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(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, California 94607 (US).

(72) Inventors: **EIBEN, Christopher B.**; 2150 Shattuck Ave., Suite 510, Berkeley, California 94704 (US). **KEASLING, Jay, D.**; 2150 Shattuck Ave., Suite 510, Berkeley, California 94704 (US).

(74) Agent: **OSMAN, Richard, Aron**; 530 Lawrence Expy #332, Sunnyvale, California 94085 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR CONVERTING METHANOL INTO HYDROGEN PEROXIDE AND CARBON DIOXIDE

(57) Abstract: The present invention provides for a method for producing hydrogen peroxide comprising: (a) contacting a methanol with methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or a formate oxidase bound with a FAD cofactor, such that the formaldehyde is oxidized into formate and the FAD is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD. The present invention also provides for a fusion protein comprising any two or all of methanol oxidase, formate oxidase, and formaldehyde dismutase.



Compositions and methods for converting methanol into hydrogen peroxide and carbon dioxide

[0001] This Application claims the benefit of U.S. Provisional Application No. 62/913,627, filed on October 10, 2019, which is incorporated in its entirety by this reference.

[0002] The invention was made with government support under Contract Nos. DE-AC02-05CH11231 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Hydrogen peroxide is a commodity chemical produced at 4.5 billion kilos per year via the anthraquinone process. The current process for producing hydrogen peroxide is the anthraquinone process, which requires large capital-intensive facilities in order to produce hydrogen peroxide economically. This means customers must ship hydrogen peroxide long distances and store it on site, which is expensive and potentially dangerous due to its chemical instability. What is needed is an alternate process to make hydrogen peroxide that should enable hydrogen peroxide production on site with less capital-intensive equipment.

SUMMARY OF THE INVENTION

[0004] The present invention provides for a method for producing hydrogen peroxide comprising: (a) contacting a methanol with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or a formate oxidase bound with a FAD cofactor, such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD; or (a) contacting a methanol with a fusion protein of the present invention comprising (i) a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, and (ii) a formate oxidase bound with a FAD cofactor, such that the methanol oxidase oxidizes the methanol into a formaldehyde and the FAD cofactor bound to the methanol oxidase is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or the formate oxidase, such that the formaldehyde is oxidized into a formate and the FAD of an unreduced methanol oxidase or formate oxidase is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD. In preferred variations, methanol oxidase and formate oxidase are enzymes extracted from distinct organisms (e.g., methanol oxidase is extracted from *Phanerochaete chrysosporium* and formate oxidase is extracted from

Schwanniomyces vanrijiae) or are functional variants of the organism enzymes (e.g. methanol oxidase from *Phanerochaete chrysosporium* expressed in *Escherichia coli* recombinantly and/or formate oxidase from *Schwanniomyces vanrijiae* expressed in *Escherichia coli* recombinantly).

[0005] The present invention provides for a fusion protein comprising any two or all of methanol oxidase, formate oxidase, and formaldehyde dismutase, wherein each enzyme is linked via a linker to another enzyme. In preferred variations, methanol oxidase, formate oxidase, and/or formaldehyde dismutase are enzymes extracted from distinct organisms (e.g., methanol oxidase is extracted from *Phanerochaete chrysosporium*, formate oxidase is extracted from *Schwanniomyces vanrijiae*, and/or formaldehyde dismutase is extracted from *Pseudomonas putida*) or are functional variants of the organism extracted enzymes.

[0006] In some embodiments, the method for producing hydrogen peroxide comprises: (a) contacting a methanol with a fusion protein comprising a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or a formate oxidase bound with a FAD cofactor, such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD.

[0007] The present invention provides for an *in vitro* composition comprising a methanol oxidase and a formate oxidase, or a fusion protein of the present invention, or a mixture thereof.

[0008] The present invention provides for a host cell comprising a polynucleotide encoding a methanol oxidase, formate oxidase, and/or formaldehyde dismutase, and/or a fusion protein of the present invention, each operatively linked to separate promoters or one promoter.

[0009] The present invention provides for a method of producing a methanol oxidase, formate oxidase, and/or formaldehyde dismutase comprising: (a) providing a host cell of the present invention in a nutrient medium, and (b) culturing or growing the host cell in the nutrient medium such that the methanol oxidase, formate oxidase, and/or formaldehyde dismutase is expressed or produced.

[0010] The present invention may be implemented as a method for producing hydrogen peroxide from a methanol source as part of an at least three step reaction, wherein the method leverages enzymatic properties of a methanol oxidase, a formaldehyde dismutase, and/or a formate oxidase to complete the reactions, and wherein the steps comprise: (a) oxidizing methanol to a formaldehyde by reducing FAD to FADH₂, (b) oxidizing the formaldehyde to a formate by reducing FAD to an FADH₂, and (c) reducing an O₂ to hydrogen peroxide by oxidizing the FADH₂ to an FAD. In this manner, the method may be implemented for general hydrogen peroxide production (e.g. as an industrial chemical product). Additionally, the method

may be implemented as part of a paper production process.

[0011] The method may be alternatively implemented as a one step, or multiple steps, of the methanol to hydrogen peroxide process. In a first implementation, oxidizing methanol to a formaldehyde while reducing FAD to FADH₂, using either methanol oxidase and/or formate oxidase, may be implemented as a standalone process. This implementation may be useful for the production of formaldehyde and/or the removal/breakdown of methanol.

[0012] In a second implementation: oxidizing formaldehyde to formate while reducing FAD to FADH₂, using methanol oxidase and/or formate oxidase, may be implemented as a standalone process. This second implementation may be useful for the production of formate.

[0013] In a third implementation: converting oxygen into hydrogen peroxide in conjunction with oxidizing FADH₂ into FAD, using the reduced form (i.e. bound to FADH₂) of methanol oxidase and/or formate oxidase, may be implemented as a standalone process. This third implementation may be useful for the production of hydrogen peroxide from available FADH₂.

[0014] In a fourth implementation: converting formate into carbon dioxide while reducing FAD to FADH₂, using methanol oxidase and/or formate oxidase, may be implemented as a standalone process. This fourth implementation may be used for formate decontamination.

[0015] In a fifth implementation, comprising a multi-step process: converting formaldehyde to hydrogen peroxide, the method may be implemented for formaldehyde decontamination.

[0016] The foregoing aspects and others will be readily appreciated by the skilled artisan from the following description of illustrative embodiments when read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1. Comparison of the amino acid sequences of *Phanerochaete chrysosporium* methanol oxidase (SEQ ID NO:1) and *Komagataella phaffii* strain GS115 alcohol oxidase (SEQ ID NO:37).

[0018] Figure 2. Putative NAD⁺-binding domain of formaldehyde dismutase and sequence similarity of nucleotide-binding domains. The conserved glycine residues found in the nucleotide-binding domains are outlined in black. The conserved aspartic acid residue is shown below the asterisk. The conserved hydrophobic amino acid residues are below the dots. A “-” represents gaps in the sequences made for alignment of amino acids. 1, Formaldehyde dismutase from *P. putida* F61 (188-218 of amino acid numbers) (SEQ ID NO:3); 2, alcohol dehydrogenase from horse liver (194-224) (SEQ ID NO:38); 3, lactate dehydrogenase from dogfish muscle (22-53) (SEQ ID NO:39); 4, glyceraldehyde-3-phosphate dehydrogenase from lobster (2-23) (SEQ ID NO:40). (Figure from: Yanase et al., *Biosci. Biotech. Biochem.*, 59:197-202, 1995.)

[0019] Figure 3. Comparison of the amino acid sequence of the *P. putida* formaldehyde dismutase (SEQ ID NO:3) and E subunit of alcohol dehydrogenase from horse liver (SEQ ID NO:38). The putative ligands of a catalytic Mg^{2+} or Zn^{2+} atom and/or a second Mg^{2+} or Zn^{2+} atom are indicated by closed and open triangles, respectively. The predicted NAD^+ -binding domain is enclosed in parallel lines. (Figure from: Yanase et al., *Biosci. Biotech. Biochem.*, 59:197-202, 1995.)

DETAILED DESCRIPTION OF THE INVENTION

[0020] Before the invention is described in detail, it is to be understood that, unless otherwise indicated, this invention is not limited to particular sequences, expression vectors, enzymes, host microorganisms, or processes, as such may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

[0021] In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

[0022] The terms "optional" or "optionally" as used herein mean that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where it does not.

[0023] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0024] The term "about" refers to a value including 10% more than the stated value and 10% less than the stated value.

[0025] The term "functional variant" refers to a protein, such as an enzyme or transcription factor, that has an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the amino acid sequence of any one of the proteins described in this specification or in an incorporated reference. The functional variant retains amino acids residues that are

recognized as conserved for the protein. The functional variant may have non-conserved amino acid residues replaced or found to be of a different amino acid, or amino acid(s) inserted or deleted, but which does not affect or has insignificant effect on the enzymatic activity of the functional variant. The functional variant has an enzymatic or biological activity that is identical or essentially identical to the enzymatic or biological activity any one of the proteins described in this specification or in an incorporated reference. The functional variant may be found in nature or be an engineered mutant thereof. The mutant may have one or more amino acids substituted, deleted or inserted, or a combination thereof, as compared to the protein described in this specification or in an incorporated reference. The term "functional variant" can also refer to a nucleotide sequence, such as a promoter, that has a nucleotide sequence that is at least 70%, 75%, 80%, 85%, 90%, 95% or 99% identical to the nucleotide sequence of any one of the nucleotide sequence, such as a promoter, described in this specification or in an incorporated reference.

[0026] As used herein, the term "promoter" refers to a polynucleotide sequence capable of driving transcription of a DNA sequence in a cell. Thus, promoters used in the polynucleotide constructs of the invention include cis- and trans-acting transcriptional control elements and regulatory sequences that are involved in regulating or modulating the timing and/or rate of transcription of a gene. For example, a promoter can be a cis-acting transcriptional control element, including an enhancer, a promoter, a transcription terminator, an origin of replication, a chromosomal integration sequence, 5' and 3' untranslated regions, or an intronic sequence, which are involved in transcriptional regulation. These cis-acting sequences typically interact with proteins or other biomolecules to carry out (turn on/off, regulate, modulate, etc.) gene transcription. Promoters are located 5' to the transcribed gene, and as used herein, include the sequence 5' from the translation start codon.

