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(54) Title: ENZYMATIC IN SITU PREPARATION OF PERACID-BASED REMOVABLE ANTIMICROBIAL COATING COMPOSITIONS AND METHODS OF USE

(57) Abstract: *In-situ* enzymatic preparation of peracid-based removable antimicrobial coating compositions and their methods of use for controlling microorganisms at various loci are disclosed.

TITLE

**ENZYMATIC *IN SITU* PREPARATION OF PERACID-BASED  
REMOVABLE ANTIMICROBIAL COATING COMPOSITIONS AND  
METHODS OF USE**

5 This application claims the benefit of the four United States  
Provisional Applications 61/228732, 61/228735, 61/228737, and  
61/228746 all filed on July 27, 2009.

FIELD OF INVENTION

10 This invention relates to a method for controlling microorganisms  
comprising coating a surface with a removable, antimicrobial film-forming  
composition that contains *in situ* enzymatically generated peroxyacids and  
methods of applying said compositions.

BACKGROUND

15 Chemical disinfectants are now used in an increasing number of  
industries to ensure food safety and to comply with more stringent health  
and safety regulations. Compositions for decontamination, disinfection  
and sterilization must have excellent microbiocidal efficacy, act rapidly, be  
non-corrosive and be effective in minimal quantities. The ideal  
composition must possess multiple mechanisms for destroying  
20 microorganisms, thus providing efficacy against a broad range of  
microorganisms and reducing the possibility of leading to evolution of  
antimicrobial-resistant microorganisms.

25 Compositions containing peroxide, especially hydrogen peroxide  
(HP) and peracetic acid (also called peroxyacetic acid, PAA) have been  
proven to be very effective antimicrobial agents. Many such preparations  
have passed the necessary tests and are registered products as  
sanitizers, disinfectants, and sporicides. Many of these  
peroxide/peroxyacid compositions are liquid solutions, which can be used  
for treating aqueous solutions, surfaces and objects. Some are approved  
30 for food contact surfaces and for sanitization of some food products.

Some are also registered as disinfectants or sterilants as vapor phase treatments.

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).

There are several methods of preparing peracids. For example WO2006/076334 describes a microbicidal and decontaminant composition comprising an aqueous solution of peroxides and peracids; U.S. patent 5,130,124 describes an aqueous antimicrobial film-forming composition with hydrogen peroxide; WO1996/022687 describes an oxidizing film-forming composition containing hydrogen peroxide. Enzyme catalysts may also be used to facilitate the generation of peracids. Commonly owned co-pending U.S. Patent Application 2008/0176299A1, which is herein incorporated by reference on its entirety, describes a process for enzymatic production of peracids. Use of enzymes such as esterases (U.S. Patent 3,974,082), proteases (U.S. Patent 5,364,554), esterases and lipases (U.S. Patent 5,296,161) for enzymatic generation of peracids have also been described.

Despite the descriptions of peracid manufacture set forth above, a need exists for an *in situ* enzymatically-generated, peracid-based, easily removable, homogenous antimicrobial coating composition providing both short-term and extended long-term (or residual) antimicrobial efficacy after application to a surface.

#### SUMMARY OF INVENTION

The present invention addresses the problems identified above by providing a method and composition that is antimicrobial and which also

provides extended effectiveness against microorganisms by forming an antimicrobial coating on a target surface.

In one aspect the present invention is directed to method of providing control of microorganisms at a locus comprising the steps:

- 5            a) forming a composition by combining components comprising:
  - i) a film-forming water soluble or water-dispersible agent;
  - ii) an inert solvent;
  - 10            iii) a carboxylic acid ester substrate;
  - iv) a peroxygen source;
  - v) an enzyme catalyst having perhydrolase activity thereby forming at least one peroxyacid to provide at least a first antimicrobial agent; and
  - 15            vi) a rheology modifier;
- b) applying said composition to said locus;
- c) allowing said composition to dry thereby forming a coating on said locus.

Another aspect of the invention is directed to a method of providing 20 control of microorganisms at a locus comprising the steps:

- (a) providing a first premixed component comprising an inert solvent and a film-forming agent;
- (b) providing a second premixed component comprising an enzyme catalyst having perhydrolase activity, a carboxylic acid ester substrate and a peroxygen source;
- 25            (c) mixing said first premixed component and said second premixed component to obtain a liquid coating composition comprising a first antimicrobial agent comprising a peroxyacid;
- (d) applying said coating composition to the locus; and
- (e) allowing said coating composition to dry thereby forming a coating on said locus.

Another aspect of the invention is directed to an antimicrobial composition comprising components which comprise:

- a) a film-forming water soluble or water-dispersible agent;
- b) an inert solvent;
- 5 c) a carboxylic acid ester substrate;
- d) a peroxygen source;
- e) an enzyme catalyst having perhydrolase activity; and
- f) a rheology modifier

wherein, upon combining said components, a peroxyacid is formed.

10 **BRIEF DESCRIPTION OF BIOLOGICAL SEQUENCES**

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 15 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.

20 SEQ ID NO: 1 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus subtilis* ATCC31954.

SEQ ID NO: 2 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus subtilis* subsp. *subtilis* strain 168 (GenBank Accession # NP\_388200).

25 SEQ ID NO: 3 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus subtilis* ATCC6633 (GenBank Accession # YP\_077621.1).

SEQ ID NO: 4 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus licheniformis* ATCC14580 (GenBank Accession 30 # YP\_077621.1).

SEQ ID NO: 5 is the amino acid sequence of the acetyl xylan esterase from *Bacillus pumilis* (GenBank Accession # CAB76451.2).

SEQ ID NO: 6 is the amino acid sequence of the acetyl xylan esterase from *Clostridium thermocellum* ATCC27405 (GenBank Accession # ZP\_00504991).

SEQ ID NO: 7 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga neapolitana* GenBank Accession # AAB70869.1).

SEQ ID NO: 8 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga maritima* MSB8 (GenBank Accession # NP\_227893.1).

SEQ ID NO: 9 is the amino acid sequence of the acetyl xylan esterase from *Thermoanaerobacterium* sp. (GenBank Accession # AAB68821.1).

SEQ ID NO: 10 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus* sp. NRRL B14911 (GenBank Accession # ZP\_01168674).

SEQ ID NO: 11 is the amino acid sequence of the acetyl xylan esterase from *Bacillus halodurans* C-125 (GenBank Accession # NP\_244192).

SEQ ID NO: 12 is the amino acid sequence of the cephalosporin C deacetylase from *Bacillus clausii* KSM-K16.

SEQ ID NO: 13 is the amino acid sequence of the acetyl xylan esterase from *Thermoanaerobacterium saccharolyticum*.

SEQ ID NO: 14 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga lettingae*.

SEQ ID NO: 15 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga petrophilia*.

SEQ ID NO: 16 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga* sp. RQ2"a".

SEQ ID NO: 17 is the amino acid sequence of the acetyl xylan esterase from *Thermotoga* sp. RQ2"b".

SEQ ID NO: 18 is the nucleotide sequence for primer f27405 used in Example 1.

SEQ ID NO: 19 is the nucleotide sequence for primer r27405 used in Example 1.

SEQ ID NO: 20 is the nucleic acid sequence of the nucleic acid product amplified by SEQ ID NO: 18 and 19 that was used to prepare 5 plasmid pSW196 used in Example 1.

SEQ ID NO: 21 is the contiguous amino acid sequence of the 4 conserved motifs found in CE-7 carbohydrate esterases.

SEQ ID NO: 22 is the nucleotide sequences for the forward primer [TAACTGCAGT AAGGAGGAAT AGGACATGGC CTTCTTCGAT 10 TTACCCACTC] used in Example 8.

SEQ ID NO: 23 is the nucleotide sequence for the reverse primer [TGATCTAGAT TAGCCTTTCT CAAATAGTTT TTTCAAGA] used in Example 8.

SEQ ID NO: 24 is the nucleic acid sequence of the nucleic acid 15 product amplified by SEQ ID NO: 22 and 23 that was used to prepare plasmid pSW207.

SEQ ID NO: 25 is the nucleotide sequences for the forward primer [TAAGAATTCT AAGGAATAGG ACATGGCGTT TCTTCGACCT GCCTCTG] used in Example 8.

20 SEQ ID NO: 26 is the nucleotide sequences for the reverse primer [AAACTGCAGT TAGCCCTTCT CAAACAGTTT CTTCAG] used in Example 8.

SEQ ID NO: 27 the nucleic acid sequence of the nucleic acid 25 product amplified by SEQ ID NO: 25 and 26 that was used to prepare plasmid pSW228 used in Example 8.

SEQ ID NO: 28 is the nucleotide sequence for forward primer Tma\_C277Sf [GGACAAACATCTCACCTCCTTCTA] used in Example 9.

SEQ ID NO: 29 is the forward reverse primer Tma\_C227Sr [TAGAAGGAGGTGAGATGTTGTCC] used in Example 9.

30 SEQ ID NO: 30 is the codon optimized sequence of a *T. maritima* acetyl xylan esterase in plasmid pSW228 used in Example 9.

## DETAILED DESCRIPTION OF THE INVENTION

Unless stated otherwise, all percentages, parts, ratios, etc., are by weight. Trademarks are shown in upper case. Further, when an amount, concentration, or other value is disclosed as either a range, preferred 5 range or a list of preferred upper and lower values, such disclosure is to have the same effect as if each individual value within the specified range – and any range obtained from a combination of any two individual values within the disclosed range – has been specifically disclosed, even if the individual values are not uniquely or individually disclosed herein. Where 10 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. Unless specified, it is not intended that the scope of the invention be limited to the specific values recited when defining a range.

15 For clarity, terms used herein are to be understood as described herein or as such term would be understood by one of ordinary skill in the art of the invention. Additional explanation of certain terms used herein, are provided below:

“Peracid” is a carboxylic acid in which an acidic -OH group has 20 been replaced by an -OOH group. As used herein, “peracid” is synonymous with peroxyacid, peroxy acid, percarboxylic acid and peroxyoic acid. As is commonly known, peracid includes peracetic acid. The term “peracetic acid” or “PAA”, as used herein, is synonymous with peroxyacetic acid, ethaneperoxoic acid and all other synonyms of CAS 25 Registry Number 79-21-0.

“Triacetin” is synonymous with glycerin triacetate; glycerol triacetate; glyceryl triacetate, 1,2,3-triacetoxyp propane, 1,2,3-propanetriol triacetate and all other synonyms of CAS Registry Number 102-76-1.

“Peroxygen source” refers to any peroxide compound or compound 30 containing hydrogen peroxide that may be released in solution.

“Shear rate” refers to the velocity gradient in a flowing material and is measured in SI units of reciprocal seconds ( $s^{-1}$ ).

“Shear-thinning properties” or “pseudoplastic properties” refers to a fluid that exhibits a decrease in viscosity with an increase in shear rate.

“Readily removable” refers to easily removing the coatings formed after application of the liquid coating composition to the surface of interest,

5 but not so easily removed as to be removed by inadvertent physical contact.

“Non-volatile” refers to a compound whose vapor pressure at 25 °C is below 1000 Pascals.

“Inert solvent or aqueous solvent” refers to water or any other

10 solvent that facilitates application of the coating composition to the locus. An aqueous solvent may also be employed to rinse coated surfaces to remove the coating as needed.

“Film-forming agent” or “water soluble or water dispersible coating agent”, which may be used interchangeably herein, refer to agents that

15 form a film and are employed to provide protective coating to the surface of interest. These agents are either water soluble or water dispersible. These agents are described in further detail below.

“Metal chelator” or “sequestrant” refers to agents that bind metals or metal-containing impurities and prevent their decomposition catalysis of

20 hydrogen peroxide or peroxyacids.

“Rheology modifier” or “rheology agent” refers to compounds that increase viscosity and/or provide shear-thinning properties to a composition and cause the aqueous treatment or coating composition to cling to the surface of interest.

25 “wt%” refers to the weight percent relative to the total weight of the solution or dispersion.

“Liquid coating composition” refers to the composition comprising at least a water soluble film-forming agent, an inert solvent, a carboxylic acid ester substrate, a peroxygen source, and an enzyme catalyst having

30 perhydrolase activity, wherein at least one peroxyacid is produced.

“Antimicrobial” or “antimicrobial properties” as used herein refers to the ability of an agent of killing microorganisms, blocking or preventing

microbial contamination (such as forming a barrier), or suppressing or preventing growth of microorganisms, trapping microorganisms for killing, preventing biofilm formation or eliminating/removing biofilm.

5 "Antimicrobial agent" as used herein refers to a compound or substance having antimicrobial properties.

"Microorganism" is meant to include any organism comprised of the phylogenetic domains of bacteria and archaea, as well as unicellular (e.g., yeasts) and filamentous (e.g., molds) fungi, unicellular and filamentous algae, unicellular and multicellular parasites, viruses, viroses and viroids.

10 "Biocide", as used herein, 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.

15 "Biofilm" refers to a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface.

"Drying" refers to a process by which the inert solvent or any other liquid present in the formulation is removed by evaporation.

20 "Disinfectant" means an agent which provides antimicrobial activity. According to an official disinfectant test, a disinfectant is a chemical that kills 99.9% of the specific test microorganisms in 10 minutes under the conditions of the test. (Germicidal and Detergent Sanitizing Action of Disinfectants, Official Methods of Analysis of the Association of Official Analytical Chemists, paragraph 960.09 and applicable sections, 15th

25 Edition, 1990 (EPA Guideline 91-2)).

"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 micromole of peracid product per minute at a specified temperature.

30 "Half-time" or " $\tau$ " is defined as the time at which the PAA concentration has decayed to half its maximum value.

"Enzyme catalyst" and "perhydrolase catalyst" are used interchangeably and refer to a catalyst comprising an enzyme having

perhydrolysis activity, that is, an enzyme that exhibits the ability to catalyze the reaction between a peroxide source and a substrate to form a peracid 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, 5 partially purified enzyme, or purified enzyme.

“Acetyl xylan esterase” refers to an enzyme (E.C. 3.1.1.72; AXEs) that catalyzes the deacetylation of acetylated xylans and other acetylated saccharides and also has significant perhydrolytic activity suitable for producing percarboxylic acids from carboxylic acid esters.

10 “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.

15 “Isolated nucleic acid molecule” and “isolated nucleic acid fragment” may be used interchangeably and refer 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.

20 “Expression” refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid molecule of the invention. Expression may also refer to translation of mRNA into a polypeptide.

25 “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.

30 “ATCC” refers to the American Type Culture Collection. A repository of microorganisms located in Virginia, USA.

“First antimicrobial agent” refers to the antimicrobial agent generated *in situ* in coating compositions according to the methods of the invention. In a preferred embodiment, the first antimicrobial agent is a peroxyacid.

5 “Second antimicrobial agent” refers to an additional antimicrobial agent other than a peroxyacid that is present in the coating composition. According to the current method, this agent is not generated *in situ*. In a preferred embodiment, the second antimicrobial agent is a quaternary ammonium compound.

10 “RGQ” refers to a highly conserved motif of amino acid residues at positions 118-120 of SEQ ID NO: 1 that is diagnostic for the CE-7 enzyme family.

15 “GXSQG” refers to a highly conserved motif of amino acid residues at positions 179-183 of SEQ ID NO: 1 that is diagnostic for the CE-7 enzyme family.

“HE” refers to a highly conserved motif of amino acid residues at positions 298-299 of SEQ ID NO:1 that is diagnostic for the CE-7 enzyme family.

20 “LXD” refers to a highly conserved motif of amino acid residues at positions 267-269 of SEQ ID NO: 1 that is diagnostic for the CE-7 enzyme family.

25 “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.

“Signature motif”, “CE-7 signature motif”, and “diagnostic motif” refer to conserved structures shared among a family of enzymes having a defined activity such as perhydrolase activity.

