subtilis</i>         |
| pSM CG-SOx<br>(ES2 Acid.) | <i>Acidothermus cellulolyticus</i> 11B<br>Q2E2H5 (synthetic gene)<br>amino acid sequence SEQ ID<br>NO:6 | Cgt signal<br>sequence.<br>SEQ ID NO:<br>36 | <i>Bacillus subtilis</i>         |

For extracellular expression of POx, the following signal sequences were fused to the mature form of the POx enzyme as described herein.

MGFGSAPIALCPLRTRRNALKRLLALLATGVSIVGLTALAGPPAQA (SEQ ID NO:31)

5 secreted endoglucanase from *Streptomyces coelicolor* SCO7363

MHEPHLDRRLFLKGTAVTGAALALGATAAPTASA (SEQ ID NO:39) possible secreted protein *Streptomyces coelicolor* SCO0624

10 MKKFLKSTAALALGLSLTFGLFSPAQA (SEQ ID NO:36) amino acid signal sequence of the *Bacillus circulans* cyclomaltodextrin glucanotransferase (cgt) precursor (accession P43379).

15

### Example 10

#### Construction of *Streptomyces lividans* & *Bacillus subtilis* Strains Expressing Polyol Oxidase (POx) from *Acidothermus cellulolyticus* 11B

[00277] In this example, experiments were conducted to functionally characterize  
20 the putative polyol oxidase (xylitol oxidase) Q2E2H5 from *Acidothermus cellulolyticus* 11B. The putative POx protein was expressed in two different host organisms (*Streptomyces lividans* and *Bacillus subtilis*). The POx protein sequence was retrieved from the database (SEQ ID NO:6). A synthetic polynucleotide encoding the  
25 *Acidothermus cellulolyticus* 11B Q2E2H5 POx protein was obtained from GeneArt (Germany).

[00278] The *Acidothermus* POx synthetic gene was designed with a truncated signal sequence ALAGPPAQA (SEQ ID NO:32) from a secreted endoglucanase from *Streptomyces coelicolor* SCO7363. *Acidothermus* POx was expressed in *Streptomyces* as a fusion protein (SEQ ID NO:35), resulting in the N-terminal extension

5 MGFGSAPIALCPLRTRRNALKRLLALLATGVSIVGLTALAGPPAQA (SEQ ID NO:31)  
(from SCO7363 signal sequence) fused to the mature POx sequence (SEQ ID NO:6).  
The amino acid sequence encoded by the synthetic *Acidothermus* POx gene (SEQ ID NO:33) is as follows.

10 ALAGPPAQAMDGGKRCRDGTPQPPAPSEQVTPSAAASLRAAYDVEVSAPRLRNWAGN  
IAFRPRRYVQPRDLDELVEIIRVSDQVRVLGTGHSFNPIADTTGTLSLDHLPREVRVMPG  
RTAVSAGTRYGDLAFPLHEAGWALANVGSLPHISAGACATATHGSGDRNGCLATAVAG  
MTGVDGTCRVFHLTAESPEFFGAVVHLGALGAVTEIELVTEPTFTVRQWVYEDAPLDNV  
FADLDDVTSAAYSVSIFTTWDPPPTARQIWLKERVAAGRPDPPARRWGGRLAERDHNPV  
15 PGMPPENCTPQLGRIGPWHERLPHFRLDVTSPAGDELQSEYFVPRAAAVEAYRALRHIG  
SRIAPVLQISEIRTVADELWLSPAYHRPSVAFHFTWIADEEAVRPVVSEVERALAPLQPR  
PHWGLFTMDPAVVRAAYPRFDDFVALAERYDPEGKFQNDFLRRFFAG (SEQ ID  
NO:33)

20 The polynucleotide sequence of the synthetic *Acidothermus* POx gene (SEQ ID NO:34) is as follows.

GCGCTAGCGGGCCCGCCGGCCAGGCCATGGATGGCGGCAAGCGCTGCCGCGAC  
GGCACCCCGCAGCCGCGCGGCCCGTCCGAGCAGGTACCCCGTCGGCCGCGCCG  
TCCCTGCGGGCCGCCTACGACGTGGAGGTCTCCGCCCCGCGCCTGCGCAACTGGG  
25 CCGGCAACATCGCCTTCCGCCCCGCGCCGCTACGTCCAGCCGCGCGACCTCGACGA  
GCTGGTTCGAGATCATCCGGGTCTCCGACCAGGTCCGCGTCCTGGGCACCGGCCAC  
TCCTTCAACCCCATCGCCGACACCACCGGCACCCTGATCTCCCTGGACCACCTGCC  
GCGCGAGGTCCGCGTCATGCCGGGCCGACCGCGGTCTCCGCGGCACCCGCTA  
CGGCGACCTGGCCTTCCCGCTGCACGAGGCCGGCTGGGCCCTGGCCAACGTTCGG  
30 CTCCCTGCCGCACATCTCCATCGCCGGCGCCTGCGCCACGGCCACCCACGGCTCC  
GGCGACCGCAACGGCTGCCTGGCCACCGCCGTCGCCGGCATGACCGGCGTCGAC  
GGCACCTGCCGCGTGTTCCACCTGACCGCCGAGTCCCCGAGTTCCCGGGCGCCG  
TCGTCCACCTGGGCGCCCTGGGCGCCGTCACCGAGATCGAGCTGGTCACCGAGCC  
GACCTTCACCGTCCGCCAGTGGGTCTACGAGGACGCCCCGCTGGACAACGTGTTC  
35 GCCGACCTGGACGACGTACCTCCGCCGCCTACTCGGTCTCCATCTTACACACCTG  
GGACCCGCCGACCGCCCGGCAGATCTGGCTGAAGGAGCGCGTCGCCGCCGGCCG  
CCCGGACCCGCCGGCCCGCCGCTGGGGCGGCCGCTCGCCGAGCGCGACCAAA

CCCC GTCCCGGGGATGCCGCCCCGAGAACTGCACCCCCCAGCTGGGCGGCATCGGC  
 CCGTGGCACGAGCGCCTGCCGCACTTCCGCCTGGACGTCACCCCCTCCGCGGGCG  
 ACGAGCTGCAGTCCGAGTACTTCGTCCCGCGCGCCGCGCCGTGAGGCCTACCG  
 CGCCCTGCGCCACATCGGCTCCCGCATCGCCCCGGTCCTGCAGATCTCCGAGATC  
 5 CGCACCGTCGCCGCGGACGAGCTGTGGCTGTCCCCGGCCTACCACCGCCCGTCCG  
 TCGCCTTCCACTTCACCTGGATCGCCGACGAGGAGGCCGTCCGCCCCGGTGGTCTC  
 CGAGGTCGAGCGCGCCCTGGCCCCGCTGCAGCCGCGCCCGCACTGGGGCAAGCT  
 GTTCACGATGGACCCGGCCGTCTGTCGCGCCGCTACCCGCGCTTCGACGACTTC  
 GTCGCCCTGGCCGAGCGCTACGACCCCGAGGGCAAGTTCAGAACGACTTCCTGC  
 10 GCCGCTTCTTCGCCGGCTAAGGATCC (SEQ ID NO:34).

