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(54) **Title**: ENZYMATIC PERACID GENERATION FOR USE IN ORAL CARE PRODUCTS

(57) **Abstract**: Disclosed herein are compositions and methods to treat an oral cavity surface with a peracid-based benefit agent. The peracid benefit agent can be use for oral surface bleaching, whitening, disinfecting, destaining, deodorizing, decreasing or removing biofilm, and combinations thereof. The peracid is enzymatically generated from a carboxylic acid ester substrate using a CE-7 carbohydrate esterase having perhydrolytic activity (perhydrolase) in the presence of a source of peroxygen. A fusion protein comprising the perhydrolase coupled to a peptidic component having affinity for an oral cavity surface, either directly or through an optional linker, may be used to target the perhydrolytic activity to the oral cavity surface.

TITLE

ENZYMATIC PERACID GENERATION FOR USE IN ORAL CARE PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Patent Application No. 61/424,903, filed December 20, 2010, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

This invention relates to the field of personal care products comprising at least one peracid as an oral care benefit agent. The peracid is enzymatically produced in the presence of at least one suitable carboxylic acid ester substrate and a source of peroxygen. Specifically, an enzyme catalyst having perhydrolytic activity is used to produce a peracid benefit agent for use in an oral care product. The perhydrolytic enzyme may be in the form of a fusion protein (a "targeted perhydrolase") engineered to contain at least one peptidic component having affinity for an oral cavity surface such that the enzymatically produced peracid is produced on or near the desired surface.

BACKGROUND OF THE INVENTION

Peroxycarboxylic acids ("peracids") are effective antimicrobial agents. Methods to clean, disinfect, and/or sanitize hard surfaces, food products, living plant tissues, and medical devices against undesirable microbial growth have been described (*e.g.*, U.S. Patent 6,545,047; U.S. Patent 6,183,807; U.S. Patent 6,518,307; U.S. Patent 5,683,724; and U.S. Patent 6,635,286). Peracids have also been reported to be useful in preparing bleaching compositions for laundry detergent applications (*e.g.*, U.S. Patent 3,974,082; U.S. Patent 5,296,161; and U.S. Patent No 5,364,554).

Oral care compositions comprising a peracid have also been disclosed. U.S. Patent 5,302,375 to Viscio, D., discloses oral compositions for whitening

teeth comprising peracetic acid dissolved in a vehicle, wherein the peracetic acid is generated within the vehicle *in situ* by combining water, acetylsalicylic acid, and a water soluble alkali metal percarbonate. U.S. Patent 5,279,816 to Church *et al.* discloses the use of a composition comprising peracetic acid to whiten stained or discolored teeth. U.S. Patents 6,221,341 and 7,189,385 to Montgomery, R., disclose peroxy acid tooth-whitening compositions suitable for use in a method to whiten teeth. More specifically, a peracetic acid composition is produced by combining a hydrogen peroxide precursor, an acetic acid ester of glycerin, and water to generate, via chemical perhydrolysis, peracetic acid. Enzymatic perhydrolysis is not described.

U.S. Patent Application Publication No. 2009-0311198 to Concar *et al.* discloses an oral composition comprising an *M. smegmatis* enzyme having perhydrolytic activity to bleach teeth. The use of a CE-7 perhydrolase to produce a peracid benefit agent is not disclosed. Concar *et al.* is also silent on the use of a targeted perhydrolytic enzyme in an oral care composition.

The inclusion of specific variant subtilisin Carlsberg proteases having perhydrolytic activity in a body care product is disclosed in U.S. Patent 7,510,859 to Wieland *et al.* Perhydrolytic enzymes beyond the specific variant proteases are not described nor are there any working examples demonstrating the enzymatic production of peracid as a personal care benefit agent.