30 In one aspect, the present invention is a method comprising the use of perhydrolases in combination with a peroxygen source and a carboxylic acid ester to form a peroxyacid. Perhydrolases are enzymes that catalyze

the reaction of a peroxygen source, (e.g., hydrogen peroxide) with a selected substrate to form a peracid. As indicated above, enzymes possessing perhydrolase activity include, but are not limited to lipases, esterases, proteases, non-heme haloperoxidases, and carbohydrate esterases of the CE-7 family, such as acetyl xylan esterases or cephalosporin C deacetylases.

5 Application of these enzymes for their perhydrolytic activity has been described in commonly owned U.S. Patent Application 2009/0005590A1, U.S. Patent Application 2008/0176299A1 and U.S. 10 Patent Application 2007/042924A1, and U.S. Patent Application 61/102,520, which are herein incorporated by reference in their entirety.

15 Certain enzymes belonging to the structural family of CE-7 esterases, such as cephalosporin C deacetylase (CAHs) and acetyl xylan esterases (AXEs), exhibit significant perhydrolysis activity for converting carboxylic acid esters in the presence of an inorganic source of peroxygen (e.g., hydrogen peroxide, sodium percarbonate, sodium perborate) into 20 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 (e.g., as described in the commonly owned U.S. Application Publication 2008/0176299A1).

25 Perhydrolase catalysts may be used as 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.

#### Carbohydrates esterases of the CE-7 family

30 Members of the CE-7 family, cephalosporin C deacetylases (E.C. 3.1.1.41; systematic name cephalosporin C acetylhydrolases; CAHs) and acetyl xylan esterases (E.C. 3.1.1.72) 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, 63:4807-4811, 1997); Sakai *et al.*, 5 J. Ferment. Bioeng. 85:53-57, 1998); Lorenz, W. and Wiegel, J., J. Bacteriol., 179:5436-5441, 1997); Cardoza *et al.*, Appl. Microbiol. Biotechnol., 54:406-412, 2000); Mitshushima *et al.*, *supra*, Abbott, B. and Fukuda, D., Appl. Microbiol. 30:413-419, 1975); Vincent *et al.*, J. Mol. Biol., 330:593-606, 2003, Takami *et al.*, NAR, 28:4317-4331, 2000); Rey 10 *et al.*, Genome Biol., 5: 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 family members having significant homology to SEQ ID NO: 1 is provided in SEQ ID NOs 1-18.

15 As members of the CE-7 family of carbohydrate esterases (Vincent *et al.*, *supra*), both cephalosporin C deacetylases (E.C. 3.1.1.41) and acetyl xylan esterases (E.C. 3.1.1.72) hydrolyze the acetyl group on acetylated polymeric xylan, acetylated xylose, acetylated glucose, and acetylated cellulose. As such, acetylated carbohydrates may be suitable 20 substrates for generating percarboxylic acids using the present process (i.e., in the presence of a peroxygen source). Examples of acetylated carbohydrates include, but are not limited to acetylated glucose (such as glucose pentaacetate), acetylated mannose, acetylated xylose (such as xylose tetraacetate), and acetylated cellulose.

25 Some enzymes, such as those suitable for application in the current method possess signature motifs that 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 discontinuous, 30 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 signature motifs as described below.

The CE-7 family members are unusual in that they typically exhibit ester hydrolysis activity for both acetylated xylooligosaccharides and 5 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.*, *supra*). Vincent *et al.* describe the structural similarity among the members of this family and define signature sequence motifs characteristic of the CE-7 family (SEQ ID NO: 21). A CE-10 7 signature motif that aligns with a reference sequence SEQ ID NO: 1 using CLUSTALW has several highly conserved motifs that are diagnostic for the family, which include residues 118-120 (RGQ) of SEQ ID NO: 1, residues 179-183 (GXSQG) of SEQ ID NO: 1, residues 298-299 (HE) of SEQ ID NO: 1 and residues 267-269 (LXD) of SEQ ID NO: 1. Multiple 15 alignment of the amino acid sequences may be performed using the Clustal method of alignment Higgins and Sharp, *CABIOS*, 5:151-153 (1989) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method are typically KTUPLE 1, GAP PENALTY=3, WINDOW=5 20 and DIAGONALS SAVED=5.

Perhydrolase enzymes having amino acid sequences that are least 50%, preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, yet even more preferably at least 90%, still yet even more preferably at least 95%, and most preferably at least 99% to 25 the sequence reported herein wherein the resulting enzyme retains the present functional properties (i.e., perhydrolytic activity) are suitable for the present method. The term "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 of 30 the amino acid sequences reported herein (e.g., SEQ ID NOs:1-17 and 30). It is therefore understood that the invention encompasses more than the specific exemplary sequences.

A comparison of the overall percent identity among perhydrolases exemplified herein indicates that enzymes having as little as 33% identity to SEQ ID NO: 1 (while retaining the signature motif) exhibit significant perhydrolase activity and are structurally classified as CE-7 carbohydrate esterases.

5 In one embodiment, one of the perhydrolase catalysts useful for this method comprises an enzyme having a CE-7 signature motif that aligns with a reference sequence SEQ ID NO: 1 using CLUSTALW, said signature motifs comprising:

10 i) an RGQ motif at amino acid positions 118-120 of SEQ ID NO:1;  
ii) a GXSQG motif at amino acid positions 179-183 of SEQ ID NO:1; and  
iii) an HE motif at amino acid positions 298-299 of SEQ ID NO:1;  
wherein said enzyme also comprises at least 30% amino acid identity to  
15 SEQ ID NO: 1.

In a further embodiment, the signature motif additionally comprises a fourth conserved motif defined as an LXD motif at amino acid residues 267-269 when aligned to reference sequence SEQ ID NO: 1 using CLUSTALW.

20 In another embodiment, the perhydrolase catalyst comprises an enzyme having perhydrolase activity selected from the group consisting of SEQ ID NOs: 1-17 and 30 or a substantially similar enzyme having perhydrolase activity derived by substituting, deleting or adding one or more amino acids to said amino acid sequence.

25 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 NOs: 1-17 and 30.

30 In another embodiment, the perhydrolase catalyst comprises an enzyme having at least 30%, preferably at least 36%, amino acid identity to a contiguous signature motif as defined as SEQ ID NO: 1 wherein the

signature motifs described above (e.g., RGQ, GXSQG, and HE, and optionally LXD) are conserved.

In yet further embodiments, the perhydrolase system of the present method is used in conjunction with additional enzymes, including but not limited to proteases, amylases, etc. Indeed, it is contemplated that various enzymes will find use in conjunction with the present invention, including but not limited to microbial cell wall-degrading and glycoprotein-degrading enzymes, lysozyme, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, 5 keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, 13-glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, endoglucanases, PNGases, amylases, etc., as well as or mixtures thereof. In some embodiments, enzyme stabilizers find use in the present method. 10 15 It is contemplated that by using combinations of enzymes, there will be a concurrent reduction in the amount of chemicals needed in the method.

Although in one preferred embodiment, the perhydrolysis activity of the structurally related members of the carbohydrate family esterase family CE-7 have been used for the current method, any perhydrolase 20 enzyme obtained from any source, which in the presence of hydrogen peroxide converts an ester substrate into mostly peracid, may also be used in the present method.

Additionally, some hydrolase enzymes may be used in the current method. Suitable hydrolases for this purpose include: (1) proteases 25 belonging to the peptidyl-peptide hydrolase class (e.g., pepsin, pepsin B, rennin, trypsin, chymotrypsin A, chymotrypsin B, elastase, enterokinase, cathepsin C, papain, chymopapain, ficin, thrombin, fibrinolysin, renin, subtilisin, aspergillopeptidase A, collagenase, clostridiopeptidase B, kallikrein, gastrisin, cathepsin D, bromelin, keratinase, chymotrypsin C, 30 pepsin C, aspergillopeptidase B, urokinase, carboxypeptidase A and B, and aminopeptidase); (2) carboxylate ester hydrolase including carboxyl esterase, lipase, pectin esterase, and chlorophyllase; and (3) enzymes

having high perhydrolysis to hydrolysis ratios. Especially effective among them are lipases, as well as esterases that exhibit high perhydrolysis to hydrolysis ratios, as well as protein engineered esterases, cutinases, and lipases, using the primary, secondary, tertiary, and/or quaternary structural 5 features of the perhydrolases of the present method.

Suitable enzymatic reaction mixture

“Suitable enzymatic reaction mixture”, “components suitable for *in situ* generation of a peracid”, “suitable reaction components”, and “suitable aqueous reaction mixture” are used interchangeably herein and refer to 10 the materials and the solution 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 15 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 for combining multiple active components are known in the art and generally will depend upon the 20 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 No. 5,398,846; U.S. Patent No. 5,624,634; U.S. Patent No. 6,391,840; EP Patent No. 0807156B1; U.S. Patent Appl. 25 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 30 combinations of solid-liquid components, such as powders (e.g., many commercially available bleaching compositions, U.S. Patent No. 5,116,575), multi-layered tablets (U.S. Patent No. 6,210,639), water

dissolvable packets having multiple compartments (U.S. Patent No. 6,995,125) and solid agglomerates that react upon the addition of water (U.S. Patent No. 6,319,888).

In another aspect, a suitable system for combining reactive components is use of a twin-nozzle bottle as disclosed in U.S. Patent Application Pub. No. 2005/014427. An alternative device suitable for use with the method of the invention is a dual compartment trigger-activated fluid dispenser as disclosed in EP Patent No. 0715899B1.

In another aspect, a suitable system for mixing the suitable reaction components may be a container with a membrane separating the reactive components where upon rupturing the membrane by mechanical force, the reaction components are combined before use. In another aspect, a suitable device may be a bag-within-a-bag.

In another aspect, the means for combining or mixing the suitable enzymatic reaction mixture for use according to the methods of this invention include systems, devices, containers, kits, multi-packs, bags, dispensers, and applicators known to those skilled in the art that are used to keep reactive components separated before use.

In another aspect, the enzymatic perhydrolysis product composition 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 No. 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; acetic acid/triethanolamine; and sodium bicarbonate/sodium carbonate. Examples of surfactants include, but are not limited to a) non-ionic surfactants such as block copolymers of

5 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

10 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

15 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

20 (e.g., polyethylene glycol, PEG), and detergent builders.

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.

In another aspect, the enzymatic perhydrolysis product may be pre-mixed to generate the desired concentration of peroxycarboxylic acid and 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 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 5 reaction components are delivered or applied to the locus and subsequently mix or combine to generate the desired peroxyacid.

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 concentration of catalyst in 10 perhydrolysis reactions typically ranges from 0.0001 mg to 10 mg per mL of total reaction volume, preferably from 0.0005 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; 15 Humana Press, Totowa, N.J., 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.

20 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 the desired application at a desired pH. In another aspect, the present method provides combinations of enzymes and enzyme 25 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 30 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.

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, 5 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 10 preferably within 1 minute of initiating the perhydrolysis reaction. In another aspect, the concentration of peracid generated by the perhydrolysis catalyst is at least about 3000 ppm, preferably at least 5000 ppm, more preferably 8000 ppm, most preferably at least 10000 ppm within one hour, more preferably within 30 minutes, most preferably within 15 5 minutes of initiating the perhydrolysis reaction. 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 5 minutes, and most preferably in about 20 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 25 minute to 8 hours, or within 1 minute to 2 hours of combining said reaction components, or any such time interval therein.

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 30 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.

The pH of the final reaction mixture containing peracid is from about 5 to about 9.5, preferably from about 6 to about 8, more preferably from 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 mixture during the

5 reaction or after completion of the reaction 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.

10 In one embodiment of the present invention, at least some of the components are premixed. Premixed refers to the combination of one or more individual components of the coating composition, but not all of the desired components of the film-forming composition are combined. Additional components of the coating composition must be added to the

15 premixed component prior to final application or use at a particular locus. In another embodiment, two premixed components must be combined to form the desired coating composition according to the methods of this invention.

The components of the coating composition of the present invention

20 can be contained in a multicompartiment containment system, also referred to herein as a multicompartiment system. A multicompartiment system refers to the means of keeping the two or more reactive components of the multicomponent system coating system separated before use. In one aspect, a multicompartiment system comprises at least

25 two compartments and may contain a multi-chamber dispenser bottle or a two-phase system used to combine reactive compounds in liquid form. In another aspect, powders, multi-layered tablets, or water dissolvable packets having multiple compartments, can be used for compounds in solid form or a combination of solid and liquid forms. In another aspect,

30 any kind of system, device, container, package, bag, kit, multi-pack, dispenser, or applicator that is used to keep reactive components

separated before use can be used according to the methods of this method.

Coatings of the present invention can be removed, after application, using an aqueous solution. An aqueous solution effective for coating

5 removal is any solution containing 60 to 100 wt% water, the remaining components being dissolved or dispersed components. Dissolved or dispersed components may include but are not limited to solvents such as alcohols, solubilizing agents, surfactants, salts, chelators, acids and bases.

10 Antimicrobial coatings of the present invention are durable coatings. Durable in the context of the present invention is the characteristic or property of the applied antimicrobial coating, after drying, of remaining on the surface under typical use conditions until its removal is purposely initiated or allowed to occur. Use conditions are the ambient

15 environmental conditions prevalent during the period the coating remains on the target surface for the application areas of this invention and may include inadvertent contact with water.

It can be preferable that the antimicrobial coatings applied to target surfaces be continuous or substantially continuous. Continuous, or

20 substantially continuous, in the context of the present invention refers to a coating that covers the target surface without voids, breaks, uncovered areas, or coating defects that leave unintentionally exposed surface areas.

Coatings of the present invention are preferably homogeneous or substantially homogeneous. Homogeneous, or substantially homogenous,

25 in the context of the present invention refers to a coating with only small thickness variations across the coating surface, with the standard deviation of the coating thickness across a coated surface being in the range of 0-40% of the coating thickness. Coatings that are not homogeneous or not substantially homogenous will not provide even

30 antimicrobial and removal properties across the whole surface the coating is applied to and typically the appearance of inhomogeneous coatings is considered unattractive for many applications.

Pseudoplastic index or shear thinning index (STI) provides an indication of the resistance to sagging and dripping that the composition may exhibit. The value recorded at the lower shear rate is divided by the value at the higher shear rate to obtain the STI. Generally, the higher the 5 STI, the higher the resistance to sagging and dripping the coating material will have. In this disclosure the shear thinning index is defined as the ratio of the viscosity measured at a first shear rate and a second shear rate, wherein said second shear rate is 10 times the value of said first shear rate. Alternatively, if the shear rates in  $s^{-1}$  are not known, the shear- 10 thinning index can also be calculated by measuring the viscosity using a rotational viscometer at a first rpm and a second rpm, wherein said second rpm is 10 times the value of said first rpm. Without being limited to specific first and second shear rates used or to specific first and second rpm values used to calculate the STI, in the Examples, the shear thinning 15 index was calculated as the ratio of the viscosities measured at 1 rpm and 10 rpm.

The antimicrobial coating of the present invention can be used as a sanitizer. As defined herein, a sanitizer is a chemical or chemical mixture that can be either (i) a food-contact sanitizer if the intention is to control 20 microorganisms on surfaces which actually or potentially come in contact with food, or (ii) a non-food-contact sanitizer if the surfaces are not intended to come into contact with food. As defined herein, a food-contact sanitizer kills at least 99.999% of the specific test microorganisms in 30 seconds under the conditions of the test method according to EPA policy 25 DIS/TSS-4: “Efficacy data requirements – Sanitizing rises for previously cleaned food-contact surfaces”, United States Environmental Protection Agency, January 30, 1979. A non-food contact sanitizer as defined herein kills at least 99.9% of the specific test microorganisms in 5 minutes under the conditions of the method according to ASTM standard E 1153–03: 30 “Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces”, edition April 10, 2003 and published July 2003.

A coating composition of the present invention can exhibit a residual antimicrobial efficacy, and exhibit self-sanitizing properties. “Residual antimicrobial efficacy” or “self-sanitizing properties” refers to the property of coatings formed as described herein which remain

5 antimicrobially active after drying. The antimicrobial activity of dry coatings can be measured using the residual self-sanitizing (RSS) test.