**[00279]** A restriction site for *nhe1* (**GCTAGC**) was introduced at the 5' end of the synthetic gene to allow fusion to the truncated SCO7363 endoglucanase signal sequence. The *Bam*H1 (**GGATCC**) restriction site was added at the 3' end of the gene.

15 Restriction digestion with *nhe1* plus *Bam* H1 resulted in the DNA fragment which was ligated with the *nhe1*/*Bam*H1 cut *Streptomyces* expression plasmid pKB 105. The cloning resulted in the expression construct pSMM-ES2 with a full length signal peptide for expression of secreted POx proteins in *Streptomyces lividans* (SEQ ID NO: 35).

20 MGFGSAPIALCPLRTRRNALKRLLALLATGVSIVGLTALAGPPAQAMDGGKRCRDGTPQ  
 PPAPSEQVTPSAAASLRAAYDVEVSAPRLRNWAGNIAFRPRRYVQPRDLDELVEIRVSD  
 QVRVLGTGHSFNPIADTTGTLISLDHLPREVRVMPGRTAVSAGTRYGDLAFLPLHEAGWA  
 LANVGSLPHISIAGACATATHGSGDRNGCLATAVAGMTGVDGTCRVFHLTAESPEFPGA  
 VVHLGALGAVTEIELVTEPTFTVRQWVYEDAPLDNVFADLDDVTSAAYSVSIFTTWDPTT  
 25 ARQIWLKERVERAAGRPDPARRWGGRLAERDHNVPVGMPPENCTPQLGRIGPWHERLP  
 HFRLDVTPSAGDELQSEYFVPRAAAVEAYRALRHIGSRIAPVLQISEIRTVAADELWLSPA  
 YHRPSVAFHFTWIADEEAVRPVVSEVERALAPLQPRPHWGKLFMDPAVVRAAYPRFD  
 DFVALAERYDPEGKFQNDLRRFFAG (SEQ ID NO:35)

30 **[00280]** The DNA fragment encoding the mature POx protein from *Acidothermus* (SEQ ID NO:6) (without a signal peptide) was also generated (SEQ ID NO:42).

**CCATGGATGGCGGCAAGCGCTGCCGCGACGGCACCCCGCAGCCGCCGGCCCCGT**  
 CCGAGCAGGTCACCCCGTCCGCCGCGCCTCCCTGCGGGCCGCCTACGACGTGGA  
 GGTCTCCGCCCGCGCCTGCGCAACTGGGCCGGCAACATCGCCTTCCGCCCGCGC  
 35 CGCTACGTCCAGCCGCGCGACCTCGACGAGCTGGTCGAGATCATCCGGGTCTCCG  
 ACCAGGTCCGCGTCCTGGGCACCGGCCACTCCTTCAACCCCATCGCCGACACCACC  
 GGCACCCTGATCTCCCTGGACCACCTGCCGCGCGAGGTCCGCGTCATGCCGGGGCC

GCACCGCGGTCTCCGCCGGCACCCGCTACGGCGACCTGGCCTTCCCGCTGCACGA  
 GGCCGGCTGGGCCCTGGCCAACGTCGGCTCCCTGCCGCACATCTCCATCGCCGGC  
 GCCTGCGCCACGGCCACCCACGGCTCCGGCGACCGCAACGGCTGCCTGGCCACC  
 GCCGTCGCCGGCATGACCGGCGTCGACGGCACCTGCCGCGTGTTCCACCTGACCG  
 5 CCGAGTCCCCCGAGTTCCCGGGCGCCGTCGTCCACCTGGGCGCCCTGGGCGCCGT  
 CACCGAGATCGAGCTGGTCACCGAGCCGACCTTCACCGTCCGCCAGTGGGTCTAC  
 GAGGACGCCCCGCTGGACAACGTGTTGCGCCGACCTGGACGACGTCACCTCCGCCG  
 CCTACTCGGTCTCCATCTTCACCACCTGGGACCCGCCGACCGCCCGGCAGATCTGG  
 CTGAAGGAGCGCGTCGCCGCCGGCCGCCCGGACCCGCCGGCCCGCCGCTGGGGC  
 10 GGCCGCCTCGCCGAGCGCGACCAACAACCCCGTCCCGGGGATGCCGCCCGAGAACT  
 GCACCCCCCAGCTGGGCCGCATCGGCCCGTGGCACGAGCGCCTGCCGCACTTCCG  
 CCTGGACGTCACCCCCTCCGCGGGCGACGAGCTGCAGTCCGAGTACTTCGTCCCG  
 CGCGCCGCCGCCGTCGAGGCCTACCGCGCCCTGCGCCACATCGGCTCCCGCATCG  
 CCCCAGTCTGAGATCTCCGAGATCCGCACCGTCGCCGCCGACGAGCTGTGGCT  
 15 GTCCCCGGCCTACCACCGCCCGTCCGTGCGCTTCCACTTCACCTGGATCGCCGACG  
 AGGAGGCCGTCCGCCCGGTGGTCTCCGAGGTGCGAGCGCGCCCTGGCCCCGCTGC  
 AGCCGCGCCCGCACTGGGGCAAGCTGTTACGATGGACCCGGCCGTGTCGCGCGC  
 CGCCTACCCGCGCTTCGACGACTTCGTGCGCCCTGGCCGAGCGCTACGACCCCGAG  
 GGCAAGTTCCAGAACGACTTCCTGCGCCGCTTCTTCGCCGGCTAAGGATCC (SEQ  
 20 ID NO:42).

[00281] Restriction digestion with Nco1 and BamH1 resulted in a DNA  
 fragment which was ligated to the Nco1 partial digest plus BamH1 cut pKB 105 vector .  
 Cloning resulted in the expression construct pSMM-POx(intra), for the intracellular  
 25 expression of the mature *Acidothrmus* POx protein.

[00282] The expression plasmids pSMM-ES2 and pSMM-POx(intra) were  
 transformed into *Streptomyces lividans* strain g3s3 and 10 transformants each were  
 selected and grown in TS medium for 2- 3 days in the presence of 50 ug/ml thiostrepton  
 at 30°C. Cells were then transferred to a production medium free of antimicrobials and  
 30 growth was continued for another three days. Then, 1 ml cultures was collected and  
 centrifuged under conditions sufficient to separate the cells from the supernatants. The  
 supernatants and cell pellets obtained were tested in enzyme activity assays.

[00283] The Nco1/BamH1 digested DNA fragment was also used to construct the  
 expression plasmid pSM CG-SOx (ES2 *Acid.*). The *Bacillus subtilis* vector pCG (Danisco  
 35 A/S) was digested with Nco1/BamH1 and ligated to the POx DNA fragment. The  
 resulting fusion protein (SEQ ID NO:37) comprises the mature POx gene to the 27 amino

acids signal peptide MKKFLKSTAALALGLSLTFGLFSPAQA (SEQ ID NO: 36) of the *Bacillus circulans* cyclomaltodextrin glucanotransferase precursor (accession P43379).