U.S. Patent Application Publication Nos. 2008-0176783 A1; 2008-0176299 A1; 2009-0005590 A1; and 2010-0041752 A1 to DiCosimo *et al.* disclose enzymes structurally classified as members of the CE-7 family of carbohydrate esterases (*i.e.*, cephalosporin C deacetylases [CAHs] and acetyl xylan esterases [AXEs]) that are characterized by significant perhydrolytic activity for converting carboxylic acid ester substrates (in the presence of a suitable source of peroxygen, such as hydrogen peroxide) into peroxycarboxylic acids at concentrations sufficient for use as a disinfectant and/or a bleaching agent. Some members of the CE-7 family of carbohydrate esterases have been demonstrated to have perhydrolytic activity sufficient to produce 4000 – 5000 ppm peracetic acid from acetyl esters of alcohols, diols, and glycerols in 1 minute

and up to 9000 ppm between 5 minutes and 30 minutes once the reaction components were mixed (DiCosimo *et al.*, U.S. 2009-0005590 A1). U.S. Patent application publication No. 2010-0087529 A1 describes variant CE-7 enzymes having improved perhydrolytic activity. Although the CE-7 perhydrolases have exceptional perhydrolytic activity, their use in personal care products has not been disclosed. As such, a problem to be solved is to provide personal care compositions and methods comprising the use of at least one CE-7 perhydrolase for the production of a peracid benefit agent.

Peracids are strong oxidizing agents that may be reactive towards a variety of materials, including materials not targeted for the desired benefit. As such, certain personal care applications may benefit from the ability to target/focus the peracid benefit agent to the desired body surface by localizing peracid production on or near the desired target body surface. Enzymatic peracid production may benefit by targeting the perhydrolase to the body surface. An additional benefit can be achieved by targeting the perhydrolase to a delivery material so as to limit enzyme concentration and exposure of the user.

Oral care compositions and/or methods of treating an oral care surface with an enzyme coupled to an oral cavity material have been reported. U.S. patent 4,138,476 to Simonson *et al.* discloses a process for treating plaque comprising the use of a glucan-degrading enzyme covalently coupled, via a complexing reagent, to a phosphate carrier group having affinity for the surface of a tooth. The enzymatic degradation of the glucan deposits is said to promote dissolution and dispersion of plaque material.

U.S. Patent Application Publication No. 2005-0158253, U.S. Patent 6,830,745 to Budny *et al.* discloses a two-component composition comprising an anchor enzyme complex to enzymatically degrade biofilm structures and a second anchor enzyme component capable of acting directly upon bacteria. The biofilm-degrading enzymes are those that directly degrade the exopolysaccharide backbone structures.

U.S. patent 5,871,714 to Budny, J., discloses a composition for controlling bacterial growth/colonization (*e.g.*, reducing dental plaque) comprising an

enzyme that degrades the plaque matrix coupled to an anchor molecule. The use of a targeted perhydrolase is not disclosed.

United States Patent 5,490,988 and EP 0479,600 B1 to Beggs *et al.* discloses the use of antibody fragments as a means for binding to a target site, wherein a therapeutic agent is connected through an additional peptide appended to the antibody fragment to attach the therapeutic agent to the target site. An oral care product is disclosed comprising a modified antibody fragment having affinity to an antigentic component of bacteria in dental plaque to delivery a therapeutic agent. The therapeutic agent may be a cytotoxic agent produced by an enzyme or a combination of enzymes, such as an oxidase in combination with a peroxidase to form oxidized halides. The use of a targeted perhydrolase to produce a peracid benefit agent is not described.

EP 0450,800 B1 to Beggs *et al.* discloses the utilization of two different enzymes working together to attack species occurring in the oral microflora. The first enzyme generates an intermediate product that is used as a substrate for the second enzyme to produce an agent active against a target within the mouth. Each enzyme is attached to an antibody or antibody fragment having affinity to a target surface within the mouth, whereby in use the enzymes are coupled to the target site in proximity to each other. Exemplified is a combination of a glucose oxidase to produce hydrogen peroxide which then may be converted by a peroxidase, in the presence of a halide or thiocyanate, to produce a hypohalite or hypothiocyanate, respectively. The use of a targeted perhydrolase to produce a peracid benefit agent is not described.

EP 0451,972 B1 to Beggs *et al.* describes a product comprising a two enzymes, the product comprising a first enzyme for generating an active agent against a target and a second enzyme for generating an intermediate which is a substrate for the first enzyme; said product further comprising a linking means (*i.e.*, an antibody or antibody fragment) attached or attachable to both enzymes to couple the enzymes together, thereby forming a complex which binds to a target cell. Exemplified is an oxidase (capable of generating hydrogen peroxide)

coupled to a peroxidase which catalyzes the formation of a hypohalite or hypothiocyanate active agent.

