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(54) Title: PRODUCTION OF PERACIDS USING AN ENZYME HAVING PERHYDROLYSIS ACTIVITY

(57) Abstract: A process is provided for producing peroxycarboxylic acids from carboxylic acid esters. More specifically, carboxylic acid esters are reacted with an inorganic peroxide, such as hydrogen peroxide, in the presence of an enzyme catalyst having perhydrolysis activity. The present perhydrolase catalysts are classified as members of the carbohydrate esterase family 7 (CE-7) based on the conserved structural features. Further, disinfectant formulations comprising the peracids produced by the processes described herein are provided.

PRODUCTION OF PERACIDS USING AN ENZYME HAVING PERHYDROLYSIS ACTIVITY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Patent Application No. 11/943872, filed November 21, 2007, the entire contents of which are incorporated herein by reference. This application is related to U.S. Patent Application No. 11/743354, filed May 2, 2007, U.S. Patent Application No. 11/638,635, filed December 12, 2006, U.S. Provisional Application No. 60/750,092, filed December 13, 2005, and U.S. Provisional Application No. 60/853,065, filed October 20, 2006, each of which in its entirety is incorporated herein by reference.

TECHNICAL FIELD

[0002] This invention relates to the field of peracid biosynthesis and *in situ* enzyme catalysis. Specifically, a process is provided to produce peracids using the perhydrolysis activity of enzymes identified structurally as belonging to the CE-7 family of carbohydrate esterases, including cephalosporin acetyl hydrolases (CAHs; E.C. 3.1.1.41) and acetyl xylan esterases (AXEs; E.C. 3.1.1.72). The enzymatic process produces percarboxylic acids from carboxylic acid ester substrates. Further, disinfectant formulations comprising the peracids produced by the processes described herein are provided.

BACKGROUND

[0003] Peracid compositions have been reported to be effective antimicrobial agents. Methods to clean, disinfect, and/or sanitize hard surfaces, meat products, living plant tissues, and medical devices against undesirable microbial growth have been described (U.S. patent 6,545,047; U.S. patent 6,183,807; U.S. patent 6,518,307; U.S. patent application publication 20030026846; and U.S. patent 5,683,724). Peracids have also been reported to be useful in preparing bleaching compositions for laundry detergent applications (U.S. patent 3,974,082; U.S. patent 5,296,161; and U.S. patent 5,364,554).

[0004] Peracids can be prepared by the chemical reaction of a carboxylic acid and hydrogen peroxide (see Organic Peroxides, Daniel Swern, ed., Vol. 1, pp 313-516; Wiley Interscience, New York, 1971). The reaction is

usually catalyzed by a strong inorganic acid, such as concentrated sulfuric acid. The reaction of hydrogen peroxide with a carboxylic acid is an equilibrium reaction, and the production of peracid is favored by the use of an excess concentration of peroxide and/or carboxylic acid, or by the removal of water. There are several disadvantages to the chemical reaction for peracid production: 1) the high concentration of carboxylic acid used to favor production of peracid can result in an undesirable odor when using the peracid-containing solution, 2) the peracid is oftentimes unstable in solution over time, and the concentration of peracid in the solution decreases during storage prior to use, and 3) the formulation is often strongly acidic due to the use of a concentrated sulfuric acid as catalyst.

[0005] One way to overcome the disadvantages of the chemical production of peracids is to employ an enzyme catalyst in place of a strong acid catalyst. The use of an enzyme catalyst allows for the rapid production of peracid at the time of use and/or application, avoiding problems associated with storage of peracid solutions and variations in peracid concentrations over time. The high concentrations of carboxylic acids typically used to produce peracid via the direct chemical reaction with hydrogen peroxide are not required for enzymatic production of peracid, where the enzyme-catalyzed reaction can use a carboxylic acid ester as substrate at a much lower concentration than is typically used in the chemical reaction. The enzyme reaction can be performed across a broad range of pH, dependent on enzyme activity and stability at a given pH, and on the substrate specificity for perhydrolysis at a given pH.

[0006] Esterases, lipases, and some proteases have the ability to catalyze the hydrolysis of alkyl esters to produce the corresponding carboxylic acids (Formula 1):

Formula 1

$$R_1COOR_2 + H_2O \xrightarrow{\text{Lipase, esterase, or protease}} R_1COOH + HOR_2.$$

[0007] Some esterases, lipases, and proteases also exhibit perhydrolysis activity, catalyzing the synthesis of peracids from alkyl esters (Formula 2):

Formula 2

$$R_1COOR_2 + H_2O_2 \xrightarrow{\text{Lipase, esterase, or protease}} R_1COOOH + HOR_2.$$

[0008] O. Kirk *et al*. (*Biocatalysis*, 11:65-77 (1994)) investigated the ability of hydrolases (lipases, esterases, and proteases) to catalyze perhydrolysis of acyl substrates with hydrogen peroxide to form peroxycarboxylic acids, and reported that perhydrolysis proceeds with a very low efficiency in aqueous systems. Furthermore, they found that lipases and esterases degraded percarboxylic acid to the corresponding carboxylic acid and hydrogen peroxide. They also found that proteases neither degraded nor catalyzed perhydrolysis of carboxylic acid esters in water. The authors concluded that esterases, lipases and proteases are, in general, not suitable for catalyzing perhydrolysis of simple esters, such as methyl octanoate and trioctanoin, in an aqueous environment.

[0009] U.S. patent 3,974,082 describes the production of bleaching compositions for laundry detergent applications by contacting the material to be bleached with an aqueous solution containing an oxygen-releasing inorganic peroxygen compound, an acyl alkyl ester, and an esterase or lipase capable of hydrolyzing the ester.

[0010] U.S. patent 5,364,554 describes an activated oxidant system for *in situ* generation of peracid in aqueous solution using a protease enzyme, a source of hydrogen peroxide, and an ester substrate that is preferably chemically non-perhydrolyzable. A method of bleaching and a method of forming peracid are also disclosed.

[0011] U.S. patent 5,296,161 describes production of peracid in an aqueous solution comprising one or more specific esterases and lipases, a source of hydrogen peroxide, and a functionalized ester substrate suitable for use in a bleaching composition. However, the concentration of peracid produced was generally insufficient for use in many commercial disinfectant applications.

[0012] Most known methods for preparing peracids from the corresponding carboxylic acid esters using enzyme catalysts do not produce and accumulate a peracid at a sufficiently high concentration to be efficacious for disinfection in a variety of applications. Several protease and lipase combinations have recently been reported to generate peracids (*e.g.*, peracetic

acid) *in situ* at concentrations suitable for use as a disinfectant and/or commercial bleaching agent (see co-owned U.S. Patent Applications Nos. 11/413,246 and 11/588,523; herein incorporated by reference). However, there remains a need to identify additional perhydrolase catalysts capable of producing peracids *in situ*.

[0013] U.S. patent 4,444,886 describes a strain of *Bacillus subtilis* (ATCC 31954™) having ester hydrolase activity (described as a "diacetinase") that has high specificity for hydrolyzing glycerol esters having acyl groups having 2 to 8 carbon atoms. U.S. patent 4,444,886 does not describe, discuss or predict that the ester hydrolase activity of this strain has perhydrolase activity towards carboxylic acid esters, including glycerol esters.

[0014] The problem to be solved is to provide a process to enzymatically produce peracids *in situ* at concentrations suitable for use in a variety of disinfectant applications and/or bleaching applications. Preferably, the substrates used to produce the peracid compositions should be relatively non-toxic and inexpensive, such as carboxylic acid esters, especially mono-, di-, and triacylglycerols, wherein the acyl group has 1- 8 carbon atoms, as well as acetylated sugars and C1 to C6 polyol esters.

SUMMARY

[0015] The stated problems have been solved by the discovery that enzymes belonging to the structural family of CE-7 esterases (e.g., cephalosporin C deacetylases [CAHs] and acetyl xylan esterases [AXEs]) exhibit significant perhydrolysis activity for converting the present carboxylic acid esters (in the presence of an inorganic source of peroxygen such as hydrogen peroxide) into peracids at concentrations sufficient for use as a disinfectant and/or bleaching agent. The system achieves efficiency by producing the peracid in high concentrations without requiring a high concentration of peroxygen.

[0016] Specific examples of perhydrolases are exemplified from *Bacillus subtilis* (ATCC 31954™), *B. subtilis* BE1010 (Payne and Jackson, *J. Bacteriol.* 173:2278-2282 (1991)), *B. subtilis* ATCC 6633™ (U.S. 6,465,233), *B. subtilis* ATCC 29233™; *B. licheniformis* ATCC 14580™ (Rey *et al.*, *Genome Biol.*, 5(10):article 77 (2004)), *Clostridium thermocellum* ATCC 27405™ (Copeland *et al.*, GENBANK® ZP_00504991, *B. pumilus* PS213 (Degrassi *et al.*, *Microbiology*, 146:1585-1591 (2000)), *Thermotoga neapolitana* (GENBANK® AAB70869.1), *Bacillus clausii* KSM-K16

(GENBANK® YP_175265), *Thermotoga maritima* MSB8 (GENBANK® NP_227893.1), *Thermoanaerobacterium saccharolyticum* (GENBANK® S41858), *Thermotoga lettingae* (GENBANK® CP000812), *Thermotoga petrophila* (GENBANK® CP000702), and *Thermotoga* sp. RQ2 (GENBANK® CP000969).

[0017] Each of the present perhydrolases described herein share conserved structural features (i.e. a conserved signature motif) as well as superior perhydrolysis activity when compared to other α/β-hydrolases, (such as commercially available lipases; see comparative Examples 26 and 28), making this unique family of enzymes particularly suitable for generating peracids *in situ* at concentrations sufficient for use as a disinfectant and/or bleaching agent. Suitable perhydrolases useful in the present process can be identified by a conserved signature motif found within the CE-7 family of carbohydrate esterases.

[0018] In one aspect, a process is provided for producing a peroxycarboxylic acid from a carboxylic acid ester comprising

a) providing a set of reaction components comprising:

1) at least one substrate selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula R_6-C(O)O

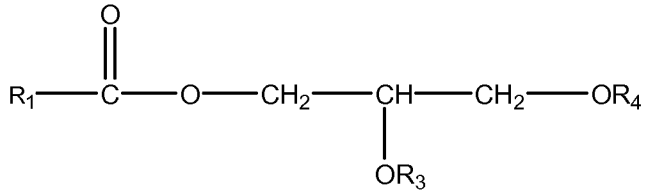
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

2) a source of peroxygen; and

3) an enzyme catalyst having perhydrolysis activity, wherein said enzyme catalyst comprises an enzyme having a CE-7 signature motif that aligns with a reference sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

i) an RGQ motif at amino acid positions 118-120 of SEQ ID NO:2;

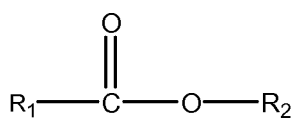
ii) a GXSQG motif at amino acid positions 179-183 of SEQ ID NO:2; and

iii) an HE motif at amino acid positions 298-299 of SEQ ID NO:2; and

wherein said enzyme comprises at least 30% amino acid identity to SEQ ID NO: 2; and

b) combining said reaction components under suitable aqueous reaction conditions whereby a peroxycarboxylic acid is produced.

[0019] In another embodiment, suitable substrates also include esters of the formula:



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_2= C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2\text{-}O)_nH$ or $(CH_2CH(CH_3)\text{-}O)_nH$ and n=1 to 10.

[0020] In another aspect, a process to disinfect a surface or inanimate object is provided, said process comprising in addition to the above process the step of:

c) contacting a surface or inanimate object with the peroxycarboxylic acid produced in step (b) whereby said surface or said inanimate object is disinfected.

[0021] In another aspect, a process to disinfect a hard surface or inanimate object using an enzymatically produced peroxycarboxylic acid composition is provided, the process comprising:

a) combining on said hard surface or inanimate object under suitable aqueous reaction conditions a set of reaction components comprising:

1) at least one substrate selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula $R_6C(O)O$

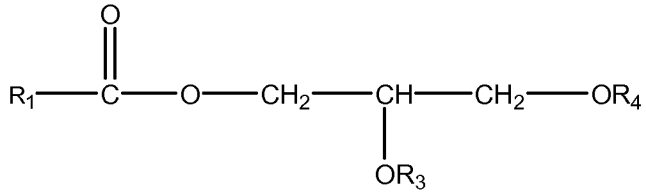
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

2) a source of peroxygen; and

3) an enzyme catalyst having perhydrolysis activity, wherein said enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO:82, SEQ ID NO:90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence,

thereby generating peroxycarboxylic acid, whereby said hard surface or inanimate object is disinfected.

[0022] In some embodiments, the peroxycarboxylic acid produced is diluted.

[0023] In a further embodiment, the above process comprises an enzyme catalyst comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 26, SEQ ID NO: 70, SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence.

[0024] In a further embodiment, the substantially similar enzyme having perhydrolase activity is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to one or more amino acid sequences selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

[0025] In a preferred embodiment, the substrate is selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose

pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; and mixtures thereof.

BRIEF DESCRIPTION OF THE FIGURE

[0026] Figure 1 (panels a-c) is a CLUSTALW alignment of perhydrolases of the present invention. Each of the perhydrolases are structurally classified members of the carbohydrate esterase family 7 (CE-7) and share certain conserved domains. Several conserved motifs (underlined) have been identified that together form the signature motif for CE-7 carbohydrate esterases. An additional motif (LXD; amino acid residues 267-269 of SEQ ID NO: 2) is also underlined and may be used to further characterize the signature motif.

BRIEF DESCRIPTION OF THE BIOLOGICAL SEQUENCES

[0027] The following sequences comply with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the European Patent Convention (EPC) and the Patent Cooperation Treaty (PCT) Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

[0028] SEQ ID NO: 1 is the nucleic acid sequence of the cephalosporin C deacetylase (*cah*) coding region from *Bacillus subtilis* ATCC 31954™.

[0029] SEQ ID NO: 2 is the deduced amino acid sequence of the cephalosporin C deacetylase from *Bacillus subtilis* ATCC 31954™.

[0030] SEQ ID NOs: 3 and 4 are primers used to PCR amplify perhydrolase genes from *Bacillus subtilis* species for construction of pSW186, pSW187, pSW188, and pSW190.

[0031] SEQ ID NO: 5 is the nucleic acid sequence of the cephalosporin C deacetylase coding region from *B. subtilis subsp. subtilis str.* 168.

[0032] SEQ ID NO: 6 is the deduced amino acid sequence of the cephalosporin C deacetylase from *B. subtilis subsp. subtilis str.* 168, and is identical to the deduced amino acid sequence of the cephalosporin C deacetylase from *B. subtilis* BE1010.

[0033] SEQ ID NO: 7 is the nucleic acid sequence of the cephalosporin acetylesterase coding region from *B. subtilis* ATCC 6633™.

[0034] SEQ ID NO: 8 is the deduced amino acid sequence of the cephalosporin acetylesterase from *B. subtilis* ATCC 6633™.

[0035] SEQ ID NO: 9 is the nucleic acid sequence of the cephalosporin C deacetylase coding region from *B. licheniformis* ATCC 14580™.

[0036] SEQ ID NO: 10 is the deduced amino acid sequence of the cephalosporin C deacetylase from *B. licheniformis* ATCC 14580™.

[0037] SEQ ID NO: 11 is the nucleic acid sequence of the acetyl xylan esterase coding region from *B. pumilus* PS213.

[0038] SEQ ID NO: 12 is the deduced amino acid sequence of the acetyl xylan esterase from *B. pumilus* PS213.

[0039] SEQ ID NO: 13 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Clostridium thermocellum* ATCC 27405™.

[0040] SEQ ID NO: 14 is the deduced amino acid sequence of the acetyl xylan esterase from *Clostridium thermocellum* ATCC 27405™.

[0041] SEQ ID NO: 15 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga neapolitana*.

[0042] SEQ ID NO: 16 is the deduced amino acid sequence of the acetyl xylan esterase from *Thermotoga neapolitana*.

[0043] SEQ ID NO: 17 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga maritima* MSB8.

[0044] SEQ ID NO: 18 is the deduced amino acid sequence of the acetyl xylan esterase from *Thermotoga maritima* MSB8.

[0045] SEQ ID NO: 19 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermoanaerobacterium sp.* JW/SL YS485.

[0046] SEQ ID NO: 20 is the deduced amino acid sequence of the acetyl xylan esterase from *Thermoanaerobacterium sp.* JW/SL YS485.

[0047] SEQ ID NO: 21 is the nucleic acid sequence of the cephalosporin C deacetylase coding region from *Bacillus sp.* NRRL B-14911.

[0048] SEQ ID NO: 22 is the deduced amino acid sequence of the cephalosporin C deacetylase from *Bacillus sp.* NRRL B-14911.

[0049] SEQ ID NO: 23 is the nucleic acid sequence of the cephalosporin C deacetylase coding region from *Bacillus halodurans* C-125.

[0050] SEQ ID NO: 24 is the deduced amino acid sequence of the cephalosporin C deacetylase from *Bacillus halodurans* C-125.

[0051] SEQ ID NO: 25 is the nucleic acid sequence of the cephalosporin C deacetylase coding region from *Bacillus clausii* KSM-K16.

[0052] SEQ ID NO: 26 is the deduced amino acid sequence of the cephalosporin C deacetylase from *Bacillus clausii* KSM-K16.

[0053] SEQ ID NOs: 27 and 28 are primers used to PCR amplify perhydrolase genes from *Bacillus subtilis* species for construction of pSW194 and pSW189.

[0054] SEQ ID NO: 29 is the nucleic acid sequence of the PCR product cloned into pSW194.

[0055] SEQ ID NO: 30 is the nucleic acid sequence of the PCR product cloned into pSW189.

[0056] SEQ ID NO: 31 is the nucleic acid sequence of the *Bacillus subtilis* ATCC 29233™ cephalosporin C deacetylase (*cah*) gene cloned into pSW190.

[0057] SEQ ID NO: 32 is the deduced amino acid sequence of the *Bacillus subtilis* ATCC 29233™ cephalosporin C deacetylase (CAH).

[0058] SEQ ID NOs: 33 and 34 are primers used to PCR amplify the *Bacillus licheniformis* ATCC 14580™ cephalosporin C deacetylase gene for construction of pSW191.

[0059] SEQ ID NOs: 35 and 36 are primers used to PCR amplify the *Clostridium thermocellum* ATCC 27405™ acetyl xylan esterase gene for construction of pSW193.

[0060] SEQ ID NOs: 37 and 38 are primers used to PCR amplify the *Bacillus pumilus* PS213 acetyl xylan esterase coding sequence (GENBANK® AJ249957) for construction of pSW195.

[0061] SEQ ID NOs: 39 and 40 are primers used to PCR amplify the *Thermotoga neapolitana* acetyl xylan esterase gene (GENBANK® 58632) for construction of pSW196.

[0062] SEQ ID NO: 41 is the nucleic acid sequence of the codon-optimized version of the *Thermotoga neapolitana* acetyl xylan esterase gene in plasmid pSW196.

[0063] SEQ ID NO: 42 is the nucleic acid sequence of the kanamycin resistance gene.

[0064] SEQ ID NO: 43 is the nucleic acid sequence of plasmid pKD13.

[0065] SEQ ID NOs: 44 and 45 are primers used to generate a PCR product encoding the kanamycin gene flanked by regions having homology to the *katG* catalase gene in *E. coli* MG1655. The product was used to disrupt the endogenous *katG* gene.

[0066] SEQ ID NO: 46 is the nucleic acid sequence of the PCR product encoding the kanamycin resistance gene flanked by regions having homology to the *kat*G catalase gene in *E. coli* MG1655. The product was used to disrupt the endogenous *kat*G gene.

[0067] SEQ ID NO: 47 is the nucleic acid sequence of the *katG* catalase gene in *E. coli* MG1655.

[0068] SEQ ID NO: 48 is the deduced amino acid sequence of the KatG catalase in *E. coli* MG1655.

[0069] SEQ ID NO: 49 is the nucleic acid sequence of plasmid pKD46.

[0070] SEQ ID NOs: 50 and 51 are primers used to confirm the disruption of the *katG* gene.

[0071] SEQ ID NO: 52 is the nucleic acid sequence of plasmid pCP20.

[0072] SEQ ID NOs: 53 and 54 are primers used to generate a PCR product encoding the kanamycin gene flanked by regions having homology to the *kat*E catalase gene in E. coli MG1655. The product was used to disrupt the endogenous *kat*E gene.

[0073] SEQ ID NO: 55 is the nucleic acid sequence of the PCR product encoding the kanamycin resistance gene flanked by regions having homology to the *kat*E catalase gene in *E. coli* MG1655. The product was used to disrupt the endogenous *kat*E gene.

[0074] SEQ ID NO: 56 is the nucleic acid sequence of the *katE* catalase gene in *E. coli* MG1655.

[0075] SEQ ID NO: 57 is the deduced amino acid sequence of the KatE catalase in *E. coli* MG1655.

[0076] SEQ ID NOs: 58 and 59 are primers used to confirm disruption of the *katE* gene in the single knockout strain *E. coli* MG1655 Δ*kat*E, and in the double-knockout strain *E. coli* MG1655 Δ*kat*G Δ*kat*E, herein referred to as *E. coli* KLP18.

[0077] SEQ ID NO: 60 is the nucleic acid sequence of the codon optimized version of the *Bacillus pumilus* PS213 encoding the amino acid sequence SEQ ID NO: 12.

[0078] SEQ ID NO: 61 is the amino acid sequence of the region encompassing amino acids residues 118 through 299 of SEQ ID NO: 2.

[0079] SEQ ID NOs: 62 and 63 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Bacillus clausii* KSM-K16 cephalosporin-C deacetylase.

[0080] SEQ ID NO: 64 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Bacillus clausii* KSM-K16 cephalosporin-C deacetylase coding sequence.

[0081] SEQ ID NO: 65 is the nucleic acid sequence of the codon-optimized *Bacillus clausii* KSM-K16 cephalosporin-C deacetylase coding sequence.

[0082] SEQ ID NOs: 66 and 67 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermoanaerobacterium saccharolyticum* acetyl xylan esterase coding region (GENBANK® Accession No. S41858).

[0083] SEQ ID NO: 68 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermoanaerobacterium saccharolyticum* acetyl xylan esterase coding sequence.

[0084] SEQ ID NO: 69 is the nucleic acid sequence of the codon-optimized version of the *Thermoanaerobacterium saccharolyticum* acetyl xylan esterase coding sequence.

[0085] SEQ ID NO: 70 is the deduced amino acid sequence of the acetyl xylan esterase from *Thermoanaerobacterium saccharolyticum* (GENBANK® Accession No. S41858).

[0086] SEQ ID NOs: 71 and 72 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga maritima* MSB8 (GENBANK® Accession No. NP_227893.1) acetyl xylan esterase coding sequence.

[0087] SEQ ID NO: 73 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga maritima* MSB8 acetyl xylan esterase coding sequence.

[0088] SEQ ID NO: 74 is the nucleic acid sequence of the codon-optimized version of the *Thermotoga maritima* MSB8 acetyl xylan esterase coding sequence.

[0089] SEQ ID NOs: 75 and 76 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga lettingae* (GENBANK® Accession No. CP000812) acetyl xylan esterase coding sequence.

[0090] SEQ ID NO: 77 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga lettingae* acetyl xylan esterase coding sequence.

[0091] SEQ ID NOs: 78 and 79 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga lettingae* (GENBANK® Accession No. CP000812) acetyl xylan esterase coding sequence.

[0092] SEQ ID NO: 80 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga lettingae* acetyl xylan esterase coding sequence.

[0093] SEQ ID NO: 81 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga lettingae.*

[0094] SEQ ID NO: 82 is the deduced amino acid sequence of the acetyl xylan esterase from *Thermotoga lettingae.*

[0095] SEQ ID NOs: 83 and 84 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga petrophila* (GENBANK® Accession No. CP000702) acetyl xylan esterase coding sequence.

[0096] SEQ ID NO: 85 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga petrophila* acetyl xylan esterase coding sequence.

[0097] SEQ ID NOs: 86 and 87 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga petrophila* (GENBANK® Accession No. CP000702) acetyl xylan esterase coding sequence.

[0098] SEQ ID NO: 88 is the nucleic acid sequence of the PCR product encoding a codon-optimized version of the *Thermotoga petrophila* acetyl xylan esterase coding sequence.

[0099] SEQ ID NO: 89 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga petrophila.*

[0100] SEQ ID NO: 90 is the deduced amino acid sequence of an acetyl xylan esterase from *Thermotoga petrophila.*

[0101] SEQ ID NOs: 91 and 92 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga* sp.

RQ2 "RQ2(a)" (GENBANK® Accession No. CP000969) acetyl xylan esterase coding sequence.

[0102] SEQ ID NO: 93 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(a)" acetyl xylan esterase coding sequence.

[0103] SEQ ID NOs: 94 and 95 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(a)" (GENBANK® Accession No. CP000969) acetyl xylan esterase coding sequence.

[0104] SEQ ID NO: 96 is the nucleic acid sequence of the PCR product encoding a codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(a)" acetyl xylan esterase coding sequence.

[0105] SEQ ID NO: 97 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga* sp. RQ2 identified herein as "RQ2(a)".

[0106] SEQ ID NO: 98 is the deduced amino acid sequence of an acetyl xylan esterase (GENBANK® Accession No. ACB09222) from *Thermotoga* sp. RQ2 identified herein as "RQ2(a)".

[0107] SEQ ID NOs: 99 and 100 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(b)" (GENBANK® Accession No. CP000969) acetyl xylan esterase coding sequence.

[0108] SEQ ID NO: 101 is the nucleic acid sequence of the PCR product encoding the codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(b)" acetyl xylan esterase coding sequence.

[0109] SEQ ID NOs: 102 and 103 are the nucleic acid sequences of the primers used to PCR amplify a codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(b)" (GENBANK® Accession No. CP000969) acetyl xylan esterase coding sequence.

[0110] SEQ ID NO: 104 is the nucleic acid sequence of the PCR product encoding a codon-optimized version of the *Thermotoga* sp. RQ2 "RQ2(b)" acetyl xylan esterase coding sequence.

[0111] SEQ ID NO: 105 is the nucleic acid sequence of the acetyl xylan esterase coding region from *Thermotoga* sp. RQ2 identified herein as "RQ2(b)".

[0112] SEQ ID NO: 106 is the deduced amino acid sequence of an acetyl xylan esterase (GENBANK® Accession No. ACB08860) from *Thermotoga* sp. RQ2 identified herein as "RQ2(b)".

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0113] The stated problems have been solved by the discovery that enzymes belonging to the CE-7 carbohydrate esterase family exhibit significant perhydrolysis activity for converting carboxylic acid ester substrates to peracids. This family of structurally related enzymes can be used to generate concentrations of peracids with high efficiency for disinfection and/or bleaching applications.

[0114] In this disclosure, a number of terms and abbreviations are used. The following definitions apply unless specifically stated otherwise.

[0115] As used herein, the term “comprising” means the presence of the stated features, integers, steps, or components as referred to in the claims, but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

[0116] As used herein, the term "about" modifying the quantity of an ingredient or reactant employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or use solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term “about” also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities.

[0117] As used herein, the term “peracid” is synonymous with peroxyacid, peroxycarboxylic acid, peroxy acid, percarboxylic acid and peroxoic acid.

[0118] As used herein, the term “peracetic acid” is abbreviated as “PAA” and is synonymous with peroxyacetic acid, ethaneperoxoic acid and all other synonyms of CAS Registry Number 79-21-0.

[0119] As used herein, the term “monoacetin” is synonymous with glycerol monoacetate, glycerin monoacetate, and glyceryl monoacetate.