MKKFLKSTAALALGLSLTFGLFSPAQAMDGGKRCRDGTPQPPAPSEQVTPSAAASLRA  
 5 AYDVEVSAPRLRNWAGNIAFRPRRYVQPRDLDELVEIRVSDQVRVLGTGHSFNPIADTT  
 GTLISLDHLPREVRVMPGRTAVSAGTRYGDLAFPLHEAGWALANVGSLPHISIAGACATA  
 THGSGDRNGCLATAVAGMTGVDGTCRVFHLTAESPEFPGAVVHLGALGAVTEIELVTEP  
 TFTVRQWVYEDAPLDNVFADLDDVTSAAYSVSIFTTWDPPTARQIWLKERVERAAGRDPDP  
 ARRWGGRLAERDHNPVPGMPPENCTPQLGRIGPWHERLPHFRLDVTPSAGDELQSEY  
 10 FVPRAAAVEAYRALRHIGSRIAPVLQISEIRTVADELWLSPAYHRPSVAFHFTWIADEEA  
 VRPVVSEVERALAPLQPRPHWGKLFTMDPAVVRAAYPRFDDFVALAERYDPEGKFQND  
 FLRRFFAG (SEQ ID NO:37).

[00284] The pSM CG-SOx (ES2 *Acid.*) and pSM CG-SOx plasmids were used for  
 15 transformation of the *Bacillus subtilis* OS21 strain. Transformants were isolated and  
 grown in 5 ml LB media containing 50mg/li Kanamycin. The cultures were cultivated at  
 30°C with shaking at 180 rpm for 24 hours. SOx activities were determined from culture  
 supernatants of host cells secreting the SOx and cell lysates of host cells that produced  
 SOx intracellularly.

20 [00285] The data obtained from the experiments showed that the SOx produced from  
 host cells that had been transformed with a polynucleotide encoding the mature form of  
 the SOx enzyme (i.e. lacking the sequence encoding the signal peptide) had greater SOx  
 activity than the SOx produced by host cells that had been transformed with a  
 polynucleotide encoding the fusion SOx protein (i.e. a SOx fusion protein comprising the  
 25 mature form operably linked to a signal peptide).

### Example 13

#### Construction of *Streptomyces lividans* Strains Expressing Polyol Oxidase (POx) 30 from *Arthrobacter sp.* FB 24

[00286] In this example, experiments were conducted to functionally characterize  
 the putative polyol oxidase (FAD linked oxidase) (Q4NJLO; SEQ ID NO:11) from  
*Arthrobacter sp.* FB 24 are described. The putative POx protein was expressed in  
 35 *Streptomyces lividans*. The POx protein sequence was retrieved from the database.  
 Synthetic gene encoding the *Arthrobacter sp.* FB 24 (Q4NJLO) POx protein was obtained  
 from GeneArt (Germany).

[00287] The *Arthrobacter sp.* FB 24 POx synthetic gene was designed with an N-terminal extension MHEPHLDRRLFLKGTAVTGAALALGATAAPTASA (SEQ ID NO:39) derived from a possible secreted *Streptomyces coelicolor* A3(2) protein SCO0624 resulting in the protein sequence below (SEQ ID NO:40). Two glycine residues were inserted after the initiator methionine of the signal sequence and before the start methionine of the mature POx protein.

MGHEPHLDRRLFLKGTAVTGAALALGATAAPTASAGMRTVSELPGLSGSTGAGSSAPE  
 LNWAGNYRYTAASIHRPRTLEEVQEVVAGASKIRALGSRHSFNIAIDSPGSLVSLEDLDP  
 10 GIRIDAATRTVTVSGGTRYGTAEQLESAGFALSNLASLPHISVAGAIATATHGSGDANG  
 NLATSVAALELVAADGTVHRLNRGSSPGFDGAVVGLGALGVVTKVTLTDIEPTFTVRQDV  
 FEALPWDTV LGNFDAVTSSAYSVSLFTDWSGDDVAQAWLKSRLSGSAASSDAGSTLAG  
 EAFAAGTFFGGTRAGVARHPLPGVSAENCTEQLGVPGSWSERLAHFRMAFTPSSGEEL  
 QSEFFVRREHAVAAIGELRALSDRITPLLLVSEIRTVAADKLWLSTAYGQDSVGFHFTWK  
 15 QRQDEVEKVLPMEEALAPFNARPHWGKLFHAGADAVAELYPRFSDFKDLAERMDPE  
 QKFRNEFLARKVFGN (SEQ ID NO:40)

[00288] The two glycine residues resulted from incorporating restriction sites Nco1 and sph1 as shown in the synthetic gene sequence below (SEQ ID NO:41). The synthetic  
 20 POx gene was cut with the restriction enzymes Nco1 and BamH1 and the resulting DNA fragment was ligated to the Nco1 partial and BamH1 digested *Streptomyces* vector pKB105. This resulted in the expression vector pSM-ES3. This plasmid was transformed into *Streptomyces lividans* strain g3s3 and 10 transformants were selected and grown in TS medium for 2- 3 days in the presence of 50 ug/ml thiostrepton at 30°C. Cells were  
 25 then transferred to a production medium free of antimicrobials and growth was continued for another three days. Then, 1 ml cultures was collected and centrifuged under conditions sufficient to separate the cells from the supernatants. The supernatants and cell pellets obtained were tested in enzyme activity assays.

30 ACCATGGGCCACGAGCCGCACCTGGACCGCCGCCTGTTCTCTGAAGGGCACCGCCG  
TCACCGGCGCCGCCCTGGCCCTGGGCGCCACCGCCGCCCGACCGCCTCCGCCG  
GCATGCGCACGGTCTCCGAGCTGCCGGGCCTGTCTGGGCTCCACCGGCGCCGGCT  
 CCTCCGCCCCCGAGCTGAACTGGGCGGCACTACCGCTACACCGCCGCCTCCAT  
 CCACCGCCCCGCGCACCCCTCGAGGAGGTCCAGGAGGTCTGTCGGGGCGCCTCCAA  
 35 GATCCGCGCCCTGGGCTCCCGCCACTCCTTCAACGCCATCGCCGACTCCCCGGGC  
 AGCCTGGTCTCCCTCGAGGACCTGGACCCGGGCATCCGCATCGACGCCGCCACCC  
 GCACCGTCACGGTCTCGGGCGGCACGCGCTACGGCACCCCTGGCCGAGCAGCTCGA