[0027] A polynucleotide or amino acid sequence is "heterologous" to an organism or a second polynucleotide or amino acid sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, when a polynucleotide encoding a polypeptide sequence is said to be operably linked to a heterologous promoter, it means that the polynucleotide coding sequence encoding the polypeptide is derived from one species whereas the promoter sequence is derived from another, different species; or, if both are derived from the same species, the coding sequence is not naturally associated with the promoter (e.g., is a genetically engineered coding sequence, e.g., from a different gene in the same species, or an allele from a different ecotype or variety, or a gene that is not naturally expressed in the target tissue).

[0028] The term "operably linked" refers to a functional relationship between two or more

polynucleotide (e.g., DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a promoter or enhancer sequence is operably linked to a DNA or RNA sequence if it stimulates or modulates the transcription of the DNA or RNA sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

[0029] The terms "host cell" and "host microorganism" are used interchangeably herein to refer to a living biological cell, such as a microbe, that can be transformed via insertion of an expression vector. Thus, a host organism or cell as described herein may be a prokaryotic organism (e.g., an organism of the kingdom Eubacteria) or a eukaryotic cell. As will be appreciated by one of ordinary skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus.

[0030] The terms "expression vector" or "vector" refer to a compound and/or composition that transduces, transforms, or infects a host cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. An "expression vector" contains a sequence of nucleic acids (ordinarily RNA or DNA) to be expressed by the host cell. Optionally, the expression vector also comprises materials to aid in achieving entry of the nucleic acid into the host cell, such as a virus, liposome, protein coating, or the like. The expression vectors contemplated for use in the present invention include those into which a nucleic acid sequence can be inserted, along with any preferred or required operational elements. Further, the expression vector must be one that can be transferred into a host cell and replicated therein. Particular expression vectors are plasmids, particularly those with restriction sites that have been well documented and that contain the operational elements preferred or required for transcription of the nucleic acid sequence. Such plasmids, as well as other expression vectors, are well known to those of ordinary skill in the art.

[0031] The terms "polynucleotide" and "nucleic acid" are used interchangeably and refer to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs may be used that may have alternate backbones, comprising, *e.g.*, phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphosphoroamidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press); positive backbones; non-ionic backbones, and non-ribose

backbones. Thus, nucleic acids or polynucleotides may also include modified nucleotides that permit correct read-through by a polymerase. "Polynucleotide sequence" or "nucleic acid sequence" includes both the sense and antisense strands of a nucleic acid as either individual single strands or in a duplex. As will be appreciated by those in the art, the depiction of a single strand also defines the sequence of the complementary strand; thus the sequences described herein also provide the complement of the sequence. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc.

[0032] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0033] The present invention provides for a process to make hydrogen peroxide that enables hydrogen peroxide production on site with less capital-intensive equipment. In addition, this process uses a cheaper feedstock than the anthraquinone process which should make this process cost competitive at scale as well. The process uses a two enzyme pathway (comprising methanol oxidase and formate oxidase) to convert one methanol molecule into one carbon dioxide (CO₂) molecule and result producing up to three hydrogen peroxide molecules.

[0034] The present invention alternatively provides a process for waste removal, wherein the process enables the removal/breakdown of methanol, formaldehyde and/or formic acid. A process that may be beneficial for waste management, particularly for water treatment. This process may use a one, two, or three enzyme pathway, wherein formaldehyde and/or formate are oxidized along a pathway to convert formaldehyde (or formate) into carbon dioxide, thereby producing hydrogen peroxide as one end product. This process may additionally include catalases (*e.g.* KatE or KatG) and/or peroxidases to further break down the hydrogen peroxide.

[0035] The present invention provides for a method for producing hydrogen peroxide comprising: (a) contacting a methanol with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, such that the methanol is oxidized into a formaldehyde and the

FAD cofactor is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or a formate oxidase bound with a FAD cofactor, such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD. In some embodiments, the method further comprises: (d) contacting the formaldehyde with a formaldehyde dismutase to convert formaldehyde into methanol and formate. In preferred variations, methanol oxidase and formate oxidase are enzymes extracted from distinct organisms (e.g., methanol oxidase is extracted from *Phanerochaete chrysosporium* and formate oxidase is extracted from *Schwanniomyces vanrijiae*) or are functional variants of the organism extracted enzymes.

[0036] The method may also function as a waste management process. That is, this enzyme pathway may also be used to detoxify methanol, formaldehyde, and/or formate, into CO₂ and water. In these variations, the method may further include the addition of catalase enzyme(s) and/or peroxidase(s) enzyme. In the case of catalase, the enzyme net reaction is to convert two H₂O₂ into two H₂O and one O₂ end products. In the case of the peroxidase enzyme, the method first reduces H₂O₂ to water, producing an oxidized peroxidase active site. The peroxidase may then oxidize an organic molecule, including, but not limited to, methanol, formaldehyde, or formate, in order to regenerate the peroxidase active site to its original state. In some variations the method may additionally or alternatively include a hydroperoxidase, wherein hydroperoxidase is a catalase enzyme that further has peroxidase activity. Catalase enzymes may use heme-based cofactors, or alternatively manganese in their active site. In some variations the catalase enzyme may comprise *Escherichia coli* native catalases (e.g. KatE or KatG). Additionally or alternatively, other catalase or peroxidase enzymes may be implemented as desired.

[0037] As the process herein may be implemented at different conditions (e.g. different pH), formaldehyde and hydrated formaldehyde (also referred to as: methanediol, formaldehyde monohydrate, or methylene glycol with the chemical formula CH₂(OH)₂), are considered as functional variants. Unless stated otherwise, any reference to formaldehyde may equally refer to hydrated formaldehyde. Additionally, in the same manner, formate may equally refer to formate or formic acid.

[0038] The present invention provides for an *in vitro* composition comprising a methanol oxidase and a formate oxidase. In some embodiments, the composition is a solution suitable for the methanol oxidase and the formate oxidase (and optionally formaldehyde dismutase) to catalyze their respective enzymatic reactions. In some embodiments, the solution comprises a suitable salt buffer and has a pH having a value from about 4.0, 5.0, 6.0, or 7.0 to about 7.2, 7.3,

7.4, 7.5, 7.6, 7.7, 7.8, 7.9, or 8.0. In some embodiments, the suitable salt buffer is a sodium phosphate, sodium pyrophosphate, or sodium metasilicate buffer. In some embodiments, the suitable salt buffer has a concentration of about 1 μ M, 10 μ M, 100 μ M, or 1 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 200 mM, 300 mM, 400 mM, or 500 mM. In some embodiments, the suitable salt buffer has a concentration of at least about 1 μ M, 10 μ M, 100 μ M, 1 mM, 10 mM, 20 mM, 30 mM, 40 mM, or 50 mM. In some embodiments, the suitable salt buffer has a concentration up to about 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 200 mM, 300 mM, 400 mM, or 500 mM. In some embodiments, the suitable salt buffer is an about 50 mM sodium phosphate buffer. In some embodiments, the solution comprises a suitable salt buffer and has a pH having a value from about 6.0 to about 8.0. In some embodiments, the solution comprises a suitable salt buffer and has a pH having a value from about 6.6 to about 7.8. In some embodiments, the solution comprises a suitable salt buffer and has a pH having a value from about 6.8 to about 7.6. In some embodiments, the solution comprises a suitable salt buffer and has a pH having a value from about 7.0 to about 7.4. In some embodiments, the pH is about 7.2. In some embodiments, the composition further comprises FAD cofactor. In some embodiments, the composition further comprises methanol and oxygen. In some embodiments, the composition further comprises: a formaldehyde dismutase. In some embodiments, the methanol oxidase, formate oxidase, and/or formaldehyde dismutase are isolated or purified.

[0039] In some embodiments, the methanol oxidase comprises the amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase, or a functional variant thereof. In one variation, the methanol oxidase matches the amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase by at least 90%. In a second variation, the methanol oxidase matches the amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase by at least 80%. In a third variation, the methanol oxidase matches the amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase by at least 70%. Dependent on implementation, the methanol oxidase may be purified and extracted from a microorganism (e.g. from *Phanerochaete chrysosporium* or from an organism with a methanol oxidase that is a functional variant), the methanol oxidase may be synthetically engineered, and/or the methanol oxidase may be produced (e.g. the amino acid sequence is expressed recombinantly in another organism, such as *Escherichia coli*). The methanol oxidase may, additionally or alternatively, be produced using any other applicable method. Examples of functional variant amino sequences may include, but are not limited to: synthetic variants, the amino acid sequence for the hypothetical protein PHACADRAFT_252324 from *Phanerochaete*, the amino acid sequence for the hypothetical protein PHLGIDRAFT 120749 from *Phlebiopsis gigantea*, the amino acid

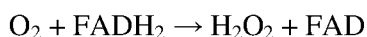
sequence for GMC oxidoreductase from *Trametes coccinea*, the amino acid sequence for alcohol oxidase from *Obba rivulosa*, the amino acid sequence for alcohol oxidase from *Gelatoporia subvermispota*, the hypothetical protein EIP91_001657 from *Steccherinum ochraceum*, the amino acid sequence for the hypothetical protein EUX98_g4623 from *Antrodia citrinella*, the amino acid sequence for alcohol oxidase from *Gloeophyllum trabeum* ATCC 11539, and the amino acid sequence for the hypothetical protein EW026_g1138 from *Phlebia centrifuga*.