[0120] As used herein, the term “diacetin” is synonymous with glycerol diacetate; glycerin diacetate, glyceryl diacetate, and all other synonyms of CAS Registry Number 25395-31-7.

[0121] As used herein, the term “triacetin” is synonymous with glycerin triacetate; glycerol triacetate; glyceryl triacetate, 1,2,3-triacetoxypropane, 1,2,3-

propanetriol triacetate and all other synonyms of CAS Registry Number 102-76-1.

[0122] As used herein, the term "monobutyrin" is synonymous with glycerol monobutyrate, glycerin monobutyrate, and glyceryl monobutyrate.

[0123] As used herein, the term "dibutyrin" is synonymous with glycerol dibutyrate and glyceryl dibutyrate.

[0124] As used herein, the term "tributyrin" is synonymous with glycerol tributyrate, 1,2,3-tributyrylglycerol, and all other synonyms of CAS Registry Number 60-01-5.

[0125] As used herein, the term "monopropionin" is synonymous with glycerol monopropionate, glycerin monopropionate, and glyceryl monopropionate.

[0126] As used herein, the term "dipropionin" is synonymous with glycerol dipropionate and glyceryl dipropionate.

[0127] As used herein, the term "tripropionin" is synonymous with glyceryl tripropionate, glycerol tripropionate, 1,2,3-tripropionylglycerol, and all other synonyms of CAS Registry Number 139-45-7.

[0128] As used herein, the term "ethyl acetate" is synonymous with acetic ether, acetoxyethane, ethyl ethanoate, acetic acid ethyl ester, ethanoic acid ethyl ester, ethyl acetic ester and all other synonyms of CAS Registry Number 141-78-6.

[0129] As used herein, the term "ethyl lactate" is synonymous with lactic acid ethyl ester and all other synonyms of CAS Registry Number 97-64-3.

[0130] As used herein, the terms "acetylated sugar" and "acetylated saccharide" refer to mono-, di- and polysaccharides comprising at least one acetyl group. Examples include, but are not limited to glucose pentaacetate, xylose tetraacetate, acetylated xylan, acetylated xylan fragments, β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, and tri-O-acetyl-glucal.

[0131] As used herein, the terms "hydrocarbyl", "hydrocarbyl group", and "hydrocarbyl moiety" is meant a straight chain, branched or cyclic arrangement of carbon atoms connected by single, double, or triple carbon to carbon bonds and/or by ether linkages, and substituted accordingly with hydrogen atoms. Such hydrocarbyl groups may be aliphatic and/or aromatic. Examples of hydrocarbyl groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, cyclopropyl, cyclobutyl, pentyl, cyclopentyl, methylcyclopentyl, hexyl, cyclohexyl, benzyl, and phenyl. In a preferred embodiment, the

hydrocarbyl moiety is a straight chain, branched or cyclic arrangement of carbon atoms connected by single carbon to carbon bonds and/or by ether linkages, and substituted accordingly with hydrogen atoms.

[0132] As used herein, the terms "monoesters" and "diesters" of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol, refer to said compounds comprising at least one ester group of the formula RC(O)O, wherein R is a C1 to C7 linear hydrocarbyl moiety.

[0133] As used herein, the terms "suitable enzymatic reaction mixture", "components suitable for *in situ* generation of a peracid", "suitable reaction components", and "suitable aqueous reaction mixture" refer to the materials and water in which the reactants and enzyme catalyst come into contact. The components of the suitable aqueous reaction mixture are provided herein and those skilled in the art appreciate the range of component variations suitable for this process. In one embodiment, the suitable enzymatic reaction mixture produces peracid *in situ* upon combining the reaction components. As such, the reaction components may be provided as a multicomponent system wherein one or more of the reaction components remains separated until use. The design of systems and means for separating and combining multiple active components are known in the art and generally will depend upon the physical form of the individual reaction components. For example, multiple active fluids (liquid-liquid) systems typically use multichamber dispenser bottles or two-phase systems (U.S. Patent Application Pub. No. 2005/0139608; U.S. Patent 5,398,846; U.S. Patent 5,624,634; U.S. Patent 6,391,840; E.P. Patent 0807156B1; U.S. Patent Appln. Pub. No. 2005/0008526; and PCT Publication No. WO 00/11713A1) such as found in some bleaching applications wherein the desired bleaching agent is produced upon mixing the reactive fluids. Other forms of multicomponent systems used to generate peracid may include, but are not limited to those designed for one or more solid components or combinations of solid-liquid components, such as powders (e.g., many commercially available bleaching composition, U.S. Patent 5,116,575), multi-layered tablets (U.S. Patent 6,210,639), water dissolvable packets having multiple compartments (U.S. Patent 6,995,125) and solid agglomerates that react upon the addition of water (U.S. Patent 6,319,888).

[0134] In one embodiment, a formulation is provided as two individual mixtures whereby a peroxycarboxylic acid disinfectant is generated upon combining the two mixtures.

[0135] In another embodiment, a formulation is provided comprising:

a) a first mixture comprising:

i) an enzyme catalyst having perhydrolase activity, said enzyme catalyst comprising an enzyme having a CE-7 signature motif; and

ii) a carboxylic acid ester substrate, said first mixture optionally comprising a component selected from the group consisting of an inorganic or organic buffer, a corrosion inhibitor, a wetting agent, and combinations thereof; and

b) a second mixture comprising a source of peroxygen and water, said second mixture optionally comprising a chelating agent.

[0136] In a further related embodiment, the carboxylic acid ester substrate in the first mixture of the formulation is selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula R_6-C(O)O

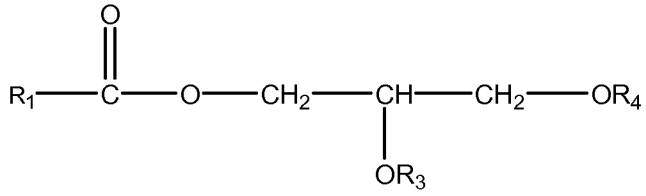
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

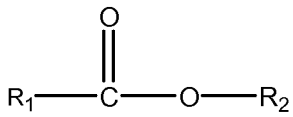
ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

In another embodiment, the carboxylic acid ester substrate in the first mixture of the formulation is defined by the following formula:



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_2= C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2\text{-}O)_nH$ or $(CH_2CH(CH_3)\text{-}O)_nH$ and n=1 to 10.

[0137] In a preferred embodiment, R_6 is C1 to C7 linear hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, optionally comprising one or more ether linkages. In a further preferred embodiment, R_6 is C2 to C7 linear hydrocarbyl moiety, optionally substituted with hydroxyl groups, and/or optionally comprising one or more ether linkages.

[0138] In another embodiment, the carboxylic acid ester substrate is selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-

propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; and mixtures thereof.

[0139] In another embodiment, the carboxylic acid ester is selected from the group consisting of monoacetin, diacetin, triacetin, and combinations thereof. In another embodiment, the carboxylic acid ester is an acetylated saccharide. In another embodiment, the substrate is a C1 to C6 polyol comprising one or more ester groups. In a preferred embodiment, one or more of the hydroxyl groups on the C1 to C6 polyol are substituted with one or more acetoxy groups (e.g. 1,3-propanediol diacetate, 1,4-butanediol diacetate, etc.). In another embodiment, the enzyme catalyst is a particulate solid. In another embodiment, the first reaction mixture described above is a solid tablet or powder.

[0140] As used herein, the term “perhydrolysis” is defined as the reaction of a selected substrate with peroxide to form a peracid. Typically, inorganic peroxide is reacted with the selected substrate in the presence of a catalyst to produce the peracid. As used herein, the term “chemical perhydrolysis” includes perhydrolysis reactions in which a substrate (a peracid precursor) is combined with a source of hydrogen peroxide wherein peracid is formed in the absence of an enzyme catalyst.

[0141] As used herein, the term “perhydrolase activity” refers to the catalyst activity per unit mass (for example, milligram) of protein, dry cell weight, or immobilized catalyst weight.

[0142] As used herein, “one unit of enzyme activity” or "one unit of activity" or “U” is defined as the amount of perhydrolase activity required for the production of 1 μmol of peracid product per minute at a specified temperature.

[0143] As used herein, the terms “enzyme catalyst” and “perhydrolase catalyst” refer to a catalyst comprising an enzyme having perhydrolysis activity and may be in the form of a whole microbial cell, permeabilized microbial cell(s), one or more cell components of a microbial cell extract, partially purified enzyme, or purified enzyme. The enzyme catalyst may also be chemically modified (e.g., by pegylation or by reaction with cross-linking reagents). The perhydrolase catalyst may also be immobilized on a soluble or insoluble support using methods well-known to those skilled in the art; see for example, Immobilization of Enzymes and Cells; Gordon F. Bickerstaff, Editor; Humana Press, Totowa, NJ, USA; 1997. As described herein, all of the present enzymes having perhydrolysis activity are structurally members of the carbohydrate family esterase family 7 (CE-7 family) of enzymes (see Coutinho,

P.M., Henrissat, B. "Carbohydrate-active enzymes: an integrated database approach" in Recent Advances in Carbohydrate Bioengineering, H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., (1999) The Royal Society of Chemistry, Cambridge, pp. 3-12.).

[0144] Members of the CE-7 family include cephalosporin C deacetylases (CAHs; E.C. 3.1.1.41) and acetyl xylan esterases (AXEs; E.C. 3.1.1.72). Members of the CE-7 esterase family share a conserved signature motif (Vincent et al., *J. Mol. Biol.*, 330:593-606 (2003)). Perhydrolases comprising the CE-7 signature motif and/or a substantially similar structure are suitable for use in the present invention. Means to identify substantially similar biological molecules are well known in the art (e.g. sequence alignment protocols, nucleic acid hybridizations, presence of a conserved signature motif, etc.). In one aspect, the enzyme catalyst in the present process comprises a substantially similar enzyme having at least 30%, preferably at least 33%, more preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, yet even more preferable at least 70%, yet even more preferably at least 80%, yet even more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to the sequences provided herein. The nucleic acid molecules encoding the present CE-7 carbohydrate esterases are also provided herein. In a further embodiment, the perhydrolase catalyst useful in the present process is encoded by a nucleic acid molecule that hybridizes stringent conditions to one of the present nucleic acid molecules.

[0145] As used herein, the terms "cephalosporin C deacetylase" and "cephalosporin C acetyl hydrolase" refers to an enzyme (E.C. 3.1.1.41) that catalyzes the deacetylation of cephalosporins such as cephalosporin C and 7-aminocephalosporanic acid (Mitsushima *et al.*, *supra*). As described herein, several cephalosporin C deacetylases are provided having significant perhydrolysis activity.

[0146] As used herein, "acetyl xylan esterases" " refers to an enzyme (E.C. 3.1.1.72; AXEs) that catalyzes the deacetylation of acetylated xylans and other acetylated saccharides. As illustrated herein, several enzymes classified as acetyl xylan esterases are provided having significant perhydrolase activity.

[0147] As used herein, the term "*Bacillus subtilis* (ATCC 31954™)" refers to a bacterial cell deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC 31954™. *Bacillus subtilis* ATCC 31954™ has been reported to have an ester hydrolase ("diacetinase") activity capable of hydrolyzing glycerol esters having 2-carbon

to 8-carbon acyl groups, especially diacetin (U.S. patent 4,444,886; herein incorporated by reference in its entirety). As described herein, an enzyme having significant perhydrolase activity has been isolated from *B. subtilis* ATCC 31954™ and is provided as SEQ ID NO: 2. The amino acid sequence of the isolated enzyme has 100% amino acid identity to the cephalosporin C deacetylase provided by GENBANK® Accession No. BAA01729.1.

[0148] As used herein, the term "*Bacillus subtilis* BE1010" refers to the strain of *Bacillus subtilis* as reported by Payne and Jackson (*J. Bacteriol.* 173:2278-2282 (1991)). *Bacillus subtilis* BE1010 is a derivative *Bacillus subtilis subsp. subtilis* strain BR151 (ATCC 33677™) having a chromosomal deletion in the genes encoding subtilisin and neutral protease. As described herein, an enzyme having significant perhydrolase activity has been isolated from B. subtilis BE1010 and is provided as SEQ ID NO: 6. The amino acid sequence of the isolated enzyme has 100% amino acid identity to the cephalosporin C deacetylase reported in Bacillus subtilis subsp. subtilis strain 168 (Kunst et al., Nature 390:249-256 (1997)).

[0149] As used herein, the term "Bacillus subtilis ATCC 29233™" refers to a strain of Bacillus subtilis deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC 29233™. As described herein, an enzyme having significant perhydrolase activity has been isolated and sequenced from B. subtilis ATCC 29233™ and is provided as SEQ ID NO: 32.

[0150] As used herein, the term "*Clostridium thermocellum* ATCC 27405™" refers to a strain of *Clostridium thermocellum* deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC 27405™. The amino acid sequence of the enzyme having perhydrolase activity from *C. thermocellum* ATCC 27405™ is provided as SEQ ID NO: 14.

[0151] As used herein, the term "*Bacillus subtilis* ATCC 6633™" refers to a bacterial cell deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC 6633™. *Bacillus subtilis* ATCC 6633™ has been reported to have cephalosporin acetylhydrolase activity (U.S. patent 6,465,233). The amino acid sequence of the enzyme having perhydrolase activity from *B. subtilis* ATCC 6633™ is provided as SEQ ID NO: 8.

[0152] As used herein, the term "*Bacillus licheniformis* ATCC 14580™" refers to a bacterial cell deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC 14580™.

Bacillus licheniformis ATCC 14580™ has been reported to have cephalosporin acetylhydrolase activity (GENBANK® YP_077621). The amino acid sequence of the enzyme having perhydrolase activity from *B. licheniformis* ATCC 14580™ is provided as SEQ ID NO: 10.

[0153] As used herein, the term "*Bacillus pumilus* PS213" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® AJ249957). The amino acid sequence of the enzyme having perhydrolase activity from *Bacillus pumilus* PS213 is provided as SEQ ID NO: 12.

[0154] As used herein, the term "*Thermotoga neapolitana*" refers to a strain of *Thermotoga neapolitana* reported to have acetyl xylan esterase activity (GENBANK® AAB70869). The amino acid sequence of the enzyme having perhydrolase activity from *Thermotoga neapolitana* is provided as SEQ ID NO: 16.

[0155] As used herein, the term "*Thermotoga maritima* MSB8" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® NP_227893.1). The amino acid sequence of the enzyme having perhydrolase activity from *Thermotoga maritima* MSB8 is provided as SEQ ID NO: 18.

[0156] As used herein, the term "*Bacillus clausii* KSM-K16" refers to a bacterial cell reported to have cephalosporin-C deacetylase activity (GENBANK® YP_175265). The amino acid sequence of the enzyme having perhydrolase activity from *Bacillus clausii* KSM-K16 is provided as SEQ ID NO: 26.

[0157] As used herein, the term "*Thermoanearobacterium saccharolyticum*" refers to a bacterial strain reported to have acetyl xylan esterase activity (GENBANK® S41858). The amino acid sequence of the enzyme having perhydrolase activity from *Thermoanearobacterium saccharolyticum* is provided as SEQ ID NO: 70.

[0158] As used herein, the term "*Thermotoga lettingae*" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® CP000812). The deduced amino acid sequence of the enzyme having perhydrolase activity from *Thermotoga lettingae* is provided as SEQ ID NO: 82.

[0159] As used herein, the term "*Thermotoga petrophila*" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® CP000702). The deduced amino acid sequence of the enzyme having perhydrolase activity from *Thermotoga lettingae* is provided as SEQ ID NO: 90.

[0160] As used herein, the term "*Thermotoga* sp. RQ2" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® CP000969). Two different acetyl xylan esterases have been idenfited from

Thermotoga sp. RQ2 and are referred to herein as "RQ2(a)" (the deduced amino acid sequence provided as SEQ ID NO: 98) and "RQ2(b)" (the deduced amino acid sequence provided as SEQ ID NO: 106).

[0161] As used herein, an "isolated nucleic acid molecule" and "isolated nucleic acid fragment" will be used interchangeably and refers to a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid molecule in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

[0162] The term "amino acid" refers to the basic chemical structural unit of a protein or polypeptide. The following abbreviations are used herein to identify specific amino acids:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid (or as defined herein)	Xaa	X

[0163] As used herein, "substantially similar" refers to nucleic acid molecules wherein changes in one or more nucleotide bases results in the

addition, substitution, or deletion of one or more amino acids, but does not affect the functional properties (i.e. perhydrolytic activity) of the protein encoded by the DNA sequence. As used herein, "substantially similar" also refers to an enzyme having an amino acid sequence that is at least 30%, preferably at least 33%, more preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, yet even more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the sequences reported herein wherein the resulting enzyme retains the present functional properties (i.e., perhydrolytic activity). "Substantially similar" may also refer to an enzyme having perhydrolytic activity encoded by nucleic acid molecules that hybridize under stringent conditions to the nucleic acid molecules reported herein. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

[0164] For example, it is well known in the art that alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded protein are common. For the purposes of the present invention substitutions are defined as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue (such as glycine) or a more hydrophobic residue (such as valine, leucine, or isoleucine). Similarly, changes which result in substitution of one negatively charged residue for another (such as aspartic acid for glutamic acid) or one positively charged residue for another (such as lysine for arginine) can also be expected to produce a functionally equivalent product. In many cases, nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein.

[0165] Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that substantially similar sequences are encompassed by the present invention. In one embodiment, substantially similar sequences are defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS, 65°C) with the sequences exemplified herein. In one embodiment, the present invention includes enzymes having perhydrolase activity encoded by isolated nucleic acid molecules that hybridize under stringent conditions to the nucleic acid molecules reported herein. In a preferred embodiment, the present invention includes an enzyme having perhydrolase activity encoded by isolated nucleic acid molecule that hybridize under stringent conditions to a nucleic acid molecule having an nucleic acid sequence selected from the group consisting of SEQ ID NO: 17; SEQ ID NO: 25; SEQ ID NO: 69, SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

[0166] As used herein, a nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single strand of the first molecule can anneal to the other molecule under appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J. and Russell, D., T. Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar molecules, such as homologous sequences from distantly related organisms, to highly similar molecules, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes typically determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of stringent hybridization conditions is 0.1X SSC, 0.1%

SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by a final wash of 0.1X SSC, 0.1% SDS, 65°C with the sequences exemplified herein.

[0167] Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T*m* for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T*m*) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T*m* have been derived (Sambrook and Russell, *supra*). For hybridizations with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (Sambrook and Russell, *supra*). In one aspect, the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferably, a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length, even more preferably at least 30 nucleotides in length, even more preferably at least 300 nucleotides in length, and most preferably at least 800 nucleotides in length. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

[0168] As used herein, the term “percent identity” is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, “identity” also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. “Identity” and “similarity” can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, NY (1991). Methods to determine identity and similarity are codified in

publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI), the AlignX program of Vector NTI v. 7.0 (Informax, Inc., Bethesda, MD), or the EMBOSS Open Software Suite (EMBL-EBI; Rice *et al.*, *Trends in Genetics* 16, (6) pp276—277 (2000)). Multiple alignment of the sequences can be performed using the Clustal method (i.e. CLUSTALW; for example version 1.83) of alignment (Higgins and Sharp, *CABIOS,* 5:151-153 (1989); Higgins *et al.*, *Nucleic Acids Res.* 22:4673-4680 (1994); and Chenna *et al.*, *Nucleic Acids Res* 31 (13):3497-500 (2003)), available from the European Molecular Biology Laboratory via the European Bioinformatics Institute) with the default parameters. Suitable parameters for CLUSTALW protein alignments include GAP Existence penalty=15, GAP extension =0.2, matrix = Gonnet (e.g. Gonnet250), protein ENDGAP = -1, Protein GAPDIST=4, and KTUPLE=1. In one embodiment, a fast or slow alignment is used with the default settings where a slow alignment is preferred. Alternatively, the parameters using the CLUSTALW method (version 1.83) may be modified to also use KTUPLE =1, GAP PENALTY=10, GAP extension =1, matrix = BLOSUM (e.g. BLOSUM64), WINDOW=5, and TOP DIAGONALS SAVED=5.

[0169] In one aspect of the present invention, suitable isolated nucleic acid molecules (isolated polynucleotides of the present invention) encode a polypeptide having an amino acid sequence that is at least about 30%, preferably at least 33%, preferably at least 40%, preferably at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 85%, even more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequences reported herein. Suitable nucleic acid molecules of the present invention not only have the above homologies, but also typically encode a polypeptide having about 300 to about 340 amino acids, more preferably about 310 to about 330 amino acids, and most preferably about 318 amino acids.

[0170] As used herein, the terms “signature motif”, “CE-7 signature motif”, and “diagnostic motif” refer to conserved structures shared among a family of enzymes having a defined activity. The signature motif can be used to define and/or identify the family of structurally related enzymes having similar enzymatic activity for a defined family of substrates. The signature motif can be a single contiguous amino acid sequence or a collection of discontiguous, conserved motifs that together form the signature motif.

Typically, the conserved motif(s) is represented by an amino acid sequence. As described herein, the present perhydrolases belong to the family of CE-7 carbohydrate esterases. This family of enzymes can be defined by the presence of a signature motif (Vincent et al., *supra*).

[0171] As used herein, "codon degeneracy" refers to the nature of the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. Accordingly, the present invention relates to any nucleic acid molecule that encodes all or a substantial portion of the amino acid sequences encoding the present microbial polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

[0172] As used herein, "synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as pertaining to a DNA sequence, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequences to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

[0173] As used herein, "gene" refers to a nucleic acid molecule that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged

in a manner different from that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

[0174] As used herein, "coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, RNA processing site, effector binding site and stem-loop structure.

[0175] As used herein, "promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0176] As used herein, the "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences (normally limited to eukaryotes) and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts (normally limited to eukaryotes) to the 3' end of the mRNA precursor.

[0177] As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid molecule so that the function of one is affected by the other. For example, a promoter is

operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence, *i.e.*, that the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0178] As used herein, the term "expression" refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid molecule described herein. Expression may also refer to translation of mRNA into a polypeptide.

[0179] As used herein, "transformation" refers to the transfer of a nucleic acid molecule into the genome of a host organism, resulting in genetically stable inheritance. In the present invention, the host cell's genome includes chromosomal and extrachromosomal (e.g. plasmid) genes. Host organisms containing the transformed nucleic acid molecules are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[0180] As used herein, the terms "plasmid", "vector" and "cassette" refer to an extrachromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[0181] As used herein, the term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to, the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), BLASTP, BLASTN, BLASTX (Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), and DNASTAR (DNASTAR, Inc. 1228 S. Park St. Madison, WI 53715 USA), CLUSTALW (for example, version 1.83; Thompson *et al.*, *Nucleic Acids*

Research, 22(22):4673-4680 (1994), and the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.,* [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Publisher: Plenum, New York, NY), Vector NTI (Informax, Bethesda, MD) and Sequencher v. 4.05. Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters set by the software manufacturer that originally load with the software when first initialized.

[0182] As used herein, the term "biological contaminants" refers to one or more unwanted and/or pathogenic biological entities including, but not limited to microorganisms, spores, viruses, prions, and mixtures thereof. The process produces an efficacious concentration of at least one percarboxylic acid useful to reduce and/or eliminate the presence of the viable biological contaminants. In a preferred embodiment, the microbial contaminant is a viable pathogenic microorganism.

[0183] As used herein, the term "disinfect" refers to the process of destruction of or prevention of the growth of biological contaminants. As used herein, the term "disinfectant" refers to an agent that disinfects by destroying, neutralizing, or inhibiting the growth of biological contaminants. Typically, disinfectants are used to treat inanimate objects or surfaces. As used herein, the term "antiseptic" refers to a chemical agent that inhibits the growth of disease-carrying microorganisms. In one aspect of the embodiment, the biological contaminants are pathogenic microorganisms.

[0184] As used herein, the term "virucide" refers to an agent that inhibits or destroys viruses, and is synonymous with "viricide". An agent that exhibits the ability to inhibit or destroy viruses is described as having "virucidal" activity. Peracids can have virucidal activity. Typical alternative virucides known in the art which may be suitable for use with the present invention include, for example, alcohols, ethers, chloroform, formaldehyde, phenols, beta propiolactone, iodine, chlorine, mercury salts, hydroxylamine, ethylene oxide, ethylene glycol, quaternary ammonium compounds, enzymes, and detergents.

[0185] As used herein, the term "biocide" refers to a chemical agent, typically broad spectrum, which inactivates or destroys microorganisms. A chemical agent that exhibits the ability to inactivate or destroy microorganisms is described as having "biocidal" activity. Peracids can have biocidal activity. Typical alternative biocides known in the art, which may be suitable for use in

the present invention include, for example, chlorine, chlorine dioxide, chloroisocyanurates, hypochlorites, ozone, acrolein, amines, chlorinated phenolics, copper salts, organo-sulphur compounds, and quaternary ammonium salts.

[0186] As used herein, the phrase "minimum biocidal concentration" refers to the minimum concentration of a biocidal agent that, for a specific contact time, will produce a desired lethal, irreversible reduction in the viable population of the targeted microorganisms. The effectiveness can be measured by the $\log_{10}$ reduction in viable microorganisms after treatment. In one aspect, the targeted reduction in viable microorganisms after treatment is at least a 3-log reduction, more preferably at least a 4-log reduction, and most preferably at least a 5-log reduction. In another aspect, the minimum biocidal concentration is at least a 6-log reduction in viable microbial cells.

[0187] As used herein, the terms "peroxygen source" and "source of peroxygen" refer to compounds capable of providing hydrogen peroxide at a concentration of about 1 mM or more when in an aqueous solution including, but not limited to hydrogen peroxide, hydrogen peroxide adducts (e.g., urea-hydrogen peroxide adduct (carbamide peroxide)), perborates, and percarbonates. As described herein, the concentration of the hydrogen peroxide provided by the peroxygen compound in the aqueous reaction mixture is initially at least 1 mM or more upon combining the reaction components. In one embodiment, the hydrogen peroxide concentration in the aqueous reaction mixture is at least 10 mM. In another embodiment, the hydrogen peroxide concentration in the aqueous reaction mixture is at least 100 mM. In another embodiment, the hydrogen peroxide concentration in the aqueous reaction mixture is at least 200 mM. In another embodiment, the hydrogen peroxide concentration in the aqueous reaction mixture is 500 mM or more. In yet another embodiment, the hydrogen peroxide concentration in the aqueous reaction mixture is 1000 mM or more. The molar ratio of the hydrogen peroxide to enzyme substrate, e.g. triglyceride, (H_2O_2:substrate) in the aqueous reaction mixture may be from about 0.002 to 20, preferably about 0.1 to 10, and most preferably about 0.5 to 5.

Suitable Reaction Conditions for the Enzyme-catalyzed Preparation of Peracids from Carboxylic Acid Esters and Hydrogen Peroxide

[0188] In one aspect, a process is provided to produce an aqueous mixture comprising a peracid by reacting carboxylic acid esters and an inorganic peroxide, not limited to hydrogen peroxide, sodium perborate or

sodium percarbonate, in the presence of an enzyme catalyst having perhydrolysis activity. In one embodiment, the enzyme catalyst comprises a perhydrolase having a structure belonging to the CE-7 carbohydrate esterase family. In another embodiment, the perhydrolase catalyst is structurally classified as a cephalosporin C deacetylase. In another embodiment, the perhydrolase catalyst is structurally classified as an acetyl xylan esterase.

[0189] In one embodiment, the perhydrolase catalyst comprises an enzyme having a CE-7 signature motif that aligns with a reference sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

i) an RGQ motif at amino acid positions 118-120 of SEQ ID NO:2;

ii) a GXSQG motif at amino acid positions 179-183 of SEQ ID NO:2; and

iii) an HE motif at amino acid positions 298-299 of SEQ ID NO:2;

wherein said enzyme also comprises at least 30% amino acid identity to SEQ ID NO: 2.