EP 0453,097 B1 to Beggs *et al.* describes the delivery of an active agent to a target site using a plurality of antibodies or antibody fragments which can self assemble to form a bridge between the agent and the target site. The active agent is glucose oxidase or a combination of a glucose oxidase and a peroxidase. The use of targeted perhydrolase to produce a peracid benefit agent is not described.

The use of antibodies, antibody fragments (F_{ab}), single chain fused variable region antibodies (scFc), *Camelidae* antibodies, and large scaffold display proteins as peptidic affinity materials may not be suitable for some personal care applications due to their size and cost. As such, there remains a need in certain low cost cosmetic applications to use shorter, less expensive peptidic affinity materials for targeted delivery of a benefit agent.

The use of shorter, biopanned peptides having strong affinity for a body surface to target a cosmetic benefit agent to a body surface has been described (U.S. Patent Nos. U.S. 7,220,405; 7,309,482; 7,285,264 and 7,807,141; U.S. Patent Application Publication Nos. 2005-0226839 A1; 2007-0196305 A1; 2006-0199206 A1; 2007-0065387 A1; 2008-0107614 A1; 2007-0110686 A1; 2006-0073111 A1; 2010-0158846; 2010-0158847; and 2010-0247589; and published PCT applications WO2008/054746; WO2004/048399, and WO2008/073368). U.S. Patent 7,807,141 to Huang *et al.* discloses peptide-based oral care surface reagents suitable to couple an oral care benefit agent to a tooth surface. The use of a peptidic material having affinity for an oral cavity surface to couple an active CE-7 perhydrolase (*i.e.*, "targeted perhydrolases") for the production of a peracid benefit agent has not been described.

As such, an additional problem to be solved is to provide compositions and methods suitable to target enzymatic peracid production to an oral cavity surface.

SUMMARY OF THE INVENTION

Methods and compositions comprising components to enzymatically produce and deliver a peracid-based benefit agent to an oral cavity surface are provided.

In one embedment, oral care compositions and methods are provided that use a CE-7 perhydrolase to enzymatically produce a peracid benefit agent for use in oral care applications such as oral cavity surface bleaching, teeth whitening, disinfecting, destaining, deodorizing, treating dental caries, preventing of dental caries, reducing oral bacteria associated with dental caries, and treating or removing oral biofilms (*e.g.*, dental plaque).

In one embodiment, a method is provided comprising:

1) providing a set of reaction components comprising:

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

i) esters having the structure

$$[X]_mR_5$$

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

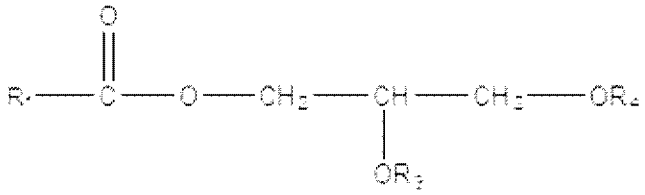
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

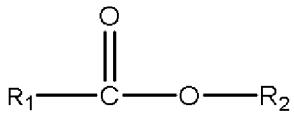
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



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

iii) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

b) a source of peroxygen; and

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

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

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

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

2) combining the reaction components of (1) under suitable reaction condition whereby at least one peracid is enzymatically produced; and

3) contacting an oral cavity surface with the at least one peracid whereby the oral cavity surface receives a peracid-based benefit selected from the group consisting of bleaching, teeth whitening, disinfecting, destaining, deodorizing, decreasing or removing biofilm, and combinations thereof.

In one embodiment, the oral cavity surface is tooth enamel, tooth pellicle, a soft tissue within the oral cavity (*e.g.* gums, tongue), or an oral cavity biofilm (*e.g.*, oral plaque).

In another embodiment, compositions and methods are provided comprising the use of a fusion protein (*i.e.*, a "targeted perhydrolase") comprising a perhydrolytic enzyme and a peptidic component having affinity for an oral cavity surface, wherein the two components may be optionally separated by a peptide spacer.