GTCCGCCGGCTTCGCCCTGTCCAACCTGGCCTCCCTGCCGCACATCTCCGTCGCCG  
 GCGCCATCGCCACCGCCACCCACGGCTCCGGCGACGCCAACGGCAACCTGGCCAC  
 CTCCGTGCGCCGCCCTCGAGCTGGTCGCGGCCGACGGCACCGTCCACCGCCTGAAC  
 CGCGGCTCCTCCCCGGGCTTCGACGGCGCGGTCTGTCGGCCTGGGCGCCCTGGGC  
 5 GTCGTCACCAAGGTCACCCTGGACATCGAGCCGACCTTCACCGTCCGCCAGGACGT  
 GTTCGAGGCCCTGCCGTGGGACACCGTCCTGGGCAACTTCGACGCCGTACCTCCT  
 CCGCCTACTCCGTGTCCCTGTTACCGACTGGTCCGGCGACGACGTGCCCCAGGC  
 CTGGCTGAAGTCCCGCCTGTCCGGCTCCGCCGCCTCCTCCGACGCCGGCTCCACC  
 CTGGCCGGCGAGGCCTTCGCCGCCGGCACCTTCTTCGGCGGCACCCGCGCCGGC  
 10 GTCGCCCCGCCACCCGCTGCCGGGCGTGTCCGCCGAGAACTGCACCGAGCAGCTGG  
 GCGTCCCGGGCTCCTGGTCCGAGCGCCTGGCCCACTTCCGCATGGCCTTCACCCC  
 GTCCTCCGGCGAGGAGCTGCAGTCCGAGTTCTTCGTCCGCCGCGAGCACGCCGTG  
 GCCGCCATCGGCGAGCTGCGCGCCCTGTCCGACCGCATCACCCCGCTGCTGCTGG  
 TCTCCGAGATCCGCACCGTCGCCGCCGACAAGCTGTGGCTGTCCACCGCCTACGG  
 15 CCAGGACTCCGTCCGCTTCCACTTCACCTGGAAGCAGCGCCAGGACGAGGTGAG  
 AAGGTCCTGCCGGTCATGGAGGAGGCCCTGGCCCCGTTCAACGCCCGCCCGCACT  
 GGGGCAAGCTGTTCCACGCCGGCGCCGACGCCGTGCGCGAGCTGTACCCGCGCTT  
 CTCCGACTTCAAGGACCTGGCCGAGCGCATGGACCCCGAGCAGAAGTTCCGCAAC  
 GAGTTCCTGGCCCGCAAGGTGTTCCGGCAACTGATGAGGATCCG (SEQ ID NO:41).

20

#### Example 14

##### Bleaching Performance of SO<sub>x</sub> in combination with perhydrolase in HDL Laundry Wash Conditions

[00289] The effect of a cleaning composition containing a PO<sub>x</sub> enzyme and a  
 25 perhydrolase in bleaching a stained fabric is tested.

[00290] The effect of bleaching a stained fabric with a composition comprising  
 perhydrolase *M. smegmatis* perhydrolase, variant S54V described in PCT/US05/056782  
 in combination with SO<sub>x</sub> is tested on fabric stained with tea, or wine or blueberry  
 essentially as described in Example 5 but without TAED. The bleaching effect of SO<sub>x</sub>  
 30 alone on the stained fabric swatches is performed as described in Example 5. To  
 determine the bleaching effect of a combination of SO<sub>x</sub> and perhydrolase on fabric  
 swatches stained in the same manner as those tested for SO<sub>x</sub> alone, 1 ppm of  
 perhydrolase and 5mM perhydrolase substrate (ester) are combined with the sorbitol  
 oxidase and added to the stained swatch disks in the microplate. The percent soil  
 35 release is determined and the bleaching performance of SO<sub>x</sub> in combination with  
 perhydrolase is calculated.

[00291] The bleaching effect of the combination of perhydrolase with SOx is significantly greater than the bleaching effect obtained with SOx alone.

Therefore, this example illustrates the usefulness of combining a POx and a perhydrolase in cleaning compositions.

5

[00292] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

10 [00293] Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

15 [00294] Those of skill in the art readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The compositions and methods described herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. It is readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

20 [00295] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features,

25 modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

30 [00296] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

35

**CLAIMS**

We claim:

- 5 1. An isolated chimeric polynucleotide comprising a sequence encoding a mature polyol oxidase protein (POx), said sequence being operably linked to a sequence encoding a secretory signal peptide, said signal peptide being derived from a prokaryotic microorganism.
- 10 2. The isolated polynucleotide of Claim 1, wherein said sequence encoding a mature POx is derived from a *Streptomyces sp.*, an *Acidothermus sp.* or an *Arthrobacter sp.*
3. The chimeric polynucleotide of Claim 1, wherein said chimeric polynucleotide has a sequence selected from SEQ ID NO:35, 37, 38, and 40.
- 15 4. The chimeric polynucleotide of Claim 1, wherein said sequence encoding said secretory signal peptide is selected from the signal sequence encoded by the *S. coelicolor* gene SCO7637, the signal sequence encoding the *S. lividans* gene SCO 0624 and the signal sequence encoding the *B. subtilis* gene P43379.
- 20 5. A recombinant expression vector comprising the isolated chimeric polynucleotide of Claim 2.
6. A host cell comprising the recombinant expression vector of Claim 5.
- 25 7. A host cell comprising a polynucleotide encoding a POx polypeptide, wherein said Pox polypeptide is selected from SEQ ID NO: 6, 35, 37, 38 and 40.
8. The host cell of Claim 7, wherein said POx has sorbitol oxidase and/or xylitol oxidase activity.
- 30 9. The host cell of Claim 7, wherein said polynucleotide is present in the genome of said host cell or in a vector that autonomously replicates in said host cell.
- 35 10. The host cell of Claim 7, wherein said host cell is an *S. lividans*, a *B. subtilis* or an *Acidothermus cellulolyticus* host cell.

11. A method for producing a polypeptide having POx activity comprising:

(a) transforming a host cell with a recombinant expression vector, said expression vector comprising a polynucleotide sequence encoding a POx polypeptide having a sequence selected from SEQ ID NO:6, 35, 37, 38, and 40;

5 (b) growing said host cell comprising said recombinant expression vector under conditions suitable for the expression of said POx polypeptide; and

(c) recovering said POx polypeptide.

12. The method of Claim 11, wherein said POx activity is sorbitol and/or xylitol activity.

10

13. The method of Claim 11, wherein said host cell is selected from Bacillus cells or Streptomyces cells.

14. The method of Claim 11, wherein said expression of said POx is extracellular.

15

15. The method of Claim 11, wherein said expression of said POx is intracellular.

16. A cleaning composition comprising an effective amount of an isolated POx comprising an amino acid sequence that is at least about 70% identical to a POx having a sequence selected from SEQ ID NO:2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14.

20

17. The cleaning composition of Claim 16, wherein said cleaning composition is a detergent.

25

18. The cleaning composition of Claim 17, further comprising a bleach activator.

19. The cleaning composition of Claim 16, further comprising at least one additional enzyme.

30

20. The cleaning composition of Claim 19, wherein said at least one additional enzyme is selected from hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, oxidoreductases, perhydrolases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, mannanases,  $\beta$ -glucanases, arabinosidases, hyaluronidases, chondroitinases, laccases, and amylases, or mixtures thereof.

35

21. The cleaning composition of Claim 19, wherein said at least one additional enzyme is a perhydrolase.

5 22. The cleaning composition of Claim 19, wherein said at least one additional enzyme is a glucose oxidase.

23. The cleaning composition of Claim 19, wherein said cleaning composition is a bleaching composition.

10 24. A method of cleaning, comprising the step of contacting a hard surface and/or an article comprising a fabric with the cleaning composition of Claim 16.

25. The method of Claim 24, further comprising the step of rinsing said surface and/or article after contacting said surface or article with said cleaning composition.