[0040] In some embodiments, the formate oxidase comprises the amino acid sequence of *Schwanniomyces vanrijae* formate oxidase, or a functional variant thereof. In one variation, the formate oxidase matches the amino acid sequence of *Schwanniomyces vanrijae* formate oxidase by at least 90%. In a second variation, the formate oxidase matches the amino acid sequence of *Schwanniomyces vanrijae* formate oxidase by at least 80%. In a third variation, the formate oxidase matches the amino acid sequence of *Schwanniomyces vanrijae* formate oxidase by at least 70%. Dependent on implementation, the formate oxidase may be purified and extracted from a microorganism (e.g. from *Schwanniomyces vanrijae* or from an organism with a formate oxidase that is a functional variant), the formate oxidase may be synthetically engineered, and/or the formate oxidase may be produced (e.g. the amino acid sequence is expressed recombinantly in another organism, such as *Escherichia coli*). The formate oxidase may, additionally or alternatively, be produced using any other applicable method. Examples of functional variant amino acid sequences may include, but are not limited to: synthetic variants, the amino acid sequence for a choline dehydrogenase from *Zygosaccharomyces bailii*, the amino acid sequence for GMC oxidoreductase from *Hyaloscypha variabilis*, the amino acid sequence for the hypothetical protein B7463_g2234 from *Scytalidium lignicola*, the amino acid sequence for LAFE_0F18206g1_1 from *Lachancea fermentati*, the amino acid sequence for the hypothetical protein TDEL_0B00110 from *Torulaspora delbrueckii*, the amino acid sequence for the hypothetical protein FDECE_1716 from *Fusarium decemcellulare*, the amino acid sequence for the hypothetical protein CHU98_g3475 from *Xylaria longipes*, the amino acid sequence for the uncharacterized protein NECHADRAFT_85374 from *Fusarium vanettenii*, the amino acid sequence for the hypothetical protein PV04_09157 from *Phialophora americana*, the amino acid sequence for the hypothetical protein PV07_09250 from *Cladophialophora immunda*, the amino acid sequence for the hypothetical protein ABW21_db0200298 from *Drechslerella brochopaga*, the amino acid sequence for the hypothetical protein ABW19_dt0209074 from *Dactylella cylindrospora*, the amino acid sequence for a glucose-methanol-choline oxidoreductase from *Aspergillus bombycis*, the amino acid sequence for the hypothetical protein BDV34DRAFT_235406 from *Aspergillus parasiticus*, the amino acid sequence for a glucose-methanol-choline oxidoreductase from *Aspergillus nomiae*, the amino acid sequence of a

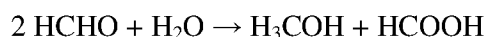
predicted protein from *Byssochlamys spectabilis*, the amino acid sequence for the hypothetical protein CEP54_015966 from *Fusarium sp.*, and the amino acid sequence for the uncharacterized protein BDV37DRAFT_284707 from *Aspergillus pseudonomius*.

[0041] In some embodiments, the formaldehyde dismutase comprises the amino acid sequence of *Pseudomonas putida* formaldehyde dismutase, or functional variant thereof. In one variation, the formaldehyde dismutase matches the amino acid sequence of *Pseudomonas putida* formaldehyde dismutase by at least 90%. In a second variation, the formaldehyde dismutase matches the amino acid sequence of *Pseudomonas putida* formaldehyde dismutase by at least 80%. In a third variation, the formaldehyde dismutase matches the amino acid sequence of *Pseudomonas putida* formaldehyde dismutase by at least 70%. Dependent on implementation, the formaldehyde dismutase may be purified and extracted from a microorganism (e.g. from *Pseudomonas putida* or from an organism with a formaldehyde dismutase that is a functional variant), the formaldehyde dismutase may be synthetically engineered, and/or the formaldehyde dismutase may be produced (e.g. the amino acid sequence is expressed recombinantly in another organism, such as *Escherichia coli*). The formaldehyde dismutase may, additionally or alternatively, be produced using any other applicable method. Examples of functional variant amino acid sequences may include, but are not limited to: synthetic variants, the amino acid sequence for the alcohol dehydrogenase catalytic domain-containing protein from *Pseudomonas monteilii*, the amino acid sequence for the alcohol dehydrogenase catalytic domain-containing protein from *Phaeobacter piscinae*, and the amino acid sequence for the aldehyde dehydrogenase from *Sinorhizobium sp.*.

[0042] Oxygen regenerates the FAD cofactor and is converted into hydrogen peroxide through the following reaction:



[0043] Formaldehyde dismutase catalyzes the following reaction:



[0044] To convert methanol to hydrogen peroxide and CO₂, two enzymes, comprising methanol oxidase and formate oxidase, are used. Methanol oxidase uses a tightly bound FAD cofactor to oxidize methanol to formaldehyde. Oxygen regenerates the cofactor to FAD and releases hydrogen peroxide in the process. Both methanol oxidase and formate oxidase can convert formaldehyde to formate by the same process as before via a tightly bound FAD cofactor yielding a hydrogen peroxide. Formate oxidase then converts formate into CO₂ and releases a hydrogen peroxide in the process. A third enzyme, formaldehyde dismutase, can be added to the process which may be advantageous at high substrate concentrations. Formaldehyde dismutase converts two formaldehyde and one water into one methanol and one formate. The terms

formate and the conjugate base, formic acid are used interchangeably herein. Since methanol oxidase and formate oxidase did not evolve specifically to use formaldehyde as a substrate, formaldehyde dismutase may help keep a low formaldehyde concentration without hurting the stoichiometry of the process.

[0045] The amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase comprises the following:

MGHP E EVDVIVCGGGPAGCVVAGRLAYADPTLKVMLIEGGANNRDDPWVYRPGIYVRNMQRNGI
NDKATFYTDTMASSYLGRRSIVPCANILGGGSSINFQMYTRASASDWDDFKTEGWTCKDLLPL
MKRLENYQKPCNNDTHGYDGPIAISNGGQIMPVAQDFLRAAHAIGVPYSDDIQDLTTAHGAEI
WAKYINRHTGRRSDAATAYVHSVMDVQDNFLRCNARVSRVLFDDNNKAVGVAYVPSRNRTHGGK
LHETIVKARKMVVLSSGTLGTPQILERSGVNGELLRLGLIKIVSDLPGVGEQYQDHYTTLSIY
RVSNESITDDFLRGVKDVQRELFTWEVVSPEKARLSSNAIDAGFKIRPTEEELKEMGPEFNE
LWNRIFYKDKPDKPVMFGSIVAGAYADHTLLPPGKYITMFQYLEYPASRGKIHKSQNPYVEPF
FD SGFMNNKADFAPIRWSYKKTREVARRMDAFRGELTSHHPRFHPASPAACKDIDIETAKQIY
PDGLTVGIHMGSWHQPSEPYKHDKVIEDIPYTEEDDKAIDDWADHVETTWHS LGTCAMKPREQ
GGVVDKRLNVYGTQNLKCVDSLICPDNLGTNTYSSALLVGEKGADLIAEELGLKIKTPHAPV
PHAPVPTGRPATQQVR (SEQ ID NO:1)

[0046] The amino acid sequence of the enzyme expressed by pHP30 is as follows:

MGSSHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKT
PLRRLMEAFAKRQ GKEMDSLRF LYDGIRIQADQTPEDLDMEDNDIEAHREQIGGHMGHP E
EVDVIVCGGGPAGCVVAGRLAYADPTLKVMLIEGGANNRDDPWVYRPGIYVRNMQRNGI
NDKATFYTDTMASSYLGRRSIVPCANILGGGSSINFQMYTRASASDWDDFKTEGWTCKDLLPL
MKRLENYQKPCNNDTHGYDGPIAISNGGQIMPVAQDFLRAAHAIGVPYSDDIQDLTTAHGAEI
WAKYINRHTGRRSDAATAYVHSVMDVQDNFLRCNARVSRVLFDDNNKAVGVAYVPSRNRTH
GGKLHETIVKARKMVVLSSGTLGTPQILERSGVNGELLRLGLIKIVSDLPGVGEQYQDHYTT
LSIYRVSNESITDDFLRGVKDVQRELFTWEVVSPEKARLSSNAIDAGFKIRPTEEELKEMG
PEFNE LWNRIFYKDKPDKPVMFGSIVAGAYADHTLLPPGKYITMFQYLEYPASRGKIHKSQ
NPYVEPF FDSGFMNNKADFAPIRWSYKKTREVARRMDAFRGELTSHHPRFHPASPAACKDI
DIETAKQIYPDGLTVGIHMGSWHQPSEPYKHDKVIEDIPYTEEDDKAIDDWADHVETTWHS
LGTCAMKPREQGGVVDKRLNVYGTQNLKCVDSLICPDNLGTNTYSSALLVGEKGADLIAEEL
GLKIKTPHAPVPHAPVPTGRPATQQVR (SEQ ID NO:4)

[0047] In some embodiments, the methanol oxidase has an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:1. In some embodiments, the methanol oxidase comprises at least one or more, or all, of the following conserved sequences:

GRRXIVPCANILGGGSSINFXMYTRXSASDXDD (SEQ ID NO:5), LLPLXK (SEQ ID

NO:6), QDFLRA (SEQ ID NO:7), TAHGAE (SEQ ID NO:8), GRRSD (SEQ ID NO:9), LPGVGXXXQDH (SEQ ID NO:10), AGXKIRPTXEE (SEQ ID NO:11), KPDKP (SEQ ID NO:12), LEYPXSRL (SEQ ID NO:13), YKKXREXARRM (SEQ ID NO:14), GEXTSHHP (SEQ ID NO:15), EEDDXAI (SEQ ID NO:16), ETTWHXLGTC (SEQ ID NO:17), and DLSXCPDNXGXNTY (SEQ ID NO:18); wherein “X” is any amino acid residue. Certain of these conserved sequences are disclosed in Figure 1.