In one embodiment, a method is provided comprising:

1) providing a set of reaction components comprising:

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

i) esters having the structure

$$[X]_mR_5$$

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

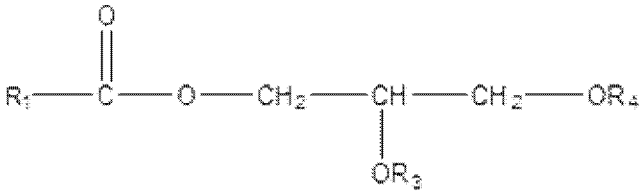
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

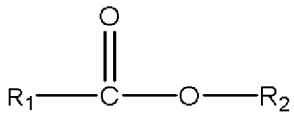
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



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

iii) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl,

alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

b) a source of peroxygen; and

c) an enzyme catalyst having perhydrolytic activity, wherein said enzyme catalyst comprises a fusion protein having the following general structure:

$PAH\text{-}[L]_y\text{-}OCBD$

or

$OCBD\text{-}[L]_y\text{-}PAH$

wherein

PAH is the enzyme having perhydrolytic activity;

OCBD is a peptidic component having affinity for the oral cavity surface; and

L is an optional peptide linker ranging from 1 to 100 amino acids in length; and

y is 0 or 1;

2) combining the reaction components of (1) under suitable reaction condition whereby at least one peracid is enzymatically produced; and

3) contacting an oral cavity surface with the at least one peracid whereby the oral cavity surface receives a peracid-based benefit selected from the group consisting of bleaching, teeth whitening, disinfecting, destaining, deodorizing, decreasing or removing biofilm, and combinations thereof.

The fusion protein may comprise a perhydrolytic enzyme selected from the group consisting of lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations thereof.

In one embodiment, the fusion protein comprises a perhydrolytic aryl esterase (ArE) from *Mycobacterium smegmatis*. In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the S54V *Mycobacterium smegmatis* aryl esterase provided as SEQ ID NO: 460.

In one embodiment, the fusion protein comprises a perhydrolytic esterase from *Pseudomonas fluorescens*. In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the *Pseudomonas fluorescens* esterase provided as SEQ ID NO: 477.

In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 424, 425, 426, 427, 428, 429, 430, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 476, 477, 478, and 479.

In another embodiment, the fusion protein comprises a CE-7 perhydrolase having a CE-7 signature motif that aligns with a reference sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

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

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

iii) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2.

In another embodiment, the peptidic component having affinity for an oral cavity surface is preferably a single chain peptide comprising at least one oral cavity surface-binding peptide. In yet a further embodiment, the oral cavity

surface-binding peptide is a peptide having affinity for tooth enamel, tooth pellicle or both tooth enamel and tooth pellicle.

In another embodiment, a fusion protein is provided comprising the following general structure:

$PAH\text{-}[L]_y\text{-}OCBD$

or

$OCBD\text{-}[L]_y\text{-}PAH$

wherein

1) PAH is an enzyme having perhydrolytic activity;

2) OCBD is a peptidic component having affinity for an oral cavity surface;

3) L is an optional peptide linker ranging from 1 to 100 amino acids in length; and

4) y is 0 or 1.

In another embodiment, a fusion protein is provided comprising the general structure:

$PAH\text{-}[L]_y\text{-}OCBD$

or

$OCBD\text{-}[L]_y\text{-}PAH$

wherein

a) PAH is a CE-7 carbohydrate esterase having perhydrolytic activity; the PAH having a CE-7 signature motif that aligns with a reference

sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

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

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

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

b) OCBD is a peptidic component having affinity for an oral cavity surface;

c) L is an optional peptide linker ranging from 1 to 100 amino acids in length; and

d) y is 0 or 1.

In another embodiment, an oral care product is provided comprising:

1) an enzyme catalyst comprising any of the above perhydrolytic fusion proteins;

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

a) esters having the structure

$$[X]_mR_5$$

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

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

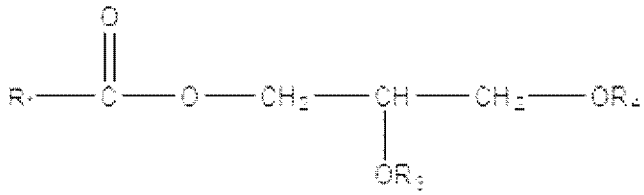
R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more

than one hydroxyl group or no more than one ester group or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

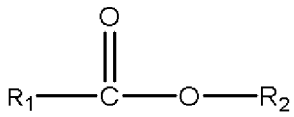
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

b) glycerides having the structure



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

c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

3) a source of peroxygen; and

4) an orally-acceptable carrier medium.