[0190] In a further embodiment, the signature motif additional comprises a forth conserved motif defined as an LXD motif at amino acid residues 267-269 when aligned to reference sequence SEQ ID NO: 2 using CLUSTALW.

[0191] In another embodiment, the perhydrolase catalyst comprises an enzyme having perhydrolase activity selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 26, SEQ ID NO: 32, SEQ ID NO: 70, SEQ ID NO: 82, SEQ ID NO :90, SEQ ID NO: 98, and SEQ ID NO: 106.

[0192] In another embodiment, the enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence.

[0193] In another embodiment, substantially similar enzyme having perhydrolase activity is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to one or more amino acid sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 26, SEQ ID NO: 70, SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

[0194] In a further embodiment, the enzyme having perhydrolase activity is selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

[0195] In another embodiment, the perhydrolase catalyst comprises an enzyme having an amino acid sequence encoded by a nucleic acid molecule that hybridizes to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17; SEQ ID NO: 25; SEQ ID NO: 69, SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105 under stringent hybridization conditions.

[0196] In another embodiment, the perhydrolase catalyst comprises an enzyme having an amino acid sequence encoded by a nucleic acid molecule that hybridizes to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105 under stringent hybridization conditions.

[0197] In another embodiment, the perhydrolase catalyst comprises an enzyme having at least 30%, preferably at last 36%, amino acid identity to a contiguous signature motif defined as SEQ ID NO: 61 wherein the conserved motifs described above (e.g. RGQ, GXSQG, and HE, and optionally, LXD) are conserved.

[0198] In one embodiment, suitable substrates include esters provided by the following formula:

$$[X]_mR_5$$

wherein X = an ester group of the formula $R_6C(O)O$

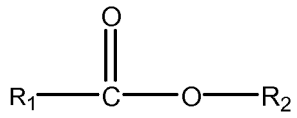
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

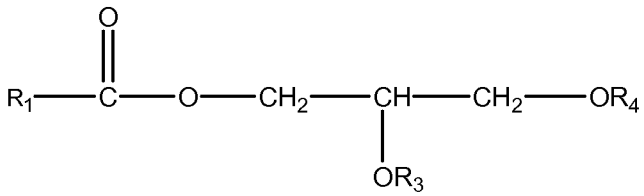
wherein said esters have a solubility in water of at least 5 ppm at 25 °C.

[0199] In another embodiment, suitable substrates also include esters of the formula:



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_2= C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2\text{-}O)_nH$ or $(CH_2CH(CH_3)\text{-}O)_nH$ and n=1 to 10.

[0200] In another embodiment, suitable substrates include glycerides of the formula:



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$.

[0201] In another embodiment, R_6 is C1 to C7 linear hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, optionally comprising one or more ether linkages. In a further preferred embodiment, R_6 is C2 to C7 linear hydrocarbyl moiety, optionally substituted with hydroxyl groups, and/or optionally comprising one or more ether linkages.

[0202] In another embodiment, suitable substrates also include acetylated saccharides selected from the group consisting of acetylated mono-, di-, and polysaccharides. In a preferred embodiment, the acetylated saccharides include acetylated mono- , di-, and polysaccharides. In another

embodiment, the acetylated saccharides are selected from the group consisting of acetylated xylan, fragments of acetylated xylan, acetylated xylose(such as xylose tetraacetate), acetylated glucose (such as glucose pentaacetate), β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, and tri-O-acetyl-D-glucal, and acetylated cellulose. In a preferred embodiment, the acetylated saccharide is selected from the group consisting of β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, and tri-O-acetyl-D-glucal, and acetylated cellulose . As such, acetylated carbohydrates may be suitable substrates for generating percarboxylic acids using the present process (i.e., in the presence of a peroxygen source).

[0203] In one embodiment, the substrate is selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; and mixtures thereof.

[0204] In a preferred embodiment, the substrate is selected from the group consisting of ethyl acetate, methyl lactate, ethyl lactate, methyl glycolate, ethyl glycolate, methyl methoxyacetate, ethyl methoxyacetate, methyl 3-hydroxybutyrate, ethyl 3-hydroxybutyrate, triethyl 2-acetyl citrate, glucose pentaacetate, gluconolactone, glycerides (mono-, di-, and triglycerides) such as monoacetin, diacetin, triacetin, monopropionin, dipropionin (glyceryl dipropionate), tripropionin (1,2,3-tripropionylglycerol), monobutyrin, dibutyrin (glyceryl dibutyrate), tributyrin (1,2,3-tributyrylglycerol), acetylated saccharides, and mixtures thereof.

[0205] In a further preferred aspect, the carboxylic acid ester substrates are selected from the group consisting of monoacetin, diacetin, triacetin, monopropionin, dipropionin, tripropionin, monobutyrin, dibutyrin, tributyrin, ethyl acetate, and ethyl lactate. In yet another aspect, the carboxylic acid ester substrates are selected from the group consisting of diacetin, triacetin, ethyl acetate, and ethyl lactate. In a preferred aspect, the carboxylic acid ester is a glyceride selected from the group consisting of monoacetin, diacetin, triacetin, and mixtures thereof.

[0206] The carboxylic acid ester is present in the reaction mixture at a concentration sufficient to produce the desired concentration of peracid upon

enzyme-catalyzed perhydrolysis. The carboxylic acid ester need not be completely soluble in the reaction mixture, but has sufficient solubility to permit conversion of the ester by the perhydrolase catalyst to the corresponding peracid. The carboxylic acid ester is present in the reaction mixture at a concentration of 0.0005 wt % to 40 wt % of the reaction mixture, preferably at a concentration of 0.1 wt % to 20 wt % of the reaction mixture, and more preferably at a concentration of 0.5 wt % to 10 wt % of the reaction mixture. The wt % of carboxylic acid ester may optionally be greater than the solubility limit of the carboxylic acid ester, such that the concentration of the carboxylic acid ester is at least 0.0005 wt % in the reaction mixture that is comprised of water, enzyme catalyst, and source of peroxide, where the remainder of the carboxylic acid ester remains as a second separate phase of a two-phase aqueous/organic reaction mixture. Not all of the added carboxylic acid ester must immediately dissolve in the aqueous reaction mixture, and after an initial mixing of all reaction components, additional continuous or discontinuous mixing is optional.

[0207] The peroxygen source may include, but is not limited to, hydrogen peroxide, hydrogen peroxide adducts (e.g., urea-hydrogen peroxide adduct (carbamide peroxide)) perborate salts and percarbonate salts. The concentration of peroxygen compound in the reaction mixture may range from 0.0033 wt % to about 50 wt %, preferably from 0.033 wt % to about 40 wt %, more preferably from 0.33 wt % to about 30 wt %.

[0208] Many perhydrolase catalysts (whole cells, permeabilized whole cells, and partially purified whole cell extracts) have been reported to have catalase activity (EC 1.11.1.6). Catalases catalyze the conversion of hydrogen peroxide into oxygen and water. In one aspect, the perhydrolysis catalyst lacks catalase activity. In another aspect, a catalase inhibitor is added to the reaction mixture. Examples of catalase inhibitors include, but are not limited to, sodium azide and hydroxylamine sulfate. One of skill in the art can adjust the concentration of catalase inhibitor as needed. The concentration of the catalase inhibitor typically ranges from 0.1 mM to about 1 M; preferably about 1 mM to about 50 mM; more preferably from about 1 mM to about 20 mM. In one aspect, sodium azide concentration typically ranges from about 20 mM to about 60 mM while hydroxylamine sulfate is concentration is typically about 0.5 mM to about 30 mM, preferably about 10 mM.

[0209] In another embodiment, the enzyme catalyst lacks significant catalase activity or is engineered to decrease or eliminate catalase activity. The catalase activity in a host cell can be down-regulated or eliminated by

disrupting expression of the gene(s) responsible for the catalase activity using well known techniques including, but not limited to, transposon mutagenesis, RNA antisense expression, targeted mutagenesis, and random mutagenesis. In a preferred embodiment, the gene(s) encoding the endogenous catalase activity are down-regulated or disrupted (i.e. knocked-out). As used herein, a "disrupted" gene is one where the activity and/or function of the protein encoded by the modified gene is no longer present. Means to disrupt a gene are well-known in the art and may include, but are not limited to insertions, deletions, or mutations to the gene so long as the activity and/or function of the corresponding protein is no longer present. In a further preferred embodiment, the production host is an *E. coli* production host comprising a disrupted catalase gene selected from the group consisting of *kat*G (SEQ ID NO: 47) and *kat*E (SEQ ID NO: 56). In another embodiment, the production host is an *E. coli* strain comprising a down-regulation and/or disruption in both *kat*g1 and a *kat*E catalase genes. An *E. coli* strain comprising a double-knockout of *kat*G and *kat*E is provided herein (see Example 15; *E. coli* strain KLP18).

[0210] The catalase negative *E. coli* strain KLP18 (*kat*G and *kat*E double knockout) that was constructed (Example 15) was demonstrated to be a superior host for large scale (10-L and greater) production of perhydrolase enzymes compared to the catalase negative strain UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT), as determined by growth under fermenter conditions (Examples 17-19). Although both KLP18 and UM2 are catalase-negative strains, UM2 is known to have numerous nutritional auxotrophies, and therefore requires media that is enriched with yeast extract and peptone. Even when employing enriched media for fermentation, UM2 grew poorly and to a limited maximum cell density (OD). In contrast, KLP18 had no special nutritional requirements and grew to high cell densities on mineral media alone or with additional yeast extract (Example 20).

[0211] The concentration of the catalyst in the aqueous reaction mixture depends on the specific catalytic activity of the catalyst, and is chosen to obtain the desired rate of reaction. The weight of catalyst in perhydrolysis reactions typically ranges from 0.0005 mg to 10 mg per mL of total reaction volume, preferably from 0.010 mg to 2.0 mg per mL. The catalyst may also be immobilized on a soluble or insoluble support using methods well-known to those skilled in the art; see for example, Immobilization of Enzymes and Cells; Gordon F. Bickerstaff, Editor; Humana Press, Totowa, NJ, USA; 1997. The use of immobilized catalysts permits the recovery and reuse of the catalyst in subsequent reactions. The enzyme catalyst may be in the form of whole

microbial cells, permeabilized microbial cells, microbial cell extracts, partially-purified or purified enzymes, and mixtures thereof.

[0212] In one aspect, the concentration of peracid generated by the combination of chemical perhydrolysis and enzymatic perhydrolysis of the carboxylic acid ester is sufficient to provide an effective concentration of peracid for bleaching or disinfection at a desired pH. In another aspect, the present methods provide combinations of enzymes and enzyme substrates to produce the desired effective concentration of peracid, where, in the absence of added enzyme, there is a significantly lower concentration of peracid produced. Although there may in some cases be substantial chemical perhydrolysis of the enzyme substrate by direct chemical reaction of inorganic peroxide with the enzyme substrate, there may not be a sufficient concentration of peracid generated to provide an effective concentration of peracid in the desired applications, and a significant increase in total peracid concentration is achieved by the addition of an appropriate perhydrolase catalyst to the reaction mixture.

[0213] The concentration of peracid generated (*e.g.* peracetic acid) by the perhydrolysis of at least one carboxylic acid ester is at least about 2 ppm, preferably at least 20 ppm, preferably at least 100 ppm, more preferably at least about 200 ppm peracid, more preferably at least 300 ppm, more preferably at least 500 ppm, more preferably at least 700 ppm, more preferably at least about 1000 ppm peracid, most preferably at least 2000 ppm peracid within 10 minutes, preferably within 5 minutes, and most preferably within 1 minute of initiating the perhydrolysis reaction. The product mixture comprising the peracid may be optionally diluted with water, or a solution predominantly comprised of water, to produce a mixture with the desired lower concentration of peracid. In one aspect, the reaction time required to produce the desired concentration of peracid is not greater than about two hours, preferably not greater than about 30 minutes, more preferably not greater than about 10 minutes, even more preferably not greater than about 50 minutes, and most preferably in about 1 minute or less. In other aspects, a hard surface or inanimate object contaminated with a concentration of a microbial population is contacted with the peracid formed in accordance with the processes described herein within about 1 minute to about 168 hours of combining said reaction components, or within about 1 minute to about 48 hours, or within about 1 minute to 2 hours of combining said reaction components, or any such time interval therein.

[0214] The temperature of the reaction is chosen to control both the reaction rate and the stability of the enzyme catalyst activity. The temperature of the reaction may range from just above the freezing point of the reaction mixture (approximately 0 °C) to about 75 °C, with a preferred range of reaction temperature of from about 5 °C to about 55 °C.

[0215] The pH of the final reaction mixture containing peracid is from about 2 to about 9, preferably from about 3 to about 8, more preferably from about 5 to about 8, even more preferably about 6 to about 8, and yet even more preferably about 6.5 to about 7.5. In another embodiment, the pH of the reaction mixture is acidic (pH <7). The pH of the reaction, and of the final reaction mixture, may optionally be controlled by the addition of a suitable buffer, including, but not limited to phosphate, pyrophosphate, bicarbonate, acetate, or citrate. The concentration of buffer, when employed, is typically from 0.1 mM to 1.0 M, preferably from 1 mM to 300 mM, most preferably from 10 mM to 100 mM.

[0216] In another aspect, the enzymatic perhydrolysis reaction mixture may contain an organic solvent that acts as a dispersant to enhance the rate of dissolution of the carboxylic acid ester in the reaction mixture. Such solvents include, but are not limited to, propylene glycol methyl ether, acetone, cyclohexanone, diethylene glycol butyl ether, tripropylene glycol methyl ether, diethylene glycol methyl ether, propylene glycol butyl ether, dipropylene glycol methyl ether, cyclohexanol, benzyl alcohol, isopropanol, ethanol, propylene glycol, and mixtures thereof.

[0217] In another aspect, the enzymatic perhydrolysis product may contain additional components that provide desirable functionality. These additional components include, but are not limited to buffers, detergent builders, thickening agents, emulsifiers, surfactants, wetting agents, corrosion inhibitors (e.g., benzotriazole), enzyme stabilizers, and peroxide stabilizers (*e.g.*, metal ion chelating agents). Many of the additional components are well known in the detergent industry (see for example U.S. Patent 5,932,532; hereby incorporated by reference). Examples of emulsifiers include, but are not limited to polyvinyl alcohol or polyvinylpyrrolidone. Examples of thickening agents include, but are not limited to LAPONITE® RD, corn starch, PVP, CARBOWAX®, CARBOPOL®, CABOSIL®, polysorbate 20, PVA, and lecithin. Examples of buffering systems include, but are not limited to sodium phosphate monobasic/sodium phosphate dibasic; sulfamic acid/triethanolamine; citric acid/triethanolamine; tartaric acid/triethanolamine; succinic acid/triethanolamine; and acetic acid/triethanolamine. Examples of

surfactants include, but are not limited to a) non-ionic surfactants such as block copolymers of ethylene oxide or propylene oxide, ethoxylated or propoxylated linear and branched primary and secondary alcohols, and aliphatic phosphine oxides b) cationic surfactants such as quaternary ammonium compounds, particularly quaternary ammonium compounds having a C8-C20 alkyl group bound to a nitrogen atom additionally bound to three C1-C2 alkyl groups, c) anionic surfactants such as alkane carboxylic acids (e.g., C8-C20 fatty acids), alkyl phosphonates, alkane sulfonates (e.g., sodium dodecylsulphate "SDS") or linear or branched alkyl benzene sulfonates, alkene sulfonates and d) amphoteric and zwitterionic surfactants such as aminocarboxylic acids, aminodicarboxylic acids, alkybetaines, and mixtures thereof. Additional components may include fragrances, dyes, stabilizers of hydrogen peroxide (*e.g.*, metal chelators such as 1-hydroxyethylidene -1,1-diphosphonic acid (DEQUEST® 2010, Solutia Inc., St. Louis, MO and ethylenediaminetetraacetic acid (EDTA)), TURPINAL® SL, DEQUEST® 0520, DEQUEST® 0531, stabilizers of enzyme activity (*e.g.*, polyethyleneglycol (PEG)), and detergent builders.

[0218] In another aspect, the enzymatic perhydrolysis product may be pre-mixed to generate the desired concentration of peroxycarboxylic acid prior to contacting the surface or inanimate object to be disinfected.

[0219] In another aspect, the enzymatic perhydrolysis product is not pre-mixed to generate the desired concentration of peroxycarboxylic acid prior to contacting the surface or inanimate object to be disinfected, but instead, the components of the reaction mixture that generate the desired concentration of percarboxylic acid are contacted with the surface or inanimate object to be disinfected, generating the desired concentration of peroxycarboxylic acid. In some embodiments, the components of the reaction mixture combine or mix at the locus. In some embodiments, the reaction components are delivered or applied to the locus and subsequently mix or combine to generate the desired concentration of peroxycarboxylic acid.

In Situ Production of Peracids using a Perhydrolase Catalyst

[0220] Cephalosporin C deacetylases (E.C. 3.1.1.41; systematic name cephalosporin C acetylhydrolases; CAHs) are enzymes having the ability to hydrolyze the acetyl ester bond on cephalosporins such as cephalosporin C, 7-aminocephalosporanic acid, and 7-(thiophene-2-acetamido)cephalosporanic acid (Abbott, B. and Fukuda, D., *Appl. Microbiol.* 30(3):413-419 (1975)). CAHs belong to a larger family of structurally related enzymes referred to as the carbohydrate esterase family seven (CE-7; see

Coutinho, P.M., Henrissat, B. "Carbohydrate-active enzymes: an integrated database approach" in Recent Advances in Carbohydrate Bioengineering, H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., (1999) The Royal Society of Chemistry, Cambridge, pp. 3-12.)

[0221] The CE-7 family includes both CAHs and acetyl xylan esterases (AXEs; E.C. 3.1.1.72). CE-7 family members share a common structural motif and are quite unusual in that they typically exhibit ester hydrolysis activity for both acetylated xylooligosaccharides and cephalosporin C, suggesting that the CE-7 family represents a single class of proteins with a multifunctional deacetylase activity against a range of small substrates (Vincent *et al.*, *J. Mol. Biol.*, 330:593-606 (2003)). Vincent *et al.* describes the structural similarity among the members of this family and defines a signature sequence motif characteristic of the CE-7 family.

[0222] Members of the CE-7 family are found in plants, fungi (e.g., *Cephalosporidium acremonium*), yeasts (e.g., *Rhodosporidium toruloides*, *Rhodotorula glutinis*), and bacteria such as *Thermoanaerobacterium* sp.; *Norcardia lactamdurans*, and various members of the genus *Bacillus* (Politino *et al.*, *Appl. Environ. Microbiol.,* 63(12):4807-4811 (1997); Sakai *et al.*, *J. Ferment. Bioeng*. 85:53-57 (1998); Lorenz, W. and Wiegel, J., *J. Bacteriol* 179:5436-5441 (1997); Cardoza *et al.*, *Appl. Microbiol. Biotechnol.*, 54(3):406-412 (2000); Mitshushima *et al.*, *supra*, Abbott, B. and Fukuda, D., *Appl. Microbiol.* 30(3):413-419 (1975); Vincent *et al.*, *supra*, Takami *et al.*, *NAR*, 28(21):4317-4331 (2000); Rey *et al.*, *Genome Biol.*, 5(10): article 77 (2004); Degrassi *et al.*, *Microbiology*., 146:1585-1591 (2000); U.S. Patent 6,645,233;. U.S. Patent 5,281,525; U.S. Patent 5,338,676; and WO 99/03984. A non-comprehensive list of CE-7 carbohydrate esterase family members having significant homology to SEQ ID NO: 2 are provided in Table 1.

Table 1. Example of CE-7 Enzymes Having Significant Homology to SEQ ID NO:2.

Source Organism (GENBANK® Accession No. of the CE-7 enzyme)	Nucleotide Sequence (SEQ ID NO:)	Amino Acid Sequence (SEQ ID NO:)	% Amino Acid Identity to SEQ ID NO: 2.	Reference
B. subtilis ATCC 31954™	1	2	100	*B. subtilis* SHS 0133 Mitshushima *et al*. *supra*
B. subtilis subsp. subtilis str. 168 (NP_388200) *B. subtilis* BE1010	5	6	98	Kunst *et al*., *supra*. WO99/03984 Payne and Jackson, *J. Bacteriol*. 173:2278-2282 (1991))
B. subtilis ATCC 6633 (YP_077621.1)	7	8	96	U.S. 6,465,233
B. subtilis ATCC 29233	31	32	96	Abbott and Fukuda, *supra*
B. licheniformis ATCC 14580 (YP_077621.1)	9	10	77	Rey *et al*., *supra*
B. pumilus PS213 (CAB76451.2)	11, 60	12	76	Degrassi *et al*., *supra*
Clostridium thermocellum ATCC 27405 (ZP_00504991)	13	14	57	Copeland *et al*. US Dept. of Energy Joint Genome Institute (JGI-PGF) Direct

				Submission GENBANK® ZP_00504991
Thermotoga neapolitana (AAB70869.1)	15, 41	16	42	See GENBANK® AAB70869.1
Thermotoga maritima MSB8 (NP_227893.1)	17,74	18	42	Nelson *et al.*, *Nature* 399 (6734):323-329 (1999)
Bacillus sp. NRRL B-14911 (ZP_01168674)	21	22	40	Siefert *et al.* J. Craig Venter Institute. Direct Submission Under GENBANK® ZP_01168674
Thermoanaerobac -terium sp. (AAB68821.1)	19	20	37	Lorenz and Wiegel, *supra*
Bacillus halodurans C-125 (NP_244192)	23	24	36	Takami *et al.*, *supra*
Thermoanearobac -terium saccharolyticum (S41858)	69	70	35	Lee, Y.E. and Zeikus, J.G., *J Gen Microbiol.* (1993), 139 Pt 6:1235-1243
Bacillus clausii KSM-K16 (YP_175265)	25, 65	26	33	Kobayashi *et al.*, *Appl. Microbiol. Biotechnol*. 43 (3), 473-481 (1995)

Thermotoga lettingae (CP000812)	77, 80, and 81	82	37	Copeland *et al.* US Dept. of Energy Joint Genome Institute Direct Submission GENBANK® CP000812
Thermotoga Petrophila (CP000702)	85, 88, and 89	90	41	Copeland *et al.* US Dept. of Energy Joint Genome Institute Direct Submission GENBANK® CP000702
Thermotoga sp. RQ2 RQ2(a) (CP000969)	93, 96, and 97	98	42	Copeland *et al.* US Dept. of Energy Joint Genome Institute Direct Submission GENBANK® CP000969
Thermotoga sp. RQ2 RQ2(b) (CP000969)	101, 104, and 105	106	42	Copeland *et al.* US Dept. of Energy Joint Genome Institute Direct Submission GENBANK® CP000969

[0223] The present perhydrolases are all members of the CE-7 carbohydrate esterase family. As described by Vincent *et al.* (*supra*), members of the family share a common signature motif that is characteristic of this family. A CLUSTALW alignment of the present perhydrolases illustrates that

all of the members belong to the CE-7 carbohydrate esterase family (Figure 1, panels A-C). A comparison of the overall percent amino acid identity amount the present perhydrolases is provided in Table 2.

Table 2: Percent Amino Acid Identity Between Perhydrolases[1]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	100														
2	99	100													
3	99	99	100												
4	96	96	97	100											
5	77	76	77	76	100										
6	76	76	76	76	68	100									
7	57	57	57	56	56	56	100								
8	42	43	43	43	43	42	41	100							
9	42	43	42	43	43	42	42	72	100						
10	42	43	43	43	44	42	43	71	91	100					
11	41	43	43	43	45	42	43	71	97	91	100				
12	41	42	42	42	43	41	42	71	98	91	97	100			
13	37	37	37	36	39	38	38	64	65	67	66	65	100		
14	34	36	35	36	35	36	33	36	32	34	34	33	36	100	
15	33	34	33	33	32	34	32	30	30	32	31	31	32	34	100

[1]=Percent identity determined using blast2seq algorithm using BLOSUM62, gap open=11, gap extension = 1, x_drop = 0, expect=10, and wordsize=3. Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", *FEMS Microbiol Lett*. 174:247-250

1. *B. subtilis* ATCC 31954™
2. *B. subtilis* BE1010
3. *B. subtilis* ATCC 29233
4. *B. subtilis* ATCC 6633
5. *B. licheniformis* 14580
6. *B. pumilus* PS213
7. *C. thermocellum* ATCC 27405
8. *Thermotoga* sp.RQ2(b)
9. *Thermotoga* sp.RQ2(a)
10. *T. neapolitana*
11. *T. maritima*
12. *T. petrophila*
13. *T. lettingae*
14. *T. saccharolyticum*
15. *B. clausii*

[0224] Although variation is observed in terms of overall percent amino acid identity (*i.e.* the *Clostridium thermocellum* ATCC 27405™ perhydrolase; SEQ ID NO: 14 shares only 57% amino acid identity with the *Bacillus subtilis* ATCC 31954™ perhydrolase; SEQ ID NO: 2, while the *Bacillus clausii* perhydrolase (SEQ ID NO: 26) shares only 33% identity with SEQ ID NO: 2), each of the present perhydrolase enzymes share the CE-7 signature motif.

Accordingly, the perhydrolase catalyst of the present invention is an enzyme structurally classified as belonging to the CE-7 carbohydrate esterase family. Each of the present perhydrolase enzymes comprises the CE-7 signature (diagnostic) motif.

[0225] Vincent *et al*. (*supra*) analyzed the structure CE-7 esterases and identified several highly conserved motifs that are diagnostic for the family. As shown in Figure 1, the highly conserved motifs (underlined in Figure 1; position numbering relative to SEQ ID NO: 2) include the Arg118-Gly119-Gln120 (RGQ), Gly179-Xaa180-Ser181-Gln182-Gly183 (GXSQG), and His298-Glu299 (HE). In addition, Figure 1 illustrates an additional highly conserved Lys267-Xaa268-Asp269 (LXD) motif that may be used to further characterize the signature motif. All of the numbering is relative to the numbering of a reference sequence (*B. subtilis* ATCC 31954™ perhydrolase; SEQ ID NO: 2).

[0226] In one embodiment, suitable perhydrolytic enzymes can be identified by the presence of the CE-7 signature motif (Vincent *et al*., *supra*). In a preferred embodiment, perhydrolases comprising the CE-7 signature motif are identified using a CLUSTALW alignment against the *Bacillus subtilis* ATCC 31954™ perhydrolase (SEQ ID NO: 2; i.e. the reference sequence used for relative amino acid position numbering). As per the amino acid residue numbering of SEQ ID NO: 2, the CE-7 signature motif comprises 3 conserved motifs defined as:

a) Arg118-Gly119-Gln120;
b) Gly179-Xaa180-**Ser181**-Gln182-Gly183; and
c) **His298**-Glu299.

Typically, the Xaa at amino acid residue position 180 is glycine, alanine, proline, tryptophan, or threonine. Two of the three amino acid residues belonging to the catalytic triad are in bold. In one embodiment, the Xaa at amino acid residue position 180 is selected from the group consisting of glycine, alanine, proline, tryptophan, and threonine.

[0227] Further analysis of the conserved motifs within the CE-7 carbohydrate esterase family indicates the presence of an additional conserved motif (LXD at amino acid positions 267-269 of SEQ ID NO: 2) that may be to further define a perhydrolase belonging to the CE-7 carbohydrate esterase family (Figures 1a-c). In a further embodiment, the signature motif defined above includes a forth conserved motif defined as:

Leu267-Xaa268-**Asp269**.

The Xaa at amino acid residue position 268 is typically isoleucine, valine, or methionine. The forth motif includes the aspartic acid residue (bold) that is the third member of the catalytic triad (Ser181-Asp269-His298).