15

26. The method of Claim 24, wherein said surface and/or article comprising a fabric is stained with a substance containing at least one polyol.

20 27. The method of Claim 26, wherein said polyol is D-sorbitol, D-xylitol, D-mannitol, D-ribitol, myo-inositol, glycerol, 1,3,-propanediol or 1,2-propanediol.

28. The method of Claim 26, wherein said surface and/or said fabric article is soiled with juice, wine and/or tea.

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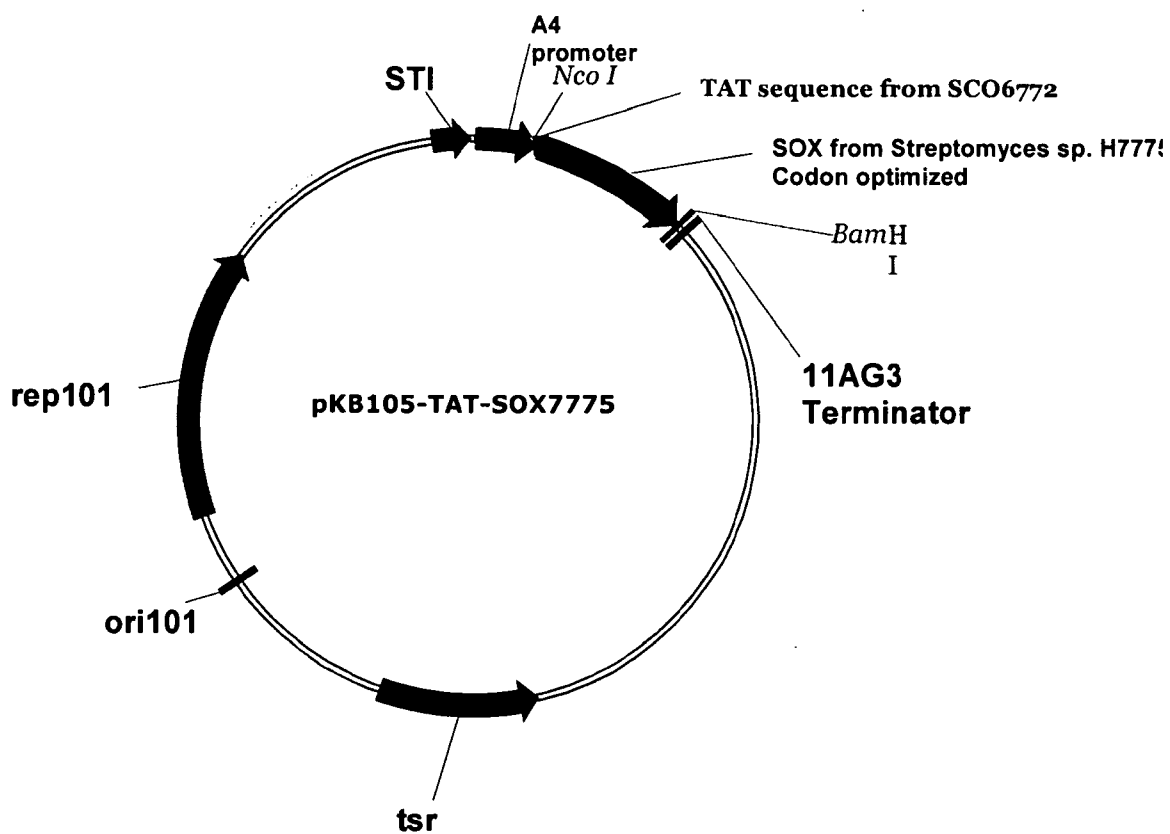


Figure 1

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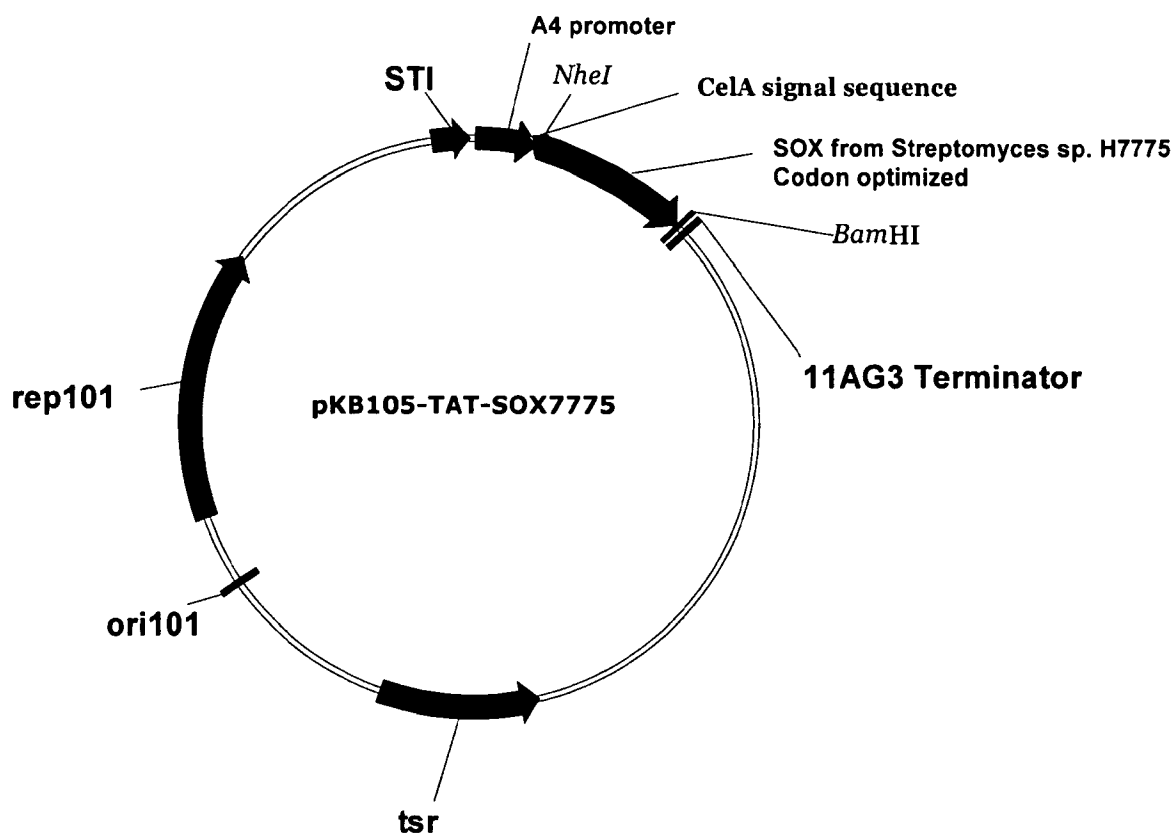