There has been a longstanding need for antimicrobial agents having improved antimicrobial efficacy and improved speed of action. The specific requirements for such agents vary according to the intended

10 application (e.g., sanitizer, disinfectant, sterilant, aseptic packaging treatment, etc.) and the applicable public health requirements. For example, as set out in Germicidal and Detergent Sanitizing Action of Disinfectants, Official Methods of Analysis of the Association of Official Analytical Chemists, paragraph 960.09 and applicable sections, 15th

15 Edition, 1990 (EPA Guideline 91-2), a sanitizer should provide a 99.999% reduction (5-log order reduction) within 30 seconds at room temperature (23-27 °C), against several test organisms.

The *in situ* enzymatically produced peracid-based, removable antimicrobial coating composition useful for the invention may be used as

20 a replacement or a supplement for standard sanitation products (such as diluted quaternary ammonium compound solutions, peracid foams, and the like), and may be used for daily sanitation as protective coatings for equipment in use or not-in use, as well as for longer term protection (weeks or months).

25 The *in situ* enzymatically produced peracid-based, removable antimicrobial coating composition of this disclosure provides several advantages including, but not limited to killing both free-living or planktonic microorganisms and microorganisms harbored in biofilms, reducing or preventing the growth of microorganisms by preventing the formation of

30 biofilms and by trapping microorganisms in, beneath or otherwise in contact with the coating.

The coating composition disclosed herein may be modified by formulating the composition with rheology modifiers to coat vertical, inclined, geometrically complex or hard-to-reach surfaces. This enables application of the antimicrobial agent to surfaces on or in equipment

5 otherwise not accessible by application of conventional antimicrobial solutions with traditional shear-viscosity profiles and viscosities below about 0.01 Pascal-seconds at 25 °C. Horizontal and vertical surfaces may be covered with a thin layer of protective coating without waste of antimicrobial agent as dripping is prevented or greatly reduced by the

10 rheology modifier. By formulating compositions with appropriate rheology modifier and degree of cross-linking, coating compositions with various coating properties may be prepared that will vary in the degree of surface finish and protection as well as ease of removal.

The coating composition of the present invention offers several

15 mechanisms of protection against contamination of microbial or non-microbial origin, such as soiling. For example, planktonic or loosely adhering cells on the surface are killed, or alternatively growth is reduced or prevented, by the antimicrobial agent in the coating formulation as the liquid coating composition is applied.

20 Further, after application of the liquid antimicrobial coating composition of the present invention, cells harbored by biofilms on the surface will be killed, or growth can be reduced or prevented, by diffusion of the antimicrobial(s) into the hydrated biofilm before the applied film-forming composition completely dries to provide an antimicrobial film. For

25 sustained antimicrobial activity it is desirable that the antimicrobial films of the present invention be semi-permeable. The antimicrobial film thus formed constitutes a reservoir of antimicrobial agent providing much longer contact time than conventional sanitary rinse solutions that typically drip off within seconds or minutes.

30 The long lasting activity while the coating present on the locus is especially beneficial in a variety of applications. A film-forming antimicrobial composition of the present invention does not drip off of the

target surface quickly, and is not easily removed by incidental contact, for example. The variation of film flexibility, viscosity, strength, and adhesion of the coating of the present invention permits it to be tailored to specific applications, thus making sustained antimicrobial protection available in

5 numerous situations where such sustained activity (residual benefit) was not previously available.

The film-forming water soluble or water dispersible agent useful in the practice of the present invention may be at least one of any agent, as described below, that is durable and removable. A film of the present

10 invention is designed to be removable under relatively mild conditions. For example, a film of the present invention can be removed when subjected to treatment with an aqueous solution at a temperature above 15 °C, preferably above 30 °C. Suitable film-forming agents are selected from, but are not limited to, polyvinyl alcohols, polyvinyl alcohol

15 copolymers, polyvinyl pyrrolidones, polyacrylic acid, acrylate copolymers, ionic hydrocarbon polymers, polyurethanes, polysaccharides, functionalized polysaccharides, arabinoxylanes, glucomannanes, guar gum, gum arabic, johannistree gums, cellulose, methyl cellulose, ethyl cellulose, hydroxymethyl cellulose, hydroxyethyl cellulose, carboxymethyl

20 cellulose, carboxyethyl cellulose starch, hydroxyethyl starch, xanthan gum, carrageenan, curdlan, pullulan, gelatin, dextran, chitosan, glycerol, sodium alginate, sodium alginate cross-linked with calcium salt, carrageenan, ethyleneoxide/propylene oxide/ethyleneoxide block copolymers, and combinations there of. One skilled in the art may easily select the range of

25 suitable molecular weights in order to provide a range of water solubility to provide a readily removable coating according to the methods of this invention.

Polyvinyl alcohol, sometimes referred to as poly(vinyl alcohol), is made from polyvinyl acetate by hydrolysis, and is suitable for use herein.

30 The physical properties of polyvinyl alcohol are controlled by the molecular weight and the degree of hydrolysis. The most commonly available grades of polyvinyl alcohol, ranked by degree of hydrolysis, are an 87-89%

grade (containing 11-13 mol% residual vinylacetate units), a 96% hydrolysis grade (containing 4 mol% residual vinyl acetate units), and the "fully hydrolyzed" and "superhydrolyzed" grades, which are about 98% and greater-than-99% hydrolyzed, respectively. Lower degrees of hydrolysis 5 (e.g., 74% and 79%) are also commercially available. Some preferred degrees of hydrolysis are greater than 85 mol%, or greater than 92 mol%. The polyvinyl alcohol component of the present invention may also be a copolymer of vinyl alcohol, such as one obtained by hydrolyzing a copolymer of vinyl acetate with small amounts (up to about 15 mol%) of 10 other monomers. Suitable co-monomers are e.g., esters of acrylic acid, methacrylic acid, maleic or fumaric acids, itaconic acid, etc. Also, copolymerization of vinyl acetate with hydrocarbons e.g., alpha-olefins such as ethylene, propylene or octadecene, etc., with higher vinyl esters such as vinyl butyrate, 2-ethyl hexoate, stearate, trimethyl acetate, or 15 homologues thereof ("VV-10" type of vinyl esters sold by Shell Chem. Co.), etc. gives copolymers that may be hydrolyzed to suitable polyvinyl alcohol copolymers. Other suitable comonomers are N-substituted acrylamides, vinyl fluoride, allyl acetate, allyl alcohol, etc. Also the free unsaturated acids such as acrylic acid, methacrylic acid, monomethyl 20 maleate, etc. may act as co-monomers.

Because of the variety of grades either known in the literature or commercially available, one skilled in the art may formulate a polyvinyl alcohol solution having an average degree of hydrolysis ranging from 74 to more than 99% simply by blending the known or commercial grades in any 25 desired ratios.

Film flexibility, water sensitivity, ease of solvation, viscosity, film strength and adhesion of the polyvinyl alcohol film may be varied by adjusting molecular weight and degree of hydrolysis.

In one embodiment, the polyvinyl alcohol for use in the process of 30 this invention has a degree of hydrolysis from about 85% to greater than 99%. In another embodiment, the polyvinyl alcohol has a degree of hydrolysis from about 87% to greater than 89%.

In one embodiment, the polyvinyl alcohol has a number-averaged molecular weight (Mn) that falls in the range of between about 4,000 to about 200,000, or about 4,000 to about 150,000, or 10,000 to about 100,000.

5 In one embodiment, the polyvinyl alcohol has a molecular weight that falls in the range of between about 10,000 and 130,000. In another embodiment, the polyvinyl alcohol of various molecular weights may be blended to give the desired properties.

10 In one embodiment, the polyvinyl alcohol is used at about 2% to about 30% by weight of the weight of the solution. In a more specific embodiment, the polyvinyl alcohol is used at about 2% to about 15% by weight of the weight of the solution. In an even more specific embodiment, the polyvinyl alcohol is used at about 5% to about 12% by weight of the weight of the solution.

15 The film-forming composition of the present invention may contain PVP at a concentration of about 0.25 to about 50% by weight. Suitable grades of PVP are available from International Specialty Products (Wayne, NJ, USA). Such grades include: K-15, having a molecular weight (Mw) average range of about 6,000 to about 15,000; K-30, having a molecular weight range of about 40,000 to about 80,000; K-60, having a molecular weight range of about 240,000 to about 450,000; K-90, having a molecular weight range of about 900,000 to about 1,500,000; and K-120, having a molecular weight range of about 2,000,000 to about 3,000,000. Mixtures of PVP's may be employed, as may combinations of PVP and other film-forming compounds. Suitable grades are described in the commonly owned and co-pending U.S. Application Nos: U.S. 2007/0275101 A1 and U.S. 2008/0026026A1 which are herein incorporated by reference on their entirety.

30 Typically, lower molecular weight PVP will give a less viscous product than a higher molecular weight PVP at the same concentration. For a given concentration of PVP, as the molecular weight range increases, the viscosity will also increase. The present invention may

employ PVP having any of a number of molecular weight ranges. It is preferred, however, to use PVP with a molecular weight distribution between about 15,000 and about 3,000,000 g/mol. PVP having this molecular weight distribution typically gives a film-forming composition

5 with a viscosity, which may be easily adjusted and washes off a surface easily with no visible signs of interaction with a painted surface. In a preferred embodiment, PVP with a molecular weight distribution between about 15,000 and about 3,000,000 g/mol is present at a concentration of between about 0.25% and about 40% by weight. In another preferred

10 embodiment, PVP with a molecular weight distribution between about 30,000 and about 1,200,000 g/mol is present at a concentration of between about 0.25% and about 10% by weight.

One class of molecules which is widely recognized as including highly effective disinfectants is that of the organic peroxyacids. In recent

15 years research has been directed at these compounds, aimed not least in providing a stable and safe means of their delivery. Amongst the peroxyacids, the one which has been the subject of closest attention has been peroxyacetic acid (often referred to as peracetic acid or PAA). This particular peroxyacid is rarely isolated as a pure compound and is

20 normally encountered in solution in both aqueous and non-aqueous media. Peroxyacetic acid is often supplied as an equilibrium mixture including primarily water, acetic acid and hydrogen peroxide, together with a number of other minor components such as stabilizers (WO 2006/076334; U.S. Patent 5,508,046). However, even equilibrium

25 systems of peroxyacetic acid are inherently thermodynamically unstable due to the decomposition of peroxyacetic acid into acetic acid and oxygen. Such decomposition may be slowed down if special measures are taken but cannot be completely prevented. A further property of peroxyacids which makes them difficult to use or store is that they may decompose

30 rapidly and violently, particularly peroxyacids of low molecular weight and of high purity. To solve this problem, peroxyacids are often generated *in situ* to perform the particular desired function. Such *in situ* generation has

an advantage in that the amount of peroxyacid produced may be stoichiometrically controlled through selecting the relative composition of the starting materials. Moreover, higher concentrations of peroxyacid may be achieved than are available from equilibrium systems due to the non-equilibrium nature of the *in situ* systems.

5 There is also a need to generate the peroxyacid at the required rate: too slow a rate may result in a lack of performance. The present method provides combinations of enzymes and enzyme substrates to produce the desired effective concentration of peracid, where, in the  
10 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  
15 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.

One solution to the inherent lack of stability of the peracids is to provide a system in which the reaction components that produce or react  
20 to form the peracids are maintained separately until required (WO 2006/016145). Such a solution to the problem is contemplated in the method described herein.

In the present invention a film-forming component is combined with other components, including a carboxylic acid ester substrate, an enzyme  
25 catalyst having perhydrolase activity, and a peroxygen source. The film-forming component and one or more of the following components, carboxylic acid ester substrate, enzyme catalyst, and peroxygen source, can be stored together in one compartment, or stored in separate compartments, prior to combining to form the antimicrobial coating  
30 composition and application to a target surface. For example, the film forming component can be stored in a separate compartment from the carboxylic acid ester substrate, enzyme catalyst and peroxygen source,

and combined just prior to application to the target surface or, alternatively, the film-forming component can be stored together with the carboxylic acid ester substrate but separately from the enzyme catalyst or peroxygen source until such time as they are combined and applied to a 5 target surface.

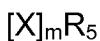
The present method provides for *in situ* enzymatic production of efficacious concentrations of peracids in a durable, continuous and easily removable film-forming composition using the perhydrolase activity of an enzyme from the CE-7 family of carbohydrate esterases. As described 10 herein, the genes encoding said enzymes (e.g., enzymes with amino acid sequence SEQ ID NO: 7 isolated from a *Thermotoga neapolitana*, amino acid sequence SEQ ID NO: 8 isolated from a *Thermotoga maritima*, or a substantially similar amino acid sequence) are suitable for application in the current method. As disclosed above, other enzymes, from various 15 sources, having perhydrolase activity may also be used in the present method disclosed herein.

In a preferred embodiment, the source of inorganic peroxide is hydrogen peroxide. It is not intended that the present invention be limited to inorganic hydrogen peroxide. Hydrogen peroxide may be generated 20 using an enzyme catalyst and a suitable substrate. Any enzyme that generates hydrogen peroxide and acid with a suitable substrate finds use in the present method. For example, lactate peroxidase from *Lactobacillus* species, which are known hydrogen peroxide producers from lactic acid and oxygen may be used in the current method.

25 Other enzymes (e.g., glucose oxidase, alcohol oxidase, ethylene glycol oxidase, glycerol oxidase, amino acid oxidase, etc.) that can generate hydrogen peroxide also find use with ester substrates in combination with the perhydrolase enzyme of the present method to generate peracids. Enzymes that generate acid from substrates without 30 the generation of hydrogen peroxide also find use in the present invention. Examples of such enzymes include, but are not limited to proteases.

In another aspect, the peroxygen source may be any peroxide compound and may be selected from, but not limited to, the following: hydrogen peroxide, sodium perborate monohydrate, sodium perborate tetrahydrate, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate, 5 sodium percarbonate, sodium peroxide and mixtures thereof.

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



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

10  $R_6=C1$  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  $C7$ ;

15  $R_5=a$   $C1$  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;

20  $m=1$  to the number of carbon atoms in  $R_5$ ; and wherein said esters have a solubility in water of at least 5 ppm at  $25^\circ C$ .

In another embodiment, suitable substrates also include esters of the formula:  $R_1-COO-R_2$  wherein  $R_1=a$   $C1$  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, 25 alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl,  $-(CH_2CH_2-O)_nH$  or  $-(CH_2CH(CH_3)-O)_nH$  and  $n=1$  to 10.

In another embodiment, suitable substrates include glycerides of the formula:  $R_1-COO-CH_2-CH(OR_3)-CH_2-OR_4$  wherein  $R_1=C1$  to  $C7$  straight chain or branched chain alkyl optionally substituted with an 30 hydroxyl or a  $C1$  to  $C4$  alkoxy group and  $R_3$  and  $R_4$  are individually H or  $R_1C(O)$ .

In another embodiment, R<sub>6</sub> 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<sub>6</sub> is C2 to C7 linear hydrocarbyl moiety, optionally

5 substituted with hydroxyl groups, and/or optionally comprising one or more ether linkages.

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

10 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),  $\beta$ -D-ribofuranose-1,2,3,5-tetraacetate, tri-O-

15 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  $\beta$ -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

20 percarboxylic acids using the present process (i.e., in the presence of a peroxygen source).

In one embodiment, the substrate is selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyryin; dibutyryin; tributyryin; glucose pentaacetate;

25 xylose tetraacetate; acetylated xylan; acetylated xylan fragments;  $\beta$ -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.

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-hydroxy- butyrate, ethyl 3-hydroxybutyrate, triethyl 2-acetyl citrate, glucose pentaacetate, gluconolactone, glycerides (mono-, di-, and triglycerides) such as monoacetin, diacetin, triacetin, monopropionin, dipropionin 5 (glyceryl dipropionate), tripropionin (1,2,3-tripropionylglycerol), monobutyrin, dibutyrin (glyceryl dibutyrate), tributyrin (1,2,3-tributyrylglycerol), acetylated saccharides, and mixtures thereof.

In a further preferred aspect, the carboxylic acid ester substrates are selected from the group consisting of monoacetin, diacetin, triacetin, 10 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 15 monoacetin, diacetin, triacetin, and mixtures thereof.