[0048] The amino acid sequence of *Schwanniomyces vanrijiae* formate oxidase comprises the following:

MVQSHYDFVIVGGGTAGNTVAGRLAENPNVTVLVVEAGVANSADLPEITTPSNAMNLRGSKHDW
 AYKTTLVKRDDYERIEKPNTRGKALGGSSSLNYFTWIPGCKPTFDRWAEYGGEEWTWDFLVPYL
 RKSATYHDDTGLYNPELKKLGAGGPIPIHSELVEELEPFRENLIKAWKSTGKPFTENIYDGEM
 IGLNHCISTIIYHGKRSGLFVKNRPNITIIPEVHSKNLIIDASNTAKGVVVIDKEGNEHSFYA
 TREVILSQGVFESPKLLMLSGVGPRKELESNGIEVKVESRHVGQNLLDHPGVFVLQVKDDICV
 DDILMRQNEKNKAAHVQYQKDGSGPVGSGLLLELVGFPRIDEYFEKDPLYRERKAANGGKDPFCP
 EGQPHFELDFVGMGTAFQWHFPTPKKGSHITIVVDLVRPVSEGGEVTLNSADPLEQPKINLNF
 FADELDIVGMREGIRFTYDLLTKDGFKDLVKEFPWEMPLDDDKEMRRAVLDRQCQTAHFPCGT
 NRLSKNIEQGVVDPALKVHGVKNLRVIDASIIIPVIPDCRIQNSVYMIGEKGADLIKAAHKDLYN
 (SEQ ID NO:2)

[0049] The amino acid sequence of the enzyme expressed by pHP2 is as follows:

MVQSHYDFVIVGGGTAGNTVAGRLAENPNVTVLVVEAGVANSADLPEITTPSNAMNLRGSKHDW
 AYKTTLVKRDDYERIEKPNTRGKALGGSSSLNYFTWIPGCKPTFDRWAEYGGEEWTWDFLVPYL
 RKSATYHDDTGLYNPELKKLGAGGPIPIHSELVEELEPFRENLIKAWKSTGKPFTENIYDGEM
 IGLNHCISTIIYHGKRSGLFVKNRPNITIIPEVHSKNLIIDASNTAKGVVVIDKEGNEHSFYA
 TREVILSQGVFESPKLLMLSGVGPRKELESNGIEVKVESRHVGQNLLDHPGVFVLQVKDDICV
 DDILMRQNEKNKAAHVQYQKDGSGPVGSGLLLELVGFPRIDEYFEKDPLYRERKAANGGKDPFCP
 EGQPHFELDFVGMGTAFQWHFPTPKKGSHITIVVDLVRPVSEGGEVTLNSADPLEQPKINLNF
 FADELDIVGMREGIRFTYDLLTKDGFKDLVKEFPWEMPLDDDKEMRRAVLDRQCQTAHFPCGT
 NRLSKNIEQGVVDPALKVHGVKNLRVIDASIIIPVIPDCRIQNSVYMIGEKGADLIKAAHKDLYN
 LEHHHHHH (SEQ ID NO:19)

[0050] In some embodiments, the formate oxidase has an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:2. In some embodiments, the formate oxidase comprises at least one or more, or all, of the following conserved sequences:

SHXDFVIVGGGTAGNTVAGRLAE (SEQ ID NO:20), SH(Y or F)DFVIVGGGTAGNTVAGRLAE (SEQ ID NO:21), DWAYK (SEQ ID NO:22), TFDXWXEXGGXEWTWD (SEQ ID NO:23), TFD(R or Q)W(A or E)E(Y or F)GG(E or

K)EWTWD (SEQ ID NO:24), EWTWDPLVPYLR (SEQ ID NO:25), and DLY (SEQ ID NO:26); wherein “X” is any amino acid residue. Certain of these conserved sequences are disclosed in Maeda et al., *Biosci. Biotech. Biochem.*, 72:1999-2004, 2008.

[0051] The amino acid sequence of *Pseudomonas putida* formaldehyde dismutase comprises the following:

MAGNKSVVYHGTRDLRVETVPYPKLEHNNRKLEHAVILKVVSTNICGSDQHIYRGRFIVPKGHV
LGHEITGEVVEKGSDELMDIGDLVSVFPNVACGRCRNCKEARSDVCENNLVNPADLGAFGFD
LKGWSGGQAEYVLVPYADYMLLKFGDKEQAMEKIKDLTLISDILPTGFHGCVSAGVKPGSHVYI
AGAGPVGRCAAAGARLLGAACVIVGDQNPRLKLLSDAGFETIDLRNSAPLRDQIDQILGKPEV
DCGVDAVGFEAHGLGDEANTETPNGALNSLFDVVRAGGAIGIPGIYVGSDDPVPNKDAGSGRLH
LDFGKMWTKSIRIMTGMAPVTNYNRHLTEAILWDQMPYLSKVMNIEVITLDQAPDGYAKFDKGS
PAKFVIDPHGMLKNKL (SEQ ID NO:3)

[0052] The amino acid sequence of the enzyme expressed by pHP23 is as follows:

MAGNKSVVYHGTRDLRVETVPYPKLEHNNRKLEHAVILKVVSTNICGSDQHIYRGRFIVPKGHV
LGHEITGEVVEKGSDELMDIGDLVSVFPNVACGRCRNCKEARSDVCENNLVNPADLGAFGFD
LKGWSGGQAEYVLVPYADYMLLKFGDKEQAMEKIKDLTLISDILPTGFHGCVSAGVKPGSHVYI
AGAGPVGRCAAAGARLLGAACVIVGDQNPRLKLLSDAGFETIDLRNSAPLRDQIDQILGKPEV
DCGVDAVGFEAHGLGDEANTETPNGALNSLFDVVRAGGAIGIPGIYVGSDDPVPNKDAGSGRLH
LDFGKMWTKSIRIMTGMAPVTNYNRHLTEAILWDQMPYLSKVMNIEVITLDQAPDGYAKFDKGS
PAKFVIDPHGMLKNKLEHHHHHH (SEQ ID NO:27)

[0053] In some embodiments, the formaldehyde dismutase has an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:3. In some embodiments, the formaldehyde dismutase comprises at least one or more, or all, of the following conserved domains:

(a) a NAD⁺-binding domain comprising one of the following conserved sequences:

GXGXXG (SEQ ID NO:28), GXGXXGX₁₈D (SEQ ID NO:29), GXGGXXGX₁₈D (SEQ ID NO:30), GXGGXXGX₁₉D (SEQ ID NO:31), GXGXVGX₅GX₄GAAXXIXXD (SEQ ID NO:32);

(b) ligands for binding a first catalytic zinc comprising one of the following conserved sequences: CXSXXHX₁₅H (SEQ ID NO:33) or CXSDXHX₃GX₄PX₅GH (SEQ ID NO:34); and,

(c) ligands for binding a second catalytic zinc comprising one of the following conserved sequences: CXXCXXCX₇C (SEQ ID NO:35) or CGXCRXCKX₆C (SEQ ID NO:36);

wherein “X” is any amino acid residue. Certain of these conserved sequences are disclosed in Figures 2 and 3.

[0054] In some embodiments, the fusion protein comprises a methanol oxidase linked via a linker to a formate oxidase. In some embodiments, the fusion protein comprises a methanol oxidase linked via a linker to a formaldehyde dismutase. In some embodiments, the fusion protein comprises a formate oxidase linked via a linker to a formaldehyde dismutase. In some embodiments, the fusion protein comprises a methanol oxidase (i) linked via a first linker to a formate oxidase and (ii) linked via a second linker to a formaldehyde dismutase. In some embodiments, the fusion protein comprises a formate oxidase (i) linked via a first linker to a methanol oxidase and (ii) linked via a second linker to a formaldehyde dismutase. In some embodiments, the fusion protein comprises a formaldehyde dismutase (i) linked via a first linker to a methanol oxidase and (ii) linked via a second linker to a formate oxidase. In all embodiments, the fusion protein may comprise functional variants of the methanol oxidase, formate oxidase, and/or formaldehyde dismutase, wherein (as mentioned above) the functional variant comprises sequences that are at least 70% functionally equivalent.

[0055] In some embodiments, the fusion protein comprises the amino acid sequence of SEQ ID NO:41, 42, or 43.

[0056] A particular embodiment of a fusion protein comprises formate oxidase and methanol oxidase with SUMO tag and having the following amino acid sequence:

MVQSHYDFVIVGGGTAGNTVAGRLAENPNVTVLVVEAGVANSADLPEITTPSNAMNLRGSKHDW
 AYKTTTLVKRDDYERIEKPNTRGKALGGSSSLNYFTWIPGCKPTFDRWAEYGGEEWTWDP LVPYL
 RKSATYHDDTGLYNPELKKLGAGGPIPI SHSELVEELEPFRENLIKAWKSTGKPFTENIYDGEM
 IGLNHCISTIYHGKRSGSFLFVKNRPNITIIPEVHSKNLIIDASNTAKGVVVIDKEGNEHSFYA
 TREVILSQGVFESPKLLMLSGVGPRKELESNGIEVKVESRHVGQNLLDHPGVPFVLQVKDDICV
 DDILMRQNEKNKAAHVQYQKDGSGPVGSGLLELVGFPRIDEYFEKDPLYRERKAANGGKDPFCP
 EGQPHFELDFVGMGTAFQWHFPTPKKGSHTIVVDLVRPVSEGGEVTLNSADPLEQPKINLNF
 FADELDIVGMREGIRFTYDLLTKGDGFKDLVVEKFPWEMPLDDDKEMRRAVLDRQCQTAHFPCGT
 NRLSKNIEQGVVDPALKVHGVKNLRVIDASIIIPVIPDCRIQNSVYMIGEKGADLIKAAHKDLYN
 GSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAF
 KRQKEMDSLRFYDGIQADQTPEDLDMEDNDII EAHREQIGGHMGHP EEDVIVCGGGPAG
 CVVAGRLAYADPTLKVMLIEGGANNRDPWVYRPGIYVRNMQRNGINDKATFYTDTMASSYL
 RRSIVPCANILGGGSSINFQMYTRASASDWDDFKTEGWTCKDLLPLMKRLENYQKPCNNDTHGY
 DGPIAISNGGQIMPVAQDFLRAAHAIGVPYSDDIQDLTTAHGAEIWAKYINRHTGRRSDAATAY
 VHSVMDVQDNFLRCNARVSRVLFDDNNKAVGVAYVPSRNRTHGGKLHETIVKARKMVVLSSGT
 LGTPQILERSGVNGELLRQLGIKIVSDLPVGVEQYQDHYTTLSIYRVSNESITDDFLRGVKD
 VQRELFTWEVSPEKARLSSNAIDAGFKIRPTEELKEMGPEFNELWNRYFKDKPKPVMFGSI
 VAGAYADHTLLPPGKYITMFQYLEYPASRGKIHKSQNPYVEPFFDSGFMNNKADFAPIRWSYK

KTREVARRMDAFRGELTSHHPRFHPASPAACKDIDDIETAKQIYPDGLTVGIHMGSWHQPSEPYK
HDKVIEDIPYTEEDDKAIDDWVADHVETTWHSLGTCAMKPREQGGVVDKRLNVYGTQNLKCVDL
SICPDNLGTNTYSSALLVGEKGADLIAEELGLKIKTPHAPVPHAPVPTGRPATQQVR (SEQ
ID NO:41) .