[0228] Any number of well-known global alignment algorithms (i.e. sequence analysis software) may be used to align two or more amino acid sequences (representing enzymes having perhydrolase activity) to determine the existence of the present signature motif (for example, CLUSTALW or Needleman and Wunsch (*J. Mol. Biol*., 48:443-453 (1970)). The aligned sequence(s) is compared to the reference sequence (SEQ ID NO: 2). In one embodiment, a CLUSTAL alignment (CLUSTALW) using a reference amino acid sequence (as used herein the CAH sequence (SEQ ID NO: 2) from the *Bacillus subtilis* ATCC 31954™) is used to identify perhydrolases belonging to the CE-7 esterase family. The relative numbering of the conserved amino acid residues is based on the residue numbering of the reference amino acid sequence to account for small insertions or deletions (5 amino acids or less) within the aligned sequence.

[0229] A comparison of the overall percent identity among perhydrolases exemplified herein indicates that enzymes having as little as 33% identity to SEQ ID NO: 2 (while retaining the signature motif) exhibit significant perhydrolase activity and are structurally classified as CE-7 carbohydrate esterases. In one embodiment, the present perhydrolases include enzymes comprising the present signature motif and at least 30%, preferably at least 33%, more preferably at least 40%, even more preferably at least 42%, even more preferably at least 50%, even more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, and most preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO: 2.

[0230] All of the present perhydrolases are comprised of the above signature motif as shown in Table 3.

Table 3. Conserved motifs found within the present enzymes having perhydrolase activity.

Perhydrolase Sequence	RGQ motif[a] (Residue #s)	GXSQG motif[a] (Residue #s)	LXD motif[b] Residue #s)	HE motif[a] (Residue #s)
SEQ ID NO: 2	118-120	179-183	267-269	298-299
SEQ ID NO: 6	118-120	179-183	267-269	298-299

SEQ ID NO: 8	118-120	179-183	267-269	298-299
SEQ ID NO: 10	119-121	180-184	268-270	299-300
SEQ ID NO: 12	118-120	179-183	267-269	298-299
SEQ ID NO: 14	119-121	181-185	269-271	300-301
SEQ ID NO: 16	118-120	186-190	272-274	303-304
SEQ ID NO: 18	118-120	186-190	272-274	303-304
SEQ ID NO: 26	117-119	180-184	270-272	301-302
SEQ ID NO: 32	118-120	179-183	267-269	298-299
SEQ ID NO: 70	117-119	180-184	270-272	301-302
SEQ ID NO: 82	118-120	186-190	272-274	303-304
SEQ ID NO: 90	118-120	186-190	272-274	303-304
SEQ ID NO. 98 RQ2(a)	118-120	186-190	272-274	303-304
SEQ ID NO. 106 RQ2(b)	119-121	187-191	273-275	304-305

[a]= Conserved motifs defined by Vincent *et al.*, *supra* used to define the signature motif.

[b]= an additional motif identified herein useful in further defining the signature motif defined by Vincent et al., *supra*.

[0231] Alternatively, a contiguous signature motif (SEQ ID NO: 61) comprising the 4 conserved motifs (RGQ, GXSQG, LXD, and HE; Amino acids residues 118-299 of SEQ ID NO: 2) may also be used as a contiguous signature motif to identify CE-7 carbohydrate esterases (Figure 1, panels A-C). As such, suitable enzymes expected to have perhydrolase activity may also be identified as having at least 30% amino acid identify, preferably at least 36%, more preferably at least 40%, even more preferably at least 50%, yet more preferably at least 60%, yet even more preferably at least 70%, yet even more preferably at least 80%, yet even more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO: 61 (the 4 conserved motifs found in CE-7 carbohydrate esterases are underlined).

RGQQSSEDTSISLHGHALGWMTKGILDKDTYYYRGVYLDAVRALEVISSFDE VDETRIGVTGGSQGGGLTIAAAALSDIPKAAVADYPYLSNFERAIDVALEQPYL EINSFFRRNGSPETEVQAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTV FAAYNHLETEKELKVYRYFGHE (SEQ ID NO: 61).

[0232] A comparison using the contiguous signature sequence against the present CE-7 esterases having perhydrolase activity is provided in Table 4. BLASTP using default parameters was used.

Table 4. Percent Amino Acid Identity of Various CE-7 Carbohydrate Esterases having Perhydrolysis Activity Versus the Contiguous Signature Sequence (SEQ ID NO: 61).

Perhydrolase Sequence	% Identity using BLASTP	E-score (expected)
SEQ ID NO: 2	100	3e-92
SEQ ID NO: 6	98	6e-91
SEQ ID NO: 8	98	4e-98
SEQ ID NO: 10	78	1e-78
SEQ IDNO: 12	80	3e-76
SEQ ID NO: 14	63	2e-56
SEQ ID NO: 16	51	1e-41
SEQ ID NO: 18	50	6e-35
SEQ ID NO: 26	36	7e-21
SEQ ID NO: 32	99	2e-90
SEQ ID NO: 70	40	2e-26
SEQ ID NO: 82	40	3e-30
SEQ ID NO: 90	46	6e-35
SEQ ID NO. 98	46	6e-35
SEQ ID NO. 106	48	9e-36

[0233] Alternatively, the percent amino acid identity to the complete length of one or more of the present perhydrolases may also be used. Accordingly, suitable enzymes having perhydrolase activity have at least 30%, preferably at least 33%, preferably at least 40%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, yet even more preferably at least 90%, and most preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO: 2. In a further embodiment, suitable perhydrolase catalysts comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO:

16, SEQ ID NO: 18, SEQ ID NO: 26, SEQ ID NO: 32, SEQ ID NO: 70, SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106. In preferred embodiments, suitable enzymes having perhydrolase activity having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% amino acid identity to SEQ ID NO: 82 or to SEQ ID NO: 90 or to SEQ ID NO: 98 or to SEQ ID NO: 106 may be used.

[0234] Suitable perhydrolase enzymes may also include enzymes having one or more deletions, substitutions, and/or insertions to one of the present perhydrolase enzymes (e.g. SEQ ID NOs. 82, 90, 98, and 106). As shown in Table 2, CE-7 carbohydrates esterases having perhydrolase activity share as little as 32% overall amino acid identity. Based on the data provided in the present examples, additional enzymes having perhydrolase activity belonging to the CE-7 carbohydrate esterase family may have even lower percent identity, so long as the enzyme retains the conserved signature motif. As such, the numbers of deletions, substitutions, and/or insertions may vary so long as the conserved signature motifs (see Table 3) are found in their relative positions within the enzyme.

[0235] Additionally, it is well within one of skill in the art to identity suitable enzymes according to the structural similarity found within the corresponding nucleic acid sequence. Hybridization techniques can be used to identity similar gene sequences. Accordingly, suitable perhydrolase catalysts of the present invention comprise an amino acid sequence encoded by a nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule having a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17; SEQ ID NO: 25; SEQ ID NO: 31, SEQ ID NO: 41, SEQ ID NO: 60, SEQ ID NO: 65, SEQ ID NO: 69, SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

[0236] In another embodiment, the perhydrolase catalyst comprises an enzyme having an amino acid sequence encoded by a nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

[0237] The present method produces industrially useful, efficacious concentrations of peracids *in situ* under aqueous reaction conditions using the perhydrolase activity of an enzyme belonging to the CE-7 family of carbohydrate esterases. In one embodiment, the enzyme having perhydrolase activity is also classified structurally and functionally as a cephalosporin C deacetylase (CAH). In another embodiment, the enzyme having perhydrolase activity is classified structurally and functionally as an acetyl xylan esterase (AXE).

[0238] The peracids produced are quite reactive and may decrease in concentration over extended periods of time, depending on variables that include, but are not limited to, temperature and pH. As such, it may be desirable to keep the various reaction components separated, especially for liquid formulations. In one aspect, the hydrogen peroxide source is separate from either the substrate or the perhydrolase catalyst, preferably from both. This can be accomplished using a variety of techniques including, but not limited to the use of multicompartment chambered dispensers (U.S. patent 4,585,150) and at the time of use physically combining the perhydrolase catalyst with an inorganic peroxide and the present substrates to initiate the aqueous enzymatic perhydrolysis reaction. The perhydrolase catalyst may optionally be immobilized within the body of reaction chamber or separated (e.g. filtered, etc.) from the reaction product comprising the peracid prior to contacting the surface and/or object targeted for treatment. The perhydrolase catalyst may be in a liquid matrix or in a solid form (i.e., powdered, tablet) or embedded within a solid matrix that is subsequently mixed with the substrates to initiate the enzymatic perhydrolysis reaction. In a further aspect, the perhydrolase catalyst may be contained within a dissolvable or porous pouch that may be added to the aqueous substrate matrix to initiate enzymatic perhydrolysis. In an additional further aspect, a powder comprising the enzyme catalyst is suspended in the substrate (*e.g.*, triacetin), and at time of use is mixed with a source of peroxygen in water.

HPLC Assay Method for Determining the Concentration of Peracid and Hydrogen Peroxide.

[0239] A variety of analytical methods can be used in the present method to analyze the reactants and products including, but not limited to titration, high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), capillary electrophoresis (CE), the analytical procedure described by U. Karst *et al.*, (*Anal. Chem.,* 69(17):3623-3627 (1997)), and the 2,2'-azino-bis (3-ethylbenzothazoline)-6-sulfonate (ABTS)

assay (S. Minning, *et al., Analytica Chimica Acta* 378:293-298 (1999) and WO 2004/058961 A1) as described in the present examples.

Determination of Minimum Biocidal Concentration of Peracids

[0240] The method described by J. Gabrielson, *et al.* (*J. Microbiol. Methods* 50: 63-73 (2002)) can be employed for determination of the Minimum Biocidal Concentration (MBC) of peracids, or of hydrogen peroxide and enzyme substrates. The assay method is based on XTT reduction inhibition, where XTT ((2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium, inner salt, monosodium salt) is a redox dye that indicates microbial respiratory activity by a change in optical density (OD) measured at 490 nm or 450 nm. However, there are a variety of other methods available for testing the activity of disinfectants and antiseptics including, but not limited to viable plate counts, direct microscopic counts, dry weight, turbidity measurements, absorbance, and bioluminescence (see, for example Brock, Semour S., Disinfection, Sterilization, and Preservation, 5th edition, Lippincott Williams & Wilkins, Philadelphia, PA, USA; 2001).

Uses of Enzymatically Prepared Peracid Compositions

[0241] The enzyme catalyst-generated peracid produced according to the present methods can be used in a variety of hard surface/inanimate object applications for reduction of concentrations of microbial, fungal, prion-related, and viral contamination, such as decontamination of medical instruments (e.g., endoscopes), textiles (e.g., garments, carpets), food preparation surfaces, food storage and food-packaging equipment, materials used for the packaging of food products, chicken hatcheries and grow-out facilities, animal enclosures, and spent process waters that have microbial and/or virucidal activity. The enzyme-generated peracids may be used in formulations designed to inactivate prions (e.g. certain proteases) to additionally provide biocidal activity. In a preferred aspect, the present peracid compositions are particularly useful as a disinfecting agent for non-autoclavable medical instruments and food packaging equipment. As the peracid-containing formulation may be prepared using GRAS or food-grade components (enzyme, enzyme substrate, hydrogen peroxide, and buffer), the enzyme-generated peracid may also be used for decontamination of animal carcasses, meat, fruits and vegetables, or for decontamination of prepared foods. The enzyme-generated peracid may be incorporated into a product whose final form is a powder, liquid, gel, film, solid or aerosol. The enzyme-generated peracid may be diluted to a concentration that still provides an efficacious decontamination.

[0242] The compositions comprising an efficacious concentration of peracid can be used to disinfect surfaces and/or objects contaminated (or suspected of being contaminated) with viable pathogenic microbial contaminants by contacting the surface or object with the products produced by the present processes. As used herein, "contacting" refers to placing a disinfecting composition comprising an effective concentration of peracid in contact with the surface or inanimate object suspected of contamination with a disease-causing entity for a period of time sufficient to clean and disinfect. Contacting includes spraying, treating, immersing, flushing, pouring on or in, mixing, combining, painting, coating, applying, affixing to and otherwise communicating a peracid solution or composition comprising an efficacious concentration of peracid, or a solution or composition that forms an efficacious concentration of peracid, with the surface or inanimate object suspected of being contaminated with a concentration of a microbial population. The disinfectant compositions may be combined with a cleaning composition to provide both cleaning and disinfection. Alternatively, a cleaning agent (*e.g.*, a surfactant or detergent) may be incorporated into the formulation to provide both cleaning and disinfection in a single composition.

[0243] The compositions comprising an efficacious concentration of peracid can also contain at least one additional antimicrobial agent, combinations of prion-degrading proteases, a virucide, a sporicide, or a biocide. Combinations of these agents with the peracid produced by the claimed processes can provide for increased and/or synergistic effects when used to clean and disinfect surfaces and/or objects contaminated (or suspected of being contaminated) with pathogenic microorganisms, spores, viruses, fungi, and/or prions. Suitable antimicrobial agents include carboxylic esters (e.g., *p*-hydroxy alkyl benzoates and alkyl cinnamates), sulfonic acids (e.g., dodecylbenzene sulfonic acid), iodo-compounds or active halogen compounds (e.g., elemental halogens, halogen oxides (e.g., NaOCl, HOCl, HOBr, ClO_2), iodine, interhalides (e.g., iodine monochloride, iodine dichloride, iodine trichloride, iodine tetrachloride, bromine chloride, iodine monobromide, or iodine dibromide), polyhalides, hypochlorite salts, hypochlorous acid, hypobromite salts, hypobromous acid, chloro- and bromo-hydantoins, chlorine dioxide, and sodium chlorite), organic peroxides including benzoyl peroxide, alkyl benzoyl peroxides, ozone, singlet oxygen generators, and mixtures thereof, phenolic derivatives (e.g., *o*-phenyl phenol, *o*-benzyl-*p*-chlorophenol, *tert*-amyl phenol and C_1-C_6 alkyl hydroxy benzoates), quaternary ammonium compounds (e.g., alkyldimethylbenzyl ammonium chloride, dialkyldimethyl

ammonium chloride and mixtures thereof), and mixtures of such antimicrobial agents, in an amount sufficient to provide the desired degree of microbial protection. Effective amounts of antimicrobial agents include about 0.001 wt% to about 60 wt% antimicrobial agent, about 0.01 wt% to about 15 wt% antimicrobial agent, or about 0.08 wt% to about 2.5 wt% antimicrobial agent.

[0244] In one aspect, the peracids formed by the present process can be used to reduce the concentration of viable microbial contaminants (e.g. a viable microbial population) when applied on and/or at a locus. As used herein, a "locus" comprises part or all of a target surface suitable for disinfecting or bleaching. Target surfaces include all surfaces that can potentially be contaminated with microorganisms, viruses, spores, fungi, prions or combinations thereof. Non-limiting examples include equipment surfaces found in the food or beverage industry (such as tanks, conveyors, floors, drains, coolers, freezers, equipment surfaces, walls, valves, belts, pipes, drains, joints, crevasses, combinations thereof, and the like); building surfaces (such as walls, floors and windows); non-food-industry related pipes and drains, including water treatment facilities, pools and spas, and fermentation tanks; hospital or veterinary surfaces (such as walls, floors, beds, equipment, (such as endoscopes) clothing worn in hospital/veterinary or other healthcare settings, including clothing, scrubs, shoes, and other hospital or veterinary surfaces); restaurant surfaces; bathroom surfaces; toilets; clothes and shoes; surfaces of barns or stables for livestock, such as poultry, cattle, dairy cows, goats, horses and pigs; hatcheries for poultry or for shrimp; and pharmaceutical or biopharmaceutical surfaces (*e.g.*, pharmaceutical or biopharmaceutical manufacturing equipment, pharmaceutical or biopharmaceutical ingredients, pharmaceutical or biopharmaceutical excipients). Additional hard surfaces also include food products, such as beef, poultry, pork, vegetables, fruits, seafood, combinations thereof, and the like. The locus can also include water absorbent materials such as infected linens or other textiles. The locus also includes harvested plants or plant products including seeds, corms, tubers, fruit, and vegetables, growing plants, and especially crop growing plants, including cereals, leaf vegetables and salad crops, root vegetables, legumes, berried fruits, citrus fruits and hard fruits.

[0245] Non-limiting examples of hard surface materials are metals (e.g., steel, stainless steel, chrome, titanium, iron, copper, brass, aluminum, and alloys thereof), minerals (e.g., concrete), polymers and plastics (e.g., polyolefins, such as polyethylene, polypropylene, polystyrene,

poly(meth)acrylate, polyacrylonitrile, polybutadiene, poly(acrylonitrile, butadiene, styrene), poly(acrylonitrile, butadiene), acrylonitrile butadiene; polyesters such as polyethylene terephthalate; and polyamides such as nylon). Additional surfaces include brick, tile, ceramic, porcelain, wood, vinyl, linoleum, and carpet.

Recombinant Microbial Expression

[0246] The genes and gene products of the instant sequences may be produced in heterologous host cells, particularly in the cells of microbial hosts. Preferred heterologous host cells for expression of the instant genes and nucleic acid molecules are microbial hosts that can be found within the fungal or bacterial families and which grow over a wide range of temperature, pH values, and solvent tolerances. For example, it is contemplated that any of bacteria, yeast, and filamentous fungi may suitably host the expression of the present nucleic acid molecules. The perhydrolase may be expressed intracellularly, extracellularly, or a combination of both intracellularly and extracellularly, where extracellular expression renders recovery of the desired protein from a fermentation product more facile than methods for recovery of protein produced by intracellular expression. Transcription, translation and the protein biosynthetic apparatus remain invariant relative to the cellular feedstock used to generate cellular biomass; functional genes will be expressed regardless. Examples of host strains include, but are not limited to bacterial, fungal or yeast species such as *Aspergillus, Trichoderma, Saccharomyces, Pichia, Phaffia, Candida, Hansenula, Yarrowia, Salmonella, Bacillus, Acinetobacter, Zymomonas, Agrobacterium, Erythrobacter, Chlorobium, Chromatium, Flavobacterium, Cytophaga, Rhodobacter, Rhodococcus, Streptomyces, Brevibacterium, Corynebacteria, Mycobacterium, Deinococcus, Escherichia, Erwinia, Pantoea, Pseudomonas, Sphingomonas, Methylomonas, Methylobacter, Methylococcus, Methylosinus, Methylomicrobium, Methylocystis, Alcaligenes, Synechocystis, Synechococcus, Anabaena, Thiobacillus, Methanobacterium, Klebsiella,* and *Myxococcus.* In one embodiment, bacterial host strains include *Escherichia*, *Bacillus*, and *Pseudomonas*. In a preferred embodiment, the bacterial host cell is *Escherichia coli*.

[0247] Large-scale microbial growth and functional gene expression may use a wide range of simple or complex carbohydrates, organic acids and alcohols or saturated hydrocarbons, such as methane or carbon dioxide in the case of photosynthetic or chemoautotrophic hosts, the form and amount of nitrogen, phosphorous, sulfur, oxygen, carbon or any trace micronutrient

including small inorganic ions. The regulation of growth rate may be affected by the addition, or not, of specific regulatory molecules to the culture and which are not typically considered nutrient or energy sources.

[0248] Vectors or cassettes useful for the transformation of suitable host cells are well known in the art. Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the transformed host cell and/or native to the production host, although such control regions need not be so derived.

[0249] Initiation control regions or promoters, which are useful to drive expression of the present cephalosporin C deacetylase coding region in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the present invention including but not limited to *CYC1*, *HIS3*, *GAL1*, *GAL10*, *ADH1*, *PGK*, *PHO5*, *GAPDH*, *ADC1*, *TRP1*, *URA3*, *LEU2*, *ENO*, *TPI* (useful for expression in *Saccharomyces*); *AOX1* (useful for expression in *Pichia*); and *lac*, *ara*, *tet*, *trp*, IP_L, IP_R, *T7*, *tac*, and *trc* (useful for expression in *Escherichia coli*) as well as the *amy*, *apr*, *npr* promoters and various phage promoters useful for expression in *Bacillus*.

[0250] Termination control regions may also be derived from various genes native to the preferred host cell. In one embodiment, the inclusion of a termination control region is optional. In another embodiment, the chimeric gene includes a termination control region derived the preferred host cell.

Industrial Production

[0251] A variety of culture methodologies may be applied to produce the present perhydrolase catalysts. For example, large-scale production of a specific gene product overexpressed from a recombinant microbial host may be produced by both batch and continuous culture methodologies.

[0252] A classical batch culturing method is a closed system where the composition of the media is set at the beginning of the culture and not subject to artificial alterations during the culturing process. Thus, at the beginning of the culturing process, the media is inoculated with the desired organism or organisms and growth or metabolic activity may occur without adding anything further to the system. Typically, however, a “batch” culture is

batch with respect to the addition of carbon source and attempts are often made to control factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. Within batch cultures cells moderate 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 will eventually die. Cells in log phase are often responsible for the bulk of production of end product or intermediate in some systems. Stationary or post-exponential phase production can be obtained in other systems.

[0253] A variation on the standard batch system is the fed-batch system. Fed-batch culture processes are also suitable in the present invention and comprise a typical batch system except that the substrate is added in increments as the culture progresses. 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 substrate in the media. Measurement of the actual substrate concentration in fed-batch systems is difficult and 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_2. Batch and fed-batch culturing methods are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989) and Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227 (1992).

[0254] Commercial production of the desired perhydrolase catalysts may also be accomplished with a continuous culture. Continuous cultures are an open system where a defined culture media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high liquid phase density where cells are primarily in log phase growth. Alternatively, continuous culture may be practiced with immobilized cells where carbon and nutrients are continuously added and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials.

[0255] Continuous or semi-continuous culture allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting

nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the culture. Methods of modulating nutrients and growth factors for continuous culture processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

[0256] Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, disaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally, the carbon substrate may also be one-carbon substrates such as carbon dioxide, methane or methanol (for example, when the host cell is a methylotrophic microorganism). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter *et al.*, *Arch. Microbiol.,* 153:485-489 (1990)). Hence, it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing substrates and will only be limited by the choice of organism.

[0257] Recovery of the desired perhydrolase catalysts from a batch or fed batch fermentation, or continuous culture, may be accomplished by any of the methods that are known to those skilled in the art. For example, when the perhydrolase catalyst is produced intracellularly, the cell paste is separated from the culture medium by centrifugation or membrane filtration, optionally washed with water or an aqueous buffer at a desired pH, then a suspension of the cell paste in an aqueous buffer at a desired pH is homogenized to produce a cell extract containing the desired perhydrolase catalyst. The cell extract may optionally be filtered through an appropriate filter aid such as celite or silica to remove cell debris prior to a heat-treatment step to precipitate undesired protein from the perhydrolase catalyst solution. The solution containing the desired perhydrolase catalyst may then be separated from the precipitated cell debris and protein by membrane filtration or centrifugation, and the resulting partially-purified perhydrolase catalyst solution concentrated by additional membrane filtration, then optionally mixed with an appropriate

carrier (for example, maltodextrin, phosphate buffer, citrate buffer, or mixtures thereof) and spray-dried to produce a solid powder comprising the desired perhydrolase catalyst.

[0258] Applicants specifically incorporate the entire contents of all cited references in this disclosure. Further, when an amount, concentration, or other value or parameter is given either as a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope be limited to the specific values recited when defining a range.

GENERAL METHODS

[0259] The following examples are provided to demonstrate preferred embodiments. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[0260] All reagents and materials were obtained from DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), TCI America (Portland, OR), Roche Diagnostics Corporation (Indianapolis, IN) or Sigma/Aldrich Chemical Company (St. Louis, MO), unless otherwise specified.

[0261] The following abbreviations in the specification correspond to units of measure, techniques, properties, or compounds as follows: "sec" or "s" means second(s), "min" means minute(s), "h" or "hr" means hour(s), "μL" means microliters, "mL" means milliliters, "L" means liters, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "ppm" means parts per million, "wt" means weight, "wt%" means weight percent, "g" means grams, "μg" means micrograms, "*g*" means gravity, "HPLC" means high performance liquid chromatography, "dd H_2O" means distilled and deionized water, "dcw" means dry cell weight, "ATCC" or "ATCC®" means the American Type Culture

Collection (Manassas, VA), "U" means units of perhydrolase activity, "rpm" means revolutions per minute, and "EDTA" means ethylenediaminetetraacetic acid.

EXAMPLE 1

Growth of *Bacillus subtilis* ATCC 31954™ and Preparation of Cell Extract

[0262] A culture of *Bacillus subtilis* (ATCC 31954™) was revived following suspension of the dried culture in 5 mL of nutrient broth (DIFCO; 0003-01-6) and incubation for 3 days at 30 °C. Following the third day of incubation, an aliquot of the culture was streaked onto a trypticase soy agar culture plate (Becton, Dickinson, and Company; Franklin Lakes, NJ) and incubated at 35 °C for 24 h. Several single colonies were scraped onto a 1 microliter inoculation loop (Becton Dickinson; catalog # 220215) and transferred into 50 mL of *Lactobacillus* MRS broth (Hardy Diagnostics, Santa Maria, CA; catalog # C5931). The culture was then grown at 30 °C and a 200-rpm agitation rate for 12 h. After 12 h of growth, 2 mL of the culture was transferred into an unbaffled 500-mL shake flask containing 100 mL of MRS broth for growth at 30 °C and 200-rpm agitation for 12-14 h. The cells were subsequently harvested by centrifugation at 15,000 x g for 25 min at 5 °C and the resulting cell paste stored at -80 °C.

[0263] For cell extract preparation, 0.9 g of cell paste was suspended at 25 wt % (wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) and EDTA (1 mM). The cell suspension was passed twice through a French press having a working pressure of 16,000 psi. The crude extract was then centrifuged at 20,000 x g to remove cellular debris, producing a clear cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT), then frozen and stored at - 80 °C.

EXAMPLE 2

Determination of Perhydrolysis Activity of *Bacillus subtilis* ATCC 31954™ Semi-purified Cell Extract

[0264] A 1.0-mL aliquot of *Bacillus subtilis* (ATCC 31954™) cell extract (10 mg total protein/mL, prepared as described in Example 1) was diluted with an equal volume of 50 mM phosphate buffer (pH 7.0) and filtered through a 100,000 Molecular Weight Cutoff (MWCO) Centricon membrane unit (Millipore Corp, Bedford, MA). The resulting filtrate (semi-purified cell extract) contained

1.5 mg total protein/mL assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma catalog # BCA1-KT), and an assay of this filtrate indicated no measurable catalase activity.

[0265] A 1-mL reaction mixture containing triacetin (250 mM), hydrogen peroxide (2.5 M) and 0.100 mL of semi-purified cell extract (0.15 mg extract total protein) in 50 mM phosphate buffer (pH 6.5) was mixed at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for semi-purified cell extract to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added semi-purified cell extract.

[0266] Determination of the concentration of peracetic acid in the reaction mixture was performed according to the method described by Karst *et al.*. Aliquots (0.250 mL) of the reaction mixture were removed at 10 min and 30 min and filtered using an Ultrafree® MC-filter unit (30,000 Normal Molecular Weight Limit (NMWL), Millipore cat # UFC3LKT 00) by centrifugation for 2 min at 12,000 rpm; removal of the protein component of the aliquot by filtration terminated the reaction. An aliquot (0.100 mL) of the resulting filtrate was transferred to 1.5-mL screw cap HPLC vial (Agilent Technologies, Palo Alto, CA; #5182-0715) containing 0.300 mL of deionized water, then 0.100 mL of 20 mM MTS (methyl-*p*-tolyl-sulfide) in acetonitrile was added, the vials capped, and the contents briefly mixed prior to a 10 min incubation at ca. 25 °C in the absence of light. To each vial was then added 0.400 mL of acetonitrile and 0.100 mL of a solution of triphenylphosphine (TPP, 40 mM) in acetonitrile, the vials re-capped, and the resulting solution mixed and incubated at ca. 25 °C for 30 min in the absence of light. To each vial was then added 0.100 mL of 10 mM N,N-diethyl-*m*-toluamide (DEET; HPLC external standard) and the resulting solution analyzed by HPLC as described below. The peracetic acid concentrations produced in 10 min and 30 min are listed in Table 5.