Inert solvents useful for the invention include water or solutions comprising at least 50 wt% water. Additional solvents include mono alcohols, monofunctional and polyfunctional alcohols, preferably containing from about 1 to about 6 carbon atoms and from 1 to about 6 20 hydroxy groups. Examples include ethanol, isopropanol, n-propanol, 1,2-propanediol, 1,2-butanediol, 2-methyl-2,4-pentanediol, mannitol and glucose. Also useful are the higher glycols, polyglycols, polyoxides, glycol ethers and propylene glycol ethers. Additional solvents include the free acids and alkali metal salts of sulfonated alkylaryls such as toluene, 25 xylene, cumene and phenol or phenol ether or diphenyl ether sulfonates; alkyl and dialkyl naphthalene sulfonates and alkoxylation derivatives.

The compositions useful for the present invention may also contain one or more surfactants. While not being bound by theory, it is believed that a surfactant will aid wetting of the surface to be covered and will aid 30 even coverage by the film. The surfactant is also believed to aid foaming by the film when removed, thereby aiding removal of the film and washing of the protected surface. Suitable surfactants have a preferred

hydrophilic-lipophilic balance (HLB) of from about 9 to about 17. Suitable surfactants include, but are not limited to: amphoteric surfactants, such as Amphoteric N from Tomah Products; silicone surfactants, such as BYK 348 available from BYK Chemie (BYK-Chemie GmbH, Wesel, Germany);

5       fluorinated surfactants such as Zonyl® FS300 from DuPont (Wilmington, DE, USA); and nonylphenoxypolyethoxyethanol based surfactants, such as Triton N-101 available from Dow (Midland, MI, USA). Other suitable surfactants include ethoxylated decynediols such as Surfynol 465 available from Air Products & Chemicals (Allentown, PA, USA); alkylaryl

10      polyethers such as Triton CF-10 available from Dow; octylphenoxy polyethoxy ethanols such as Triton X-100 available from Dow; ethoxylated alcohols such as Neodol 23-5 or Neodol 91-8 available from Shell (The Hague, the Netherlands); Tergitol 15-S-7 available from Dow; Steol-4N, a 28% sodium laureth sulfate from Stepan Company (Northfield, IL, USA);

15      amine oxides such as Ammonyx® LO available from Stepan; EO/PO block copolymers such as Pluronic® 17R4 available from BASF (Parsippany, NJ, USA); sorbitan derivatives such as Tween 20 or Tween 60 from Uniqema (New Castle, DE, USA); and quaternary ammonium compounds, such as benzalkonium chloride. Other suitable surfactants include

20      organo-silicone surfactants such as Silwet®L-77 from Setre Chemical Company (Memphis, TN, USA); DowCorning® Q2-5211 from DowCorning Silicones (Midland, MI, USA); or Silsurf® A008 by Siltech Corporation (Toronto, ON, Canada).

It can be important for flexibility and integrity of the protective film

25      that the resultant film be plasticized. Plasticization of the film has been accomplished for the purposes of this invention by the incorporation of a suitable plasticizing agent such as polyethylene glycol or glycerol. Other plasticizers suitable for the invention include, but are not limited, to solvents, polyols, polyethylene glycols of and average molecular weight

30      between 200 and 800 g/mol and sorbitol. PEG is preferred over glycerol since glycerol is easily metabolized by microorganisms potentially resulting in microbial growth.

Inclusion of a plasticizer generally also allows the film to retain a slightly tacky surface feel. As the plasticizer level increases, the resulting film will also exhibit an increasing degree of tackiness. Such tackiness may be desirable at low levels in order to capture airborne particles and

5 soil or other materials. If plasticizer levels are too high, however, the coating becomes too tacky and will show low resistance to accidental mechanical removal, by wiping, for example.

The preferred plasticizer amount is from about 1 wt% to about 20 wt% of the weight of the film former, and more preferably from about 5

10 10 wt% to about 10 wt%.

The composition useful for the invention may also contain one or more rheology modifiers employed to enhance viscosity, or thicken and cause the aqueous treatment or coating composition to cling to the surface. Clinging enables the composition to remain in contact with

15 transient and resident microorganisms for longer periods of time, promoting microbiological efficacy and resisting waste because of excessive dripping. The rheology modifier may be a film former or act cooperatively with a film-forming agent to form a barrier that provides additional protection. Water soluble or water dispersible rheology

20 modifiers that are useful may be classified as inorganic or organic. The organic thickeners may further be divided into natural and synthetic polymers with the latter still further subdivided into synthetic natural-based and synthetic petroleum-based.

Inorganic thickeners are generally compounds such as colloidal

25 magnesium aluminum silicate (VEEGUM®), colloidal clays (Bentonites), or silicas (CAB-O-SIL®) which have been fumed or precipitated to create particles with large surface to size ratios. Natural hydrogel thickeners of use are primarily vegetable derived exudates. For example, tragacanth, karaya, and acacia gums; and extractives such as carrageenan, locust

30 bean gum, guar gum and pectin; or, pure culture fermentation products such as xanthan gum are all potentially useful in this invention.

Chemically, all of these materials are salts of complex anionic

polysaccharides. Synthetic natural-based thickeners having application are cellulosic derivatives wherein the free hydroxyl groups on the linear anhydro-glucose polymers have been etherified or esterified to give a family of substances which dissolve in water and give viscous solutions.

5 This group of materials includes the alkyl and hydroxyl- alkylcelluloses, specifically methylcellulose, hydroxyethylmethylcellulose, hydroxypropylmethylcellulose, hydroxybutylmethylcellulose, hydroxyethyl-cellulose, ethylhydroxyethylcellulose, hydroxypropylcellulose, and carboxy- methylcellulose. Another preferred group of thickeners include

10 polyacrylates such as the proprietary Acusol thickeners, (e.g., Acusol 823, Rohm and Haas, Philadelphia, PA, USA), and Carbopol thickeners, such as Carbopol 934 or Carbopol Aqua-30 Polymer (B F Goodrich, Cleveland, OH, USA). Additional preferred acrylate-based rheology modifiers include the proprietary cationic Rheovis® thickeners (Ciba, Basel, Switzerland). A

15 polyacrylate thickener may be used at concentrations of up to about 3 wt% of the film former weight. Mixtures of thickening agents may also be employed where the total amount may be up to about 3 wt% depending on the thickeners used and the desired viscosity of the final product.

Other potential thickeners for this application include dextrin,

20 cornstarch and hydrous magnesium silicates, such as sodium magnesium silicate sold under the trade name Laponite XLG (Southern Clay Products, Inc., Gonzales, TX, USA).

In addition to the *in situ* generated peroxyacid, one or more additional antimicrobial agents may be present in the composition. The

25 antimicrobial agent useful for the invention may be either an inorganic or organic agent, or a mixture thereof.

The term "inorganic antimicrobial agent" used herein is a general term for inorganic compounds which contain a metal or metal ions, such as silver, zinc, copper and the like which have antimicrobial properties.

30 The invention is not to be limited to the selection of any particular antimicrobial agent, and any known water-soluble or water-dispersible antimicrobial may be included in the compositions of the invention such as

5 antimicrobials, mildewcides, antiseptics, disinfectants, sanitizers, germicides, algicides, antifouling agents, preservatives, and combinations of the foregoing and the like provided that the antimicrobial agent is chemically compatible with other components in the composition. Suitable classes of antimicrobial agents are described below.

Examples of useful antimicrobial agents include chlorhexidine, chlorhexidine gluconate, glutaral, halazone, hexachlorophene, nitrofurazone, nitromersol, thimerosal, C1-C5 -parabens, hypochlorite salts, clofucarban, clorophen, phenolics, mafenide acetate, aminacrine hydrochloride, quaternary ammonium salts, chlorine and bromine release compounds (e.g., alkali and alkaline earth hypochlorites and hypobromites, isocyanurates, chlorinated derivatives of hydantoin, sulfamide, amine, etc.), peroxide and peroxyacid compounds (e.g., peracetic acid, peroctanoic acid), protonated short chain carboxylic acids, oxychlorosene, metabromsalan, merbromin, dibromsalan, glyceryl laurate, sodium and/or zinc pyrithione, trisodium phosphates, (dodecyl)(diethylenediamine)glycine and/or (dodecyl) (aminopropyl)glycine and the like. Useful quaternary ammonium salts include the N-C<sub>10</sub>-C<sub>24</sub> - alkyl-N-benzyl-quaternary ammonium salts which comprise water solubilizing anions such as halide, e.g., chloride, bromide and iodide; sulfate, methosulfate and the like and the heterocyclic imides such as the imidazolinium salts. Quaternary ammonium salts may also include other non-halogenated anions such as propionates and saccharinates and the like. Useful phenolic germicides include phenol, m-cresol, o-cresol, p-cresol, o-phenyl-phenol, 4-chloro-m-cresol, chloroxylenol, 6-n-amyl-m-cresol, resorcinol, resorcinol monoacetate, p-tert-butylphenol and o-benzyl-p-chlorophenol. Useful antimicrobial agents known to be effective in preventing the visible growth of mildew colonies, include, for example, 3-iodo-2-propynyl butylcarbamate, 2-(4-thiazolyl)benzimidazole, diiodomethyl-p-tolylsulfone, tetrachloroisophthalonitrile, the zinc complex of 2-pyridinethiol-1-oxide (including salts thereof) as well as combinations

of the foregoing. The coating composition comprising the antimicrobial agent offers protection against diverse microorganisms.

In one embodiment, the coating composition protects against Gram positive or Gram negative bacteria. Gram positive bacteria which are

5 inhibited or killed by the coating include, but are not limited to, *Clostridium tetani*, *C. perfringens*, *C. botulinum*, other *Clostridium* species, *Mycobacterium tuberculosis*, *M. bovis*, *M. typhimurium*, *M. bovis* strain *BCG*, *BCG* substrains, *M. avium*, *M. intracellulare*, *M. africanum*, *M. kansasii*, *M. marinum*, *M. ulcerans*, *M. avium* subspecies *paratuberculosis*,

10 *Staphylococcus aureus*, *S. epidermidis*, *S. equi*, *Streptococcus pyogenes*, *S. agalactiae*, *Listeria monocytogenes*, *L. ivanovii*, *Bacillus anthracis*, *B. subtilis*, *Nocardia asteroides*, and other *Nocardia* species, *Streptococcus viridans* group, *Peptococcus* species, *Peptostreptococcus* species, *Actinomyces israelii* and other *Actinomyces* species, *Propionibacterium acnes*, and *Enterococcus* species. Gram negative bacteria which are

15 inhibited or killed by the coating include, but are not limited to, *Pseudomonas aeruginosa*, other *Pseudomonas* species, *Campylobacter* species, *Vibrio cholerae*, *Ehrlichia* species, *Actinobacillus pleuropneumoniae*, *Pasteurella haemolytica*, *P. multocida*, other

20 *Pasteurella* species, *Legionella pneumophila*, other *Legionella* species, *Salmonella typhi*, other *Salmonella* species, *Shigella* species, *Brucella abortus*, other *Brucella* species, *Chlamydia trachomatis*, *C. psittaci*, *Coxiella burnetti*, *Neisseria meningitidis*, *N. gonorrhoea*, *Haemophilus influenzae*, *H. ducreyi*, other *Haemophilus* species, *Yersinia pestis*, *Y. enterolitica*, other *Yersinia* species, *Escherichia coli*, *E. hirae* and other *Escherichia* species, as well as other Enterobacteriaceae, *Burkholderia cepacia*, *B. pseudomallei*, *Francisella tularensis*, *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Provetella* species, *Cowdria ruminantium*, *Klebsiella* species, and *Proteus* species.

30 In another embodiment, the coating provides protection against fungi, including but are not limited to *Alternaria alternata*, *Aspergillus niger*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Drechslera*

*australiensis*, *Gliomastix cerealis*, *Monilia grisea*, *Penicillium commune*, *Phoma fimetari*, *Pithomyces chartarum*, and *Scolecobasidium humicola*.

Trace amounts of impurities, especially metals, may react with hydrogen peroxide and peroxyacids and cause decomposition. Therefore, 5 many peroxide/peroxyacid compositions include stabilizing ingredients, such as compounds that sequester metals and metal-containing impurity materials. Examples of preferred chelating agents include, but are not limited to, alkylideneaminophosphonic acids or salts thereof, some of which are marked under the name Dequest® (Thermphos, Vlissingen, the 10 Netherlands), 1-hydroxyethylidene-1,1-diphosphonic acids and salts thereof, some of which are marked under the name Turpinal® (Thermphos), and 2-phosphono-1,2,4-butanetricarboxylic acids and salts thereof, available from LANXESS Corporation (Pittsburgh, PA, USA) under Bayhibit®. A further class of compounds suitable for use is the 15 aminocarboxylic acids or salts thereof. An example is ethylenediaminetetraacetic acid.

In addition, other suitable chelating agents include dipicolinic acid, ethane-1,1,2-triphosphonic acid and ethylidene-1,1-diphosphonic acid. Furthermore, also well known in the art as chelating agents are phosphates, 20 polyphosphates, pyrophosphates, and carboxylic acids, such as citric acid or salicylic acid. The stabilizer or stabilizers should be present in sufficient amounts to inhibit breakdown of the peroxide/peroxyacids.

The present invention can optionally include colorants or dyes. Colorants useful for the present invention include dyes and pigments such 25 as food grade pigments.

Dyes useful for the invention include both water soluble and water insoluble dyes. Water soluble dyes may be formulated easily in the aqueous systems of the invention. Water insoluble dyes may be included in an oil phase that may be dispersed or suspended in the antimicrobial 30 coating compositions useful for the invention. Useful dyes for the purpose of this invention are typically organic compounds that absorb visible light resulting in the appearance of a detectable color. Fluorescent dyes may

also be used, for example, for purposes of visualizing a film by ultraviolet light.

The dyes typically useful in this invention are colorants approved for use in foods, drugs, cosmetics and medical devices. Colorants currently 5 in use and their status is as follows: Colorants permitted in foods that are (1) subject to certification: FD&C Blue No. 1, FD&C Blue No. 2, FD&C Green No. 3, FD&C Red No. 3, FD&C Red No. 40, FD&C Yellow No. 5, FD&C Yellow No. 6, Citrus Red No. 2, and Orange (B) (2) exempt from certification: annatto extract, theta-apo-8'-carotenal, canthaxanthin, 10 caramel, theta-carotene, carrot oil, cochineal extract (carmine), corn endosperm oil, dehydrated beets (beet powder), dried algae meal, ferrous gluconate, fruit juice, grape color extract, grape skin extract, paprika, paprika oleoresin, riboflavin, saffron, synthetic iron oxide, tagetes meal and extract, titanium dioxide, toasted partially defatted cooked cottonseed flour, turmeric, termeric oleoresin, ultramarine blue, and vegetable juice. Colorants permitted in drugs (including colorants permitted in foods) that are 15 (1) subject to certification: FD&C Red No. 4, D&C Blue No. 4, D&C Blue No. 9, D&C Green No. 5, D&C Green No. 6, D&C Green No. 8, D&C Orange No. 4, D&C Orange No. 5, D&C Orange No. 10, D&C Orange 20 No. 11, D&C Red No. 6, D&C Red No. 7, D&C Red No. 17, D&C Red No. 21, D&C Red No. 22, D&C Red No. 27, D&C Red No. 28, D&C Red No. 30, D&C Red No. 31, D&C Red No. 33, D&C Red No. 34, D&C Red No. 36, D&C Red No. 39, D&C Violet No. 2, D&C Yellow No. 7, D&C Yellow No. 8, D&C Yellow No. 10, D&C Yellow No. 11, and Ext. D&C 25 Yellow No. 7. Additionally canthaxanthin, beta carotene, chlorophyllin, and other colors are known. For a more detailed listing and/or discussion on approved colors, see D. M. Marmion, Handbook of U.S. Colorants, Foods, Drugs, Cosmetics and Medical Devices, John Wiley & Sons Inc., New York (1991) and U.S. Code of Federal Regulations, Title 21, parts 70-82.