[0057] A particular embodiment of a fusion protein comprises formate oxidase and methanol oxidase and having the following amino acid sequence:

MVQSHYDFVIVGGGTAGNTVAGRLAENPNVTVLVVEAGVANSADLPEITTPSNAMNLRGSKHDW
AYKTTLVKRDDYERIEKPNTRGKALGGSSSLNYFTWIPGCKPTFDRWAEYGGEEWTWDP LVPYL
RKSATYHDDTGLYNPELKKLGAGGPIPI SHSELVEELEPFRENLIKAWKSTGKPF TENIYDGEM
IGLNHCISTIYHGKRSGSFLFVKNRPNITIIPEVHSKNLIIDASNTAKGVVVIDKEGNEHSFYA
TREVILSQGVFESP KLLMLSGVGPRKELESNGIEVKVESRHVGQNLLDHPGV PFVLQVKDDICV
DDILMRQNEKNKAAHVQYQKDGS GPVGSGLLELVGFPRIDEYFEKDPLYRERKAANGGKDPFCP
EGQPHFELDFVGMYGTA FQWHFPTPKKGS HITIVVDLVRPVSEGGEVTLNSADPLEQPKINLNF
FADEL DIVGMREGIRFTYDLLTKGDGFKDLVVK EFPWEMPLDDDKEMRRAVLDR CQTAFHPCGT
NRLSKNIEQGVVDPALKVHGVKNLRVIDASIIPVIPDCRIQNSVYMIGEKGADLIKAAHKDLYN
GGHMGHP EEV DVIVCGGGPAGCVVAGRLAYADPTLKVMLIEGGANNRDPWVYRPGIYVRNMQR
NGINDKATFYTDTMAS SYLRGRRSIVPCANILGGGSSINFQMYTRASASDWDDFKTEGWTCKDL
LPLMKRLENYQKPCNN DTHGYDGP I AISNGGQIMPVAQDFLRAAHAIGVPYSDDIQDLTTA HGA
EIWAKYINRHTGRRSDAATAYVHSVMDVQDNLFLRCNARVSRVLFDDNNKAVGVAYVPSRN RTH
GGKLHETIVKARKMVVLSSGTLGTPQILERSGVNGELLRQLGIKIVSDLPGVGEQYQDHYTTL
SIYRVSNESITTD DFLRGVKDVQRELFTEW EVSPEKARLSSNAIDAGFKIRPTEELKEMGPEF
NELWNRYFKDKPKDPVMFGSIVAGAYADHTLLPPGKYITMFQYLEYPASRGKIH IKSQNPYVEP
FFDSGFMNNKADFAPIRWSYKKTREVARRMDAFRGELTSHHPRFHPASPAACKDIDDIETAKQIY
PDGLTVGIHMGSWHQPSEPYKHDKVIEDIPYTEEDDKAIDDWVADHVETTWHSLGTCAMKPREQ
GGVVDKRLNVYGTQNLKCVDLSICPDNLGTNTYSSALLVGEKGADLIAEELGLKIKTPHAPVPH
APVPTGRPATQQVR (SEQ ID NO:42) .

[0058] A particular embodiment of a fusion protein comprises formate oxidase and methanol oxidase with a SUMO tag and having the following amino acid sequence:

MVQSHYDFVIVGGGTAGNTVAGRLAENPNVTVLVVEAGVANSADLPEITTPSNAMNLRGSKHDW
AYKTTLVKRDDYERIEKPNTRGKALGGSSSLNYFTWIPGCKPTFDRWAEYGGEEWTWDP LVPYL
RKSATYHDDTGLYNPELKKLGAGGPIPI SHSELVEELEPFRENLIKAWKSTGKPF TENIYDGEM
IGLNHCISTIYHGKRSGSFLFVKNRPNITIIPEVHSKNLIIDASNTAKGVVVIDKEGNEHSFYA
TREVILSQGVFESP KLLMLSGVGPRKELESNGIEVKVESRHVGQNLLDHPGV PFVLQVKDDICV
DDILMRQNEKNKAAHVQYQKDGS GPVGSGLLELVGFPRIDEYFEKDPLYRERKAANGGKDPFCP
EGQPHFELDFVGMYGTA FQWHFPTPKKGS HITIVVDLVRPVSEGGEVTLNSADPLEQPKINLNF

FADELDIVGMREGIRFTYDLLTKGDGFKDLVVKEFPWEMPLDDDKEMRRAVLDRQCOTAFHPCGT
 NRLSKNIEQGVVDPALKVHGVKNLRVIDASIIIPVIPDCRIQNSVYMIGEKGADLIKAAHKDLYN
 GGSAGNKSVMYHGTDLRVETVPYPKLEHNNRKLEHAVILKVVSTNICGSDQHIYRGRFIVPKG
 HVLGHEITGEVVEKGSDELMDIGDLVSVPFNVACGRCRNCKEARSDDVCENNLVNPADLGAFG
 FDLKGWSGGQAEYVLVPYADYMLLKFGDKEQAMEKIKDLTLISDILPTGFHGCVSAGVKPGSHV
 YIAGAGPVGRCAAAGARLLGAACVIVGDQNPRLKLLSDAGFETIDLRNSAPLRDQIDQILGKP
 EVDCGVDAVGFEAHGLGDEANTETPNGALNSLFDVVRAGGAIGIPGIYVGSDDPVPNKDAGSGR
 LHLDFGKMWTKSIRIMTGMAPVTNYNRHLTEAILWDQMPYLSKVMNIEVITLDQAPDGYAKFDK
 GSPAKFVIDPHGMLKNKGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIF
 FKIKKTTPLRRLMEAFKRGKEMDSLRFYDGIQADQTPEDLDMEDNDIIEAHREQIGGH
 GHPEEVDVIVCGGGPAGCVVAGRLAYADPTLKVMLIEGGANNRDDPWVYRPGIYVRNMQRNGIN
 DKATFYTDTMASSYLGRRSIVPCANILGGSSINFQMYTRASASDWDDFKTEGWTCKDLLPLM
 KRLNYQKPCNNDTHGYDGP I A ISNGGQIMPVAQDFLRAAHAIGVPYSDDIQDLTTAHGAEIWA
 KYINRHTGRRSDAATAYVHSVMDVQDNFLRCNARVSRVLFDDNNKAVGVAYVPSRNRTGGKL
 HETIVKARKMVVLSSGTLGTPQILERSGVGNGELLRQLGIKIVSDLPGVGEQYQDHYTTLSIYR
 VSNESITDDFLRGVKDVQRELFTEWEVSPEKARLSSNAIDAGFKIRPTEEELKEMGPEFNE
 LWNRYFKDKPKPVMFGSIVAGAYADHTLLPPGKYITMFQYLEYPASRGKIHKSQNPYVEPFFDS
 GFMNNKADFAPIRWSYKKTREVARRMDFRGELTSHHPRFHPASPAACKDIDIETAKQIYPDGL
 TVGIIHMGSWHQPSEPYKHDKVIEDIPYTEEDDKAIDDWVADHVETTWHS LGTCAMKPREQGGV
 DKRLNVYGTQNLKCVDLSICPDNLGTNTYSSALLVGEKGADLIAEELGLKIKTPHAPVPHAPVP
 TGRPATQQVR (SEQ ID NO:43) .

[0059] In some embodiments, the linker, or first linker and/or second linker, is a covalent bond, or one or more amino acid residues. In some embodiments, the linker is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues in length. In some embodiments, the linker is up to about 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1,000 amino acid residues in length. In some embodiments, the linker is from about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, to about 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1,000 amino acid residues in length.

[0060] In some embodiments, the linker comprises a tag, such as a Small Ubiquitin-like Modifier (SUMO) tag. In some embodiments, the linker comprises an amino acid sequence as follows: GGH,

GSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAF
 KRQKGEMDSLRFYDGIQADQTPEDLDMEDNDIIEAHREQIGGH (SEQ ID NO:44), or
 GGSAGNKSVMYHGTDLRVETVPYPKLEHNNRKLEHAVILKVVSTNICGSDQHIYRGRFIVPKG
 HVLGHEITGEVVEKGSDELMDIGDLVSVPFNVACGRCRNCKEARSDDVCENNLVNPADLGAFG
 FDLKGWSGGQAEYVLVPYADYMLLKFGDKEQAMEKIKDLTLISDILPTGFHGCVSAGVKPGSHV

YIAGAGPVGRCAAAGARLLGAACVIVGDQNPRLKLLSDAGFETIDLRNSAPLRDQIDQILGKP
EVDCGVDAVGFEAHGLGDEANTETPNGALNSLFDVVRAGGAIGIPGIYVGSDDPVPNKDAGSGR
LHLDFGKMWTKSIRIMTGMAPVTNYNRHLTEAILWDQMPYLSKVMNIEVITLDQAPDGYAKFDK
GSPAKFVIDPHGMLKNKGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIF
FKIKKTTPLRRLMEAFKRQKGEMDSLRFLYDGIRIQADQTPEDLDMEDNDIEAHREQIGGH
(SEQ ID NO:45) .

[0061] The present invention provides for a host cell comprising a polynucleotide encoding a methanol oxidase, formate oxidase, and/or formaldehyde dismutase, or a fusion protein of the present invention, each operatively linked to separate promoters or one promoter.

[0062] In some embodiments, the polynucleotide is a vector capable of stably residing in the host cell. In some embodiments, the vector is a plasmid. In some embodiments, the vector is an expression vector. In some embodiments, the promoter is an inducible promoter or constitutive promoter. In some embodiments, the promoter is heterologous to the enzyme to which it is operably linked to.