In another embodiment, an oral care product is provided comprising:

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

a) an RGQ motif at positions corresponding to positions 118-120 of SEQ ID NO:2;

b) a GXSQG motif at positions corresponding to positions 179-183 of SEQ ID NO:2; and

c) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2; and

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

a) esters having the structure

$[X]_mR_5$

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

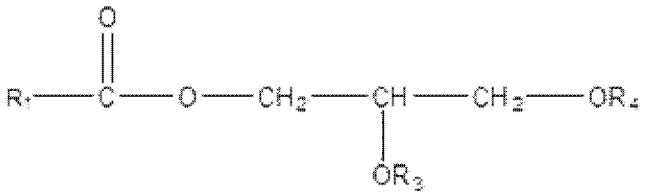
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

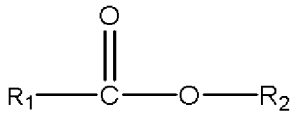
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

b) glycerides having the structure



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

c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

3) a source of peroxygen; and

4) an orally acceptable carrier medium.

In another embodiment, an isolated polypeptide having affinity for an oral cavity surface is provided, said polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, and 422.

In another embodiment, the use of a CE-7 carbohydrate esterase having perhydrolytic activity in an oral care product to produce an efficacious concentration of at least one peracid to bleach, whiten, disinfect, destain, deodorize or remove biofilm from an oral cavity material/surface is also provided.

In another embodiment, the use of a peracid generation composition is provided comprising:

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

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

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

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

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

1) esters having the structure

$$[X]_mR_5$$

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

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

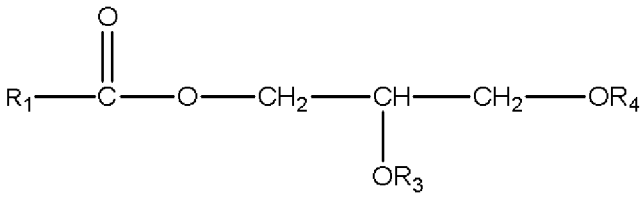
R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic or six-membered cyclic aromatic or heteroaromatic moiety optionally substituted with hydroxyl groups; wherein each carbon atom in R_5 individually comprises no more than one

hydroxyl group or no more than one ester group or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

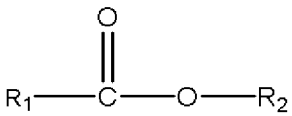
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

2) glycerides having the structure



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

3) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

c) a source of peroxygen;

whereby a peracid is formed upon mixing simultaneously, or in a step-wise manner (but in no particular order), (a), (b), and (c); for the treatment or prevention of dental caries, gingivitis, oral candidiasis, or periodontitis.

In another embodiment, the use of fusion protein comprising the following general structure in an oral cavity product is provided comprising:

$PAH\text{-}[L]_y\text{-}OCBD$

or

$OCBD\text{-}[L]_y\text{-}PAH$

wherein

1) PAH is an enzyme having perhydrolytic activity having an amino acid sequence with at least 95% amino acid identity to SEQ ID NO: 460;

2) OCBD is a peptidic component having affinity for an oral cavity surface;

3) L is a peptide linker ranging from 1 to 100 amino acids in length; and

4) y is 0 or 1.

Several of the ester substrates described herein (Table 20) were particularly susceptible to chemical perhydrolysis when reacted with hydrogen peroxide to produce peracetic acid. In another embodiment, a personal care product is provided comprising a peracid precursor selected from the group consisting of 1, 2, 3, 5-tetra-O-acetyl-ribofuranose; 1,2,3,4-tetra-O-acetyl-ribopyranose; 2-acetamido-2-deoxy-1,3,4,6-tetraacetyl-β-D-glucopyranose; β-D-glucopyranose,1,2,3,4-tetraacetate; 2,3,4,6-tetraacetyl-β-D-glucopyranose; 1,3,4,6-tetra-O-acetyl-mannopyranose; and α-D-mannopyranose pentaacetate. In a preferred embodiment, the personal care product is an oral care product.