30 The present invention may optionally include cross-linking agents. Advantages of using cross-linking agents with the film-forming composition include influencing the mechanical film properties, such as tackiness and

mechanical strength, as well as solubility of the coating. Furthermore, cross-linking decreases tackiness and prevents soil and microorganisms from physically adhering to the polymer film, which may be desirable for certain applications. The degree of cross-linking is adjusted so to achieve

5 the desired combination of properties.

Cross-linking agents suitable for use with polyvinyl alcohol and copolymers thereof include, but are not limited to: aldehydes (e.g., formaldehyde, glyoxal, glutaraldehyde), boric acid, sodium tetraborate, metal ions (e.g., ions of Zn, Fe, Al, Ni, V, Co, Cu, Zr, Ti, Mn),

10 organometallic compounds (e.g., organic titanates such as DuPont Tyzor®, organic Cr(III) complexes such as DuPont Quilon®), siloxanes (e.g., tetraethoxysilane, polydimethylsiloxane), isocyanates (e.g., of the blocked, water-soluble or dispersed type), epoxides (e.g., diglycidyl ether), dicarboxylic acid (e.g., oxalic, maleic, fumaric, phthalic), urea based cross-linkers (e.g., Sunrez 700). Bi- and trivalent metal cations (e.g., Fe(II), Fe(III), Al(III)) are preferred because they provide the formation of a coordinative linkage between the PVOH polymer chains upon film drying. This allows the cross-linker to be added to the film-forming liquid in a 'one-pot' mixture. Care must be taken to choose an adequate concentration in

15 order to efficiently cross-link the polymer without precipitating other ingredients such as particulate rheology control agents.

In most cases the cross-linking agent will be mixed with other ingredients using standard mixing techniques. The cross-linking reaction may optionally be carried out in the presence of a catalyst, as is well

20 known to those skilled in the art. In the case of the aldehydes, isocyanates, siloxanes, diglycidyl ether, and dicarboxylic acid, heat and an acid catalyst or metal catalyst may be used additionally.

The cross-linking agent concentration in the formulation may be zero to an upper limit which is either determined by the stability limit of the

25 formulation where precipitation starts to occur, or the inability of the resulting film to be removed efficiently.

The preferred cross-linking agent concentration may depend strongly on the type of cross-linking agent used and is typically below 25 wt% of the polymer content, more preferably below 10 wt% of the polymer content.

5 In addition to the foregoing components, the composition of the present invention may also comprise one or more performance enhancing additives also known as “performance enhancers”. These include flash rust inhibitors, which include any of a number of organic or inorganic materials used in a water-based system to prevent rust from forming on  
10 contact with the material and bare metal. Two examples are sodium benzoate or benzotriazole.

Another optional performance enhancing additive include one or more of an array of defoamers recommended for water-based systems, to prevent unwanted foaming of the product during application. Too much  
15 foam may disrupt the required continuous film formation of the product and result in product failure. It may also be advantageous to add a foam control product, to aid in mixing and processing the masking composition, such as Drewplus L475 from Ashland Chemical, Inc. Drew Industrial Division (Covington, KY, USA). Additional optional performance  
20 enhancing additives are antioxidants to increase the shelf life of the coating formulation. One example is butylated hydroxytoluene. Further additional additives include fragrances.

Foaming agents may additionally be added to create gas bubbles in the applied coating. Gas bubbles may function as an opacifying agent to  
25 facilitate the application and/or to allow for longer contact time with a surface; e.g., by preventing dripping from an inclined surface and/or to reduce the amount of coating formulation needed to treat a certain surface area or volume.

Application indicators may also be added. Some of these are  
30 described above, but include pigments, dyes, fluorescent dyes, pH indicators or gas bubbles generated during application.

Small amounts (typically less than 1 percent by weight) of these additional materials may be added with an appropriate adjustment of the water or other components. It is to be understood that mixtures of any one or more of the foregoing optional components may also be employed.

5 For loci comprised of fibrous substrates, an optional performance-enhancing ingredient is an agent that provides a surface effect. Such surface effects include no iron, easy to iron, shrinkage control, wrinkle free, permanent press, moisture control, softness, strength, anti-slip, antistatic, anti-snag, anti-pill, stain repellency, stain release, soil repellency, soil release, water repellency, oil repellency, odor control, antimicrobial, or sun protection.

10

The film or coating may be applied to the target surface or locus by any means, including pouring. The film or coating is applied to achieve a continuous and/or homogenous layer on a target surface. Coating

15 systems routinely used for paints and coatings, such as, but not limited to, brushes, rollers, paint pads, mats, sponges, combs, hand-operated pump dispensers, compressed air operated spray guns, airless spray guns, electric or electrostatic atomizers, backpack spray application equipment, aerosol spray cans, clothes, papers, feathers, styluses, knives, and other

20 applicator tools may be used for coating. If dipping is used as a method to apply the coating, no special equipment is required. If an aerosol spray can is used for application, the coating composition may be mixed with an aerosol propellant (such as a compressed gas) or the coating composition may be physically separated from the propellant by a barrier material such

25 as a polymer bag inside the can; if the coating composition and the propellant are mixed the mixture may constitute one or more liquid phases.

For fibrous substrates, such as textiles and carpets, the coating may be applied by exhaustion, foam, flex-nip, nip, pad, kiss-roll, beck,

30 skein, winch, liquid injection, overflow flood, roll, brush, roller, spray, dipping, immersion, and the like. The coating may also be applied by use

of the conventional beck dyeing procedure, continuous dyeing procedure or thread-line application.

In one embodiment of the current disclosure, electrostatic sprayers can be used to coat the surface. Electrostatic sprayers impart energy to

5 the aqueous coating composition via a high electrical potential. This energy serves to atomize and charge the aqueous coating composition, creating a spray of fine, charged particles. Electrostatic sprayers are readily available from suppliers such as Tae In Tech Co., South Korea and Spectrum, Houston, TX, USA.

10 In another embodiment of the invention, an airless spray gun may be used to coat the target surface. Airless spray guns use high fluid pressures and special nozzles, rather than compressed air, to convey and atomize the liquid. The liquid is supplied to an airless gun by a fluid pump at pressures typically ranging from 3.5 to 45 MPa. When the paint exits

15 the fluid nozzle at this pressure, it expands slightly and atomizes into tiny droplets without the impingement of atomizing air. The high velocity of the exiting paint propels the droplets toward the target surface. The fluid nozzle on an airless gun differs substantially from the fluid nozzle on an air atomized gun. Selection of the proper nozzle determines how much paint

20 is delivered and the fan pattern of application. The size of the airless nozzle orifice determines the quantity of paint to be sprayed. Airless fluid delivery is high, ranging from 700-2000 mL/min. Recommended gun distance is about 30 cm from the target, and depending upon the nozzle type, a fan pattern of 10 to 45 cm is possible. Thus, nozzles may be

25 selected for each application based on the size and shape of the target surface and the thickness of the coating to be applied. Airless guns create little air turbulence that may repel the liquid from "hard to reach areas", such as would be found in food processing equipment, hatcheries etc. The high flow rate makes airless advantageous in cleaning and

30 disinfecting situations, where the antimicrobial coating is to be applied over a large surface area and multiple surfaces.

The thickness of the applied and dried film will depend on a variety of factors. These factors include the concentration of the film forming agent, the concentration of rheology control additives and/or other additives, as well as the application temperature and humidity. Film thickness and film uniformity also depend, at least in part, on parameters of the application equipment, such as fluid delivery, spray orifice diameter, air pressure or piston pump pressure in the case of airless application, and the distance of the spray applicator to the target surface. Therefore, the liquid formulation may be adjusted to yield the desired film thickness. The atomization of the coating solution is chosen such that a thin film is applied homogeneously to the target area.

Target surfaces (loci) include all surfaces that may potentially be contaminated with microorganisms, including surfaces typically difficult to apply a disinfectant or sanitizer to (such as hard-to-reach surfaces).

Examples of target surfaces include equipment surfaces found in the food or beverage industry (such as tanks, conveyors, floors, drains, coolers, freezers, refrigerators, equipment surfaces, ceilings, walls, valves, belts, pipes, drains, ductwork, joints, crevasses, combinations thereof, and the like); building surfaces, including buildings under construction, new home construction, and surfaces in or on seasonal properties like vacation home surfaces (such as ceilings, walls, wood frames, floors, windows, ductwork), kitchens (sinks, drains, counter-tops, refrigerators, cutting boards), bathrooms (showers, toilets, drains, pipes, ductwork, bath-tubs), (especially for mold removal), decks, wood, siding and other home exteriors, asphalt shingle roofing, patio or stone areas (especially for algae treatment); boats and boating equipment surfaces; garbage disposals, garbage cans and dumpsters or other trash removal equipment and surfaces; non-food-industry related pipes and drains; surfaces found in hospitals; or surfaces where surgery, out-patient, or veterinary services are provided (such as ceilings, walls, floors, ductwork, beds, equipment, clothing worn in hospital/veterinary or other healthcare settings, including scrubs, shoes, and other hospital or veterinary surfaces) first-responder or

other emergency services equipment and clothing; lumber-mill equipment, surfaces and wood products; restaurant surfaces; supermarket, grocery, retail and convenience store equipment and surfaces; deli equipment and surfaces and food preparation surfaces; brewery and bakery surfaces;

5       bathroom surfaces such as sinks, showers, counters, and toilets; clothes and shoes; toys; school and gymnasium equipment, ceilings, walls, floors, windows, ductwork and other surfaces; kitchen surfaces such as sinks, counters, appliances; wooden or composite decks, pool, hot tub and spa surfaces; carpet; paper; leather; animal carcasses, fur and hides; surfaces

10      of barns, or stables for livestock, such as poultry, cattle, dairy cows, goats, horses and pigs; and hatcheries for poultry or for shrimp. Surfaces within structures wherein animals are housed, such as cages and pens for example, can be coated using the antimicrobial coatings described herein. Additional surfaces also include food products, such as beef, poultry, pork,

15      vegetables, fruits, seafood, combinations thereof, and the like.

Additional loci suitable for use in the present invention comprise fibrous surface substrates and include fibers, yarns, fabrics, textiles, nonwovens, carpets, leather, or paper. The fibrous substrates are made with natural fibers such as wool, cotton, jute, sisal, sea grass, paper, coir and cellulose, or mixtures thereof; or are made with synthetic fibers such as polyamides, polyesters, polyolefins, polyaramids, acrylics and blends thereof; or blends of at least one natural fiber and at least one synthetic fiber. By "fabrics" is meant natural or synthetic fabrics, or blends thereof, composed of fibers such as cotton, rayon, silk, wool, polyester,

20      polypropylene, polyolefins, nylon, and aramids such as "NOMEX®" and "KEVLAR®." By "fabric blends" is meant fabric made of two or more types of fibers. Typically these blends are a combination of at least one natural fiber and at least one synthetic fiber, but also may be a blend of two or more natural fibers or of two or more synthetic fibers. Nonwoven

25      substrates include, for example, spunlaced nonwovens, such as SONTARA available from E. I. du Pont de Nemours and Company

(Wilmington, DE, USA), and laminated nonwovens, such as spunbonded-meltblown-spunbonded nonwovens.

Examples of surface materials are metals (e.g., steel, stainless steel, chrome, titanium, iron, copper, brass, aluminum, and alloys thereof),

5 minerals (e.g., concrete), natural or synthetic 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

10 nylon). Additional surfaces include brick, tile, ceramic, porcelain, glass, wood, vinyl, and linoleum.

Equipment or surfaces protected with a temporary coating may be in use or not in use while protected. The target surface may be hydrophobic or hydrophilic.

15 The coating system may also be one or more components, and may include a catalyst. Generally, the coating is allowed to set or dry for about greater than 5 minutes in order to form the film. However, the coating may be antimicrobially effective in a shorter time-frame, such as after 30 seconds. The coating may be removed before it is dried or

20 anytime thereafter depending on the desired use. The drying time will be partially dependent on a number of factors, including environmental conditions such as humidity and temperature. The drying time will also depend on the thickness of the applied coating.

The thickness of the film or coating applied onto the target surface

25 influences the time needed for removal and the amount of biocide per unit area applied to the surface. Thicker films increase the time interval until the film has to be re-applied to maintain the desired antimicrobial properties. Thinner films will be easier and faster to remove by rinsing. It is thus important to apply the formulation in a fashion that results in a film

30 thickness that allows both easy removal of the coating and long-lasting antimicrobial properties. As described above, the film or coating has a thickness of about 0.3 to about 300 micrometers. In a more specific

embodiment, the film or coating has a thickness of about 0.5 to about 100 micrometers. In an even more specific embodiment, the film or coating has a thickness of about 1.0 to about 30 micrometers.

This invention is directed toward films that may be removed at a

5 time determined appropriate by the user. The coatings are removed with ease and may be removed by rinsing the surface with an aqueous solvent or solution. The time of removal may be determined by either (i) the desired minimum contact time to allow for the desired antimicrobial activity, typically expressed as amount of killed or inactivated

10 microorganisms out of a starting population or (ii) the need or desire to take the coating off the surface before starting a subsequent operation or process step. Although the coating may be removed at any time, such as after drying, the film thickness, concentration of antimicrobial agent, and specific use determines the appropriate time for removal. For instance the

15 user may wish to put treated equipment back into normal operation after a period of operational shutdown. Fruits, for example, will require washing prior to eating. Upon exhaustion of the biocide in the film, the film could be removed and a fresh coating layer could be applied. For example, drains may be treated periodically such as daily, weekly or biweekly.

20 Antimicrobial activity may be measured as early as after 30 seconds, hours, days, weeks, months, even years after application of the film. Therefore, timing of removing the coating is a function of the application for which the coating is employed.

Film removal may be achieved by dissolution or dispersion of the

25 resulting coating. This may be achieved by the application of an aqueous solution onto the coating. In one embodiment, the temperature of the solution is in the range of about 15 °C to about 100 °C. In another embodiment, the temperature of the solution is from about 30 to about 80 °C. The application of the solution, or water, may be achieved by a

30 simple rinse or spray onto the surface. Coating removal may also be achieved by use of a pressure washer, facilitating removal by additional mechanical forces. Coating removal may also be achieved by washing

with water together with a cloth or sponge. Further, mild additives may be utilized or mixed with the aqueous solution to help solubilize or disperse the film-forming or water-dispersible agents, including commonly used acids or bases, chelators or detergents. Alternatively, the film may be 5 degraded, such as in a drain, by repeated washing of water and/or other components down the drain. The film may also be removed by peeling it off a surface, being abraded or brushed from the surface, or other mechanical mechanisms of removal.

Besides the intentional removal by an operator, removal also 10 includes the removal by an automated or robotic system and the non-intentional removal by a liquid continuously or periodically contacting the coating over time, e.g., in a pipe or drain, or by continuous or periodical application of mechanical forces, such as wear.

Contact time refers to the time the coating or coating composition 15 provides antimicrobial properties to microorganisms that come into contact or the vicinity of said coating or coating composition. Depending on the specific requirements for the antimicrobial formulations, the contact time would vary, as set out in Germicidal and Detergent Sanitizing Action of Disinfectants, Official Methods of Analysis of the Association of Official Analytical Chemists, paragraph 960.09 and applicable sections, 15th Edition, 1990 (EPA Guideline 91-2). For example, if the intended application of the present disclosure is use as a sanitizer for food-contact surfaces, then the composition should provide a 99.999% reduction (5-log order reduction) within 30 seconds at room temperature against several 20 test microorganisms. If the intended application is as a sanitizer for non-food contact surfaces, then the composition should provide a 99.9% reduction (3-log order reduction) within 5 minutes at room temperature against several test microorganisms. If the intention is to use the disclosure as a disinfectant, then the composition should provide a 99.9% 25 reduction (3-log order reduction) within 10 minutes. If the intended application is to provide residual antimicrobial activity, then the present 30

method would be allowed to have greater than 10 minute contact time with microorganisms.

The coating of the present invention provides a physical barrier. A physical barrier is defined herein as the film formed from the present film forming composition. The resulting film seals the treated surface from contamination from the surrounding, such as soil, fat, dust, microorganisms etc. These contaminants will remain on the surface of the coating and will wash off at the time of removal of the coating.