HPLC Method:
Supelco Discovery C8 column (10-cm X 4.0-mm, 5 μm) (cat. #569422-U) w/precolumn Supelco Supelguard Discovery C8 (Sigma-Aldrich; cat # 59590-U); 10 microliter injection volume; gradient method with CH_3CN (Sigma-Aldrich; #270717) and deionized H_2O at 1.0 mL/min and ambient temperature:

Time (min:sec)	(% CH_3CN)
0:00	40
3:00	40

3:10	100
4:00	100
4:10	40
7:00 (stop)	40

Table 5. Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (2.5 M) at pH 6.5 in the presence or absence of *B. subtilis* (ATCC 31954™) semi-purified cell extract.

B. subtilis (ATCC 31954™) semi-purified cell extract (mg total protein/mL)	peracetic acid (ppm) in 10 min	peracetic acid (ppm) in 30 min
0	641	1343
0.15	3492	3032

EXAMPLE 3

Perhydrolysis Activity of Semi-Purified Enzyme from *Bacillus subtilis* ATCC 31954™ Cell Extract

[0267] *Bacillus subtilis* ATCC 31954™ growth and extract preparation was performed as described in Example 1, except that the crude extract was not centrifuged. The crude extract was fractionated with cold *n*-propanol (- 20 °C). A flask containing the cell-free extract was stirred in an ice bath for 15 min, then the n-propanol (- 20 °C) was added drop-wise (to prevent freezing of the extract) to a concentration of 40 % (v/v). The resulting extract/propanol mixture was stirred in the ice bath for 30 min, then centrifuged at 12,000 x *g* for 10 min at 5 °C, and the supernatant returned to the flask and placed into the ice bath. Additional n-propanol (– 20 °C) was slowly added to the supernatant with stirring to a concentration of 60 % (v/v), and the resulting mixture stirred for 30 min in the ice bath and then centrifuged as before. The pellet from this second fraction was saved on ice and the supernatant returned to the flask and placed into the ice bath. Cold n-propanol was slowly added to the supernatant with stirring to a concentration of 80 % (v/v), the mixture stirred for 30 min and centrifuged as before. The pellet from the 60-80 % fraction was saved on ice. The pellets from the 40 - 60 % (v/v) n-propanol fractions and the 60 - 80 % (v/v) n-propanol fractions were dissolved in a minimum amount of 0.05 M phosphate buffer (pH 6.5) and the resulting solutions assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, catalog # BCA1-KT), then frozen and stored at - 80 °C.

[0268] A 1-mL reaction mixture containing triacetin (250 mM), hydrogen peroxide (1.0 M) and 0.10 mg/mL of total soluble protein from either the 40 - 60 % (v/v) or 60 - 80 % (v/v) n-propanol fractions of the cell extract (prepared as described above) in 50 mM phosphate buffer (pH 6.5) was mixed at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the n-propanol fractions of the cell extract containing semi-purified enzyme to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added semi-purified enzyme. The reaction mixture was assayed for peracetic acid at 5 min and 30 min using the procedure described in Example 2, and the concentrations of peracetic acid produced by added enzyme are listed in Table 6.

Table 6. Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence or absence of *B. subtilis* (ATCC 31954™) semi-purified cell extracts.

n-propanol fraction of cell extract	total protein (mg/mL reaction)	peracetic acid (ppm) in 5 min	peracetic acid (ppm) in 30 min
no extract	0	221	803
40 - 60 %	0.1	2829	4727
60 - 80 %	0.1	1832	3777

EXAMPLE 4

Identification of a Cephalosporin C Deacetylase Having Perhydrolysis Activity from *Bacillus subtilis* ATCC 31954™ Cell Extract

[0269] A 0.1 mL sample (500 µg total protein) of the 40-60% *n*-propanol fraction described in Example 3 was mixed at room temperature with an equal volume of 2x non-denaturing (native) sample buffer (Invitrogen) and loaded into the preparative sample well of a 1.5 mm 8-16% Tris-Glycine polyacrylamide mini-gel (2D gels; Invitrogen). The native gel electrophoresis was operated at 125 V for 90 min using Tris-Glycine running buffer (Invitrogen). Following electrophoresis, the gel was prepared for an *in situ* esterase activity assay using the pH indicator, bromothymol blue.

[0270] The gel was washed for 10 min x 2 with deionized water and slow mechanical mixing. The gel was then washed for 10 min using 10 mM phosphate buffer. Following the removal of the phosphate buffer, 50 mL of 10 mM phosphate buffer containing 665 µL of saturated bromothymol blue (in

water) was incubated with the gel for 10 min followed by the addition of 1 mL of neat triacetin (Sigma Aldrich). Within 10 min of incubation one yellow band at 146 kDa appeared on the gel indicating esterase activity.

[0271] The esterase-positive band was excised from the gel and transferred into a 50 mL polypropylene conical tube (Falcon). The yellow bromothymol blue stain was removed from the gel slice following two 5-mL deionized water washes with gentle mixing. The gel slice was then treated for 30 min with 0.9 mL of 2X Novex Tris-Glycine SDS sample buffer plus 100 µL of 10X NuPAGE reducing agent (Invitrogen) with gentle mixing. Following the sample treatment, the gel slice and sample buffer were incubated at 85 °C for 5 min using a hot water bath. The gel slice was then removed from the incubation tube and carefully placed in the single preparative well of a 1.5 mm 8-16% Tris-Gly mini-gel. Care was taken to exclude air bubbles and to have direct contact with the stacking gel. The gel slice was then immobilized in place following the addition of 250-300 µL of a warm 0.5 % agarose solution prepared in deionized water into the preparative well. The single molecular marker lane was loaded with 15 µL of SeeBlue® Plus2 pre-stained MW marker (Invitrogen).

[0272] The electrophoresis of the gel slice was operated at 30 V for 30 min for electro-elution of the protein from the gel slice into the slab gel. The voltage was then ramped up from 30 V to 125 V over 10 min followed by 90 min operation at 125 V. Following electrophoresis, the resolved protein bands on the gel were blotted onto a PVDF membrane as described in the XCell II™ blotting manual (Invitrogen) and the blotting buffer was 10 mM CAPS, pH 11.0. The electro-blotting procedure was operated at 25 V for 2 hr at room temperature with ice water in the jacket of the transfer apparatus.

[0273] Following the transfer, the PVDF membrane was stained with ProBlot staining solution (Applied Biosystems, Foster City, CA) for 1 min followed by de-staining with methanol:water (50:50). Six protein bands were identified and each was N-terminal sequenced. Following a Blast search of the GENBANK® amino acid sequence database, the only band having esterase-related sequence homology was identified as Band 1 and the 17 N-terminal amino acid calls had 100 % amino acid identity to a *Bacillus subtilis* cephalosporin C deacetylase (GENBANK® BAA01729; Mitsushima *et al.*, *supra*; U.S. patent 5,528,152; and U.S. patent 5,338,676).

EXAMPLE 5

Cloning and Expression of Perhydrolase from *Bacillus subtilis* ATCC 31954™

[0274] Genomic DNA was isolated from *Bacillus subtilis* ATCC 31954™ using the PUREGENE® DNA purification system (Gentra Systems, Minneapolis MN). The perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 3 and SEQ ID NO: 4. The resulting nucleic acid product (SEQ ID NO: 1) was subcloned into pTrcHis2-TOPO® (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW186. The perhydrolase gene was also amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 27 and SEQ ID NO: 28. The resulting nucleic acid product (SEQ ID NO: 29) was cut with restriction enzymes *Pst*I and *Xba*I and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW194. The plasmids pSW186 and pSW194 were used to transform *E. coli* TOP10 (Invitrogen, Carlsbad CA), *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as TOP10/pSW186, MG1655/pSW186, UM2/pSW186, KLP18/pSW186, TOP10/pSW194, MG1655/pSW194, UM2/pSW194 and KLP18/pSW194, respectively. TOP10/pSW186, MG1655/pSW186, UM2/pSW186, KLP18/pSW186, TOP10/pSW194, MG1655/pSW194, UM2/pSW194 and KLP18/pSW194 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4-0.5, at which time IPTG was added to a final concentration of 1 mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 6

Cloning and Expression of Perhydrolase from *Bacillus subtilis* BE1010

[0275] Genomic DNA was isolated from *Bacillus subtilis* BE1010 (Payne and Jackson 1991 *J. Bacteriol.* 173:2278-2282) using the PUREGENE® DNA purification system (Gentra Systems). The perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 3 and SEQ ID NO: 4. The resulting nucleic acid product (SEQ ID NO: 5) was subcloned into pTrcHis2-TOPO® (Invitrogen) to generate the plasmid identified as pSW187. The perhydrolase gene was also amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 27 and SEQ ID NO: 28. The resulting

nucleic acid product (SEQ ID NO: 30) was cut with restriction enzymes *Pst*I and *Xba*I and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW189. The plasmids pSW187 and pSW189 were used to transform *E. coli* TOP10 (Invitrogen), *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as TOP10/pSW187, MG1655/pSW187, UM2/pSW187, KLP18/pSW187, TOP10/pSW189, MG1655/pSW189, UM2/pSW189 and KLP18/pSW19, respectively. TOP10/pSW187, MG1655/pSW187, UM2/pSW187, KLP18/pSW187, TOP10/pSW189, MG1655/pSW189, UM2/pSW189 and KLP18/pSW189 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4 - 0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 7

Cloning and Expression of Perhydrolase from *Bacillus subtilis* ATCC 6633™

[0276] Genomic DNA was isolated from *Bacillus subtilis* ATCC 6633™ using the PUREGENE® DNA purification system. The perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 3 and SEQ ID NO: 4. The resulting nucleic acid product (SEQ ID NO: 7) was subcloned into pTrcHis2-TOPO® to generate the plasmid identified as pSW188. The plasmid pSW188 was used to transform *E. coli* MG1655 (ATCC 47076™) and *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) to generate the strains identified as MG1655/pSW188 and UM2/pSW188, respectively. MG1655/pSW188 and UM2/pSW188 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2 - 3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 8

Cloning and Expression of Perhydrolase from *Bacillus subtilis* ATCC 29233™

[0277] Genomic DNA was isolated from *Bacillus subtilis* ATCC 29233™ using the PUREGENE® DNA purification system. The perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min

at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 3 and SEQ ID NO: 4. The resulting nucleic acid product (SEQ ID NO: 31) was subcloned into pTrcHis2-TOPO® to generate the plasmid identified as pSW190. The plasmid pSW190 was used to transform *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as MG1655/pSW190, UM2/pSW190 and KLP18/pSW190, respectively. MG1655/pSW190, UM2/pSW190 and KLP18/pSW190 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4 - 0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 9

Cloning and Expression of Perhydrolase from *Bacillus licheniformis* ATCC 14580™

[0278] Genomic DNA was isolated from *Bacillus licheniformis* ATCC 14580™ using the PUREGENE® DNA purification system. The perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 33 and SEQ ID NO: 34. The resulting nucleic acid product (SEQ ID NO: 9) was subcloned into pTrcHis2-TOPO® to generate the plasmid identified as pSW191. The plasmid pSW191 was used to transform *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT), *E. coli* PIR1 (Invitrogen, Carlsbad CA) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as MG1655/pSW191, UM2/pSW191, PIR1/pSW191 and KLP18/pSW191, respectively. MG1655/pSW191, UM2/pSW191, PIR1/pSW191 and KLP18/pSW191 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4-0.5, at which time IPTG was added to a final concentration of 1 mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 10

Cloning and Expression of Perhydrolase from *Clostridia thermocellum* ATCC 27405™

[0279] Genomic DNA was isolated from *Clostridia thermocellum* ATCC 27405™ using the PUREGENE® DNA purification system. The

perhydrolase gene was amplified from the genomic DNA by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 35 and SEQ ID NO: 36. The resulting nucleic acid product (SEQ ID NO: 13) was subcloned into pTrcHis2-TOPO® to generate the plasmid identified as pSW193. The plasmid pSW193 was used to transform *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as MG1655/pSW193, UM2/pSW193 and KLP18/pSW193, respectively. MG1655/pSW193, UM2/pSW193 and KLP18/pSW193 were gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4-0.5, at which time IPTG was added to a final concentration of 1 mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 11

Cloning and Expression of Perhydrolase from *Bacillus pumilus* PS213

[0280] The gene encoding acetyl xylan esterase (*axe*1) from *B. pumilus* PS213 as reported in GENBANK® (accession # AJ249957) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park CA). The gene was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 37 and SEQ ID NO: 38. The resulting nucleic acid product (SEQ ID NO: 60) was subcloned into pTrcHis2-TOPO® (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW195. The plasmid pSW195 was used to transform *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as MG1655/pSW195, UM2/pSW195 and KLP18/pSW195, respectively. MG1655/pSW195, UM2/pSW195 and KLP18/pSW195 were gown in LB media at 37 °C with shaking up to OD600nm = 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 12

Cloning and Expression of Perhydrolase from *Thermotoga neapolitana*

[0281] The gene encoding acetyl xylan esterase from *Thermotoga neapolitana* as reported in GENBANK® (accession # 58632) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The gene was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 39 and SEQ ID NO: 40. The resulting nucleic acid product (SEQ ID NO: 41) was subcloned into pTrcHis2-TOPO® to generate the plasmid identified as pSW196. The plasmid pSW196 was used to transform *E. coli* MG1655 (ATCC 47076™), *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) and *E. coli* KLP18 (see EXAMPLE 15) to generate the strains identified as MG1655/pSW196, UM2/pSW196 and KLP18/pSW196, respectively. MG1655/pSW196, UM2/pSW196 And KLP18/pSW196 were gown in LB media at 37 °C with shaking up to OD600nm = 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein.

EXAMPLE 13

Construction of a *katG* Catalase Disrupted *E. coli* strain

[0282] The kanamycin resistance gene (*kan*; SEQ ID NO: 42) was amplified from the plasmid pKD13 (SEQ ID NO: 43) by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 44 and SEQ ID NO: 45 to generate the PCR product identified as SEQ ID NO: 46. The *katG* nucleic acid sequence is provided as SEQ ID NO: 47 and the corresponding amino acid sequence is SEQ ID NO: 48. *E. coli* MG1655 (ATCC 47076™) was transformed with the temperature-sensitive plasmid pKD46 (SEQ ID NO: 49), which contains the λ-Red recombinase genes (Datsenko and Wanner, 2000, *PNAS* USA 97:6640-6645), and selected on LB-amp plates for 24 h at 30 °C. MG1655/pKD46 was transformed with 50-500 ng of the PCR product by electroporation (BioRad Gene Pulser, 0.2 cm cuvette, 2.5 kV, 200 W, 25 uF), and selected on LB-kan plates for 24 h at 37 °C. Several colonies were streaked onto LB-kan plates and incubated overnight at 42 °C to cure the pKD46 plasmid. Colonies were checked to confirm a phenotype of kanR/ampS. Genomic DNA was isolated from several colonies using the PUREGENE® DNA purification system, and checked by PCR to confirm disruption of the *katG* gene using primers identified as SEQ ID NO: 50and SEQ ID NO: 51. Several *katG*-disrupted strains were transformed with

the temperature-sensitive plasmid pCP20 (SEQ ID NO: 52), which contains the FLP recombinase, used to excise the *kan* gene, and selected on LB-amp plates for 24 h at 37 °C. Several colonies were streaked onto LB plates and incubated overnight at 42 °C to cure the pCP20 plasmid. Two colonies were checked to confirm a phenotype of kanS/ampS, and called MG1655 KatG1 and MG1655 KatG2.

EXAMPLE 14

Construction of a *katE* Catalase Disrupted *E. coli* strain

[0283] The kanamycin resistance gene (SEQ ID NO: 42) was amplified from the plasmid pKD13 (SEQ ID NO: 43) by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 53 and SEQ ID NO: 54 to generate the PCR product identified as SEQ ID NO: 55. The *katE* nucleic acid sequence is provided as SEQ ID NO: 56 and the corresponding amino acid sequence is SEQ ID NO: 57. *E. coli* MG1655 (ATCC 47076™) was transformed with the temperature-sensitive plasmid pKD46 (SEQ ID NO: 49), which contains the λ-Red recombinase genes, and selected on LB-amp plates for 24 h at 30 °C. MG1655/pKD46 was transformed with 50-500 ng of the PCR product by electroporation (BioRad Gene Pulser, 0.2 cm cuvette, 2.5 kV, 200 W, 25 uF), and selected on LB-kan plates for 24 h at 37 °C. Several colonies were streaked onto LB-kan plates and incubated overnight at 42 °C to cure the pKD46 plasmid. Colonies were checked to confirm a phenotype of kanR/ampS. Genomic DNA was isolated from several colonies using the PUREGENE DNA purification system, and checked by PCR to confirm disruption of the *katE* gene using primers identified as SEQ ID NO: 58 and SEQ ID NO: 59. Several *katE*-disrupted strains were transformed with the temperature-sensitive plasmid pCP20 (SEQ ID NO: 52), which contains the FLP recombinase, used to excise the kan gene, and selected on LB-amp plates for 24 h at 37 °C. Several colonies were streaked onto LB plates and incubated overnight at 42 °C to cure the pCP20 plasmid. Two colonies were checked to confirm a phenotype of kanS/ampS, and called MG1655 KatE1 and MG1655 KatE2

EXAMPLE 15

Construction of a *katG* catalase and *katE* Catalase Disrupted *E. coli* strain (KLP18)

[0284] The kanamycin resistance gene (SEQ ID NO: 42) was amplified from the plasmid pKD13 (SEQ ID NO: 43) by PCR (0.5 min at 94 °C,

0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 53 and SEQ ID NO: 54 to generate the PCR product identified as SEQ ID NO: 55. *E. coli* MG1655 KatG1 (EXAMPLE 13) was transformed with the temperature-sensitive plasmid pKD46 (SEQ ID NO: 49), which contains the λ-Red recombinase genes, and selected on LB-amp plates for 24 h at 30 °C. MG1655 KatG1/pKD46 was transformed with 50-500 ng of the PCR product by electroporation (BioRad Gene Pulser, 0.2 cm cuvette, 2.5 kV, 200 W, 25 uF), and selected on LB-kan plates for 24 h at 37 °C. Several colonies were streaked onto LB-kan plates and incubated overnight at 42 °C to cure the pKD46 plasmid. Colonies were checked to confirm a phenotype of kanR/ampS. Genomic DNA was isolated from several colonies using the PUREGENE® DNA purification system, and checked by PCR to confirm disruption of the *katE* gene using primers identified as SEQ ID NO: 58 and SEQ ID NO: 59. Several *kat*E-disrupted strains (Δ *katE*) were transformed with the temperature-sensitive plasmid pCP20 (SEQ ID NO: 52), which contains the FLP recombinase, used to excise the kan gene, and selected on LB-amp plates for 24 h at 37 °C. Several colonies were streaked onto LB plates and incubated overnight at 42 °C to cure the pCP20 plasmid. Two colonies were checked to confirm a phenotype of kanS/ampS, and called MG1655 KatG1KatE18.1 and MG1655 KatG1KatE23. MG1655 KatG1KatE18.1 is designated *E. coli* KLP18.

EXAMPLE 16

Estimation of Perhydrolase Molecular Mass

[0285] Cell pellets obtained from shake flask growths of *E. coli* KLP18, a catalase double knockout of *E. coli* MG1655, expressing perhydrolase genes from *Bacillus subtilis*, *Bacillus licheniformis* and *Clostridium thermocellum*, were suspended in 2.2 mL of 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM). Each cell suspension was passed twice through a French press having a working pressure of 16,000 psi (~110.3 MPa). The crude extracts were centrifuged at 20,000 x *g* to remove cellular debris, producing clear crude extracts that were assayed for total soluble protein (Bicinchoninic Acid Kit [BCA] for Protein Determination, Sigma Aldrich, BCA1-KT).

[0286] Clarified crude extracts (5 µL) containing 20 µg total protein were mixed at room temperature with an equal volume of 2x non-denaturing (native) sample buffer (Invitrogen) and loaded into sample wells of a 1.5 mm x 10 well 4-12% Tris-Glycine polyacrylamide mini-gel (Invitrogen), and 7.5 µL of

NATIVEMARK™ Unstained Protein Standard (Invitrogen) was loaded into two separate wells. Native gel electrophoresis was performed at 125 V for 105 min using Tris-Glycine running buffer (Invitrogen). Following electrophoresis, the gel was prepared for an *in situ* esterase activity assay using the pH indicator bromothymol blue.

[0287] The gel was washed for 10 min x 2 with deionized water and slow mechanical mixing. The gel was then washed for 10 min using 10 mM pH 7.0 phosphate buffer and slow mechanical mixing. Following the removal of the phosphate buffer, 30 mL of 10 mM pH 7.0 phosphate buffer containing 400 µL of saturated bromothymol blue in water was incubated with the gel for 10 min followed by the addition of 1 mL of neat triacetin (Tessenderlo Fine Chemicals; Staffordshire, UK). Within 2 minutes of incubation yellow bands developed at the active perhydrolase enzyme sites. All *B. subtilis* species and *B. licheniformis* had intense bands around a molecular mass of 216 kDa. The *C. thermocellum* displayed an intense major primary band around 432 kDa and a minor secondary band around 576 kDa, indicating esterase activity. All bands were marked by punching a small hole in the gel adjacent to the bands. The gel was washed for 10 min x 2 with deionized water and slow mechanical mixing to remove the esterase activity stain. The gel was then washed for 10 min using 10 mM phosphate buffer with slow mechanical mixing to prepare for protein staining. Coomassie blue stain was added to cover the gel. After 5 minutes of slow mechanical mixing, the Coomassie blue was decanted and replaced with 40 mL de-stain (10% acetic acid, 30% methanol, 60% de-ionized water). After de-staining, the molecular masses of the active areas were estimated. The results are summarized in Table 7.

Table 7. Estimation of Perhydrolase Molecular Mass.

Transformant Strain	Perhydrolase source	Primary native gel activity stain, estimated molecular mass (kDa)	Secondary native gel activity stain, estimated molecular mass (kDa)	Calculated sub-unit molecular mass (kDa)
KLP18	none	none	none	-
KLP18/pSW186	*B. subtilis* ATCC 31954™	216	none detected	35.8
KLP18/pSW189	*B. subtilis*	216	none	35.9

KLP18/pSW190	BE1010 *B. subtilis* ATCC 29233™	216	detected none	35.8
KLP18/pSW191	*B. licheniformis* ATCC14580™	216	detected none	35.8
KLP18/pSW193	*C. thermocellum* ATCC 27405™	432	detected 648	36.0

EXAMPLE 17

Fermentation of *E. coli* UM2/pSW187 Expressing *B. subtilis* BE1010 Perhydrolase

[0288] A fermenter seed culture was prepared by charging a 2-L shake flask with 0.5 L seed medium containing LB Miller medium (DIFCO). The pH of the medium was adjusted to 6.8 and sterilized in the flask. Post-sterilization, 1 mL of ampicillin stock solution (25 mg/mL) was added. The seed medium was inoculated with a 1-mL culture of *E. coli* UM2/pSW187 in 20% glycerol, and cultivated at 36 °C and 300 rpm. The seed culture was transferred at ca. 1-2 OD_{550} to a 14L fermentor (Braun) with 8 L of medium at 35 °C containing KH_2PO_4 (3.50 g/L), $FeSO_4$ heptahydrate (0.05 g/L), $MgSO_4$ heptahydrate (2.0 g/L), sodium citrate dihydrate (1.90 g/L), yeast extract (Ambrex 695, 5.0 g/L), Biospumex153K antifoam (0.25 mL/L, Cognis Corporation), NaCl (1.0 g/L), $CaCl_2$ dihydrate (10 g/L), and NIT trace elements solution (10 mL/L). The trace elements solution contained citric acid monohydrate (10 g/L), $MnSO_4$ hydrate (2 g/L), NaCl (2 g/L), $FeSO_4$ heptahydrate (0.5 g/L), $ZnSO_4$ heptahydrate (0.2 g/L), $CuSO_4$ pentahydrate (0.02 g/L) and $NaMoO_4$ dihydrate (0.02 g/L). Post sterilization addition included 60 g fed batch solution (see below) and 16.0 mL ampicillin stock solution (25 mg/mL). A fed-batch solution contained 2.4 kg of 60% w/w glucose, 0.6L of 25 g/L yeast extract and 50 g/L Bacto peptone (DIFCO). Glucose feed was initiated when the glucose concentration decreased below 0.5 g/L, starting at 0.3 g/min, and increased progressively each hour to 0.35, 0.40, 0.47, and 0.53 g/min, respectively; the rate remained constant afterwards. Glucose concentration in the medium was monitored and if the concentration exceeded 0.1 g/L the addition rate was decreased or stopped temporarily. Induction was initiated at OD_{550} = 7 with the addition of 16 mL IPTG (0.5 M). The temperature was controlled at 36 °C, the aeration was fixed at 2 slpm (standard liters per minute) with agitation at 400 rpm. The pH was controlled at 6.8; NH_4OH (29% w/w) and H_2SO_4 (20% w/v) were used for pH control. The

head pressure was 0.5 bar. The cells were harvested by centrifugation at 8 h post IPTG addition.

EXAMPLE 18

Fermentation of *E. coli* UM2/pSW186 Expressing *B. subtilis* ATCC 31954™ Perhydrolase or *E. coli* UM2/pSW191 Expressing *B. licheniformis* ATCC 14580™ Perhydrolase

[0289] The seed culture was prepared as described in Example 17 using *E. coli* UM2/pSW186 expressing *B. subtilis* ATCC 31954™ perhydrolase or *E. coli* UM2/pSW191 expressing *B. licheniformis* ATCC 14580™ perhydrolase . The fermentation medium was LB Miller (25 g/L, DIFCO). Post sterilization additions included 50 g glucose solution (50% w/w) and 16.0 mL ampicillin stock solution (25 mg/mL). Glucose (50% w/w) was used for fed batch fermentation. Glucose feed was initiated when the glucose concentration decreased below 0.5 g/L, at a constant rate of 0.3 g/min. Glucose concentration in the medium was monitored and if the concentration exceeded 0.1 g/L the addition rate was decreased or stopped temporarily. Induction was initiated at OD_{550} = 2 with addition of 16 mL IPTG (0.5 M). The temperature was controlled at 36 °C, the aeration was fixed at 2 slpm with agitation at 400 rpm. The pH was controlled at 6.8; NH_4OH (29% w/w) and H_2SO_4 (20% w/v) were used for pH control. The head pressure was 0.5 bar. The cells were harvested by centrifugation at 8 h post IPTG addition.