In another embodiment, a method is also provided comprising:

a) providing a set of reaction components comprising
   i) a peracid precursor selected form the group consisting of 1, 2, 3, 5-tetra-O-acetyl-ribofuranose; 1,2,3,4-tetra-O-acetyl-ribopyranose; 2-acetamido-2-deoxy-1,3,4,6-tetraacetyl-β-D-glucopyranose; β-D-glucopyranose,1,2,3,4-tetraacetate; 2,3,4,6-tetraacetyl-β-D-glucopyranose; 1,3,4,6-tetra-O-acetyl-mannopyranose; and α-D-mannopyranose pentaacetate; and
   ii) a source of peroxygen;
b) contacting a body surface with an effective amount of peracetic acid produced by combining the set of reaction components in the presence of water; whereby the peracetic acid provides a benefit to the body surface. In a preferred aspect, the body surface in the above method is an oral cavity tissue, such as teeth and/or gums.

BRIEF DESCRIPTION OF THE 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 ST.25 (2009) 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.

SEQ ID NO: 1 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *Bacillus subtilis* ATCC® 31954™.

SEQ ID NO: 2 is the amino acid sequence of a cephalosporin C deacetylase from *Bacillus subtilis* ATCC® 31954™.

SEQ ID NO: 3 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *Bacillus subtilis* subsp. *subtilis* strain 168.

SEQ ID NO: 4 is the amino acid sequence of a cephalosporin C deacetylase from *Bacillus subtilis* subsp. *subtilis* strain 168.

SEQ ID NO: 5 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *B. subtilis* ATCC® 6633™.

SEQ ID NO: 6 is the acid sequence of a cephalosporin C deacetylase from *B. subtilis* ATCC® 6633™.

SEQ ID NO: 7 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *B. licheniformis* ATCC® 14580™.

SEQ ID NO: 8 is the deduced amino acid sequence of a cephalosporin C deacetylase from *B. licheniformis* ATCC® 14580™.

SEQ ID NO: 9 is the nucleic acid sequence encoding an acetyl xylan esterase from *B. pumilus* PS213.

SEQ ID NO: 10 is the deduced amino acid sequence of an acetyl xylan esterase from *B. pumilus* PS213.

SEQ ID NO: 11 is the nucleic acid sequence encoding an acetyl xylan esterase from *Clostridium thermocellum* ATCC®27405™.

SEQ ID NO: 12 is the deduced amino acid sequence of an acetyl xylan esterase from *Clostridium thermocellum* ATCC®27405™.

SEQ ID NO: 13 is the nucleic acid sequence encoding an acetyl xylan esterase from *Thermotoga neapolitana*.

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

SEQ ID NO: 15 is the nucleic acid sequence encoding an acetyl xylan esterase from *Thermotoga maritima* MSB8.

SEQ ID NO: 16 is the amino acid sequence of an acetyl xylan esterase from *Thermotoga maritima* MSB8.

SEQ ID NO: 17 is the nucleic acid sequence encoding an acetyl xylan esterase from *Thermoanaerobacterium sp.* JW/SL YS485.

SEQ ID NO: 18 is the deduced amino acid sequence of an acetyl xylan esterase from *Thermoanaerobacterium sp.* JW/SL YS485.

SEQ ID NO: 19 is the nucleic acid sequence of a cephalosporin C deacetylase from *Bacillus sp.* NRRL B-14911. It should be noted that the nucleic acid sequence encoding the cephalosporin C deacetylase from *Bacillus sp.* NRRL B-14911 as reported in GENBANK® Accession number ZP_01168674 appears to encode a 15 amino acid N-terminal addition that is likely incorrect based on sequence alignments with other cephalosporin C deacetylases and a comparison of the reported length (340 amino acids) versus the observed length of other CAH enzymes (typically 318-325 amino acids in length; see U.S. Patent Application Publication No. US-2010-0087528-A1; herein incorporated by reference). As such, the nucleic acid sequence as reported herein encodes the cephalosporin C deacetylase sequence from *Bacillus sp.* NRRL B-14911 without the N-terminal 15 amino acids reported under GENBANK® Accession number ZP_01168674.

SEQ ID NO: 20 is the deduced amino acid sequence of the cephalosporin C deacetylase from *Bacillus sp.* NRRL B-14911 encoded by the nucleic acid sequence of SEQ ID NO: 19.

SEQ ID NO: 21 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *Bacillus halodurans* C-125.

SEQ ID NO: 22 is the deduced amino acid sequence of a cephalosporin C deacetylase from *Bacillus halodurans* C-125.