All of the methods and compositions disclosed and claimed herein may be made and executed without undue experimentation in light of the present disclosure. While the methods and compositions of the present disclosure have been described in terms of various aspects of the invention and preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit, and scope of the invention. More specifically, it will be apparent that certain agents, which are chemically related, may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

### GENERAL METHODS

#### Recombinant techniques and growth of microorganisms

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984, and by Ausubel, F. M. et al., Current Protocols

*in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience, N.Y., 1987.

Materials and methods suitable for the maintenance and growth of bacterial cultures are also well known in the art. Techniques suitable for 5 use in the following Examples may be found in *Manual of Methods for General Bacteriology*, Phillip Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds., American Society for Microbiology, Washington, DC, USA, 1994, or by Thomas D. Brock in *Biotechnology: A Textbook of Industrial* 10 *Microbiology*, Second Edition, Sinauer Associates, Inc., Sunderland, MA, USA, 1989.

All reagents, restriction enzymes and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI, USA), BD Diagnostic Systems (Sparks, MD, USA), Life 15 Technologies (Rockville, MD, USA), or Sigma Chemical Company (St. Louis, MO, USA), unless otherwise specified.

#### SDS Gel electrophoresis

SDS gel electrophoresis was performed using methods well known in the art.

#### 20 Test methods for antimicrobial activity on hard surfaces

Peracids may 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, 25 hypochlorites, ozone, amines, chlorinated phenolics, copper salts, organo-sulfur compounds, and quaternary ammonium salts. Biocidal or antimicrobial efficacy of the coating compositions was measured using the two test methods described below.

Non-food contact sanitizer test: To assess the antimicrobial activity of coating compositions for a situation where a microbial contamination 30 was already present on the target surface at the time of the application of the antimicrobial coating composition the "Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact

Surfaces" according to ASTM standard E1153–03 was used. The test method is referred to as non-food contact sanitizer test or the "NFC test".

*Residual self-sanitizing test:* To assess the antimicrobial activity of coating compositions for a situation where microbial contamination comes 5 into contact with the already dry coating, the following residual self-sanitizing test method was used. The test method is referred to as the residual self-sanitizing test or the "RSS test". Non-porous, pre-cleaned, stainless steel (type SS316) coupons of 25.4 x 25.4 mm in size were used for the test. From a stock plate (transferred three times consecutively but 10 no more than 30 transfers) of the organism, a colony was selected and placed into 10 mL of AOAC Nutrient Broth (NaCl 2.5 g; Beef Extract 2.5 g; Anatone 5 g; Deionized water 500 mL). The inoculated culture was incubated under static conditions for 24 hr at 35°C. In preparation of the test inoculum, the static culture was agitated vigorously using a Vortex 15 mixer, allowed to stand for 15 min, and the upper two-thirds of the culture was transferred to a sterile tube (approximately 6 mL). An organic soil load of fetal bovine serum (FBS) was added for a final concentration of 5 wt% organic soil load to this test inoculum. The final test inoculum density was approximately  $1 \times 10^8$  CFU/mL.

20 The test coupons were placed in 70 wt% ethanol overnight, soaked in a mild detergent for at least 30 min, rinsed with tap water thoroughly and allowed to air dry. All handling of surfaces, once cleaned, was done using sterile forceps. Coupons were sprayed with 70 wt% ethanol and allowed to dry completely. An aliquot (50  $\mu$ L) of the coating composition to 25 be tested was applied to each stainless steel coupon, spread evenly with a sterile plastic spreader, placed in a sterile plastic petri dish and allowed to air dry overnight in the biological safety cabinet. Ambient air temperature and relative humidity were recorded. Uncoated coupons were used as control surfaces which were handled under the same conditions as the 30 coupons treated with the coating compositions.

Coupons were inoculated by spotting 0.01 mL of the inoculum using at least 30 spots over the surface of the coated coupon. At least two

replicate coupons were inoculated per coating composition. After 5 min, the test coupons were aseptically transferred to 20 mL of Bacto™ D/E neutralizing broth. The tubes were shaken vigorously by hand, sonicated for 10 s in a sonicating water bath at the maximum setting, and finally

5 shaken for 4 min on a rotary shaker at 250 rpm. The cell density of the culture was determined using a serial-dilution spread plate technique with Butterfield Phosphate Buffer dilution tubes and Trypticase™ Soy Agar (TSA) petri plates. All samples were enumerated within approximately 30 min of their transfer to the D/E Neutralizing Broth.

10 Average cell densities (CFU/mL) for replicate measurements for both treated and untreated coupons were calculated as the geometric mean of the individual CFU measurements. All log numbers are base-10 logarithms.

#### EXAMPLES

15 The following examples are provided to demonstrate preferred aspects of the invention. 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

20 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.

#### Abbreviations used in the Examples

25 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), "mm" means millimeter; "µL" means microliters, "mL" means milliliters, "L" means liters, "mM" means millimol per liter, "M" means mol per liter,

30 "mmol" means millimole(s), "°C" means degrees Celsius; "ppm" means parts per million and refers to mg/L (milligrams per liter) in the following examples, "wt" means weight, "wt%" indicates percent by weight, "g"

means grams, “ $\mu\text{g}$ ” means micrograms, “g” means earth gravitational constant, “HPLC” means high performance liquid chromatography, “dd  $\text{H}_2\text{O}$ ” means distilled and deionized water, “DI” means deionized, “dcw” means dry cell weight, “ATCC” or “ATCC®” means the American Type 5 Culture Collection (Manassas, VA, USA), “U” means units of perhydrolase activity, “U/mg protein” means unit of perhydrolase activity per milligram protein, “rpm” means revolutions per minute, “EDTA” means ethylenediaminetetraacetic acid, “PAA” means peracetic acid, “PEG” means polyethylene glycol, “Pa·s” means pascal seconds; “IPTG” means 10 isopropyl-beta-D-thiogalactopyranoside; “MPa” means megapascals; “NMWCO” indicates nominal molecular weight cut-off, “LB” means Luria broth medium, “OD” means optical density, “g feed/min” means gram feed per minute, “SDS” means sodium dodecyl sulfate, “o-DA” means ortho-dianisidine, “pNPA” means *para*-nitrophenyl acetate, “ $\text{L}\cdot\text{cm}^{-1}\cdot\text{mol}^{-1}$ ” means 15 liter per centimeter per mole, “mg/mL” means milligram per milliliter, “kg” means kilogram, “U/mg” means units per milligram; “CFU” means colony forming unit; “FBS” means Fetal bovine serum; “log CFU” means the base 10 logarithm of the CFU number; “CFU/mL” means CFU per milliliter; “NFC” means Non-food contact sanitizer test; “RSS” means residual self- 20 sanitizing activity; “SS316” means stainless steel, type 316 (ASTM standard); “TAED” means tetraacetylethylene diamine.

### Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Elvanol® 51-04 (partially hydrolyzed grade 25 polyvinyl alcohol, 88% grade) was from DuPont (Wilmington, DE, USA). Polyethylene glycol of an average molecular weight of 300 g/mol (Carbowax™ PEG-300) was from Dow (Midland, MI, USA). BTC® 885, Onyxide® 3300 and Biosoft® N25-7 were from Stepan (Northfield, IL, USA). BTC® 885 contains 50 wt% quaternary ammonium compounds as 30 antimicrobial agents and 50 wt% inert materials. Rheovis® FRC was obtained from Ciba (Basel, Switzerland). Glucopon® 215 UP was obtained from Cognis Corporation (Cincinnati, OH). PC 5450 NF was

from Performance Chemicals, LLC (Concord, NH). Tetraacetylenediamine (TAED B675) was from Warwick International Ltd. (Flintshire, UK). Sodium percarbonate (Provox® C) was from OCI Chemical Corp. (Decatur, AL, USA). Turpinal® SL was obtained from 5 Thermphos (Vlissingen, the Netherlands). Silwet® L-77 was obtained from Setre Chemical Company (Memphis, TN, USA). Microbial growth media were obtained from DIFCO Laboratories (Detroit, MI, USA), GIBCO/BRL (Gaithersburg, MD, USA), TCI America (Portland, OR, USA), Roche Diagnostics Corporation (Indianapolis, IN, USA) or Sigma-Aldrich 10 Chemical Company (St. Louis, MO, USA). Bacto™ D/E neutralizing broth was from Difco (Cat. No. 281910, Difco™ Laboratories, Detroit, MI, USA).

pNPA microplate assay

Into each of four wells of a 96-well microplate were added 170  $\mu$ L of potassium phosphate buffer (50 mM, pH 7.2) and either 10  $\mu$ L of 0.1 mg 15 total protein/mL of cell lysate, or 10  $\mu$ L of 0.02 mg total protein/mL of a spray-dried enzyme solution. The plate was covered and placed in a microplate reader (Molecular Devices SPECTRAmax® 384 Plus), then mixed for several seconds and equilibrated at 30 °C for 10 minutes. To each well of the plate was then simultaneously added 20  $\mu$ L of a 30 mM 20 pNPA solution prepared by mixing (immediately prior to use) 0.6 mL of 100 mM pNPA in acetonitrile with 1.4 mL of 50 mM potassium phosphate buffer (pH 7.2). The change in absorbance at 400 nm was measured over 1 minute of reaction time. The background rate of pNPA hydrolysis 25 (subtracted from the rate of hydrolysis measured in the presence of enzyme) was separately measured in four wells by substituting 10  $\mu$ L/well of potassium phosphate buffer (50 mM, pH 7.2) for cell lysate or enzyme-containing solution. The extinction coefficient for para-nitrophenol, measured at pH 7.2 in a 1-cm path length cell, was 10,909 L·cm<sup>-1</sup>·mol<sup>-1</sup>. One pNPA Unit of enzyme activity is equivalent to the amount of protein 30 that catalyzes the hydrolysis of one micromole of pNPA in one minute.

Determination of rheological properties

The rheological properties of the liquid antimicrobial formulations were assessed using a Brookfield Digital Viscometer Model DV-II (Brookfield Engineering Laboratories, Middleboro, MA, USA) with RV 5 Series spindle #6 and a tall glass beaker. Samples were loaded by pouring into the glass beaker. Viscosity measurements were taken at different rpm.

High performance chromatography (HPLC)

The concentration of PAA in the reaction mixtures was determined 10 using HPLC. A Supelco Discovery C8 column (15 cm x 4.0 mm, 5  $\mu$ m) with precolumn Supelco Superguard Discovery C8 was used. The injection volume was 10  $\mu$ L. The gradient method with acetonitrile and deionized water at 1.0 mL/min and ambient temperature is shown in the Table below.

15

Time (min:sec)	% CH <sub>3</sub> CN
0:00	41
3:00	41
3:10	100
6:00	100
6:10	41
10:00 (stop)	41

Retention times of compounds of interest, using this HPLC method, were: MTSO (methyl p-tolyl sulfoxide) = 2.34 min; DEET (N,N-diethyl-m-toluamide) = 4.40 min; TPPO = (triphenylphosphine oxide) = 5.02 min; 20 MTS = (methyl p-tolyl sulfide) = 5.99 min and TPP = (triphenylphosphine) = 6.48 min.

EXAMPLE 1CLONING AND EXPRESSION OF ACETYL XYLAN ESTERASE FROM  
*THERMOTOGA NEAPOLITANA*

A coding region encoding an acetyl xylan esterase from

5    *Thermotoga neapolitana* (GENBANK® accession # AAB70869, SEQ ID NO:7) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA, USA). The coding region 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:18 and SEQ ID NO:19.

10   The resulting nucleic acid product (SEQ ID NO:20) was subcloned into pTrcHis2-TOPO® (Invitrogen, Carlsbad, CA, USA) to generate the plasmid identified as pSW196. The plasmid pSW196 was used to transform *E. coli* KLP18 to generate the strain identified as KLP18/pSW196 (See Published U.S. Patent Application

15   No. 2008/0176299 incorporated herein by reference in its entirety). KLP18/pSW196 was grown in LB medium at 37 °C with shaking up to an OD<sub>600nm</sub> of 0.4-0.5, at which time isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation continued for 2-3 h. Cells were harvested by centrifugation and stored in

20   20 wt% glycerol at -80 °C; the expression of perhydrolase at 20-40% of total soluble protein was determined using SDS-PAGE.

EXAMPLE 2FERMENTATION OF *E. COLI* KLP18/PSW196 EXPRESSING *T.  
NEAPOLITANA* PERHYDROLASE

25   A fermentation was performed in three stages: pre-seed preparation in a flask, seed preparation in a 14-L fermenter, and scale-up in a 200-L fermenter. The seed culture was prepared by charging a 2-L shake flask with 0.5 L seed medium containing yeast extract (Difco, 1.0 g/L), K<sub>2</sub>HPO<sub>4</sub> (10.0 g/L), KH<sub>2</sub>PO<sub>4</sub> (7.0 g/L), sodium citrate dihydrate (1.0 g/L), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (4.0 g/L), MgSO<sub>4</sub> 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/pSW196 in 20% glycerol, and cultivated at 35 °C and 300 rpm.

5        The seed culture was transferred to a 14-L seed fermenter (Braun, B. Braun Biotech Inc., Allentown, PA, USA) at an optical density at 550 nm of about 1-2 with 8 L of medium containing  $\text{KH}_2\text{PO}_4$  (5.0 g/L),  $\text{FeSO}_4$  heptahydrate (0.05 g/L),  $\text{MgSO}_4$  heptahydrate (1.0 g/L), sodium citrate dihydrate (1.90 g/L), Biospumex153K antifoam (0.5 mL/L, Cognis Corporation),  $\text{NaCl}$  (1.0 g/L),  $\text{CaCl}_2$  dihydrate (0.1 g/L), and a trace elements solution (10 mL/L) containing citric acid monohydrate (10 g/L),  $\text{MnSO}_4$  hydrate (2 g/L),  $\text{NaCl}$  (2 g/L),  $\text{FeSO}_4$  heptahydrate (0.5 g/L),  $\text{ZnSO}_4$  heptahydrate (0.2 g/L),  $\text{CuSO}_4$  pentahydrate (0.02 g/L) and  $\text{NaMoO}_4$  dihydrate (0.02 g/L). Post sterilization additions included glucose solution (50 wt%, 80.0 g), ampicillin (25 mg/mL) stock solution (16.00 mL) and thiamine (3.3 g/L) stock solution (6.00 mL). 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 L/min at standard conditions). The temperature was controlled at 35 °C. The pH was controlled at 6.8.  $\text{NH}_4\text{OH}$  (29 wt%) and  $\text{H}_2\text{SO}_4$  (200 g/L) were used for pH control. The overhead pressure was maintained at 0.50 bar. When the culture density reached an  $\text{OD}_{550}$  nm of more than 5 or when glucose concentration in the broth decreased to less than 1 g/L, the 8 L culture was transferred to the 200 L Sartorius Biostet-  
20      DCU-D production fermenter.

25      The initial volume of the medium charged to the 200-L Sartorius Biostet-DCU-D production fermenter (Sartorius Stedium North America Inc., Bohemia, NY, USA) was 150 L, where the medium was prepared using the same ingredients and concentrations as in the 14-L seed fermenter. Post sterilization additions included glucose- $\text{MgSO}_4$  solution (glucose 50 wt% and  $\text{MgSO}_4$  heptahydrate 1 wt%; 1600 g), ampicillin (25 g/L) stock solution (3200 mL) and thiamine (3.3 g/L) stock solution (120

mL). The dissolved oxygen (DO) concentration was controlled at 25% of air saturation. The DO was controlled first by impeller agitation rate (200 to 450 rpm) and later by aeration rate (35 to 80 L/min at standard conditions). The temperature was controlled at 35 °C. The pH was 5 controlled at 6.8. NH<sub>4</sub>OH (29 wt%) and H<sub>2</sub>SO<sub>4</sub> (200 g/L) were used for pH control. The overhead pressure was maintained at 0.50 bar. A glucose-MgSO<sub>4</sub> solution (glucose (50 wt%) and MgSO<sub>4</sub> heptahydrate (1 wt%) was used for fed batch growth. Glucose feed was initiated when glucose concentration decreased to 0.5 g/L, starting at a rate of 6.30 g feed/min 10 and increasing progressively each hour to 7.30, 8.50, 9.8, 11.40, 13.30, 15.40, 17.2 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. Induction was initiated at an OD<sub>550</sub> nm of 75 (at 26.7 h) with 15 addition of 180 mL IPTG (0.5 M). The cells were harvested by centrifugation 16 h post IPTG addition. The fermentation was terminated at 42.7 h, and about 21 kg wet cells paste was harvested having perhydrolase specific activity of 3.4 pNPA U/mg cells dry weight.