Figure 2

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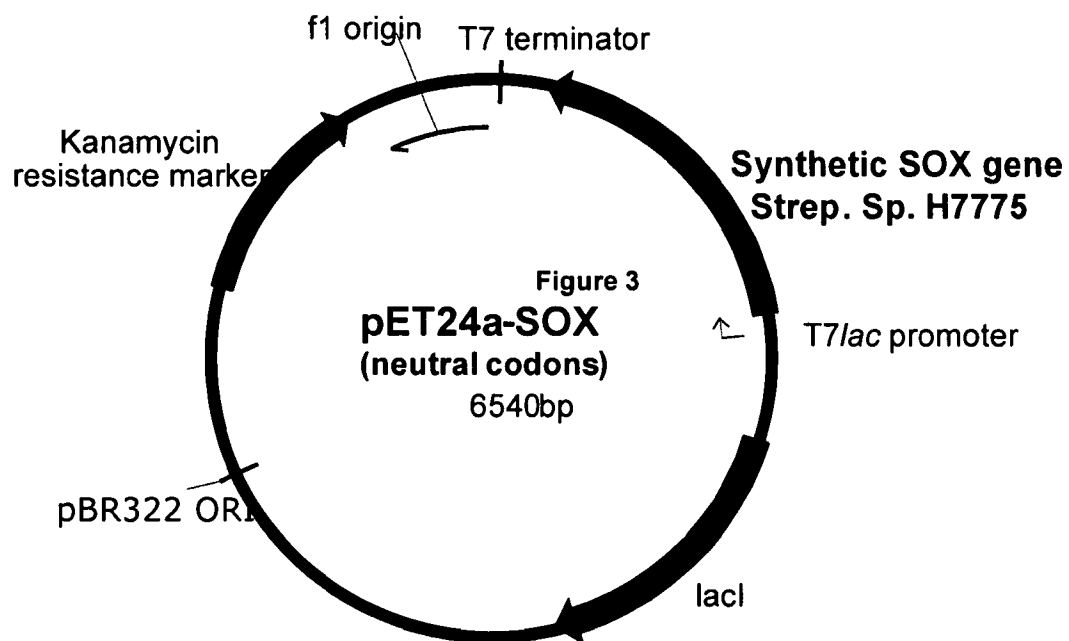


Figure 3

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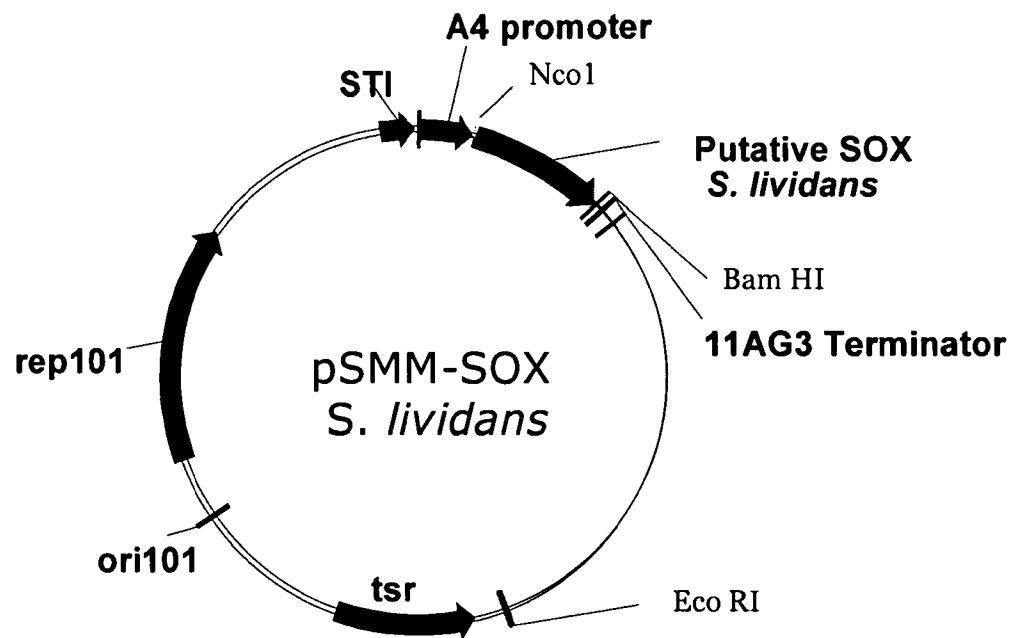


Figure 4

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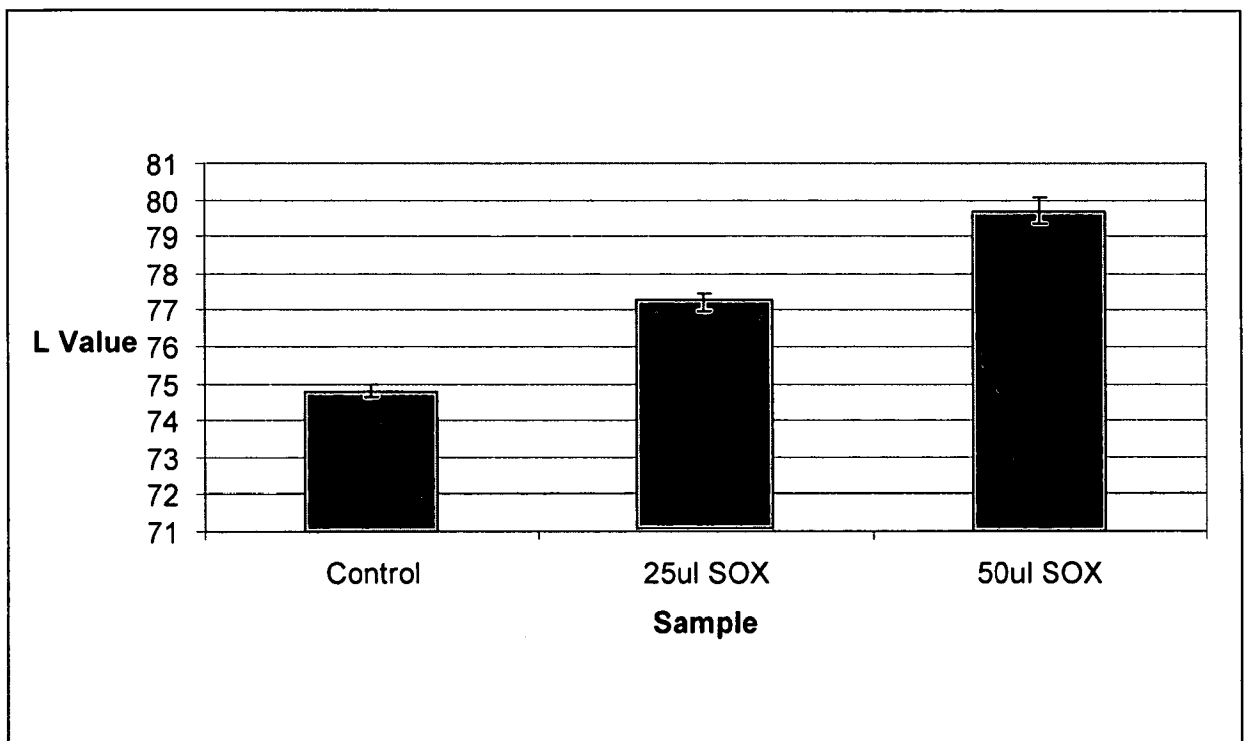