EXAMPLE 19

Fermentation of *E. coli* KLP18/PSW189 Expressing *B. subtilis* BE1010 Perhydrolase or *E. coli* KLP18/PSW191 Expressing *B. licheniformis* ATCC 14580™ Perhydrolase

[0290] A fermentor seed culture was prepared by charging a 2-L shake flask with 0.5 L seed medium containing yeast extract (Amberx 695, 5.0 g/L), K_2HPO_4 (10.0 g/L), KH_2PO_4 (7.0 g/L), sodium citrate dihydrate (1.0 g/L), $(NH_4)_2SO_4$ (4.0 g/L), $MgSO_4$ heptahydrate (1.0 g/L) and ferric ammonium citrate (0.10 g/L). The pH of the medium was adjusted to 6.8 and the medium was sterilized in the flask. Post sterilization additions included glucose (50 wt %, 10.0 mL) and 1 mL ampicillin (25 mg/mL) stock solution. The seed medium was inoculated with a 1-mL culture of *E. coli* KLP18/PSW189 or KLP18/PSW191 in 20% glycerol, and cultivated at 35 °C and 300 rpm. The seed culture was transferred at ca. 1-2 OD_{550} to a 14L fermentor (Braun) with 8 L of medium at 35 °C containing KH_2PO_4 (3.50 g/L), $FeSO_4$ heptahydrate (0.05

g/L), $MgSO_4$ heptahydrate (2.0 g/L), sodium citrate dihydrate (1.90 g/L), yeast extract (Ambrex 695, 5.0 g/L), Biospumex153K antifoam (0.25 mL/L, Cognis Corporation), NaCl (1.0 g/L), $CaCl_2$ dihydrate (10 g/L), and NIT trace elements solution (10 mL/L). The trace elements solution contained citric acid monohydrate (10 g/L), $MnSO_4$ hydrate (2 g/L), NaCl (2 g/L), $FeSO_4$ heptahydrate (0.5 g/L), $ZnSO_4$ heptahydrate (0.2 g/L), $CuSO_4$ pentahydrate (0.02 g/L) and $NaMoO_4$ dihydrate (0.02 g/L). Post sterilization additions included glucose solution (50% w/w, 80.0 g) and ampicillin (25 mg/mL) stock solution (16.00 mL). Glucose solution (50% w/w) was used for fed batch. Glucose feed was initiated when glucose concentration decreased to 0.5 g/L, starting at 0.31 g feed/min and increasing progressively each hour to 0.36, 0.42, 0.49, 0.57, 0.66, 0.77, 0.90, 1.04, 1.21, 1.41 1.63 g/min respectively; the rate remained constant afterwards. Glucose concentration in the medium was monitored and if the concentration exceeded 0.1 g/L the feed rate was decreased or stopped temporarily. For *E. coli* KLP18/PSW191, the induction was initiated at OD_{550} = 80 with addition of 16 mL IPTG (0.5 M), for *E. coli* KLP18/PSW189 the growth was slower and induction was initiated at OD_{550} = 56. The dissolved oxygen (DO) concentration was controlled at 25% of air saturation. The DO was controlled first by impeller agitation rate (400 to 1400 rpm) and later by aeration rate (2 to 10 slpm). The pH was controlled at 6.8. NH_4OH (29% w/w) and H_2SO_4 (20% w/v) were used for pH control. The head pressure was 0.5 bars. The cells were harvested by centrifugation 16 h post IPTG addition.

EXAMPLE 20

E. coli KLP18 versus *E. coli* UM2 as Fermentation Host for Perhydrolase Production

[0291] *E. coli* KLP18 (EXAMPLE 15) was used to produce transformants (EXAMPLES 5, 8, 9 and 10) that were grown in multiple 10-L fermentations following the method described in EXAMPLE 19. The final OD for these runs is compared to fermentations that produced *E. coli* UM2 transformants (EXAMPLES 5, 8, 9 and 10) expressing these same perhydrolases that were run following the fermentation methods described in EXAMPLES 17 and 18. Table 8 summarizes 10-L fermentation runs with both UM2 and KLP18 as host, and demonstrates the superior performance of KLP18 compared to UM2.

Table 8.

run ID	Host	plasmid	perhydrolase	run time, (h)	final OD_{550}
PAA25	UM2	pSW186	SEQ ID NO: 2	21.6	11.9
PAA26	UM2	pSW186	SEQ ID NO: 2	7.4	11.9
PAA42	UM2	pSW186	SEQ ID NO: 2	12.4	5.5
PAA43	UM2	pSW186	SEQ ID NO: 2	12.4	5.5
PAA48	KLP18	pSW186	SEQ ID NO: 2	33.1	181.0
PAA30	UM2	pSW190	SEQ ID NO: 32	12.1	6.2
PAA31	UM2	pSW190	SEQ ID NO: 32	12.3	8.8
PAA40	UM2	pSW190	SEQ ID NO: 32	12.7	4.6
PAA41	UM2	pSW190	SEQ ID NO: 32	12.6	5.3
PAA49	KLP18	pSW190	SEQ ID NO: 32	33.6	128.0
PAA39	UM2	pSW191	SEQ ID NO: 10	10.6	6.5
PAA46	KLP18	pSW191	SEQ ID NO: 10	33.6	140.0
PAA50	KLP18	pSW191	SEQ ID NO: 10	36.2	155.0
PAA45	UM2	pSW193	SEQ ID NO: 14	12.4	5.7
PAA51	KLP18	pSW193	SEQ ID NO: 14	35.7	147.0

EXAMPLE 21

Evaluation of *Bacillus subtilis* ATCC 31954™ Perhydrolase Expressed in *E. coli* Transformants

[0292] The three transformants *E. coli* TOP10/pSW186, *E. coli* MG1655/pSW186 and *E. coli* UM2/pSW186 described in Example 5 were grown in unbaffled shake flasks containing Miller's LB broth (50 mL; Mediatech, Inc, Herndon, VA) with ampicillin (100 µg/mL) for 14-16 h at 35-37 °C with 200 rpm agitation. Following the overnight growth of the three transformants, each culture was sub-cultured by preparing a 1:100 dilution of each culture into fresh Miller's LB broth containing ampicillin (100 µg/mL). Following a 3 h growth at 35-37 °C with 200 rpm agitation, each culture was induced by the addition of IPTG to a final concentration of 1 mM. After an additional 3 h growth under the same conditions, the cell paste from each culture was harvested by centrifugation at 26,000 x *g* for 20 min at 5 °C. Cell extracts of each of the transformants were prepared according to the procedure described in Example 1, except that the extraction buffer used to

prepare the 25 wt% wet cell suspension was composed of 0.05 M potassium phosphate (pH 7.0) and 1 mM dithiothreitol.

[0293] Separate 1-mL reactions containing triacetin (250 mM), hydrogen peroxide (1.0 M) and 50 µg of extract total protein from one of the three cell extracts (prepared as described above) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. A second set of control reactions was run using 50 µg of extract total protein prepared from extracts of untransformed *E. coli* TOP10, *E. coli* MG1655 and *E. coli* UM2 to determine the background level of peracid produced by each strain in the absence of expressed perhydrolase. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2 (Table 9).

Table 9: Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence of cell extracts of *E. coli* TOP10/pSW186, *E. coli* MG1655/pSW186 and *E. coli* UM2/pSW186.

total protein extract source	total protein (µg/mL reaction)	peracetic acid (ppm) in 5 min	peracetic acid (ppm) in 30 min
no extract	0	188	598
TOP10	50	181	654
TOP10/pSW186	50	2684	5363
MG1655	50	173	638
MG1655/pSW186	50	1354	4333
UM2	50	175	655
UM2/pSW186	50	3002	6529

EXAMPLE 22

Perhydrolytic Activity of *E. coli* TOP10/pSW186 Extract Expressing *Bacillus subtilis* ATCC 31954™ Perhydrolase

[0294] Separate 1.0 mL triacetin perhydrolysis reactions were run as described in Example 21 using the *E. coli* TOP10/pSW186 transformant extract to provide one of the following total protein concentrations in the reaction: 196 µg/mL, 98 µg/mL, 49 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.0 µg/mL, or 1.5 µg/mL total protein concentration in each reaction (Table 10).

Table 10: Dependence of peracetic acid (PAA) concentration on total protein concentration derived from *E. coli* TOP10/pSW186 transformant extract in reactions containing triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5.

total protein extract source	total protein (µg/mL reaction)	peracetic acid (ppm) in 5 min	peracetic acid (ppm) in 30 min
no extract	0	193	854
TOP10	50	181	654
TOP10/pSW186	1.5	580	1710
TOP10/pSW186	3.0	824	2233
TOP10/pSW186	6.3	1371	3029
TOP10/pSW186	12.5	2052	4587
TOP10/pSW186	25	2849	4957
TOP10/pSW186	49	4294	
TOP10/pSW186	98	4244	
TOP10/pSW186	196	4294	

EXAMPLE 23

Perhydrolytic Activity of *E. coli* UM2/pSW186 Extract Expressing *Bacillus subtilis* ATCC 31954™ Perhydrolase

[0295] An extract of *E. coli* UM2/pSW186 transformant (20 mg total protein/mL extract, prepared as described in Example 21) was employed in 1.0 mL perhydrolysis reactions (run as described in Example 21) containing triacetin (40 mM or 100 mM), hydrogen peroxide (40 mM or 100 mM) and extract total protein (0.1 mg/mL or 1.0 mg/mL) in phosphate buffer (Pi, 100 mM, 200 mM or 300 mM) at pH 6.5 or 7.5 at 25 °C each reaction (Table 11).

Table 11: Dependence of peracetic acid (PAA) concentration on triacetin and hydrogen peroxide concentrations using perhydrolase derived from *E. coli* UM2/pSW186 transformant extract at pH 6.5 or 7.5.

total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	Pi (mM)	pH	PAA (ppm) in 5 min	PAA (ppm) in 30 min
0	40	40	100	6.5	0	0
0	40	100	100	6.5	0	0
0.1	40	40	100	6.5	49	0
1	40	40	100	6.5	239	160
1	40	100	100	6.5	439	560
0	40	100	200	6.5	0	0
0	100	100	200	6.5	1	30
0	100	100	200	7.5	14	1
0	100	100	300	7.5	5	4
1	100	40	200	6.5	75	9
1	100	100	200	6.5	1150	925
1	40	100	200	7.5	290	80
1	100	100	300	7.5	332	58

EXAMPLE 24

Evaluation of Perhydrolase Expressed in *E. coli* transformants derived from *Bacillus subtilis* BE1010

[0296] The *E. coli* TOP10/pSW187, *E. coli* MG1655/pSW187 and *E. coli* UM2/pSW187 transformants described in Example 6 were grown in unbaffled shake flasks containing Miller's LB broth (50 mL; Mediatech, Inc, Herndon, VA) with ampicillin (100 µg/mL) for 14-16 h at 35-37 °C with 200 rpm agitation. Following the overnight growth of the three transformants, each culture was sub-cultured by preparing a 1:100 dilution of each culture into fresh Miller's LB broth containing ampicillin (100 µg/mL). Following a 3 hour growth at 35-37 °C with 200 rpm agitation, each culture was induced by the addition of IPTG to a final concentration of 1 mM. After an additional 3 hours growth under the same conditions, the cell paste from each culture was harvested by centrifugation at 26,000 x *g* for 20 min at 5 °C. For cell extract preparation, the procedure described in Example 1 was repeated except that the extraction buffer used to prepare the 25 wt % wet cell suspension was composed of 0.05 M potassium phosphate (pH 7.0) and 1 mM dithiothreitol.

[0297] Separate 1.0 mL reactions containing triacetin (250 mM), hydrogen peroxide (1.0 M) and 50 µg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C with each transformant extract. A control reaction was run substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin with hydrogen peroxide. A second set of control reactions was run using 50 µg of extract total protein prepared from extracts of untransformed *E. coli* TOP10, *E. coli* MG1655 and *E. coli* UM2 to determine the background level of peracid produced by each strain in the absence of expressed perhydrolase. The concentration of peracetic acid in the reaction mixtures (Table 12) was determined according to the method of Karst *et al.* as described in Example 2.

Table 12: Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence of cell extracts of *E. coli* TOP10/pSW187, *E. coli* MG1655/pSW187 and *E. coli* UM2/pSW187.

total protein extract source	total protein (µg/mL reaction)	peracetic acid (ppm) in 5 min	peracetic acid (ppm) in 30 min
no extract	0	159	626
TOP10	50	181	654
TOP10/pSW187	50	3192	6663
MG1655	50	173	638
MG1655/pSW187	50	3472	7349
UM2	50	175	655
UM2/pSW187	50	3741	7626

EXAMPLE 25

Evaluation of Perhydrolases Expressed in *E. coli* Transformants

[0298] The transformants were prepared as described in Examples 5, 6, 7, 8, 9, 10, 18 and 19. Cell extracts of each of the transformants were prepared according to the procedure described in Example 1, except that the extraction buffer used to prepare the 25 wt% wet cell suspension was composed of 0.05 M potassium phosphate (pH 7.0) and 1 mM dithiothreitol.

[0299] Separate 1-mL reactions containing triacetin (250 mM), hydrogen peroxide (1.0 M) and 50 µg of extract total protein from a cell extract (prepared as described above) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH

6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. A second set of control reactions was run using 50 µg of extract total protein prepared from extracts of untransformed *E. coli* TOP10, *E. coli* MG1655, *E. coli* UM2 and *E. coli* KLP18 to determine the background level of peracid produced by each strain in the absence of expressed perhydrolase. The concentration of peracetic acid in the reaction mixtures (Table 13) was determined according to the method of Karst *et al.* described in Example 2.

Table 13: Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence of 50 µg of total extract protein/mL from transformant cell extracts of *E. coli* TOP10, *E. coli* MG1655, *E. coli* UM2, *E. coli* PIR2, and *E. coli* KLP18.

transformant cell extract	perhydrolase source	PAA (ppm) 5 min	PAA (ppm) 5 min, no extract	PAA (ppm) 30 min	PAA (ppm) 30 min, no extract
TOP10	none (control)	181	188	654	598
MG1655	none (control)	173	188	638	598
UM2	none (control)	175	188	655	598
PIR2	none (control)	144	276	515	677
KLP18	none (control)	200	100	555	330
TOP10/pSW186	*B. subtilis* ATCC 31954™	2684	188	5363	598
MG1655/pSW186	*B. subtilis* ATCC 31954™	1354	188	4333	598
UM2/pSW186	*B. subtilis* ATCC 31954™	3002	188	6529	598
KLP18/pSW186	*B. subtilis* ATCC 31954™	1033	268	2641	792
TOP10/pSW187	*B. subtilis* BE1010	3192	159	6663	626
MG1655/pSW187	*B. subtilis*	3472	159	7349	626

	BE1010				
UM2/pSW187	*B. subtilis* BE1010	3741	159	7626	626
KLP18/pSW189	*B. subtilis* BE1010	2631	146	6579	625
UM2/pSW188	*B. subtilis* ATCC 6633™	4617	289	8742	306
UM2/pSW190	*B. subtilis* ATCC 29233™	5314	320	8845	738
UM2/pSW190a	*B. subtilis* ATCC 29233™	2622	234	3553	642
KLP18/pSW190	*B. subtilis* ATCC 29233™	1006	146	3285	625
PIR2/pSW191	*B. licheniformis* ATCC 14580™	3125	276	6338	677
UM2/pSW191	*B. licheniformis* ATCC 14580™	1632	276	4640	677
KLP18/pSW191	*B. licheniformis* ATCC 14580™	3936	146	8016	625
MG1655/pSW193	*C. thermocellum* ATCC 27405™	2279	349	3178	645
UM2/pSW193	*C. thermocellum* ATCC 27405™	2738	349	3597	645
KLP18/pSW193	*C. thermocellum* ATCC 27405™	1687	146	2407	625
UM2/pSW195	*B. pumilus PS213*	2226	360	6354	776
KLP18/pSW195	*B. pumilus PS213*	5023	100	9642	394
UM2/pSW196	*T. neapolitana*	1347	360	2553	776
KLP18/pSW196	*T. neapolitana*	878	100	2023	394

EXAMPLE 26 (COMPARATIVE)

Evaluation of Commercial Lipases for Perhydrolysis

[0300] Separate 1-mL reactions containing triacetin (250 mM), hydrogen peroxide (1.0 M) and 50 µg of commercial lipases in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. Control reactions were run without commercial lipase to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added lipase. The concentration of peracetic acid in the reaction mixtures (Table 14) was determined according to the method of Karst *et al.* described in Example 2. The commercial lipases were obtained from Sigma/Aldrich Chemical Company (St. Louis, MO), BioCatalytics (Pasadena, CA), Meito Sangyo Co. (Nagoya, Japan), Amano Enzymes (Lombard, IL), Novozymes (Franklinton, NC), Valley Research (South Bend, IN), and Enzyme Development Corporation (ENZECO®; New York, NY).

Table 14: Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence of 50 µg/mL of commercial lipases.

commercial lipase	lipase source	PAA (ppm); 5 min	PAA (ppm); 30 min
no enzyme	control	105	105
Meito MY	*Candida rugosa*	155	280
Meito OF	*Candida rugosa*	120	340
Meito AL	*Achromobacter sp.*	165	315
Meito PL	*Alcaligenes sp.*	165	430
Meito SL	*Pseudomonas cepacia*	210	440
Meito TL	*Pseudomonas stutzeri*	225	500
Meito QLC	*Alcaligenes sp.*	195	240
Meito QLM	*Alcaligenes sp.*	225	555
no enzyme	control	150	205
Amano F-DS	*Rhizopus oryzae*	180	265
Amano R	*Penicillium roqueforti*	170	160
Amano M 10	*Mucor javanicus*	255	425
Amano G 50	*Penicillium cambertii*	40	40

Amano F-AP15	*Rhizopus oryzae*	120	50
Amano AY 30	*Candida rugosa*	140	300
Amano PS	*Burkholder cepacia*	150	150
Amano DS	*Aspergillus niger*	140	125
Amano AY	*Candida rugosa*	180	390
Amano AK-20	*Pseudomonas fluorescens*	215	500
Amano LPS	*Burkholder cepacia*	315	350
Amano A 12	*Aspergillus niger*	245	490
no enzyme	control	30	55
BioCatalytics ICR 110	*Candida antarctica B*	145	245
Novozymes Lipolase 100 L type EX	*Thermomyces lanuginosus*	10	0
Novozymes Lipozyme TL 100 L	*Thermomyces lanuginosus*	125	370
Novozymes Lipozyme CALB L	*Candida antarctica*	0	180
Novozymes Palatase 20000L	*Aspergillus oryzae*	95	220
Valley Research CR	*Candida rugosa*	70	320
Valley Research MJ	*Mucor javanicus*	140	440
Valley Research AN	*Aspergillus niger*	165	240
Enzeco LC	*Candida rugosa*	105	120
Enzeco MLC	*Aspergillus niger*	140	370
Enzeco R0 20	*Rhizopus oryzae*	55	100

EXAMPLE 27

Evaluation of Perhydrolases Expressed in *E. coli* transformants

[0301] Cell extracts of transformants expressing perhydrolase were prepared according to the procedure described in Example 21. Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM) and 1 mg or 2 mg of extract total protein from a cell extract (prepared as described above) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 15) was determined according to the method of Karst *et al.* described in
Example 2.

Table 15: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 6.5 or 7.5 in the presence of 1 mg or 2 mg of total extract protein/mL from transformant cell extracts of *E. coli* MG1655, *E. coli* UM2, *E. coli* PIR2 and *E. coli* KLP18.

transformant cell extract	source of perhydrolase	total protein (mg/mL)	pH	PAA (ppm); 5 min	PAA (ppm); 30 min
No extract	control	0	6.5	0	0
No extract	control	0	7.5	8	12
UM2/pSW186	*B. subtilis* ATCC 31954™	1.0	6.5	945	1420
UM2/pSW186	*B. subtilis* ATCC 31954™	2.0	6.5	1000	1250
UM2/pSW186	*B. subtilis* ATCC 31954™	1.0	7.5	1001	1215
UM2/pSW186	*B. subtilis* ATCC 31954™	2.0	7.5	1036	1050
No extract	control	0	6.5	0	0
No extract	control	0	7.5	45	0
MG1655/pSW187	*B. subtilis* BE1010	1.0	6.5	690	265
UM2/pSW187	*B. subtilis* BE1010	1.0	6.5	730	755
UM2/pSW187	*B. subtilis* BE1010	2.0	6.5	1400	1990
UM2/pSW187	*B. subtilis* BE1010	2.0	7.5	1710	2105
KLP18/pSW189	*B. subtilis* BE1010	1.0	6.5	885	1288
KLP18/pSW189	*B. subtilis* BE1010	2.0	6.5	950	1263
No extract	control	0	6.5	0	0
UM2/pSW190	*B. subtilis* ATCC 29233™	1.0	6.5	940	685

No extract	control	0	6.5	0	0
PIR2/pSW191	*B. lichen.* ATCC 14580™	1.0	6.5	860	1305
UM2/pSW191	*B. lichen.* ATCC 14580™	1.0	6.5	675	1530
No extract	control	0	6.5	0	0
UM2/pSW195	*B. pumilus* PS213	1.0	6.5	400	850
UM2/pSW195	*B. pumilus* PS213	2.0	6.5	460	790
No extract	control	0	6.5	0	0
UM2/pSW196	*T. neapolitana*	1.0	6.5	1100	1685
UM2/pSW196	*T. neapolitana*	2.0	6.5	1190	1900

EXAMPLE 28 (COMPARATIVE)

Evaluation of Commercial Lipases for Perhydrolysis

[0302] Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM) and 1 mg of commercial lipases in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run without commercial lipase to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added lipase. The concentration of peracetic acid in the reaction mixtures (Table 16) was determined according to the method of Karst *et al.* described in Example 2.

Table 16: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 6.5 in the presence of 1 mg/mL of commercial lipases.

commercial lipase	lipase source	PAA (ppm); 5 min	PAA (ppm); 30 min
no enzyme	control	15	20

Meito MY	*Candida rugosa*	25	45
Meito SL	*Pseudomonas cepacia*	0	0
Meito QLM	*Alcaligenes sp.*	35	85
Amano F-DS	*Rhizopus oryzae*	20	50
Amano M 10	*Mucor javanicus*	20	40
Amano A 12	*Aspergillus niger*	70	140
BioCatalytics ICR 110	*Candida antarctica B*	55	110

EXAMPLE 29

B. subtilis ATCC31954™ Perhydrolase Activity with Wetting Agents

[0303] A cell extract of *E. coli* UM2/pSW186 transformant expressing *B. subtilis* ATCC 31954™ perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM), wetting agent COLATERIC® MSC-NA (mixed short chain sodium dipropionate; Colonial Chemical Co.), SURFYNOL® 2502 (an ethoxylated/propoxylated acetylenic-based surfactant; Air Products and Chemicals; Utrecht, NL), SURFYNOL® MD-20, SILWET® L7650 (a polyalkyleneoxide modified polydimethylsiloxane; Chemtura Corp, Middlebury, CT) or SILWET® L8620; a siloxane-based surfactant), and 1 mg of extract total protein in 50 mM phosphate buffer (pH 7.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 17) was determined according to the method of Karst *et al.* described in Example 2.

Table 17: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 7.5 in the presence of 1 mg of total extract protein/mL from transformant cell extracts of *E. coli* UM2/pSW186 expressing *B. subtilis* ATCC 31954™ perhydrolase.

wetting agent	wetting agent conc. (ppm)	total protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
None	0	0	130	170
COLATERIC MSC-NA	1000	0	80	70
COLATERIC MSC-NA	1000	1.0	745	1520

SURFYNOL® 2502	1000	0	35	10
SURFYNOL® 2502	1000	1.0.	650	1210
SURFYNOL® MD-20	1000	0	110	150
SURFYNOL® MD-20	1000	1.0	555	1110
SILWET® L7650	1000	0	50	0
SILWET® L7650	1000	1.0	830	1360
SILWET® L8620	1000	0	60	135
SILWET® L8620	1000	1.0	735	1145

EXAMPLE 30

B. subtilis BE1010 Perhydrolase Activity with Wetting Agents

[0304] A cell extract of *E. coli* UM2/pSW187 transformant expressing *B. subtilis* BE1010 perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM), wetting agent (PLURONIC® 17R4 (a polyoxyalkylene ether surfactant; BASF, Mount Olive, NJ), PLURONIC® L43 (a difunctional block copolymer surfactant), or SILWET® L7650), and 1 mg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 18) was determined according to the method of Karst *et al.* described in Example 2.

Table 18: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 6.5 in the presence of 1 mg of total extract protein/mL from transformant cell extracts of *E. coli* UM2/pSW187 expressing *B. subtilis* BE1010 perhydrolase.

wetting agent	wetting agent conc. (ppm)	total protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
None	0	0	0	0
None	0	1.0	975	1345
PLURONIC® 17R4	2500	0	0	0
PLURONIC®17R4	2500	1.0	860	1360

PLURONIC® L43	2500	0	0	0
PLURONIC® L43	2500	1.0	855	1360
SILWET® L7650	2500	0	0	0
SILWET® L7650	2500	1.0	975	1205

EXAMPLE 31

Perhydrolase Activity with Wetting and Chelating Agents

[0305] A cell extract of *E. coli* UM2/pSW187 transformant expressing *B. subtilis* BE1010 perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM), wetting agent (SILWET® L7650), chelating agent (TURPINAL® SL; etidronic acid; Solutia Inc., St. Louis, MO), and 1 mg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 19) was determined according to the method of Karst *et al.* described in Example 2.

Table 19: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 6.5 in the presence of 1 mg of total extract protein/mL from transformant cell extracts of *E. coli* UM2/pSW187 expressing *B. subtilis* BE1010 perhydrolase.

SILWET® L7650 (ppm)	Turpinal® SL (ppm)	total protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
0	0	0	21	50
1000	0	0	20	26
0	500	0	10	45
1000	500	0	0	100
0	0	1.0	1600	2245
1000	0	1.0	1550	2136
0	500	1.0	1520	2130
1000	500	1.0	1505	2080

EXAMPLE 32

Perhydrolase Activity with Wetting Agent, Chelating Agent and Corrosion Inhibitor

[0306] A cell extract of *E. coli* UM2/pSW187 transformant expressing *B. subtilis* BE1010 perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing triacetin (105 mM), hydrogen peroxide (78 mM), wetting agent (SILWET® L7650), chelating agent (TURPINAL® SL), corrosion inhibitor (benzotriazole) and 1 mg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 20) was determined according to the method of Karst *et al.* described in Example 2.

Table 20: Peracetic acid (PAA) produced by reaction of triacetin (105 mM) and hydrogen peroxide (78 mM) at pH 6.5 in the presence of 1 mg of total extract protein/mL from transformant cell extracts of *E. coli* UM2/pSW187 expressing *B. subtilis* BE1010 perhydrolase.

SILWET® L7650 (ppm)	Turpinal® SL (ppm)	benzotriazole (ppm)	total protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
0	0	0	0	0	0
0	0	0	1.0	795	1205
1000	500	1000	0	0	20
1000	500	1000	1.0	825	960
1000	500	2500	0	0	24
1000	500	2500	1.0	795	960
1000	2000	2500	0	0	0
1000	2000	2500	1.0	270	450

EXAMPLE 33

Peracetic Acid Production using Immobilized *B. subtilis* ATCC 31954™ or BE1010 Perhydrolase

[0307] A suspension of 0.50 g of AMBERZYME® Oxirane enzyme immobilization polymeric support (Rohm and Haas, Philadelphia, PA) in 5.0 mL

of 0.225 M sodium phosphate buffer (pH 8.0) containing 10 mg/mL of total soluble protein from extracts (prepared as described in Example 21) of either *E. coli* KLP/pSW189 (expressing *B. subtilis* BE1010 perhydrolase) or *E. coli* UM2/pSW186 (expressing *B. subtilis* ATCC 31954™ perhydrolase) was mixed on a rotating platform at room temperature for 24 h. The supernatant was then decanted from the immobilized enzyme, which was washed with four 40-mL volumes of phosphate buffer (50 mM, pH 6.5) and stored at 5 °C in this same buffer. The immobilized enzyme was dried by vacuum filtration prior to use.

[0308] Separate 1-mL reactions containing triacetin (250 mM), hydrogen peroxide (1.0 M) and either 1.5 mg/mL or 5.0 mg/ml of immobilized perhydrolase (prepared as described above) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added immobilized enzyme. The concentration of peracetic acid in the reaction mixtures (Table 21) was determined according to the method of Karst *et al.* described in Example 2.