### EXAMPLE 3

#### 20 PREPARATION OF *T. NEAPOLITANA* PERHYDROLASE

Cell paste (13 kg; *E. coli* KLP18/pSW196, prepared as described in Example 2) was suspended in 40 L of 50 mM sodium phosphate buffer at pH 7.4, the cell suspension homogenized using a APV 16.56 homogenizer (APV Fluid Handling and Homogenizers, Delavan, WI, USA) operating at 25 83 MPa and room temperature, and the resulting homogenate heated to 65 °C and held at this temperature for 30 minutes prior to cooling to room temperature. A 0.1 micrometer hollow-fiber membrane cartridge (GE Healthcare, Little Chalfont, United Kingdom) was used to separate the solids from the protein solution. The clarified protein solution was then 30 concentrated using a 30,000 NMWCO hollow-fiber membrane cartridge (GE Healthcare) to obtain 14.7 L of final concentrate which was frozen at –80 °C. This material was subsequently thawed and further concentrated

(30,000 NMWCO) to give 7.5 liters of solution containing approximately 256 grams of protein (34.15 g protein /L; determined using a Micro-BCA protein assay (Sigma Life Sciences, Sigma-Aldrich Corp., St. Louis, MO, USA). To this solution was added 500 g Maltrin M100 maltodextrin, 110 g

5 Maltrin M250 corn syrup solids, and 110 g Maltrin M040 maltodextrin (all from Grain Processing Corporation (GPC), Muscatine, IA, USA).

The resulting mixture (ca. 7.9 kg) was spray dried (inlet temperature = 225 °C, exit temperature = 76 °C, feed rate = 60 g/min) to produce a total of 786 grams of solids (68% yield) of enzyme powder: 93.2 % dry

10 weight, 20.3 wt% protein, 34.5 pNPA U/mg protein.

EXAMPLE 4

IN SITU GENERATION OF PERACETIC ACID IN THE  
PRESENCE OF POLYVINYL ALCOHOL WITH AN ENZYME HAVING  
PERHYDROLASE ACTIVITY

5        This Example illustrates the *in situ* generation of peracetic acid with an enzyme having perhydrolase activity in the presence of polyvinyl alcohol. The reaction was performed using a perhydrolase enzyme isolated from *T. neapolitana*. Into a 75 mL glass bottle equipped with a magnetic stir bar, was placed 12.3 mL of 0.2 M sodium bicarbonate buffer (pH 7.2; 44 mM), 36.2 mL of DI water, 41.7 mg of Turpinal® SL (Thermphos, Vlissingen, the Netherlands), 0.95 mL of triacetin (90 mM) and 6.0 g of Elvanol® 51-04 (20% aqueous solution, DuPont, Wilmington, DE, USA) and stirred at room temperature until the sample was homogeneous. At this time, 0.51 mL of 30 wt% hydrogen peroxide (89 mM) was added, followed immediately by 125  $\mu$ L of 20 mg/mL *T. neapolitana* perhydrolase enzyme in 50 mM sodium bicarbonate buffer (pH 7.2). The hydrogen peroxide and peracetic acid concentrations were monitored over time using the cerium sulfate/sodium thiosulfate titration analysis (Greenspan, F. P.; MacKeller, D. G. *Anal. Chem.*, 20: 1061-1063, 1948). Control reactions were performed with (12.3 mL 0.2 M sodium bicarbonate buffer, pH 7.2, 50 mM; 0.51 mL 30% hydrogen peroxide, 100 mM; 36.2 mL DI water; 41.7 mg Turpinal® SL; 0.95 mL triacetin, 0.1 M; 125  $\mu$ L of 20 mg/mL of *T. neapolitana* perhydrolase enzyme (based on wt% protein in enzyme powder) in 50 mM sodium bicarbonate buffer, pH 7.2) and without added enzyme (identical to above control conditions without enzyme). Over a 30 minute time period, the control reaction performed with triacetin and hydrogen peroxide with no added enzyme produced 114 ppm peracetic acid from the chemical reaction of triacetin and hydrogen peroxide. The control reaction performed with triacetin and hydrogen peroxide with added enzyme produced 380 ppm peracetic acid within 1 minute and increased over 30 minutes to 2280 ppm. The *in situ* enzymatic production of PAA in the presence of polyvinyl alcohol

produced 456 ppm within 1 minute and increased to 1976 ppm over 30 minutes. Table 1 shows the concentrations of PAA produced from *in situ* enzymatic production of PAA in the presence of a film-former.

**TABLE 1**

Enzymatic production of PAA in the presence of polyvinyl alcohol

	1 min (ppm)	5 min (ppm)	30 min (ppm)
Control (no enzyme)	114	114	114
Control (with enzyme)	380	1178	2280
Elvanol® 51-04	456	798	1976

5

**EXAMPLE 5**

**IN SITU GENERATION OF PERACETIC ACID IN A FILM-FORMING COMPOSITION WITH AN ENZYME HAVING PERHYDROLASE ACTIVITY**

10 This example illustrates the *in situ* generation of peracetic acid with an enzyme having perhydrolase activity in the presence of a film-forming composition with additional performance-enhancing additives. The reaction was performed using the perhydrolase enzyme from *T. neapolitana*. Into a 75 mL glass bottle equipped with a magnetic stir bar, 15 was placed 12.3 mL of 0.2 M sodium bicarbonate buffer (pH 7.2; 44 mM), 36.2 mL of deionized water, 41.7 mg of Turpinal® SL (Thermphos, Vlissingen, the Netherlands), 0.95 mL of triacetin (89 mM), 6.0 g of Elvanol® 51-04 (20 wt% aqueous solution, DuPont, Wilmington, DE, USA), 0.5 g of PEG-300 (Dow, Midland, MI, USA) and 0.25 g Silwet® L-77 20 (Setre Chemical Company, Memphis, TN, USA) and stirred at room temperature until the sample was homogeneous. At this time, 0.51 mL of 30% hydrogen peroxide (88 mM) was added, followed immediately by 125 µL of 20 mg/mL of *T. neapolitana* perhydrolase enzyme (based on wt % protein in enzyme powder) in 50 mM sodium bicarbonate buffer (pH 7.2). 25 The hydrogen peroxide and peracetic acid concentrations were monitored over time using the cerium sulfate/sodium thiosulfate titration test

(Greenspan, F. P.; MacKeller, D. G. *Anal. Chem.*, 20: 1061-1063, 1948). Control reactions were performed with (12.3 mL 0.2 M sodium bicarbonate buffer, pH 7.2, 50 mM; 0.51 mL 30% hydrogen peroxide, 100 mM; 36.2 mL DI (deionized water) water; 41.7 mg Turpinal® SL; 0.95 mL triacetin, 0.1 M; 125 µL of 20 mg/mL of *T. neapolitana* perhydrolase enzyme (based on wt % protein in enzyme powder) in 50 mM sodium bicarbonate buffer, pH 7.2) and without added enzyme (identical to above control conditions without enzyme). Over a 30 minute time period, the control reaction performed with triacetin and hydrogen peroxide with no added enzyme 5 produced 114 ppm peracetic acid from the chemical reaction of triacetin and hydrogen peroxide. The control reaction performed with triacetin and hydrogen peroxide with added enzyme produced 380 ppm peracetic acid 10 within 1 minute and increased over 30 minutes to 2280 ppm. The *in situ* enzymatic production of PAA in a film-forming composition produced 342 15 ppm within 1 minute and increased to 1900 ppm over 30 minutes. Table 2 shows the concentrations of PAA produced from *in situ* enzymatic production of PAA in the presence of a film-forming composition with additional performance-enhancing additives.

**TABLE 2**  
Enzymatic production of PAA in a film-forming composition

	1 min (ppm)	5 min (ppm)	30 min (ppm)
Control (no enzyme)	114	114	114
Control (with enzyme)	380	1178	2280
Elvanol® 51-04/PEG-300/Silwet® L-77	342	1102	1900

20

EXAMPLE 6

IN SITU ENZYMATIC GENERATION OF PERACETIC ACID IN A FILM-FORMING COMPOSITION CONTAINING A SECOND BIOCIDE AND A RHEOLOGY MODIFIER

This example illustrates the *in situ* generation of peracetic acid with 25 an enzyme having perhydrolase activity in the presence of a film-forming

composition that contains a rheology modifier and a second biocide. Into a 100 mL glass bottle equipped with a stir bar, was placed 25 g of a 20 wt% aqueous solution of Elvanol® 51-04, 14 g of deionized water, 0.8 g of Rheovis® FRC, 0.5 g of PEG 300 and 0.15 g BTC® 885 and stirred at 5 room temperature until the sample was homogeneous. At this time, 1.09 g of triacetin (100 mM) and 0.67 g of 30 wt% hydrogen peroxide (100 mM) were added and stirred to mix. The pH was adjusted to above 7.0 with addition of 8 g of 0.2 M sodium bicarbonate buffer (32 mM). Following the pH adjustment, 125 µL of *T. neapolitana* perhydrolase enzyme (20 mg/mL 10 solution (based on wt % protein in enzyme powder) in 50 mM sodium bicarbonate buffer) was added and the timer started. A control reaction was performed identical to the above conditions, except without enzyme. The peracetic acid concentrations were monitored over time at 5 minutes, 30 minutes and 1 hour using the cerium sulfate/sodium thiosulfate titration 15 test. Over a 1 hour time period, the control reaction with no added enzyme produced 487 ppm peracetic acid from the chemical reaction of triacetin and hydrogen peroxide. The *in situ* enzymatic production of PAA in a film-forming composition produced 2112 ppm within 5 minutes and increased to 2593 ppm over 1 hour. Table 3 shows the concentrations of 20 enzymatically generated PAA in a film-forming composition.

**TABLE 3**

PAA produced enzymatically in a film-forming composition containing a second biocide and a rheology modifier

	5 min (ppm)	30 min (ppm)	60 min (ppm)
No enzyme	206	258	487
<i>T. neapolitana</i>	2112	2446	2593

EXAMPLE 7SHEAR-THINNING INDEX OF A REMOVABLE ANTIMICROBIAL  
COATING COMPOSITION

The "pseudoplastic index" or "shear thinning index" (STI) 5 provides an indication as to resistance of the composition to sagging and dripping. A common measurement determines the viscosity at two different shear rates. The value recorded at the lower shear rate is divided by the value at the higher shear rate obtain the STI. Generally, the higher the STI, the higher the resistance to sagging and dripping the coating 10 material will have. The STI values as used in herein were determined by measuring the viscosities 30 minutes after addition of the enzyme. The viscosities were measured at room temperature using a Brookfield Digital Viscometer Model DV-II (Brookfield Engineering Laboratories, Middleboro, MA, USA) with RV Series spindle #6. The shear-thinning index (STI) was 15 calculated by dividing the viscosity measured at 1 rpm by the viscosity measured at 10 rpm. The STI for the inventive coating composition A (see Table 4 for composition) is given in Table 5. At 1 rpm the viscosity of film-forming composition A was 12.8 Pa·s and decreased to 4.86 Pa·s at 10 rpm. The shear-thinning index for this coating composition is 2.74.

**TABLE 4**

Coating composition A for enzymatic peracid production

Ingredient	wt%
Elvanol® 51-04 (20 wt% solution)	50
DI water	26.94
Sodium bicarbonate (0.2 M)	16
Tricetin	2.18
Rheovis® FRC	2.2
Hydrogen peroxide (30 wt%)	1.13
PEG-300	1.0
BTC® 885	0.3
<i>T. neapolitana</i> (20 mg/mL in 50 mM NaHCO <sub>3</sub> )	0.25

**TABLE 5**

5

Viscosities and shear-thinning index

Coating composition	Temperature (°C)	Viscosity at		STI
		1 rpm (Pa·s)	10 rpm (Pa·s)	
<i>A (T. neapolitana)</i>	25	12.8	4.68	2.74

**EXAMPLE 8****CLONING AND EXPRESSION OF ACETYL XYLAN ESTERASE FROM *THERMOTOGA MARITIMA***

10 A gene encoding acetyl xylan esterase from *T. maritima* MSB8 as reported in GENBANK® (accession # NP\_227893.1, SEQ ID NO: 8) was synthesized (DNA 2.0, Menlo Park, CA, USA). 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: 22 and SEQ

15 ID NO: 23. The resulting nucleic acid product (SEQ ID NO: 24) 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 pSW207. The codon optimized version of *T. maritima* MSB8 acetyl xylan esterase for expression in *E. coli* was synthesized and subsequently amplified by PCR (0.5 min at 94 °C, 0.5 min at 55 °C, 1 min at 70 °C, 30 cycles) using 5 primers identified as SEQ ID NOs:25 and 26. The resulting nucleic acid product (SEQ ID NO: 27) was cut with restriction enzymes *Eco*RI and *Pst*I and subcloned between the *Eco*RI and *Pst*I sites in pTrc99A (GENBANK® Accession no. M22744) to generate the plasmid identified as pSW228. The plasmids pSW207 and pSW228 were used to transform *E. coli* KLP18 10 (U.S. Patent Application Pub. 2008/0176299 herein incorporated by reference) to generate the strain identified as KLP18/pSW207 and KLP18/pSW228, respectively. KLP18/pSW207 and KLP18/pSW228 were grown in the LB medium at 37 °C with shaking up to OD<sub>600nm</sub> 0.4-0.5, at which time IPTG was added to a final concentration of 1mM, and 15 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

##### CONSTRUCTION OF THERMOTOGA MARITIMA ACETYL XYLAN

##### ESTERASE MUTATION AT RESIDUE C277

The C277 (Cys277) position of *T. maritima* acetyl xylan esterase was changed to Ser using an oligonucleotide primer pair (SEQ ID NOs: 28 and 29) that was designed based on the codon optimized sequence of *T. maritima* acetyl xylan esterase (SEQ ID NO: 30) in the plasmid 25 pSW228. The mutation was made using Quikchange (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's instructions. Amplified plasmids were treated with 1U of *Dpn*I at 37 °C for 1 hour. Treated plasmids were used to transform chemically competent *E. coli* XL1-Blue (Stratagene). Transformants were streaked on LB agar plates 30 supplemented with 0.1 mg ampicillin/mL and grown overnight at 37 °C.

Up to five individual colonies were picked and the plasmid DNA sequenced to confirm the expected mutation.

#### EXAMPLE 10

##### EXPRESSION OF *THERMOTOGA MARITIMA* ACETYL XYLAN

##### ESTERASE C277S MUTANT IN *E. COLI* KLP18

Plasmids with the confirmed acetyl xylan esterase C277S mutation were used to transform *E. coli* KLP18 (Example 1) to generate the strain identified as KLP18/pSW228/C277S. Transformants were grown in the LB medium at 37 °C with shaking up to OD<sub>600nm</sub> 0.4-0.5, at which time IPTG 10 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 acetyl xylan esterase C277S mutant at 20-40% of total soluble protein.

#### EXAMPLE 11

##### PREPARATION OF SPRAY-DRIED *T. MARITIMA* ACETYL XYLAN ESTERASE WILD TYPE AND ITS MUTANT C277S

Fermentation protocol used to grow *E. coli* KLP18/pSW228 (to produce *T. maritima* acetyl xylan esterase) or *E. coli* KLP18/pSW228/C277S (to produce *T. maritima* acetyl xylan esterase 20 C277S mutant) was similar to that described in Example 2, except that it was performed at a 10-L scale. Cell pastes were harvested by centrifugation at 5,000 x g for 15 minutes, and stored frozen at -80 °C for later use.