[0063] The present invention provides for a method of producing a methanol oxidase, formate oxidase, and/or formaldehyde dismutase comprising: (a) providing a host cell of the present invention in a nutrient medium, and (b) culturing or growing the host cell in the nutrient medium such that the methanol oxidase, formate oxidase, and/or formaldehyde dismutase is expressed or produced. In some embodiments, the method further comprises: (c) separating the methanol oxidase, formate oxidase, and/or formaldehyde dismutase from the rest of the host cell and/or nutrient medium. In some embodiments, the (c) separating step comprises isolating or purifying the methanol oxidase, formate oxidase, and/or formaldehyde dismutase. In some embodiments, the methanol oxidase, formate oxidase, and/or formaldehyde dismutase comprise a tag, such a histidine tag (such as a C-terminal six histidine tag), and the isolating or purifying comprises using an affinity column based on the affinity of the tag to the affinity column. In some embodiments, the tag comprises the amino acid sequence MGSSHHHHHHGGS (SEQ ID NO:46). The tag can be located at the N-terminal, C-terminal, within the enzyme or fusion protein, or within a linker of the fusion protein. In some embodiments, the tag comprises the amino acid sequence of a SUMO tag or a tag that aids solubility. The SUMO tag aids in either solubility or protein folding (or both) of the methanol oxidase protein. In some embodiments, the tag that aids solubility is amino acid sequence that aids in the increasing the solubility of the enzyme or fusion protein, including, but not limited to, maltose binding protein (MBP), glutathione-S-transferase (GST), and thioredoxin (TRX).

[0064] Any prokaryotic or eukaryotic host cell may be used in the present method so long as it remains viable after being transformed with the polynucleotide. Generally, although not

necessarily, the host microorganism is bacterial. In some embodiments, the host cell is a Gram negative bacterium. In some embodiments, the host cell is of the phylum Proteobacteria. In some embodiments, the host cell is of the class Gammaproteobacteria. In some embodiments, the host cell is of the order Enterobacteriales. In some embodiments, the host cell is of the family Enterobacteriaceae. Examples of bacterial host cells include, without limitation, those species assigned to the *Escherichia* (such as *E. coli*), *Enterobacter*, *Azotobacter*, *Erwinia*, *Bacillus*, *Pseudomonas* (such as *P. putida*), *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Rhizobia*, *Vitreoscilla*, and *Paracoccus* genera. The host cell is not adversely affected by the transduction of the necessary nucleic acid sequences, and/or the subsequent expression of the proteins (i.e., enzymes). Suitable eukaryotic cells include, but are not limited to, fungal, insect or mammalian cells. Suitable fungal cells are yeast cells, such as yeast cells of the *Saccharomyces* (such as *S. cerevisiae*) or *Rhodospiridium* (such as *R. toruloides*) genera.

[0065] The hydrogen peroxide can be used in the paper and pulp industry, in the production of sodium percarbonate and sodium perborate for laundry detergent, in the production of propylene oxide, in the waste water treatment industry, and for mining. Hydrogen peroxide is also potentially useful for ethylene oxide production. The process should enable economic production of hydrogen peroxide in smaller batches on site for hydrogen peroxide consumers. This reduces the need to ship hydrogen peroxide long distance and can be stored on site. In the long run the process might produce hydrogen peroxide cheaper than the current anthraquinone process.

[0066] It is to be understood that, while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

[0067] All patents, patent applications, and publications mentioned herein are hereby incorporated by reference in their entireties.

[0068] The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

Example 1

Materials and Methods:

Plasmids:

[0069] Each plasmid has a constitutively expressed kanamycin resistance gene for selection, a pBR322 origin of replication for plasmid maintenance, a lacI gene that expresses constitutively for T7 promoter repression, a F1 origin for potential single stranded DNA generation, a T7 promoter for overexpression of the gene of interest, and a T7 terminator to terminate mRNA

production. Genes for the hydrogen peroxide system were cloned between the T7 promoter and T7 terminator as appropriate for expression by Isopropyl β -D-1-thiogalactopyranoside (IPTG) induction. This resulted in a total of 3 plasmids named pHP2, pHP23, pHP30, each with one gene from the hydrogen peroxide generating system in it.

[0070] Plasmid pHP2 expresses a formate oxidase from *Schwanniomyces vanriijiae* with a C-terminal six histidine (6xHis) tag for purification. Plasmid pHP23 uses a T7 promoter to express formaldehyde dismutase from *Pseudomonas putida* with a C-terminal 6xHis tag for purification. Plasmid pHP30 uses a T7 promoter to express methanol oxidase from *Phanerochaete chrysosporium* with a N-terminal 6xHis followed by a small ubiquitin-like modifier (SUMO) tag followed by the coding sequence of methanol oxidase.

Plasmid expressions:

[0071] All glassware, pipet tips, media, etc. used in cell culture are either autoclaved or sterile filtered prior to use or purchased pre-sterilized. Unless otherwise noted, kanamycin supplementation is used at a concentration of 50 μ g/mL. Plasmids pHP2, pHP23, and pHP30 are transformed into *E. coli* BL21DE3* cells using the KCM method¹. Briefly, on day one BL21DE3* cells are streaked from a glycerol stock onto lysogeny broth (LB) agar plates (1.0% weight to volume (w/v) tryptone, 0.5% w/v yeast extract, 1.0% w/v sodium chloride, 1.5% w/v agar), and placed in a 37 °C incubator overnight.

[0072] On day two a single colony was picked into 10 mL of LB liquid media (as LB agar, but without the 1.5% w/v agar) in a glass test tubes and incubated overnight (approximately sixteen hours) at 37 °C, shaking at 250 rotation per minute (RPM).

[0073] On day three, 0.5 mL of the day two overnight is used to inoculate 50 mL of fresh LB liquid media in a 250 mL baffled flask and allowed to grow at 37 °C shaking at 250 RPM until the cells reach an optical density at 600 nm of approximately 0.35. The culture is then chilled on ice for 20 minutes prior to centrifugation at 8,000 relative centrifugal force (RCF) for 8 minutes in conical plastic tubes at 4 °C. Supernatant is then decanted, and the cells are resuspended in 5 mL TSS media (1.0% w/v tryptone, 0.5% w/v yeast extract, 1.0% w/v sodium chloride, 10% w/v polyethylene glycol with average mol weight 3,350, 5% dimethyl sulfoxide v/v, 20 mM MgCl_2). 100 μ L aliquots of the TSS media cell mixture are then pipetted into .6 mL plastic tubes with caps. 10 ng of appropriate plasmid is then added to the TSS media cell mixture containing tubes, resulting in 3 tubes, each with one plasmid. 2xKCM (0.06 M KCl, 0.2 M CaCl_2 , 0.1 M MgCl_2) is added and mixed into the 0.6 mL tubes. Tubes are incubated on ice for 20 minutes before heat shocking at 42 °C for 90 seconds. The tubes are returned to ice for 2 minutes before adding 200 μ L of terrific broth (TB) and are incubated at 37 °C for 1 hour. TB is composed of 1.2% w/v tryptone, 2.4% w/v yeast extract, 0.4% v/v glycerol, 72 mM K_2HPO_4 , and 17 mM KH_2PO_4 . The

full tube liquid volume, approximately 400 μ L, is then spread onto LB agar plates supplemented with kanamycin using glass beads. Plates are then incubated at 37 °C overnight, approximately sixteen hours. At this point there are 3 plates, each with cells only containing either pHP2, pHP23, or pHP30.

[0074] On day four, single colonies are picked into 10 mL LB media supplemented with kanamycin. Cultures are allowed to grow overnight, approximately sixteen hours, at 37 °C shaking at 250 RPM.

[0075] On day five, .5 mL of saturated cultures from day four are used to inoculate 50 mL of fresh TB media with kanamycin, in a 250 mL baffled flasks. At this point there are 3 baffled flasks, each with cells only containing either pHP2, pHP23, or pHP30. Cells are then grown shaking at 250 RPM at 37 °C until they reach an optical density at 600 nm of 0.8. Isopropyl- β -D-thiogalactoside (IPTG) is added at a final concentration of 1 mM to each tube and the temperature is reduced to 18 °C. Shaking otherwise remained the same. Cells are allowed to grow for 24 hours at 18 °C before harvesting in a centrifuge at 8,000 RCF for 8 minutes in conical plastic tubes at 4 °C. The supernatant is decanted, and the cell pellets are frozen at -20 °C until purification.

Enzyme Purification:

[0076] All column steps are carried out in a 4 °C room. Purification buffers (lysis buffer, wash buffer, elution buffer, dialysis buffer 1, dialysis buffer 2) are prepared using distilled and deionized water and are chilled to 4 °C before use. Lysis buffer consists of 50 mM sodium phosphate buffer pH 7.2, 25 mM imidazole, 1 mM β -mercaptoethanol (BME), 1 mg/mL lysozyme and 0.1 mg/mL of DNase. Wash buffer consists of 50 mM sodium phosphate buffer pH 7.2, 25 mM imidazole, and 1 mM BME. Elution buffer consists of 50 mM sodium phosphate buffer pH 7.2, 200 mM imidazole and 1 mM BME. Dialysis buffer 1 consists of 50 mM sodium phosphate buffer pH 7.2 with 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Dialysis buffer 2 consists of 50 mM sodium phosphate buffer pH 7.2. Cell pellets are resuspended in 5 mL of lysis buffer and sonicated on ice to disrupt the cell membrane. The sonication protocol uses a cycle of 5 seconds sonicating, 10 seconds without sonicating, and repeated 12 times per cell pellet with enough amplitude to disrupt the cells, in this case 30% power. The disrupted cells are then clarified by centrifugation at 15,000 RCF for forty five minutes at 4 °C. Concurrently columns containing approximately 1 mL of Ni-NTA agarose beads are washed with 10 mL wash buffer. Flow through is discarded. Upon completion of centrifugation, the clarified supernatant from the lysed cells is applied to the column and allowed to flow through. The flow through is discarded. The columns are then washed with 40 mL of wash buffer. Flow through is discarded. 15 mL of elution buffer is then added to the

columns, and flow through is collected in 10,000 molecular weight cut off (MWCO) conical spin filters. Samples are centrifuged at 6,000 RCF at 4 °C until concentrated to approximately 700 µL. The concentrated protein is transferred to a dialysis cassette with a 3,500 MWCO membrane. Flow through from the concentration step is discarded. Concentrated proteins are dialyzed in dialysis buffer 1 overnight, approximately sixteen hours. Concentrated proteins are then dialyzed in dialysis buffer 2 for four hours. Cells are then used immediately for assays. At this stage there are three individual concentrated and purified proteins.