Table 21: Peracetic acid (PAA) produced by reaction of triacetin (250 mM) and hydrogen peroxide (1.0 M) at pH 6.5 in the presence of immobilized *B. subtilis* ATCC 31954™ or BE1010 perhydrolase.

immobilized perhydrolase	catalyst loading (mg immob. enzyme/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
no enzyme	0	83	240
B. subtilis ATCC 31954™	1.5	185	700
B. subtilis BE1010	1.5	502	1715
no enzyme	0	99	319
B. subtilis ATCC 31954™	5.0	596	972
B. subtilis BE1010	5.0	1669	2610

EXAMPLE 34

Perhydrolysis of a Mixture of Diacetin, Triacetin, and Monoacetin Using Perhydrolases from *B. subtilis*, *B. licheniformis* and *C. thermocellum*

[0309] Separate 1-mL reactions containing a mixture of diacetin (118 mM), triacetin (42 mM) and monoacetin (90 mM), hydrogen peroxide (1.0 M) and 50 µg of extract total protein from an *E. coli* UM2 cell extract (prepared as

described Example 21) that contained *B. subtilis* or *B. licheniformis* or *C. thermocellum* perhydrolase in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of a mixture of diacetin (118 mM), triacetin (42 mM) and monoacetin (90 mM) by hydrogen peroxide in the absence of added extract protein. A second control reaction was run using 50 µg of extract total protein prepared from an extract of untransformed *E. coli* UM2 to determine the background level of peracid produced by the *E. coli* strain in the absence of expressed perhydrolase. The concentration of peracetic acid in the reaction mixtures (Table 22) was determined according to the method of Karst *et al.* described in Example 2.

Table 22: Peracetic acid (PAA) produced by reaction of a mixture of diacetin (118 mM), triacetin (42 mM) and monoacetin (90 mM) with hydrogen peroxide (1.0 M) at pH 6.5 in the presence of 50 µg of total extract protein/mL from transformant cell extracts of *E. coli* UM2 expressing perhydrolase.

transformant cell extract	perhydrolase source	PAA (ppm); 5 min	PAA (ppm); 30 min
no extract	control	76	270
UM2	none (control)	110	276
UM2/pSW186	*B. subtilis* ATCC 31954™	2352	4341
UM2/pSW187	*B. subtilis* BE1010	2710	4713
UM2/pSW188	*B. subtilis* ATCC 6633™	2685	4234
UM2/pSW190	*B. subtilis* ATCC 29233™	641	1889
UM2/pSW191	*B. licheniformis* ATCC 14580™	1183	2608
UM2/pSW193	*C. thermocellum* ATCC 27405™	1498	1708

EXAMPLE 35

Perhydrolysis of a Mixture of Diacetin, Triacetin, and Monoacetin Using Perhydrolase from *B. subtilis* BE1010

[0310] Separate 1-mL reactions containing a mixture of diacetin (49.6 mM), triacetin (17.6 mM) and monoacetin (37.8 mM), hydrogen peroxide (78 mM) and 1 mg or 2 mg of extract total protein from an *E. coli* KLP18/pSW189 cell extract (prepared as described Example 21) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM

phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of a mixture of diacetin (49.6 mM), triacetin (17.6 mM) and monoacetin (37.8 mM) by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 23) was determined according to the method of Karst *et al.* described in Example 2.

Table 23: Peracetic acid (PAA) produced by reaction of a mixture of diacetin (49.6 mM), triacetin (17.6 mM) and monoacetin (37.8 mM) and hydrogen peroxide (78 mM) at pH 6.5 in the presence of 1 mg or 2 mg of total extract protein/mL from transformant cell extracts of *E. coli* KLP18/pSW189 expressing *B. subtilis* BE1010 perhydrolase.

transformant cell extract	source of perhydrolase	total protein (mg/mL)	pH	PAA (ppm); 5 min	PAA (ppm); 30 min
No extract	control	0	6.5	0	0
KLP18/pSW189	*B. subtilis* BE1010	1.0	6.5	475	423
KLP18/pSW189	*B. subtilis* BE1010	2.0	6.5	505	463

EXAMPLE 36

Perhydrolysis of Acetylated Sugars by *B. subtilis* ATCC 31954™Perhydrolase

[0311] A cell extract of *E. coli* UM2/pSW186 transformant expressing *B. subtilis* ATCC 31954™ perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing 0.1 M acetylated sugar (β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, or tri-O-acetyl-D-glucal (Aldrich)), hydrogen peroxide (100 or 500 mM), 2 mg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of 0.1 M acetylated sugar (β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, or tri-O-acetyl-D-glucal by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 24) was determined according to the method of Karst *et al.* described in Example 2.

Table 24: Peracetic acid (PAA) produced by reaction of acetylated sugar (100 mM) and hydrogen peroxide (100 or 500 mM) at pH 6.5 in the presence of 2 mg of total extract protein/mL from transformant cell extracts of *E. coli* UM2/pSW186 expressing *B. subtilis* ATCC 31954™ perhydrolase.

acetylated sugar	hydrogen peroxide (mM)	protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
β-D-ribofuranose-1,2,3,5-tetraacetate	500	0	550	705
β-D-ribofuranose-1,2,3,5-tetraacetate	500	2.0	1115	1540
tri-O-acetyl-D-galactal	500	0	220	225
tri-O-acetyl-D-galactal	500	2.0	885	815
tri-O-acetyl-D-glucal	500	0	20	25
tri-O-acetyl-D-glucal	500	2.0	420	275
β-D-ribofuranose-1,2,3,5-tetraacetate	100	0	52	37
β-D-ribofuranose-1,2,3,5-tetraacetate	100	2.0	289	354
tri-O-acetyl-D-galactal	100	0	5	95
tri-O-acetyl-D-galactal	100	2.0	185	175
tri-O-acetyl-D-glucal	100	0	65	0
tri-O-acetyl-D-glucal	100	2.0	102	60

EXAMPLE 37

Perhydrolysis of Acetylated Sugars by *B. subtilis* BE1010 Perhydrolase

[0312] A cell extract of *E. coli* KLP18/pSW189 transformant expressing *B. subtilis* BE1010 perhydrolase was prepared according to the procedure described in Example 21. Separate 1-mL reactions containing 0.1 M acetylated sugar (β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, or tri-O-acetyl-D-glucal (Aldrich)), hydrogen peroxide (100 or 500 mM), 2 mg of extract total protein in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of 0.1 M acetylated sugar (β-D-ribofuranose-1,2,3,5-tetraacetate, tri-O-acetyl-D-galactal, or tri-O-acetyl-

D-glucal by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures (Table 25) was determined according to the method of Karst *et al.* described in Example 2.

Table 25: Peracetic acid (PAA) produced by reaction of acetylated sugar (100 mM) and hydrogen peroxide (100 or 500 mM) at pH 6.5 in the presence of 2 mg of total extract protein/mL from transformant cell extracts of *E. coli* KLP18/pSW189 transformant expressing *B. subtilis* BE1010 perhydrolase.

acetylated sugar	hydrogen peroxide (mM)	total protein (mg/mL)	PAA (ppm); 5 min	PAA (ppm); 30 min
β-D-ribofuranose-1,2,3,5-tetraacetate	500	0	550	705
β-D-ribofuranose-1,2,3,5-tetraacetate	500	2.0	1465	1950
tri-O-acetyl-D-galactal	500	0	185	375
tri-O-acetyl-D-galactal	500	2.0	880	985
tri-O-acetyl-D-glucal	500	0	10	40
tri-O-acetyl-D-glucal	500	2.0	770	405
β-D-ribofuranose-1,2,3,5-tetraacetate	100	0	52	37
β-D-ribofuranose-1,2,3,5-tetraacetate	100	2.0	360	437
tri-O-acetyl-D-galactal	100	0	102	112
tri-O-acetyl-D-galactal	100	2.0	305	262
tri-O-acetyl-D-glucal	100	0	12	17
tri-O-acetyl-D-glucal	100	2.0	240	137

EXAMPLE 38

Cloning and Expression of Perhydrolase from *Bacillus clausii* KSM-K16

[0313] The gene encoding a cephalosporin-C hydrolase from *B. clausii* KSM-K16 as reported in GENBANK® (accession # YP_175265; SEQ ID NO: 26) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park CA). The gene (SEQ ID NOs: 65) was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 66 and SEQ ID NO: 67. The

resulting nucleic acid product (SEQ ID NO: 64) was cut with restriction enzymes *Pst*I and *Xba*I and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW200. The plasmid pSW200 was used to transform *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) to generate the strain identified as UM2/pSW200, UM2/pSW200 was gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4 - 0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein. Cell paste was frozen and stored at - 80 °C.

EXAMPLE 39

Cloning and Expression of Perhydrolase from *Thermoanaerobacterium saccharolyticum*

[0314] The gene encoding acetyl xylan esterase from *Thermoanaerobacterium saccharolyticum* as reported in GENBANK® Accession No. S41858 (SEQ ID NO: 70) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park CA). The gene (SEQ ID NO: 69) was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 71 and SEQ ID NO: 72. The resulting nucleic acid product (SEQ ID NO: 68) was cut with restriction enzymes *Pst*I and *Xba*I and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW201. The plasmid pSW201 was used to transform *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) to generate the strain identified as UM2/pSW201, UM2/pSW201 was gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4 - 0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein. Cell paste was frozen and stored at - 80 °C.

EXAMPLE 40

Cloning and Expression of Perhydrolase from *Thermotoga maritima* MSB8

[0315] The gene encoding acetyl xylan esterase from *T. maritima* MSB8 as reported in GENBANK® (accession # NP_227893.1; SEQ ID NO: 18) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park CA). The gene (SEQ ID NO: 74) was subsequently amplified

by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 71 and SEQ ID NO: 72. The resulting nucleic acid product (SEQ ID NO: 73) was cut with restriction enzymes *Pst*I and *Xba*I and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW202. The plasmid pSW202 was used to transform *E. coli* UM2 (*E. coli* Genetic Stock Center #7156, Yale University, New Haven CT) to generate the strain identified as UM2/pSW202, UM2/pSW202 was gown in LB media at 37 °C with shaking up to OD_{600nm} = 0.4 - 0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 20-40% of total soluble protein. Cell paste was frozen and stored at - 80 °C.

EXAMPLE 41

Peracetic Acid Production Using Perhydrolases from *B. clausii* KSM-K16, *Thermoanaerobacterium saccharolyticum,* and *T. maritima* MSB8

[0316] Cell extracts of transformants expressing perhydrolases from *B. clausii* KSM-K16 (*E. coli* UM2/pSW200, Example 38), *Thermoanaerobacterium saccharolyticum* (*E. coli* UM2/pSW201, Example 39), and *T. maritima* MSB8 (*E. coli* UM2/pSW202, Example 40) were each prepared by passing a suspension of cell paste (25 wt % wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) twice through a French press having a working pressure of 16,000 psi (~110.32 MPa). The crude extract was then centrifuged at 20,000 x *g* to remove cellular debris, producing a clarified cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT), then frozen and stored at - 80 °C.

[0317] Reactions (5 mL) containing triacetin, hydrogen peroxide and extract total protein (prepared as described above) in 50 mM phosphate buffer (pH 6.5) were run at 25 °C. In a first set of duplicate reactions, the reactants (and corresponding concentrations) were: triacetin (250 mM), hydrogen peroxide (1.0 M) and extract total protein (50 µg/mL). In a second set of duplicate reactions, the reactants (and corresponding concentrations) were: triacetin (100 mM), hydrogen peroxide (250 mM) and extract total protein (1.0 mg/mL). A control reaction was run by substituting 50 mM phosphate buffer (pH 6.5) for the extract total protein solution to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. All reactions additionally

contained 500 ppm of TURPINAL® SL (etidronic acid; Solutia Inc., St. Louis, MO), originally added to the aqueous hydrogen peroxide as stabilizer prior to use.

[0318] Determination of the concentration of peracetic acid in the reaction mixtures was performed according to the method described by Karst *et al.*, *supra*. The peracetic acid concentrations produced in 5 min and 30 min are listed in Table 26.

Table 26: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide and extract total protein prepared from transformants expressing perhydrolases from *B. clausii* KSM-K16 (*E. coli* UM2/pSW200), *Thermoanaerobacterium saccharolyticum* (*E. coli* UM2/pSW201), and *T. maritima* MSB8 (*E. coli* UM2/pSW202) at 25 °C and pH 6.5.

cell extract (UM2/)	perhydrolase source	total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	PAA in 5 min (ppm)	PAA in 30 min (ppm)
None	control	0.00	1000	250	60	462
pSW200	*B. clausii*	0.05	1000	250	85	520
pSW200	*B. clausii*	0.05	1000	250	110	534
pSW201	*T. saccharolyticum*	0.05	1000	250	70	615
pSW201	*T. saccharolyticum*	0.05	1000	250	95	595
pSW202	*T. maritima*	0.05	1000	250	1995	3270
pSW202	*T. maritima*	0.05	1000	250	1978	3315
None	control	0.0	250	100	0	0
pSW200	*B. clausii*	1.0	250	100	105	525
pSW200	*B. clausii*	1.0	250	100	120	585
pSW201	*T. saccharolyticum*	1.0	250	100	290	790
pSW201	*T. saccharolyticum*	1.0	250	100	195	855
pSW202	*T. maritima*	1.0	250	100	4770	6025
pSW202	*T. maritima*	1.0	250	100	4770	6005

EXAMPLE 42

Cloning and Expression of Perhydrolase from *Thermotoga lettingae*

[0319] The gene encoding acetyl xylan esterase (SEQ ID NO: 82) from *Thermotoga lettingae* as reported in GENBANK® (accession #CP000812) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The gene was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70, 30 cycles) using primers identified as SEQ ID NO: 75 and SEQ ID NO: 76. The resulting nucleic acid product (SEQ ID NO: 77) was subcloned into pTrcHis2-TOPO (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW219. The gene was also amplified by PCR (0.5 min at 94°C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 78 and SEQ ID NO: 79. The resulting nucleic acid product (SEQ ID NO: 80) was cut with restriction enzymes PstI and XbaI and subcloned into pUC19 using *Pst*I and *Xba*I sites to generate pSW220. The plasmids pSW219 and pSW220 were used to transform *E. coli* KLP18 (double catalase knockout) to generate the strains identified as KLP18/PSW219 and KLP18/pSW220, respectively. KLP18/PSW219 and KLP18/pSW220 were gown in LB media at 37 °C with shaking up to OD_{600nm} of 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2 - 3 hours. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 10 - 20 % of total soluble protein.

EXAMPLE 43

Cloning and Expression of Perhydrolase from *Thermotoga petrophila*

[0320] The gene encoding acetyl xylan esterase (SEQ ID NO: 90) from *Thermotoga petrophila* as reported in GENBANK® (accession #CP000702) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The gene was subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 83 and SEQ ID NO: 84. The resulting nucleic acid product (SEQ ID NO: 85) was subcloned into pTrcHis2-TOPO (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW221. The gene was also amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 C, 1 min at 70 °C, 30 cycles) using primers identified as SEQ ID NO: 86 and SEQ ID NO: 87. The

resulting nucleic acid product (SEQ ID NO: 88) was cut with restriction enzymes PstI and XbaI and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW222. The plasmids pSW221 and pSW222 were used to transform *E. coli* KLP18 (double catalase knockout) to generate the strains identified as KLP18/PSW221 and KLP18/pSW222, respectively. KLP18/PSW221 and KLP18/pSW222 were gown in LB media at 37 °C with shaking up to OD_{600nm} of 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and incubation continued for 2 - 3 hours. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 10 - 20 % of total soluble protein.

EXAMPLE 44

Peracetic Acid Production Using Perhydrolase from *Thermotoga lettingae*

[0321] A cell extract of a transformant expressing perhydrolase (SEQ ID NO: 82) from *Thermotoga lettingae* (KLP18/pSW220, Example 42) was prepared by passing a suspension of cell paste (20 wt % wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) twice through a French press having a working pressure of 16,000 psi (~110.32 MPa). The crude extract was then centrifuged at 20,000 x *g* to remove cellular debris, producing a clarified cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT). The clarified extract was heated for 20 min at 75 °C, followed immediately by cooling in an ice/water bath. The resulting mixture was centrifuged to remove precipitated protein, and the supernatant collected and assayed for total soluble protein as before. SDS-PAGE of the supernatant indicated that the perhydrolase was at least 85 –90 % pure. The supernatant was frozen in dry ice and stored at - 80 °C.

[0322] Reactions (10 mL total volume) containing triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant (prepared as described above) in 50 mM sodium phosphate buffer (pH 7.2) or 50 mM sodium bicarbonate buffer (pH 8.5) were run at 24 °C or 10 °C. A control reaction for each reaction condition was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin

by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2. The peracetic acid concentrations produced in 1 min, 5 min and 30 min are listed in Table 27.

Table 27: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant prepared from transformant expressing perhydrolase from *Thermotoga lettingae* (*E. coli* KLP18/pSW220).

buffer (50 mM)	temp. (°C)	total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	PAA, 1 min (ppm)	PAA, 5 min (ppm)	PAA, 30 min (ppm)
phosphate	24	0	250	250	54	60	160
phosphate	24	0.10	250	250	958	2195	2647
phosphate	24	0	250	100	24	58	56
phosphate	24	0.10	250	100	464	1232	1566
phosphate	24	0	100	100	134	155	244
phosphate	24	0.10	100	100	415	1209	1659
phosphate	24	0	100	50	127	135	179
phosphate	24	0.10	100	50	312	1068	1740
phosphate	24	0	50	100	0	6	61
phosphate	24	0.10	50	100	239	602	1161
phosphate	24	0	50	50	0	11	364
phosphate	24	0.10	50	50	252	539	1018
phosphate	24	0	250	100	49	104	125
phosphate	24	0.050	250	100	510	1255	2113
phosphate	24	0	500	250	24	149	319
phosphate	24	0.050	500	250	889	2300	2960

phosphate	10	0	100	100	0	50	69
phosphate	10	0.10	100	100	94	390	709
bicarbonate	24	0	100	100	111	219	679
bicarbonate	24	0.10	100	100	380	1073	1939

EXAMPLE 45

Peracetic Acid Production Using Perhydrolase from *Thermotoga petrophila*

[0323] A cell extract of a transformant expressing perhydrolase (SEQ ID NO. 90) from *Thermotoga petrophila* (KLP18/pSW221, Example 43) was prepared by passing a suspension of cell paste (20 wt % wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) twice through a French press having a working pressure of 16,000 psi (~110.32 MPa). The crude extract was then centrifuged at 20,000 x *g* to remove cellular debris, producing a clarified cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT). The clarified extract was heated for 20 min at 75 °C, followed immediately by cooling in an ice/water bath. The resulting mixture was centrifuged to remove precipitated protein, and the supernatant collected and assayed for total soluble protein as before. SDS-PAGE of the supernatant indicated that the perhydrolase was at least 85–90 % pure. The supernatant was frozen in dry ice and stored at - 80 °C.

[0324] Reactions (2 mL total volume) containing triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant (prepared as described above) in 50 mM sodium phosphate buffer (pH 7.2) or 50 mM sodium bicarbonate buffer (pH 8.5) were run at 24 °C or 10 °C. A control reaction for each reaction condition was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2. The peracetic acid concentrations produced in 1 min, 5 min and 30 min are listed in Table 28.

Table 28: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant prepared from transformant expressing perhydrolase from *Thermotoga petrophila* (*E. coli* KLP18/pSW221).

buffer (50 mM)	temp. (°C)	total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	PAA, 1 min (ppm)	PAA, 5 min (ppm)	PAA, 30 min (ppm)
phosphate	24	0	250	250	96	179	434
phosphate	24	0.30	250	250	2780	5957	6428
phosphate	24	0	250	100	0	21	413
phosphate	24	0.30	250	100	1894	3769	3762
phosphate	24	0	100	100	86	22	62
phosphate	24	0.30	100	100	1168	2642	2800
phosphate	24	0	100	50	0	7	4
phosphate	24	0.30	100	50	719	1599	1674
phosphate	24	0	50	100	40	0	0
phosphate	24	0.300	50	100	551	1383	1702
phosphate	24	0	50	50	0	0	67
phosphate	24	0.30	50	50	408	856	981
phosphate	24	0	500	250	64	67	538
phosphate	24	0.150	500	250	3147	5902	5791
phosphate	24	0	250	100	0	121	209
phosphate	24	0.150	250	100	1214	2919	3374
phosphate	10	0	100	100	2	2	22
phosphate	10	0.30	100	100	290	949	1829
bicarbonate	24	0	100	100	170	409	517

bicarbonate	24	0.30	100	100	1208	2266	2308

EXAMPLE 46

Cloning and Expression of a First Perhydrolase from *Thermotoga* sp. RQ2

[0325] The gene encoding a first acetyl xylan esterase (SEQ ID NO: 98) from *Thermotoga* sp. RQ2 as reported in GENBANK® (accession # CP000969) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The first perhydrolase is referred to herein as "RQ2(a)". The gene was subsequently amplified by PCR (0.5 min @ 94 °C, 0.5 min @ 55 °C, 1 min @ 70°C, 30 cycles) using primers identified as SEQ ID NO: 91 and SEQ ID NO: 92. The resulting nucleic acid product (SEQ ID NO: 93) was subcloned into pTrcHis2-TOPO (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW223. The gene was also amplified by PCR (0.5 min @ 94 °C, 0.5 min @ 55 °C, 1 min @ 70 °C, 30 cycles) using primers identified as SEQ ID NO: 94 and SEQ ID NO: 95. The resulting nucleic acid product (SEQ ID NO: 96) was cut with restriction enzymes PstI and XbaI and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW224. The plasmids pSW223 and pSW224 were used to transform *E. coli* KLP18 (double catalase knockout) to generate the strains identified as KLP18/pSW223 and KLP18/pSW224, respectively. KLP18/pSW223 and KLP18/pSW224 were gown in LB media at 37 °C with shaking up to OD_{600nm} of about 0.4-0.5, at which time IPTG was added to a final concentration of 1 mM, and incubation continued for 2 to 3 hours. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 10-20 % of total soluble protein.

EXAMPLE 47

Cloning and Expression of a Second Perhydrolase from *Thermotoga* sp. RQ2

[0326] A gene encoding a second acetyl xylan esterase (SEQ ID NO: 106) from *Thermotoga* sp. RQ2 as reported in GENBANK® (accession #CP000969) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The second perhydrolase is referred to herein as

"RQ2(b)". The gene was subsequently amplified by PCR (0.5 min @ 94 °C, 0.5 min @ 55 °C, 1 min @ 70 °C, 30 cycles) using primers identified as SEQ ID NO: 99 and SEQ ID NO: 100. The resulting nucleic acid product (SEQ ID NO: 101) was subcloned into pTrcHis2-TOPO (Invitrogen, Carlsbad CA) to generate the plasmid identified as pSW225. The gene was also amplified by PCR (0.5 min @ 94 °C, 0.5 min @ 55 °C, 1 min @ 70 °C, 30 cycles) using primers identified as SEQ ID NO: 102 and SEQ ID NO: 103. The resulting nucleic acid product (SEQ ID NO: 104) was cut with restriction enzymes PstI and XbaI and subcloned between the *Pst*I and *Xba*I sites in pUC19 to generate the plasmid identified as pSW226. The plasmids pSW225 and pSW226 were used to transform *E. coli* KLP18 (double catalase knockout) to generate the strains identified as KLP18/pSW225 and KLP18/pSW226, respectively. KLP18/pSW225 and KLP18/pSW226 were gown in LB media at 37 °C with shaking up to OD_{600nm} of about 0.4-0.5, at which time IPTG was added to a final concentration of 1 mM, and incubation continued for 2 to 3 hours. Cells were harvested by centrifugation and SDS-PAGE was performed to confirm expression of the perhydrolase at 10-20 % of total soluble protein.

EXAMPLE 48

Peracetic Acid Production Using Perhydrolase Activity from a First Perhydrolase from *Thermotoga* sp. RQ2

[0327] A cell extract of a transformant expressing perhydrolase from a first acetyl xylan esterase (SEQ ID NO: 98) from *Thermotoga* sp. RQ2 referred to herein as "RQ2(a)" (KLP18/pSW223, Example 46) was prepared by passing a suspension of cell paste (20 wt % wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) twice through a French press having a working pressure of 16,000 psi (~110.32 MPa). The crude extract was then centrifuged at 20,000 x *g* to remove cellular debris, producing a clarified cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT). The clarified extract was heated for 20 min at 75 °C, followed immediately by cooling in an ice/water bath. The resulting mixture was centrifuged to remove precipitated protein, and the supernatant collected and

assayed for total soluble protein as before. SDS-PAGE of the supernatant indicated that the perhydrolase was at least 85 to 90 % pure. The supernatant was frozen in dry ice and stored at - 80 °C.

[0328] Reactions containing triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant (prepared as described above) in 50 mM sodium bicarbonate buffer (10 mL total volume, pH 8.1) or in 50 mM sodium phosphate buffer (2 mL total volume, pH 7.2) were run at 25 °C. A control reaction for each reaction condition was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2. The peracetic acid concentrations produced in 1 min, 5 min and 30 min are listed in Table 29.

Table 29: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant prepared from transformant expressing the RQ2(a) perhydrolase from *Thermotoga* sp. RQ2 (*E. coli* KLP18/pSW223).

buffer (50 mM)	total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	PAA, 1 min (ppm)	PAA, 5 min (ppm)	PAA, 30 min (ppm)
bicarbonate	0	100	100	6	69	339
bicarbonate	0.10	100	100	242	877	1396
bicarbonate	0	100	50	32	40	251
bicarbonate	0.10	100	50	197	573	880
bicarbonate	0	50	100	43	109	214
bicarbonate	0.10	50	100	188	593	975
bicarbonate	0	250	100	56	260	491
bicarbonate	0.10	250	100	558	1568	2250

phosphate	0	1000	250	62	295	-
phosphate	0.015	1000	250	327	1422	-

EXAMPLE 49

Peracetic Acid Production Using Perhydrolase Activity from a Second Perhydrolase from *Thermotoga* sp. RQ2

[0329] A cell extract of a transformant expressing perhydrolase from a second acetyl xylan esterase (SEQ ID NO: 106) from *Thermotoga* sp. RQ2 referred to herein as "RQ2(b)" (KLP18/pSW226, Example 47) was prepared by passing a suspension of cell paste (20 wt % wet cell weight) in 0.05 M potassium phosphate buffer (pH 7.0) containing dithiothreitol (1 mM) twice through a French press having a working pressure of 16,000 psi (~110.32 MPa). The crude extract was then centrifuged at 20,000 x *g* to remove cellular debris, producing a clarified cell extract that was assayed for total soluble protein (Bicinchoninic Acid Kit for Protein Determination, Sigma Aldrich, Sigma catalog # BCA1-KT). The clarified extract was heated for 20 min at 75 °C, followed immediately by cooling in an ice/water bath. The resulting mixture was centrifuged to remove precipitated protein, and the supernatant collected and assayed for total soluble protein as before. SDS-PAGE of the supernatant indicated that the perhydrolase was at least 85 to 90 % pure. The supernatant was frozen in dry ice and stored at - 80 °C.

[0330] Reactions containing triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant (prepared as described above) in 50 mM sodium bicarbonate buffer (10 mL total volume, pH 8.1) or in 50 mM sodium phosphate buffer (2 mL total volume, pH 7.2) were run at 25 °C. A control reaction for each reaction condition was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2. The peracetic acid concentrations produced in 1 min, 5 min and 30 min are listed in Table 30.