Figure 5

6/10

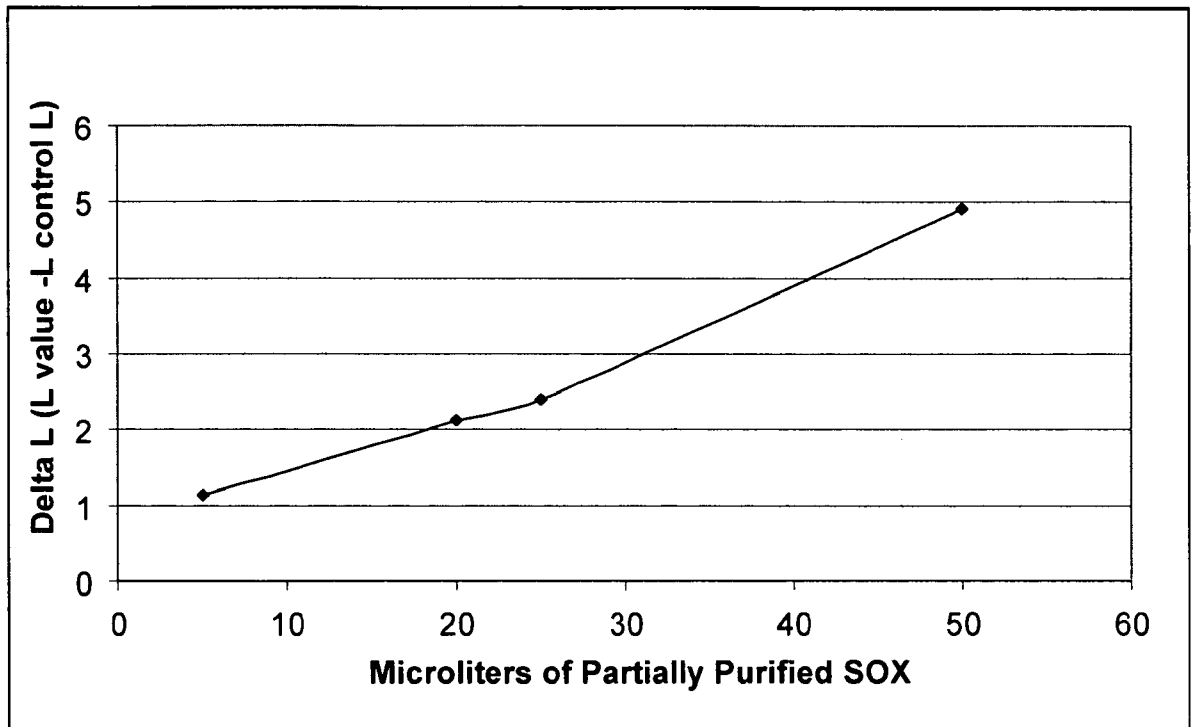


Figure 6

7/10

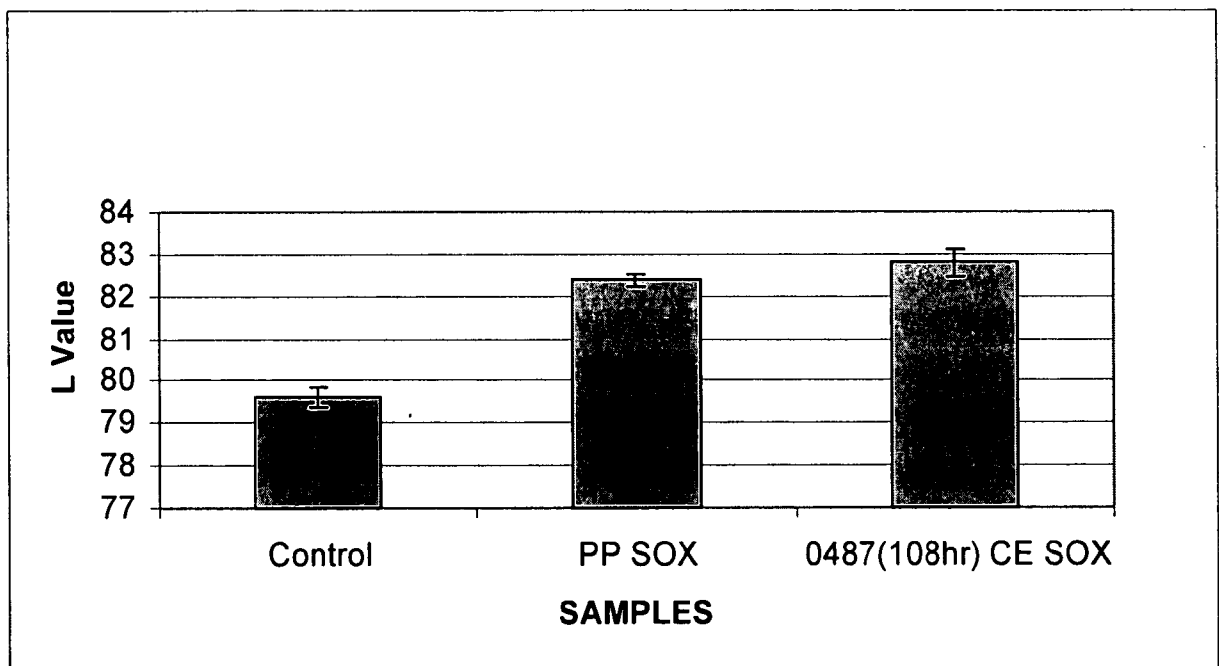


Figure 7

8/10

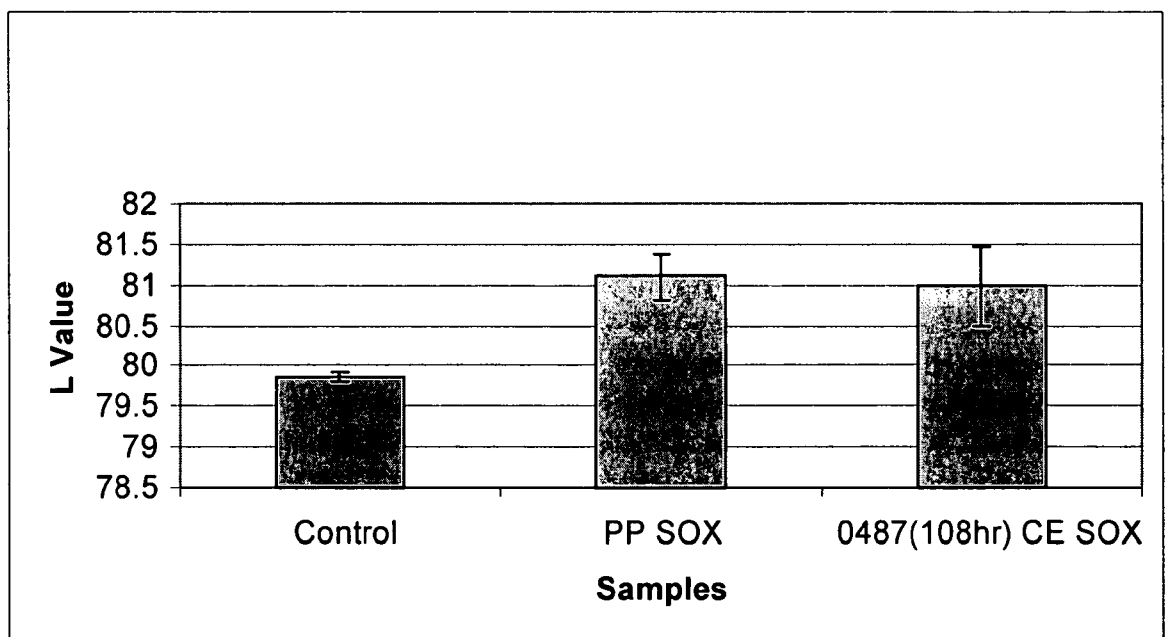