Protein Assays:

[0077] Protein assays are conducted at room temperature. Hydrogen peroxide concentrations are tested with a commercially available coulometric kit that could detect between 0 and 100 mg/L hydrogen peroxide.

[0078] Purified methanol oxidase, still with 6xHis tag and SUMO tag, is added to 50 mM sodium phosphate buffer, pH 7.2 with and without 270 mM methanol with a final volume of 500 µL in a 2 mL plastic tube. The tube is vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In the condition with methanol, the commercial kit detects between 1 and 100 mg/L hydrogen peroxide. In the no methanol condition, the kit detects 0 mg/L hydrogen peroxide.

[0079] Purified formate oxidase, still with 6xHis tag, is added to 50 mM sodium phosphate buffer, pH 7.2 with and without 270 mM sodium formate with a final volume of 500 µL in a 2 mL plastic tube. The tube is vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In the condition with sodium formate, the commercial kit detects between 1 and 100 mg/L hydrogen peroxide. In the no sodium formate condition, the kit detects 0 mg/L hydrogen peroxide.

[0080] Purified formaldehyde dismutase, still with 6xHis tag, is added to 50 mM sodium phosphate buffer, pH 7.2 with and without 30 mM formaldehyde with a final volume of 500 µL in a 2 mL plastic tube. The tubes are vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In both conditions 0 mg/L hydrogen peroxide is detected.

[0081] Purified formaldehyde dismutase, still with 6xHis tag, and methanol oxidase, still with 6xHis tag and SUMO tag, are added to 50 mM sodium phosphate buffer, pH 7.2 with and without 30 mM formaldehyde with a final volume of 500 µL in a 2 mL plastic tube. The tubes are vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In the condition with formaldehyde, the commercial kit detects between 1 and 100 mg/L hydrogen peroxide. In the condition without the formaldehyde, 0 mg/L hydrogen peroxide is detected.

[0082] Purified formaldehyde dismutase, still with 6xHis tag, and formate oxidase, still with 6xHis tag, are added to 50 mM sodium phosphate buffer, pH 7.2 with and without 30 mM

formaldehyde with a final volume of 500 μ L in a 2 mL plastic tube. The tubes are vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In the condition with formaldehyde, the commercial kit detects between 1 and 100 mg/L hydrogen peroxide. In the condition without the formaldehyde, 0 mg/L hydrogen peroxide is detected.

[0083] Purified formaldehyde dismutase, still with 6xHis tag, and formate oxidase, still with 6xHis tag, and methanol oxidase, still with 6xHis tag and SUMO tag, are added to 50 mM sodium phosphate buffer, pH 7.2 with and without 270 mM methanol with a final volume of 500 μ L in a 2 mL plastic tube. The tubes are vortexed and allowed to sit for one hour before testing for hydrogen peroxide. In the condition with methanol, the commercial kit detects between 1 and 100 mg/L hydrogen peroxide. In the condition without the methanol, 0 mg/L hydrogen peroxide is detected.

[0084] References cited:

1. Eiben, C.B. *et al.* Mevalonate pathway promiscuity enables noncanonical terpene production. *ACS Synth. Biol.* (2019).

[0085] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

What is claimed is:

1. A method for producing hydrogen peroxide comprising: (a) contacting a methanol with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or a formate oxidase bound with a FAD cofactor, such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD.
2. The method of claim 1, further comprising: (d) contacting the formaldehyde with a formaldehyde dismutase to convert formaldehyde into methanol and formate.
3. The method of claim 1, wherein the methanol oxidase comprises the amino acid sequence of *Phanerochaete chrysosporium* methanol oxidase, or a functional variant thereof.
4. The method of claim 3, wherein the methanol oxidase comprises an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:1.
5. The method of claim 4, wherein the methanol oxidase comprises at least one or more, or all, of the following conserved sequences: GRRXIVPCANILGGGSSINFXMYTRXSASDXDD (SEQ ID NO:5), LLPLXK (SEQ ID NO:6), QDFLRA (SEQ ID NO:7), TAHGAE (SEQ ID NO:8), GRRSD (SEQ ID NO:9), LPGVGXXXQDH (SEQ ID NO:10), AGXKIRPTXEE (SEQ ID NO:11), KPDKP (SEQ ID NO:12), LEYPXSRG (SEQ ID NO:13), YKKXREXARRM (SEQ ID NO:14), GEXTSHHP (SEQ ID NO:15), EEDDXAI (SEQ ID NO:16), ETTWHXLGTC (SEQ ID NO:17), and DLSXCPDNXGXNTY (SEQ ID NO:18); wherein "X" is any amino acid residue.
6. The method of claim 1, wherein the formate oxidase comprises the amino acid sequence of *Schwanniomyces vanrijae* formate oxidase, or a functional variant thereof.
7. The method of claim 6, wherein the formate oxidase comprises an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:2.
8. The method of claim 7, wherein the formate oxidase comprises at last one or more, or all, of the following conserved sequences: SHXDFVIVGGGTAGNTVAGRLAE (SEQ ID NO:20),

SH(Y or F)DFVIVGGGTAGNTVAGRLAE (SEQ ID NO:21), DWAYK (SEQ ID NO:22), TFDXWXEXGGXEWTWD (SEQ ID NO:23), TFD(R or Q)W(A or E)E(Y or F)GG(E or K)EWTWD (SEQ ID NO:24), EWTWDPLVPYLR (SEQ ID NO:25), and DLY (SEQ ID NO:26); wherein "X" is any amino acid residue.

9. The method of claim 1, wherein the formaldehyde dismutase comprises the amino acid sequence of *Pseudomonas putida* formaldehyde dismutase, or functional variant thereof.

10. The method of claim 9, wherein the formaldehyde dismutase comprises an amino acid sequence having at least 70% amino acid residue identity with SEQ ID NO:3.

11. The method of claim 10, wherein the formaldehyde dismutase comprises at last one or more, or all, of the following conserved domains: (a) a NAD⁺-binding domain comprising one of the following conserved sequences: GXGXXG (SEQ ID NO:28), GXGXXGX₁₈D (SEQ ID NO:29), GXGGXXGX₁₈D (SEQ ID NO:30), GXGGXXGX₁₉D (SEQ ID NO:31), GXGXVGX₅GX₄GAAXXIXXD (SEQ ID NO:32); (b) ligands for binding a first catalytic zinc comprising one of the following conserved sequences: CXSXXHX₁₅H (SEQ ID NO:33) or CXSDXHX₃GX₄PX₅GH (SEQ ID NO:34); and, (c) ligands for binding a second catalytic zinc comprising one of the following conserved sequences: CXXCXXCX₇C (SEQ ID NO:35) or CGXCRXCKX₆C (SEQ ID NO:36); wherein "X" is any amino acid residue.

12. An *in vitro* composition comprising a methanol oxidase and a formate oxidase.

13. The *in vitro* composition of claim 12, wherein the methanol oxidase is a methanol oxidase from *Phanerochaete chrysosporium* or its functional variant and the formate oxidase is a formate oxidase from *Schwanniomyces vanrijiae* or its functional variant.

14. The *in vitro* composition of claim 13, wherein the composition is a solution suitable for the methanol oxidase and the formate oxidase to catalyze their respective enzymatic reactions.

15. The *in vitro* composition of claim 14, wherein the solution comprises a suitable salt buffer and has a pH having a value from about 7.0 to about 7.4.

16. The *in vitro* composition of claim 15, wherein the suitable salt buffer is an about 50 mM sodium phosphate buffer.

17. The *in vitro* composition of claim 15, wherein the pH is about 7.2.
18. The *in vitro* composition of claim 13, further comprising FAD cofactor.
19. The *in vitro* composition of claim 13, further comprising methanol and oxygen.
20. The *in vitro* composition of claim 13, further comprising a formaldehyde dismutase.
21. The *in vitro* composition of claim 21, wherein the formaldehyde dismutase is a formaldehyde dismutase from *Pseudomonas putida* or its functional variant.
22. A host cell comprising a polynucleotide encoding a methanol oxidase, formate oxidase, and/or formaldehyde dismutase, each operatively linked to separate promoters or one promoter.
23. The host cell of claim 22, wherein the polynucleotide encoding the methanol oxidase, formate oxidase, and/or formaldehyde dismutase comprise sequences for:
 - a) the methanol oxidase from *Phanerochaete chrysosporium* or its functional variant,
 - b) the formate oxidase from *Schwanniomyces vanriijiae* or its functional variant, and/or
 - c) the formaldehyde dismutase from *Pseudomonas putida* or its functional variant.
24. A method of producing a methanol oxidase, formate oxidase, and/or formaldehyde dismutase comprising: (a) providing a host cell of the present invention in a nutrient medium, and (b) culturing or growing the host cell in the nutrient medium such that the methanol oxidase, formate oxidase, and/or formaldehyde dismutase is expressed or produced.
25. A fusion protein comprising any two or all of methanol oxidase, formate oxidase, and formaldehyde dismutase.
26. A method for producing hydrogen peroxide comprising: (a) contacting a methanol with a fusion protein of the present invention comprising (i) a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor, and (ii) a formate oxidase bound with a FAD cofactor, such that the methanol oxidase oxidizes the methanol into a formaldehyde and the FAD cofactor bound to the methanol oxidase is reduced into FADH₂; (b) contacting the formaldehyde with the methanol oxidase or the formate oxidase, such that the formaldehyde is oxidized into a formate

and the FAD of the methanol oxidase or formate oxidase is reduced into FADH₂; and (c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD.

27. A method for producing hydrogen peroxide comprising:

a) contacting methanol with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor,

wherein the methanol oxidase is a methanol oxidase from *Phanerochaete chrysosporium* or its functional variant,

such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂;

b) contacting the formaldehyde with an unreduced methanol oxidase or a formate oxidase bound with a FAD cofactor,

wherein the formate oxidase is a formate oxidase from *Schwanniomyces vanrijiae* or its functional variant,

such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and

c) contacting oxygen with one or more of the FADH₂ to produce hydrogen peroxide and oxidize FADH₂ into FAD.