Table 30: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide and total protein from a heat-treated, centrifuged cell extract supernatant prepared from transformant expressing the RQ2(b) perhydrolase from *Thermotoga* sp. RQ2 (*E. coli* KLP18/pSW226).

buffer (50 mM)	total protein (mg/mL)	H_2O_2 (mM)	triacetin (mM)	PAA, 1 min (ppm)	PAA, 5 min (ppm)	PAA, 30 min (ppm)
bicarbonate	0	100	100	6	69	339
bicarbonate	0.050	100	100	314	919	1659
bicarbonate	0	100	50	32	40	251
bicarbonate	0.050	100	50	230	691	1043
bicarbonate	0	50	100	43	109	214
bicarbonate	0.050	50	100	241	654	1126
bicarbonate	0	250	100	56	260	491
bicarbonate	0.050	250	100	563	1452	2077
phosphate	0	1000	250	79	311	-
phosphate	0.010	1000	250	686	2020	-

EXAMPLE 50

Peracetic Acid Production Using Sodium Percarbonate and Perhydrolase from *Thermotoga lettingae* or *Thermotoga petrophila*

[0331] The procedures described in Example 44 and Example 45 were repeated using total protein from a heat-treated, centrifuged cell extract supernatant from a transformant expressing perhydrolase (SEQ ID NO. 82) from *Thermotoga lettingae* (KLP18/pSW220, Example 42) or a transformant expressing perhydrolase (SEQ ID NO. 90) from *Thermotoga petrophila* (KLP18/pSW222, Example 43), except that sodium percarbonate (~25 wt % H_2O_2) was substituted for aqueous hydrogen peroxide to produce an initial concentration of either 100 mM or 250 mM hydrogen peroxide. Reactions

containing triacetin, sodium percarbonate and heat-treated, centrifuged cell extract supernatant in 50 mM sodium bicarbonate buffer (2 mL total volume, pH 8.1) were run at 24 °C. A control reaction for each reaction condition was run to determine the concentration of peracetic acid produced by chemical perhydrolysis of triacetin by hydrogen peroxide in the absence of added extract protein. The concentration of peracetic acid in the reaction mixtures was determined according to the method of Karst *et al.* described in Example 2. The peracetic acid concentrations produced in 1 min, 5 min and 30 min are listed in Table 31.

Table 31: Dependence of peracetic acid (PAA) concentration on concentrations of triacetin, hydrogen peroxide (from sodium percarbonate) and total protein from heat-treated, centrifuged cell extract supernatant prepared from transformants expressing perhydrolase from *Thermotoga lettingae* (KLP18/pSW220) or *Thermotoga petrophila* (KLP18/pSW222).

perhydrolase	total protein (mg/mL)	H_2O_2, from percarbonate (mM)	triacetin (mM)	PAA, 1 min (ppm)	PAA, 5 min (ppm)	PAA, 30 min (ppm)
none	0	100	100	41	41	172
T. lettingae	0.050	100	100	23	244	854
T. petrophila	0.15	100	100	312	1314	2230
none	0	250	100	275	440	1913
T. lettingae	0.050	250	100	546	1280	3593
T. petrophila	0.15	250	100	1419	2678	4556

What is claimed is:

1. A process for producing a peroxycarboxylic acid comprising:

a) providing a set of reaction components comprising:

1) at least one substrate selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula $R_6C(O)O$

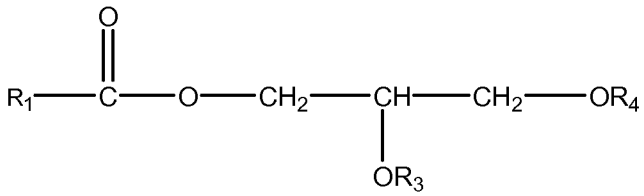
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

2) a source of peroxygen; and

3) an enzyme catalyst having perhydrolysis activity, wherein said enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence; and

b) combining said reaction components under suitable aqueous reaction conditions whereby a peroxycarboxylic acid is produced.

2. The process of claim 1 wherein the substantially similar enzyme having perhydrolase activity is at least 95% identical to one or more amino acid sequences selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

3. The process of claim 1 wherein the substrate is selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; and mixtures thereof.

4. The process of claim 1 wherein said enzyme catalyst is encoded by a nucleic acid molecule that hybridizes under stringent conditions to SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

5. The process of claim 1 wherein the peroxycarboxylic acid is produced at a concentration of at least 20 ppm within about 5 minutes to about 2 hours of combining the reaction components.

6. The process of claim 1 wherein the peroxycarboxylic acid produced is peracetic acid, perpropionic acid, perbutyric acid, perlactic acid, perglycolic acid, permethoxyacetic acid, per-β-hydroxybutyric acid, or mixtures thereof.

7. The process of claim 6 wherein the peroxycarboxylic acid produced is peracetic acid.

8. The process of claim 1 wherein the enzyme catalyst is in the form of a microbial cell, a permeabilized microbial cell, a microbial cell extract, a partially purified enzyme, or a purified enzyme.

9. The process of claim 1 wherein the enzyme catalyst lacks catalase activity.

10. A process to disinfect a hard surface or inanimate object using an enzymatically produced peroxycarboxylic acid composition, said process comprising:

providing a set of reaction components comprising:

1) at least one substrate selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula $R_6C(O)O$

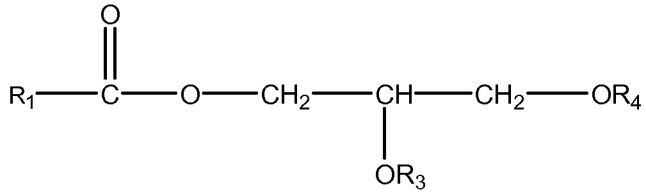
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

2) a source of peroxygen; and

3) an enzyme catalyst having perhydrolysis activity, wherein said enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO:82, SEQ ID NO:90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence; and

combining said reaction components under suitable aqueous reaction conditions whereby a peroxycarboxylic acid is produced; and

contacting said hard surface or inanimate object with the peroxycarboxylic acid whereby said surface or said inanimate object is disinfected.

11. The process of claim 10 further comprising diluting said peroxycarboxylic acid produced by said combining of said reaction components.

12. The process of claim 10 wherein said enzyme catalyst is encoded by a nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule having a sequence selected from the group consisting of SEQ ID NO: 77 SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

13. The process of claim 10 wherein the substantially similar enzyme having perhydrolase activity is at least 95% identical to one or more amino acid

sequences selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

14. The process of claim 10 wherein the peroxycarboxylic acid is produced at a concentration of at least 20 ppm within about 5 minutes to about 2 hours of combining the reaction components.

15. The process of claim 10 wherein the substrate is monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; or a mixture thereof.

16. The process of claim 10 wherein the peroxycarboxylic acid produced is peracetic acid, perpropionic acid, perbutyric acid, perlactic acid, perglycolic acid, permethoxyacetic acid, per-β-hydroxybutyric acid, or a mixture thereof.

17. The process of claim 10 wherein the peroxycarboxylic acid produced is peracetic acid.

18. The process of claim 10 wherein the hard surface or the inanimate object is contacted with the peroxycarboxylic acid within about 5 minutes to about 168 hours of combining said reaction components.

19. The process of claim 10 wherein the hard surface or the inanimate object is contacted with the peroxycarboxylic acid within about 5 minutes to about 48 hours of combining said reaction components.

20. The process of claim 10 wherein the hard surface or the inanimate object is contacted with the peroxycarboxylic acid within about 5 minutes to about 2 hours of combining said reaction components.

21. The process according to claim 10 wherein a concentration of viable biological contaminants on the hard surface or the inanimate object is reduced at least 3-log.

22. The process according to claim 10 wherein a concentration of viable biological contaminants on the hard surface or the inanimate object is reduced at least 5-log.

23. The process of claim 10 wherein the enzyme catalyst is in the form of a microbial cell, a permeabilized microbial cell, a microbial cell extract, a partially purified enzyme, or a purified enzyme.

24. The process of claim 10 wherein the enzyme catalyst lacks catalase activity.

25. A process to disinfect a hard surface or inanimate object using an enzymatically produced peroxycarboxylic acid composition, said process comprising combining on said hard surface or inanimate object under suitable aqueous reaction conditions a set of reaction components comprising:

1) at least one substrate selected from the group consisting of:

i) esters having the structure

$$[X]_mR_5$$

wherein X = an ester group of the formula $R_6C(O)O$

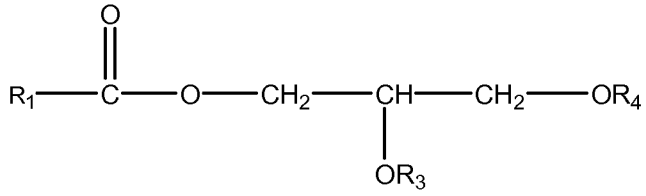
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group; wherein R_5 optionally comprises one or more ether linkages;

m=1 to the number of carbon atoms in R_5; and

wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



wherein R_1= C1 to C7 straight chain or branched chain alkyl optionally substituted with an hydroxyl or a C1 to C4 alkoxy group and R_3 and R_4 are individually H or $R_1C(O)$; and

iii) acetylated saccharides selected from the group consisting of acetylated monosaccharides, acetylated disaccharides, and acetylated polysaccharides;

2) a source of peroxygen; and

3) an enzyme catalyst having perhydrolysis activity, wherein said enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO:82, SEQ ID NO:90, SEQ ID NO: 98, and SEQ ID NO: 106 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence,

thereby generating peroxycarboxylic acid, whereby said hard surface or inanimate object is disinfected.

26. The method of claim 25 wherein said peroxycarboxylic acid is diluted.

27. The process of claim 25 wherein said enzyme catalyst is encoded by a nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule having a sequence selected from the group consisting of SEQ ID NO: 77 SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 104, and SEQ ID NO: 105.

28. The process of claim 25 wherein the substantially similar enzyme having perhydrolase activity is at least 95% identical to one or more amino acid sequences selected from the group consisting of SEQ ID NO: 82, SEQ ID NO: 90, SEQ ID NO: 98, and SEQ ID NO: 106.

29. The process of claim 25 wherein the peroxycarboxylic acid is produced at a concentration of at least 20 ppm within about 5 minutes to about 2 hours of combining the reaction components.

30. The process of claim 25 wherein the substrate is monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-glucal; monoesters or diesters of 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 2,3-butanediol, 1,4-butanediol, 1,2-pentanediol, 2,5-pentanediol, 1,6-pentanediol, 1,2-hexanediol, 2,5-hexanediol, 1,6-hexanediol; or a mixture thereof.

31. The process of claim 25 wherein the peroxycarboxylic acid produced is peracetic acid, perpropionic acid, perbutyric acid, perlactic acid, perglycolic acid, permethoxyacetic acid, per-β-hydroxybutyric acid, or a mixture thereof.

32. The process of claim 25 wherein the peroxycarboxylic acid produced is peracetic acid.

33. The process according to claim 25 wherein a concentration of viable biological contaminants on the hard surface or the inanimate object is reduced at least 3-log.

34. The process according to claim 25 wherein a concentration of viable biological contaminants on the hard surface or the inanimate object is reduced at least 5-log.

35. The process of claim 25 wherein the enzyme catalyst is in the form of a microbial cell, a permeabilized microbial cell, a microbial cell extract, a partially purified enzyme, or a purified enzyme.

36. The process of claim 25 wherein the enzyme catalyst lacks catalase activity.

37. A disinfectant or bleaching composition comprising the peroxycarboxylic acid produced by the process of claim 1.

```
SEQ ID NO.2      -MQLFDLPLDQLQTYKPEKTAPKDFSEFWKLSLEELAKVQAEPDLQPVDYPADGVKVYRL
SEQ ID NO.6      -MQLFDLPLDQLQTYKPEKTAPKDFSEFWKLSLEELAKVQAEPDLQPVDYPADGVKVYRL
SEQ ID NO.8      -MQLFDLPLDQLQTYKPEKTTPNDFSEFWKSSLDELAKVKAAPDLQLVDYPADGVKVYRL
SEQ ID NO.10     MQQPYDMPLEQLYQYKPERTAPADFKEFWKGSLEELANEKAGPQLEPHEYPADGVKVYWL
SEQ ID NO.12     -MQLFDLSLEELKKYKPKKTARPDFSDFWKKSLEELRQVEAEPTLESYDYPVKGVKVYRL
SEQ ID NO.14     MAQLYDMPLEELKKYKPALTKQKDFDEFWEKSLKELAEIPLKYQLIPYDFPARRVKVFRV
SEQ ID NO.16     -MAFFDMPLEELKKYRPERYEEKDFDEFWRETLKESEGFPLDPVFEKVDFHLKTVETYDV
SEQ ID NO.18     -MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESHLKTVEAYDV
SEQ ID NO.26     -MPLVDMPLRELLAYEGINPKPADFDQYWNRAKTEIEAIDPEVTLVESSFQCSFANCYHF
SEQ ID NO.32     -MQLFDLPLDQLQTYKPEKTAPKDFSEFWKLSLEELAKVQAEPDLQPVDYPADGVKVYRL
SEQ ID NO.70     -MGLFDMPLQKLREYTGTNPCPEDFDEYWDRALDEMRSVDPKIKMKKSSFQVPFAECYDL
SEQ ID NO.82     -MVYFDMPLEDLRKYLPQRYEEKDFDDFWKQTIHETRGYFQEPILKKVDFYLQNVETFDV
SEQ ID NO.90     -MAFFDLPLEELKKYRPERYEEKDFDEFWEGTLAENEKFPLDPVFERMESHLKTVEAYDV
SEQ ID NO.98     -MAFFDLPLEELKKYRPERYEEKDFDEFWKETLAESEKFPLDPVFERMESHLKTVEVYDV
SEQ ID NO.106    -MALFDMPLEKLRSYLPDRYEEEDFDLFWKETLEESRKFPLDPIFERVDYLLENVEVYDV
                      *:.* .*  *        **. :*  :  *         :   .    .: : .

SEQ ID NO.2      TYKSFGNARITGWYAVPDKQ--GPHPAIVKYHGYNASYDGEIHEMVNWALHGYAAFGMLV
SEQ ID NO.6      TYKSFGNARITGWYAVPDKE--GPHPAIVKYHGYNASYDGEIHEMVNWALHGYATFGMLV
SEQ ID NO.8      TYKSFGNARITGWYAVPDKE--GPHPAIVKYHGYNASYDGEIHEMVNWALHGYAAFGMLV
SEQ ID NO.10     TYRSIGGARIKGWYAVPDRQ--GPHPAIVKYHGYNASYDGDIHDIVNWALHGYAAFGMLV
SEQ ID NO.12     TYQSFGHSKIEGFYAVPDQT--GPHPALVRFHGYNASYDGGIHDIVNWALHGYATFGMLV
SEQ ID NO.14     EYLGFKGANIEGWLAVPEGE--GLYPGLVQFHGYNWAMDGCVPDVVNWALNGYAAFLMLV
SEQ ID NO.16     TFSGYRGQRIKGWLLVPKLA-EEKLPCVVQYIGYNGGR-GFPHDWLFWPSMGYICFVMDT
SEQ ID NO.18     TFSGYRGQRIKGWLLVPKLE-EEKLPCVVQYIGYNGGR-GFPHDWLFWPSMGYICFVMDT
SEQ ID NO.26     YYRSAGNAKIHAKYVQPKAG--EKTPAVFMFHGYGGRS-AEWSSLLNYVAAGFSVFYMDV
SEQ ID NO.32     TYKSFGNARITGWYAVPDKQ--GPHPAIVKYHGYNASYDGEIHEMVNWALHGYAAFGMLV
SEQ ID NO.70     YFTGVRGARIHAKYIRPKTE--GKHPALIRFHGYSSNS-GDWNDKLNYVAAGFTVVAMDA
SEQ ID NO.82     TFSGYRGQKIKGWLILPKFR-NGKLPCVVEFVGYGGGR-GFPYDWLLWSAAGYAHFIMDT
SEQ ID NO.90     TFSGYMGQRIKGWLLVPKLE-EEKLPCVVQYIGYNGGR-GFPHDWLFWPSMGYICFVMDT
SEQ ID NO.98     TFSGYRGQRIKGWLLVPKLE-EEKLPCVVQYIGYNGGR-GFPHDWLFWPSMGYICFVMDT
SEQ ID NO.106    TFSGYRGQRIKAWLILPVVKKEERLPCIVEFIGYRGGR-GFPFDWLFWSSAGYAHFVMDT
                  : .   .* .   *        * :. : **     .   . : :   *:  . * .
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FIGURE 1A

```
SEQ ID NO.2      RGQQS-SEDTSISLH------GHALGWMTKGILD-KDTYYYRGVYLDAVRALEVISSFDE
SEQ ID NO.6      RGQQS-SEDTSISPH------GHALGWMTKGILD-KDTYYYRGVYLDAVRALEVISSFDE
SEQ ID NO.8      RGQQS-SEDTSISPH------GHALGWMTKGILD-KDTYYYRGVYLDAVRALEVISSFDE
SEQ ID NO.10     RGQNS-SEDTEISHH------GHVPGWMTKGILD-PKTYYYRGVYLDAVRAVEVVSGFAE
SEQ ID NO.12     RGQGG-SEDTSVTPG------GHALGWMTKGILS-KDTYYYRGVYLDAVRALEVIQSFPE
SEQ ID NO.14     RGQQGRSVDNIVPGS------GHALGWMSKGILS-PEEYYYRGVYMDAVRAVEILASLPC
SEQ ID NO.16     RGQGSGWMKGDTPDYPEGPVDPQYPGFMTRGILD-PGTYYYRRVFVDAVRAVEAAISFPR
SEQ ID NO.18     RGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGILD-PRTYYYRRVFTDAVRAVEAAASFPQ
SEQ ID NO.26     RGQGGTSEDPGGVRG------NTYRGHIIRGLDAGPDALFYRSVFLDTVQLVRAAKTLPH
SEQ ID NO.32     RGQQS-SEDTSISPH------GHALGWMTKGILD-KDTYYYRGVYLDAVRALEVISSFDE
SEQ ID NO.70     RGQGGQSQDVGGVNG------NTLNGHIIRGLDDDADNMLFRHIFLDTAQLAGIVMNMPE
SEQ ID NO.82     RGQGSNWMKGDTPDYEDNPSDPQYPGFLTKGVLN-PETYYYRRVFMDAFMAVETISQLEQ
SEQ ID NO.90     RGQGSGWMKGDTPDYPEDPVDPQYPGFMTRGILD-PRTYYYRRVFTDAVRAVEAAASFPR
SEQ ID NO.98     RGQGSGWLKGDTPDYPEDPVDPQYPGFMTRGILD-PRTYYYRRVFTDAVRAVEAAASFPR
SEQ ID NO.106    RGQGTSRVKGDTPDYCDEPINPQFPGFMTRGILD-PRTYYYRRVFTDAVRAVETASSFPG
                 ***      .                * : :*:        :* :: *:         :

SEQ ID NO.2      VDETRIGVTGGSQGGGLTIAAAALSDIPKAAVADYPYLSNFERAIDVALEQ-PYLEINSF
SEQ ID NO.6      VDETRIGVTGGSQGGGLTIAAAALSDIPKAAVADYPYLSNFERAIDVALEQ-PYLEINSF
SEQ ID NO.8      VDETRIGVTGGSQGGGLTIAAAALSDIPKAAVADYPYLSNFERAIDVALEQ-PYLEINSF
SEQ ID NO.10     VDEKRIGVIGASQGGGLAVAVSALSDIPKAAVSEYPYLSNFQRAIDTAIDQ-PYLEINSF
SEQ ID NO.12     VDENRIGVIGGSQGGALAIAAAALSDIPKVVVADYPYLSNFERAVDVALEQ-PYLEINSY
SEQ ID NO.14     VDESRIGVTGGSQGGGLALAVAALSGIPKVAAVHYPFLAHFERAIDVAFDG-PYLEINEY
SEQ ID NO.16     VDSRKVVVAGGSQGGGIALAVSALSNRVKALLCDVPFLCHFRRAVQLVDTH-PYVEITNF
SEQ ID NO.18     VDQERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLCHFRRAVQLVDTH-PYAEITNF
SEQ ID NO.26     IDKTRLMATGWSQGGALTLACAALVPEIKRLAPVYPFLSDYKRVWQMDLAVRSYKELADY
SEQ ID NO.32     VDETRIGVTGGSQGGGLTIAAAALSDIPKAAVADYPYLSNFERAIDVALEQ-PYLEINSF
SEQ ID NO.70     IDEDRVAVMGPSQGGGLSLACAALEPKIRKVVSEYPFLSDYKRVWDLDLAKNAYQEITDY
SEQ ID NO.82     IDSQTIILSGASQGGGIALAVSALSSKVMALLCDVPFLCHYKRAVQITDSM-PYAEITRY
SEQ ID NO.90     VDHERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLCHFRRAVQLVDTH-PYAEITNF
SEQ ID NO.98     VDHERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLCHFRRAVQLVDTH-PYAEITNF
SEQ ID NO.106    IDPERIAVVGTSQGGGIALAVAALSEIPKALVSNVPFLCHFRRAVQITDNA-PYSEIVNY
                 :*    :   * ****.:::* :**           *:*..:.*. :      .* *:  :
```

FIGURE 1B

```
SEQ ID NO.2      FRRNGS-PETEVQAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETE
SEQ ID NO.6      FRRNGS-PETEVQAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETK
SEQ ID NO.8      FRRNGS-PETEEKAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETE
SEQ ID NO.10     FRRNTS-PDIEQAAMHTLSYFDVMNLAQLVKAPVLMSIGLVDTITPPSTVFAAYNHLETD
SEQ ID NO.12     FRRNSD-PKVEEKAFETLSYFDLINLAGWVKQPTLMAIGLIDKITPPSTVFAAYNHLETD
SEQ ID NO.14     LRRNSG-EEIERQVKKTLSYFDIMNLAPRIKCRTWICTGLVDEITPPSTVFAVYNHLKCP
SEQ ID NO.16     LKTHR---DKEEIVFRTLSYFDGVNFAARAKVPALFSVGLMDTICPPSTVFAAYNHYAGP
SEQ ID NO.18     LKTHR---DKEEIVFRTLSYFDGVNFAARAKIPALFSVGLMDNICPPSTVFAAYNYYAGP
SEQ ID NO.26     FRSYDPQHKRHGEIFERLGYIDVQRLADRIQGDVLMGVGLMDTECPPSTQFAAYNKIKAK
SEQ ID NO.32     FRRNGS-PETEVQAMKTLSYFDIMNLADRVKVPVLMSIGLIDKVTPPSTVFAAYNHLETE
SEQ ID NO.70     FRLFDPRHERENEVFTKLGYIDVKNLAKRIKGDVLMCVGLMDQVCPPSTVFAAYNNIQSK
SEQ ID NO.82     CKTHI---DKIQTVFRTLSYFDGVNFAARAKCPALFSVGLMDDICPPSTVFAAYNYYAGE
SEQ ID NO.90     LKTHR---DKEEIVFRTLSYFDGVNFAVRAKIPALFSVGLMDNICPPSTVFAAYNHYAGP
SEQ ID NO.98     LKTHR---DKEEIVFRTLSYFDGVNFAVRAKIPALFSVGLMDNICPPSTVFAAYNHYAGP
SEQ ID NO.106    LKVHR---DKEEIVFRTLSYFDGVNFAARAKIPALFSVALMDKTCPPSTVFAAYNHYAGP
                  :       .         *.*:*  ::*   :  . :  .*:*   **** **.**

SEQ ID NO.2      KELKVYRYFGHEYIPAFQT-EKLAFFKQHLKG-----
SEQ ID NO.6      KELKVYRYFGHEYIPAFQT-EKLAFFKQHLKG-----
SEQ ID NO.8      KELKVYRYFGHEYIPAFQT-EKLAFFKQHLKG-----
SEQ ID NO.10     KEIKVYRYFGHEYIPFFQT-EKLAFLRKHLK------
SEQ ID NO.12     KDLKVYRYFGHEFIPAFQT-EKLSFLQKHLLLST---
SEQ ID NO.14     KEISVFRYFGHEHMPGSVE-IKLRILMDELNP-----
SEQ ID NO.16     KEIRIYPYNNHEGGGSFQAIEQVKFLKRLFEEG-----
SEQ ID NO.18     KEIRIYPYNNHEGGGSFQAVEQVKFLKKLFEKG----
SEQ ID NO.26     KSYELYPDFGHEHLPGMND-HIFRFFTS---------
SEQ ID NO.32     KELKVYRYFGHEYIPAFQT-EKLAFFKQHLKG-----
SEQ ID NO.70     KDIKVYPDYGHEPMRGFGD-LAMQFMLELYS------
SEQ ID NO.82     KDIRIYPYNNHEGGGSFHTLEKLKFVKKTISMRE---
SEQ ID NO.90     KEIRIYPYNNHEGGGSFQAIEQVKFLKRLFEKG----
SEQ ID NO.98     KEIRIYPYNNHEGGGSFQAIEQVKFLKRLFEKG----
SEQ ID NO.106    KEIKVYPFNEHEGGESFQRMEELRFMKRILKGEFKA
                 *.  ::    **          . :.
```

FIGURE 1C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/067712

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/40 C12P7/62 C12N9/18

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2008/073139 A (DU PONT [US]; DICOSIMO ROBERT [US]; GAVAGAN JOHN E [US]; PAYNE MARK S) 19 June 2008 (2008-06-19) the whole document	1-37
X	WO 2007/070609 A (DU PONT [US]; DICOSIMO ROBERT [US]; GAVAGAN JOHN E [US]; PAYNE MARK [U) 21 June 2007 (2007-06-21) cited in the application page 4, line 4 - page 35, line 7; claims 1-25; table 1	1-37
X	US 5 296 161 A (WIERSEMA RICHARD J [US] ET AL) 22 March 1994 (1994-03-22)	37
A	the whole document	1-36
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 2008	16/01/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	De Kok, Ad

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VINCENT F ET AL: "Multifunctional Xylooligosaccharide/Cephalosporin C Deacetylase Revealed by the Hexameric Structure of the Bacillus subtilis Enzyme at 1.9 Angst. Resolution" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 330, no. 3, 11 July 2003 (2003-07-11), pages 593-606, XP004434207 ISSN: 0022-2836 page 594 - page 597	1-37
A	DATABASE UniProt [Online] 13 November 2007 (2007-11-13), COPELAND A ET AL.: "Thermotoga lettingae acetyl xylan esterase" XP002501372 retrieved from EBI accession no. A8F440_THELT Database accession no. A8F440 cited in the application abstract	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2008/067712

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-37 partly

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-37 partly

A process for producing a peroxycarboxylic acid comprising combining a substrate, a source of peroxygen and an enzyme catalyst, wherein said enzyme catalyst comprises an amino acid sequence consisting of SEQ ID NO: 82 or a derivative thereof having perhydrolase activity; the use of said process to disinfect a hard surface or inanimate object and a disinfectant or bleaching composition obtained by said process.

---

2. claims: 1-37 partly

A process for producing a peroxycarboxylic acid comprising combining a substrate, a source of peroxygen and an enzyme catalyst, wherein said enzyme catalyst comprises an amino acid sequence consisting of SEQ ID NO: 90 or a derivative thereof having perhydrolase activity; the use of said process to disinfect a hard surface or inanimate object and a disinfectant or bleaching composition obtained by said process.

---

3. claims: 1-37 partly

A process for producing a peroxycarboxylic acid comprising combining a substrate, a source of peroxygen and an enzyme catalyst, wherein said enzyme catalyst comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 98 and SEQ ID NO: 106 or a derivative thereof having perhydrolase activity; the use of said process to disinfect a hard surface or inanimate object and a disinfectant or bleaching composition obtained by said process.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/067712

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008073139 A	19-06-2008	NONE	
WO 2007070609 A	21-06-2007	EP 1960529 A2	27-08-2008
US 5296161 A	22-03-1994	NONE	