A sample of the cell paste was resuspended (200 g/L) in 50 mM 25 phosphate buffer pH 7.0 supplemented with 1.0 mM Dithiothreitol (DTT). Resuspended cells were passed through a French Pressure Cell twice to ensure >95% cell lysis. Lysed cells were centrifuged for 30 minutes at 12,000 xg, and the supernatant was heated at 75°C for 20 minutes, followed by quenching in an ice bath for 2 minutes. Precipitated protein 30 was removed by centrifugation for 10 minutes at 11,000 x g. SDS-PAGE

indicated that the CE-7 enzyme comprised approximately 85-90% of the total protein in the preparation.

A procedure similar to that described in Example 3 was repeated using either cell paste prepared as described in above. The resulting 5 *T. maritima* perhydrolase spray-dried enzyme powder was: 95.5 % dry weight, 27.8 wt% protein, 86.1 pNPA U/mg protein. The resulting *T. maritima* C277S perhydrolase spray-dried enzyme powder was: 95.5 % dry weight, 25.5 wt% protein, 129.5 pNPA U/mg protein.

#### EXAMPLE 12

10 IN SITU ENZYMATIC GENERATION OF PERACETIC ACID IN A FILM-FORMING COMPOSITION USING MULTIPLE PERHYDROLASES

In this Example the *in situ* enzymatic generation of PAA in a film-forming composition using different suitable perhydrolases to generate 15 PAA and the ability to use a second biocidal active and a rheology modifier in the formulation was demonstrated.

Similar coating compositions as described in Example 6 were made using the same process as described above but using the ingredients and compositions in wt% as listed in Table 6. Coating composition B generates PAA in a film-forming composition using TAED and sodium 20 percarbonate without enzyme and is used for comparison only. Coating composition C lacks enzyme and serves as a control to the enzyme-containing compositions D, E and F. The initial pH of each enzymatic reaction mixture was about 7.0. The initial pH of the TAED reaction mixture was about 8.0.

25 Determination of the concentration of PAA in the reaction mixtures were performed according to the method described by Karst *et al.* (Anal. Chem., 69: 3623-3627, 1997). At the desired time points (1 min, 5 min, etc.), an aliquot of the reaction mixture was removed (approx. 25 - 50 mg) and diluted by weight with 5 mM phosphoric acid to terminate the reaction. 30 A typical dilution was 1:35 for samples with PAA concentration greater than 2000 ppm, 1:25 for samples with PAA concentration around 1000

ppm, and 1:10 for samples with PAA concentration below 200 ppm. Approximately 200  $\mu$ L (or more to completely fill the filter cup) of the diluted aliquot was transferred to a non-sterile 10,000 NMWL (nominal molecular weight limit) filter unit (Nanosep 10K Omega, PALL Life Sciences, East Hills, NY, USA) and filtered by centrifugation at 12,000 rpm for 7 minutes. An aliquot (100  $\mu$ L) of the resulting filtrate was transferred to a 1.8-mL screw cap HPLC vial (1.8 mL ROBO Autosampler Vial, VWR International, West Chester, PA, USA) containing 300  $\mu$ L of deionized water, then 100  $\mu$ L of 20 mM MTS (methyl-p-tolyl-sulfide) in acetonitrile 5 was added, the vials capped, and the contents briefly mixed prior to a 10 minute incubation at about 25 °C in the absence of light. To each vial was then added 400  $\mu$ L of acetonitrile and 100  $\mu$ L of a solution of triphenylphosphene (120 mM) in acetonitrile, the vial recapped, and the 10 minute incubation continued. The resulting solution was then analyzed by HPLC as described above.

15

15 The PAA concentrations over time are given in Table 7. The maximum observed concentrations of PAA for all enzyme-containing as well as the TAED-containing compositions was observed between 0.5 and 20 2 hours after initiating the respective chemical reaction. After the maximum concentrations have been reached, the concentration decreases with a time profile that can be mathematically approximated by 25 an exponential decay function of the form  $c(t) = k \cdot \exp [-t/(\ln(2) \cdot \tau)]$ , wherein  $c(t)$  describes the concentration of PAA as a function of the reaction time  $t$ . The parameters  $k$  and  $\tau$  are constants that may differ for each composition with  $\tau$  indicating the approximate half-time, i.e., the time at which the PAA concentration has decayed to half its maximum value. The approximate 30 half times were calculated by fitting the PAA concentration data over time for times between 2 and 120 hours to the above exponential decay function using the method of least squares. The half times  $\tau$  are also given in Table 7. As can be seen, the half times for the enzymatically

generated PAA are similar and between about 27 and 29 hours. In contrast, the half time for the non-enzymatically generated PAA of Coating Composition B is more than 7 hours shorter. Hence, the enzymatically generated compositions provide for slower reduction of the PAA

5 concentrations in the liquid coating composition over time. This can be beneficial in situations where a specific minimum PAA concentration is desired to be present over a longer period of time.

**TABLE 6**  
Coating compositions B to F

Ingredient	Concentration (wt%)				
	Coating composition B (control, TAED)	Coating composition C (control, no enzyme)	Coating composition D	Coating composition E	Coating composition F
Elvanol® 51-04	10	10	10	10	10
Rheovis® FRC	1.6	1.6	1.6	1.6	1.6
PEG-300	1.0	1.0	1.0	1.0	1.0
BTC® 885	0.3	0.3	0.3	0.3	0.3
Triacetin	-	2.18	2.18	2.18	2.18
Hydrogen peroxide (30 wt%)	-	1.13	1.13	1.13	1.13
Sodium bicarbonate (0.2M)	-	16	16	16	16
<i>T. neapolitana</i> wild type perhydrolase (20 mg/mL)	-	-	0.25	-	-
<i>T. maritima</i> wild type perhydrolase (20 mg/mL)	-	-	-	0.25	-
<i>T. maritima</i> C277S mutant perhydrolase (20 mg/mL)	-	-	-	-	0.05
TAED B 675	0.69	-	-	-	-
Provox® C (88 wt% sodium percarbonate)	0.72	-	-	-	-
DI water	remainder	remainder	remainder	remainder	remainder

**TABLE 7**  
 PAA produced enzymatically in a film-forming composition using multiple perhydrolases

Coating composition	1 min (ppm)	5 min (ppm)	0.5 h (ppm)	1 h (ppm)	2 h (ppm)	6 h (ppm)	24 h (ppm)	48 h (ppm)	120 h (ppm)	Half-time $\tau$ (hours)
B (TAED)	2512	2864	2912	2829	2242	1568	723	263	34	20.1
C (no enzyme)	245	247	289	310	404	540	546	333	92	n.d.
D	564	1149	2151	2192	1855	1825	1533	656	115	28.4
E	954	1878	2530	2653	2513	2327	1531	787	126	27.1
F	387	1401	2399	2484	2402	1914	1206	593	111	27.1

5 n.d. indicates "not determined"

**EXAMPLE 13**

**THE EFFICACY OF THE FILM-FORMING ANTIMICROBIAL COMPOSITION G ON STAPHYLOCOCCUS AUREUS**

The antimicrobial properties of the film-forming composition G  
 10 (Table 8) were tested using the NFC test method and *Staphylococcus aureus* (ATCC 6538) as the test microorganism. The composition of G in wt% is given in Table 8. Combined reagents after mixing were 100 wt%. A volume of 0.05 mL of the coating composition was applied evenly per coupon. With a five minute exposure, a 5.6 log reduction in colony-forming units (CFU) or greater was observed, which is equivalent to a reduction of the CFU number by at least 99.999%.

15

**TABLE 8**Antimicrobial NFC test on composition G with *S. aureus*

Ingredient (wt%)	G
Elvanol® 51-04 (20 wt% solution)	50
DI water	29.87
Sodium bicarbonate (0.2 M)	16
Tricetin	0.78
Rheovis® FRC	1.6
Hydrogen peroxide (30 wt%)	0.4
PEG-300	1.0
BTC® 885	0.3
<i>T. maritima</i> C277S mutant	0.05
Perhydrolase (20 mg/mL in 50 mM NaHCO <sub>3</sub> )	
Contact time (min)	5
Log <sub>10</sub> CFU reduction	5.6

**EXAMPLE 14**

5

**THE EFFICACY OF THE FILM-FORMING ANTIMICROBIAL COMPOSITION H ON STAPHYLOCOCCUS AUREUS**

The antimicrobial properties of the film-forming composition H (Table 9) were tested using the RSS test method and *Staphylococcus aureus* (ATCC 6538) as the test microorganism. The composition of H in 10 wt% is given in Table 9. All combined reagents equaled 100 wt%. A volume of 0.05 mL of the coating composition was applied evenly per coupon. As shown in Table 9, a 3.1 log reduction in CFU was achieved for composition H, which is equivalent to a reduction of the CFU number by at least 99.9%.

15

**TABLE 9**Antimicrobial RSS test on composition H with *S. aureus*

Ingredient (wt%)	H
Elvanol® 51-04 (20 wt% solution)	50
DI water	26.4
Sodium bicarbonate (0.2 M)	16
Tricetin	2.18
Rheovis® FRC	2.2
Hydrogen peroxide (30 wt%)	1.13
PEG-300	1.0
BTC® 885	0.3
Glucopon® 215 UP	0.5
PC 5450 NF	0.2
Biosoft® N25-7	0.01
<i>T. maritima</i> C277S mutant	0.05
Perhydrolase (20 mg/mL in 50 mM NaHCO <sub>3</sub> )	
Contact time (min)	5
Log <sub>10</sub> CFU reduction	3.1

**EXAMPLE 15**5      **COMPARISON BETWEEN COMMERCIALLY AVAILABLE PAA**  
**SOLUTION AND IN SITU-GENERATED PAA IN A FILM**

This example compares the PAA concentration in a film over time between a commercially available equilibrium PAA solution doped into a film-forming composition Pack A and *in situ* generated PAA in a film-forming composition.

The film-forming compositions of H and #141B in wt% are given in Table 10. Mixing all components results in 100 wt% of the antimicrobial coating composition. For composition #141B, commercially available PAA (90 µL of a 32 wt% solution) was added to Pack A in lieu of *in situ* generation.

**TABLE 10**

Film-forming compositions H and #141B

Ingredient (wt%)	H	#141B
Elvanol® 51-04 (20 wt% solution)	50	10
DI water	26.4	84.4
Sodium bicarbonate (0.2 M)	16	
Tricetin	2.18	
Rheovis® FRC	2.2	2.2
Hydrogen peroxide (30 wt%)	1.13	
PEG-300	1.0	1.0
BTC® 885	0.3	0.3
Glucopon® 215 UP	0.5	0.5
PC 5450 NF	0.2	0.2
Biosoft® N25-7	0.01	0.01
<i>T. maritima</i> C277S mutant perhydrolase (20 mg/mL in 50 mM NaHCO <sub>3</sub> )	0.05	
PAA (32 wt%)		90 µL

After mixing all components of the compositions together, films

5 were applied to plastic panels using an 8-path wet film applicator (5 mil film depth, model No. 15, Paul N. Gardner Co. Inc., Pompano Beach, FL, USA). The PAA concentrations in the films were monitored at regular intervals as the film dried. In a typical experiment, the films were tacky to the touch within 30 minutes and completely dry by one hour.

10 Determination of the concentration of PAA in the reaction mixtures were performed according to the method described by Karst *et al.* (Anal. Chem., 69: 3623-3627, 1997). See details in example 12.

The PAA concentration in coating composition H was observed to increase from 1925 ppm PAA at 1 minute to a maximum of 2599 ppm PAA

15 at 10 minutes, until dropping to 732 ppm PAA at one hour, while the PAA concentration in composition #141B steadily dropped over one hour from a maximum of 2849 ppm PAA at one minute to 22 ppm PAA at one hour.

This example highlights the benefit of the inventive film-forming

compositions where PAA is generated in situ rather than previously prepared.

**TABLE 11**

5 PAA concentration over time in films of coating compositions H and #141B

Time	H	#141B
	ppm	ppm
1 min	1925	2849
5 min	2383	2656
10 min	2599	1741
20 min	2190	534
30 min	2001	138
1 h	732	22

**EXAMPLE 16**

**APPLICATION AND REMOVAL OF COATING COMPOSITION H**

Coating composition H (Table 9) was applied to an aluminum panel  
10 using an 8-path wet film applicator (5 mil film depth, model No. 15, Paul N. Gardner Co. Inc., Pompano Beach, FL, USA). The resulting coating after drying had an excellent appearance characterized by the absence of coating defects such as sags, foam or bubbles, craters or uncovered areas. The dry film was easily removed by rinsing the panel with 30-32 °C  
15 tap water. There was no visible residue left behind on the aluminum panel, demonstrating the good removal characteristics of the film.

CLAIMSWhat is claimed is:

1. A method of providing control of microorganisms at a locus comprising the steps:
  - 5 a) forming a composition by combining components comprising:
    - i) a film-forming water soluble or water-dispersible agent;
    - ii) an inert solvent;
    - 10 iii) a carboxylic acid ester substrate;
    - iv) a peroxygen source;
    - v) an enzyme catalyst having perhydrolase activity thereby forming at least one peroxyacid to provide at least a first antimicrobial agent; and
    - 15 vi) a rheology modifier;
  - b) applying said composition to said locus;
  - c) allowing said composition to dry thereby forming a coating on said locus;

wherein said components further comprise a second antimicrobial agent,

20 wherein said second antimicrobial agent comprises a quaternary ammonium compound.

25

2. The method of Claim 2 wherein said enzyme catalyst is selected from carbohydrate esterases of CE-7 family.

3. The method of Claim 1, wherein said peroxyacid is produced at a concentration of at least 20 ppm within about 5 minutes to about 2 hours of combining said components.

30 4. A method of providing control of microorganisms at a locus comprising the steps:

(a) providing a first premixed component comprising an inert solvent and a film-forming agent;

5 (b) providing a second premixed component comprising an enzyme catalyst having perhydrolase activity, a carboxylic acid ester substrate and a peroxygen source;

(c) mixing said first premixed component and said second premixed component to obtain a liquid coating composition comprising a first antimicrobial agent comprising a peroxyacid;

10 (d) applying said coating composition to the locus; and

(e) allowing said coating composition to dry thereby forming a coating on said locus;

wherein at least one premixed component further comprises a second antimicrobial agent wherein said second antimicrobial agent comprises a 15 quaternary ammonium compound.

5. The method of Claim 4, wherein said first and second premixed component are provided in a multi-compartment system; wherein the first and second premixed components remain separated before step (c).

20 6. The method of Claim 4, wherein one premixed component is in liquid form and one premixed component is in solid form.

25 7. The method of Claim 4 wherein said enzyme catalyst comprises carbohydrate esterases of CE-7 family.

8. An antimicrobial composition comprising components which comprise:

30 a) a film-forming water soluble or water-dispersible agent;

b) an inert solvent;

c) a carboxylic acid ester substrate;

- d) a peroxygen source;
- e) an enzyme catalyst having perhydrolase activity; and
- f) a rheology modifier

wherein, upon combining said components, a peroxyacid is formed;

5    wherein said components further comprises a component that is an antimicrobial agent comprising a quaternary ammonium compound

9.    The composition of Claim 8 wherein at least one or more said components are separately packaged from the other components in a  
10    multi-compartment system prior to their combination to form said peroxyacid.

10.    The composition of Claim 8, wherein said peroxyacid is produced at a concentration of at least 20 ppm within about 5 minutes to about 2 hours  
15    of combining said components.

11.    An article comprising a coating on at least one surface thereof with an antimicrobial composition, wherein the antimicrobial composition comprises:

20                    a) a film-forming water soluble or water-dispersible agent;  
                          b) an inert solvent;  
                          c) a carboxylic acid ester substrate;  
                          d) a peroxygen source; and  
                          e) an enzyme catalyst having perhydrolase activity

25    wherein, upon combining components of (a) through (e), a peroxyacid is formed.

12.    The article of Claim 11 wherein the article is equipment used in the  
30    food or beverage industry.