28. The method of claim 27, further comprising: contacting the formaldehyde with a formaldehyde dismutase,

wherein the formaldehyde dismutase is a formaldehyde dismutase from *Pseudomonas putida*,

such that the formaldehyde is converted into methanol and formate end products.

29. A method for waste removal comprising:

a) contacting formaldehyde with with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor or a formate oxidase bound with a FAD cofactor,

wherein the methanol oxidase is a methanol oxidase from *Phanerochaete chrysosporium* or its functional variant,

wherein the formate oxidase is a formate oxidase from *Schwanniomyces vanrijiae* or its functional variant,

such that the formaldehyde is oxidized into a formate and the FAD is reduced into FADH₂; and

b) contacting oxygen with one or more of the FADH_2 to produce hydrogen peroxide and oxidize FADH_2 into FAD.

30. The method of claim 29, further comprising:

contacting the hydrogen peroxide with a catalase, such that the hydrogen peroxide is broken down into water and oxygen.

31. The method of claim 30, wherein the catalase comprises the enzyme KatE or the enzyme KatG or a functional variant of either enzyme.

32. The method of claim 30, wherein the catalase comprises a hydroperoxidase.

33. The method of claim 29, further comprising:

contacting the hydrogen peroxide with a peroxidase,

such that the hydrogen peroxide is reduced to water, producing an oxidized peroxidase active site; and

the peroxidase oxidizes an organic molecule.

34. The method of claim 33, wherein the organic molecule is a compound selected from methanol, formaldehyde, and formate.

35. A method for producing hydrogen peroxide comprising:

a) contacting methanol with a formate oxidase bound with a FAD cofactor,

wherein the formate oxidase is a formate oxidase from *Schwanniomyces vanriijiae* or its functional variant,

such that the methanol is oxidized into formaldehyde and the FAD cofactor is reduced into FADH_2 ;

b) contacting the formaldehyde with an unreduced formate oxidase,

such that the formaldehyde is oxidized into formate and the FAD is reduced into FADH_2 ;

and

c) contacting oxygen with one or more of the FADH_2 to produce hydrogen peroxide and oxidizing FADH_2 into FAD.

36. The method of claim 35, further comprises:

contacting the methanol with a methanol oxidase bound with a flavin adenine dinucleotide (FAD) cofactor,

wherein the methanol oxidase is a methanol oxidase from *Phanerochaete chrysosporium* or its functional variant, and

wherein the method further comprises contacting the methanol with the methanol oxidase such that the methanol is oxidized into a formaldehyde and the FAD cofactor is reduced into FADH₂.

37. The method of claim 36, further comprising: contacting the formaldehyde with a formaldehyde dismutase,

wherein the formaldehyde dismutase is a formaldehyde dismutase from *Pseudomonas putida* or its functional variant,

such that the formaldehyde is converted into methanol and formate end products.

Query 1 MGHPEEVDVIVCGGGFAGCVVAGRLAYADPTLKVM LIEGGANNRDEPWVYRPGIYVRNMQ 60
M PEE D++V GGG +G +AGRLA D +LKV LIE G NN ++PWVY PGIY PNM+

Sbjct 1 MAIPPEEFBILVLGGGSSGSCIAGRLANLDHSLKVLIEAGENN LNNPWVYLPFGIYPRNMK 60

Query 61 RNGINDKATFYTDMASSYLRFPSIVPCANILGGGSSINFQMYTRASASDWDEFKTEGW 120
+ + A+FYT S +L GRP+IVPCAN+LGGGSSINF MYTR SASD+BDE+ EGW

Sbjct 61 LD--SKTASFYTSN-PSPHLNGRRRAIVPCANVLGGGSSINFMMYTFGSASDYDLEFQAEGW 117

Query 121 TKKLLPLMKPLENYQKPCNN-DTHGYDGPFAISNGGQIMFVAQDFLRAHAHAIGVPEYSD 179
KLLPLMK+ E YQ+ CNN D HG++GPI +S G PV QDFLRA+ + G+PY DD

Sbjct 118 KTKLLPLMKKTETTYQRACNNPDHGFEGPIKVSFGNYTYPVQDFLRASESQGIPYVDD 177

Query 180 IQDLTATAGAEIWAKYINPHTGRRSEAAATAYVHSVMDVQDNLFRLCNARVSEVLFEDNNK 239
++DL TATAGAE W K+INR TGRRE+A A+VHS M DNL+L CN +V +++ +D +

Sbjct 178 LEDLVATAGAEHWLKWNRDTERRESAHAFVHSTMNRHNDLYLICNTKVDKIIVEDG-R 236

Query 240 AVGVAYVPSRNRTHGGKLHETIVKAKMVVLSSGTLGTFQILERSGVNGELLRQLGIKI 299
A V VPS+ H+ I +ARK +VLS GT+ +P +L+RSG G+ LP G+K

Sbjct 237 AAVRTVPSKPLNPKKPSHK-IYPARKQIVLSCGTISSPLVLQRSGFQDPIKLRAAGVKP 295

Query 300 VSDLPGVGEQYQDHVTTLSTYRVSNEISITDDFLRGVKDVQRELFTWEVVSPEKARLSSN 359
+ +LPGVG +QDHV S YR+ + + DDE+RG ++Q+ +E +W + L++N

Sbjct 296 LVNLPGVGRNFQDHVCFPSYRIKPQYESFDDFVRGLAEIQKRVFDQWYANGT-GPLATN 354

Query 360 AIDAGFKIPTEEBLKEMGPEFNELWNPYFKKPKDKPMFGSIVAGAYADHTLLPPGKYI 419
I+AG KIRPT EEL +M F E + YF+KPKDKPM SI+AG + DHT +PPGKY+

Sbjct 355 GIEAGVKIRPTPEELSOMDESQEGYREYFELKPKDKPMHYSIIAGFFGDHTKIIPGKYM 414

Query 420 TMFQYLEYPASRQKIHIKSONPYVEPFDDSGFMNNKADFAPIRWSYKKTREVAREMBAFR 479
TMF +LEYP SRC IHI S +PY P PD GFMN++ D AP+ W+YKK+RE ARMD F

Sbjct 415 TMFHLEYPFSRQSIHITSPPDYAAPDFDPGFMNDERLMAFMVWAYKKSRETARRMHFA 474

Query 480 BELTSHHLEFHPASPAACKDIDIETAKQI-YPDGLTVGIHMGSWHP-----SEPYKH 531
GE+TSHHE F +S A ++D+ET+ P L+ G+ GSW QP +E +

Sbjct 475 BEVTSHHLEFPYSSEARALEMDLETSNAYGGPLNLSAGLAHGSWTQPLKKPTAKNEGHVT 534

Query 532 DKVIE---DIPYTEEDDKAIDDWVADHVEETTWHSLGTCAMKFE-----QGGVVDKRLN 582
+E DI Y EEDDKAI++++ +H ETTWH LGTC++ PRE GGV+D P N

Sbjct 535 SNQVELHPDIEYDEEDDKAIEYIREHTETTWHCLGTCISIGPREGSKIVKWGGVLDHRSN 594

Query 583 VYGTQNLKCVLSICPDNLGTNTYSSALLVGEKGADLIAEELGLKIKTAPHAPVPHAPVPT 642
VYG + LK DLS+CPDN+G NTY++ALL+GEK A L+ E+LG + VP + T

Sbjct 595 VYGVKGLKVGSLSVCPDNVGCNTYFTALLIGEKTATLVGEDLGYSGEALDMTVPQFKLGT 654

FIG. 1

1 H V Y I A G A G P V G R C A A A G A R L L G A A — — C V I V G D Q
 2 T C A V F G L G G V G L S V I M G C K A A G A A — — R I I G V D I
 3 K I T V V G V G A V G M A C A I S I L M K D L A D — E V A L V D V
 4 K I G I D G F G R I G R L V L R A A L S C G A Q — — V V A V N D P

FIG. 2

ADH	STAGKVIKCK	AAVLWEEKKP	FSIEEVEVAP	PKAHEVRIKM	VATGICRSDD	50
FDM	-AGNKSVMYH	GTRDLRVETV	PYPKLEHNNR	KLEHAVILKV	VSTNICGSDQ	49
					▼ ▼	
ADH	HVVSGLTVTP	LPVIAGHEAA	GIVESIGEGV	TTVRPGDKVI	PLFTPQCGKC	100
FDM	HIYRGRFIVP	KGHVLGHEIT	GEVVEKGS DV	ELMDIGDLVS	VPFNVACGRC	99
	▼	▼			▼ ▼	
ADH	RVCKHPEGNF	CLKNDLSMPR	GTMQDGTSRF	TCRGKPIHHE	LGTSTFSQYT	150
FDM	RNCKEARS DV	CENNLVNPDA	DLGAFGF DLK	GWSSGQAEYV	LVPYADYMLL	149
	▼	▼			▲ ▲	
ADH	VVDEISVAKI	DAASPLEKVC	LIGCGFSTGY	GS AVKVAKVT	QGSTCAVFGL	200
FDM	KFGDKEQAME	KIKDLTLISD	ILPTGFHGC-	-----VSAGVK	PGSHVYIAGA	194
			▲			
ADH	GGVGLSVIMG	CKAAGAARI	GVDINKDKFA	KAKEVG--AT	ECVNPQDYKK	248
FDM	GPVGRCAAAG	ARLLGAACVI	VGDQNPRLK	LLSDAGFETI	DLRNSAPLRD	244
ADH	PIQEV L-TEM	SNGGVDFS-F	EVIGRLDTMV	TALSCCQEAY	GVSIVGVPP	296
FDM	QIDQILGKPE	VDCGVDAVGF	EAHGLGDEAN	TETPNGALNS	LFDVVRAGGA	294
ADH	DSQNLSMNP	LLLSGRTWKG	AIFGGFKSKD	SVPKLVADFM	AKKFALDPLI	346
FDM	IGIPGIYVGS	DPDPVNKDAG	SGRLHLDFGK	MWTKSIRIMT	GMAPVTNYNR	344
ADH	THVLPFEKIN	EGFDLLRSGE	SIRTILTF..			374
FDM	HLTEAILWDQ	MPYLSKVMNI	EVITLDQAPD	GYAKFDKGSP	AKFVIDPHGM	394
ADH						
FDM	LKNK.....					398

FIG. 3