Figure 8

9/10

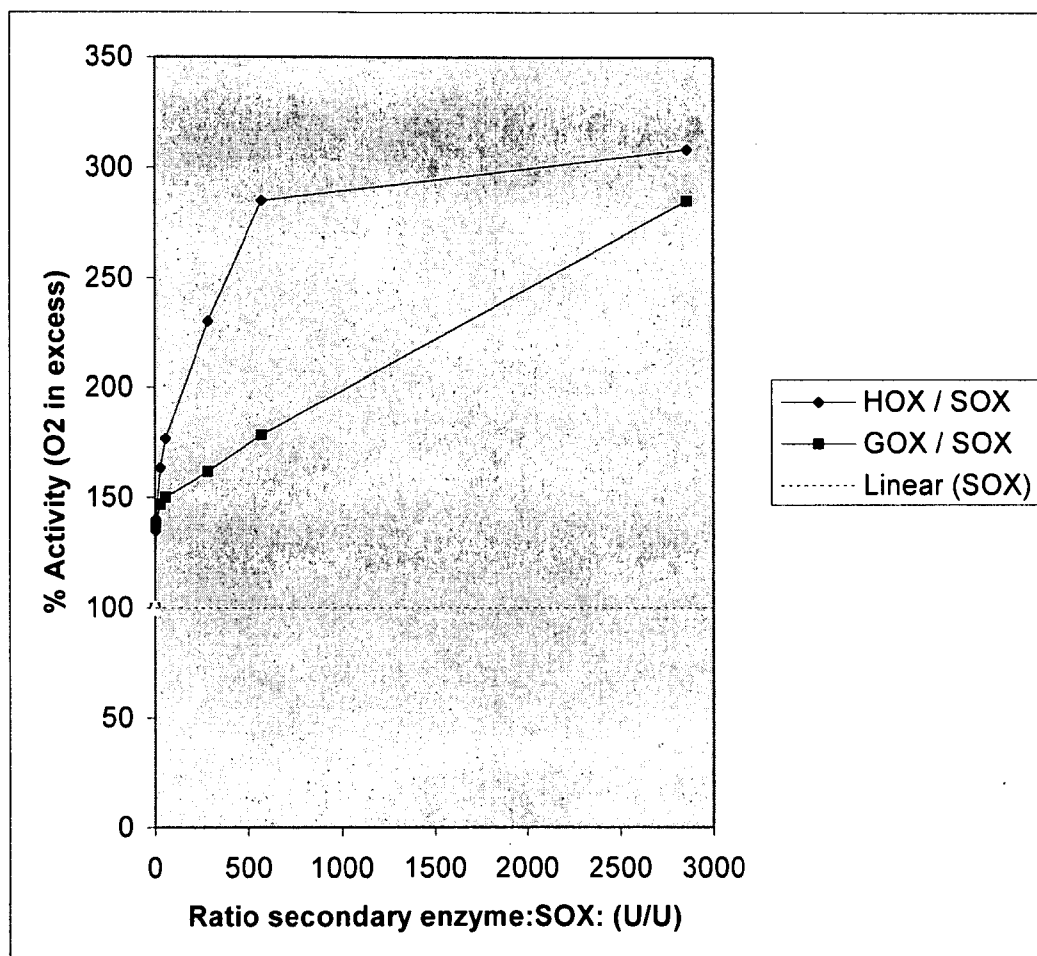


Figure 9A

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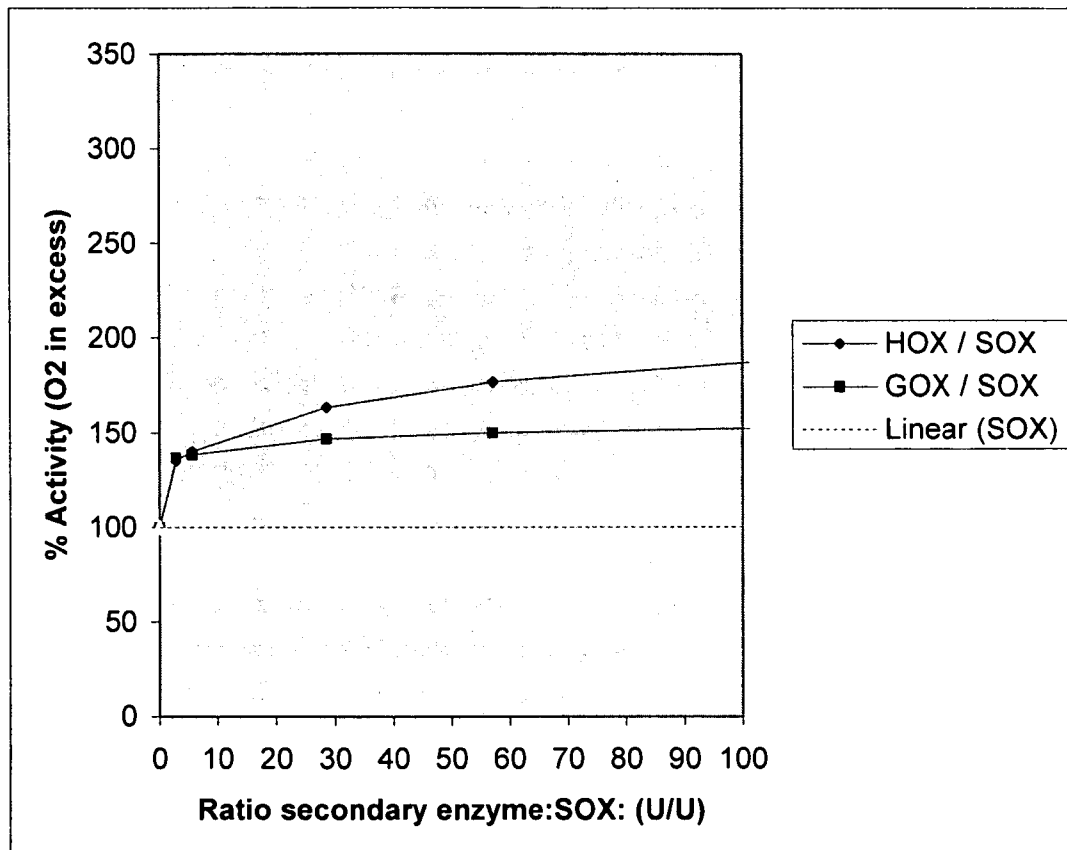


Figure 9B