SEQ ID NO: 23 is the nucleic acid sequence encoding a cephalosporin C deacetylase from *Bacillus clausii* KSM-K16.

SEQ ID NO: 24 is the deduced amino acid sequence of a cephalosporin C deacetylase from *Bacillus clausii* KSM-K16.

SEQ ID NO: 25 is the nucleic acid sequence encoding a *Bacillus subtilis* ATCC® 29233™ cephalosporin C deacetylase (CAH).

SEQ ID NO: 26 is the deduced amino acid sequence of a *Bacillus subtilis* ATCC® 29233™ cephalosporin C deacetylase (CAH).

SEQ ID NO: 27 is the deduced amino acid sequence of a *Thermotoga neapolitana* acetyl xylan esterase variant from U.S. Patent Application

Publication No. 2010-0087529 (incorporated herein by reference in its entirety), where the Xaa residue at position 277 is Ala, Val, Ser, or Thr.

SEQ ID NO: 28 is the deduced amino acid sequence of a *Thermotoga maritima* MSB8 acetyl xylan esterase variant from U.S. Patent Application Publication No. 2010-0087529, where the Xaa residue at position 277 is Ala, Val, Ser, or Thr.

SEQ ID NO: 29 is the deduced amino acid sequence of a *Thermotoga lettingae* acetyl xylan esterase variant from U.S. Patent Application Publication No. 2010-0087529, where the Xaa residue at position 277 is Ala, Val, Ser, or Thr.

SEQ ID NO: 30 is the deduced amino acid sequence of a *Thermotoga petrophila* acetyl xylan esterase variant from U.S. Patent Application Publication No. 2010-0087529, where the Xaa residue at position 277 is Ala, Val, Ser, or Thr.

SEQ ID NO: 31 is the deduced amino acid sequence of a *Thermotoga* sp. RQ2 acetyl xylan esterase variant derived from"RQ2(a)" from U.S. Patent Application Publication No. 2010-0087529, where the Xaa residue at position 277 is Ala, Val, Ser, or Thr.

SEQ ID NO: 32 is the deduced amino acid sequence of a *Thermotoga* sp. RQ2 acetyl xylan esterase variant derived from "RQ2(b)" from U.S. Patent Application Publication No. 2010-0087529, where the Xaa residue at position 278 is Ala, Val, Ser, or Thr.

SEQ ID NO: 33 is the deduced amino acid sequence of a *Thermotoga lettingae* acetyl xylan esterase.

SEQ ID NO: 34 is the deduced amino acid sequence of a *Thermotoga petrophila* acetyl xylan esterase.

SEQ ID NO: 35 is the deduced amino acid sequence of a first acetyl xylan esterase from *Thermotoga* sp. RQ2 described herein as "RQ2(a)".

SEQ ID NO: 36 is the deduced amino acid sequence of a second acetyl xylan esterase from *Thermotoga* sp. RQ2 described herein as "RQ2(b)".

SEQ ID NO: 37 is the codon optimized nucleic acid sequence encoding a *Thermoanearobacterium saccharolyticum* cephalosporin C deacetylase.

SEQ ID NO: 38 is the deduced amino acid sequence of a *Thermoanearobacterium saccharolyticum* cephalosporin C deacetylase.

SEQ ID NO: 39 is the nucleic acid sequence encoding the acetyl xylan esterase from *Lactococcus lactis* (GENBANK® accession number EU255910).

SEQ ID NO: 40 is the amino acid sequence of the acetyl xylan esterase from *Lactococcus lactis* (GENBANK® accession number ABX75634.1).

SEQ ID NO: 41 is the nucleic acid sequence encoding the acetyl xylan esterase from *Mesorhizobium loti* (GENBANK® accession number NC_002678.2).

SEQ ID NO: 42 is the amino acid sequence of the acetyl xylan esterase from *Mesorhizobium loti* (GENBANK® accession number BAB53179.1).

SEQ ID NO: 43 is the nucleic acid sequence encoding the acetyl xylan esterase from *Geobacillus stearothermophilus* (GENBANK® accession number AF038547.2).

SEQ ID NO: 44 is the amino acid sequence of the acetyl xylan esterase from *Geobacillus stearothermophilus* (GENBANK® accession number AAF70202.1).

SEQ ID NO: 45 is the nucleic acid sequence encoding a variant acetyl xylan esterase (variant “A3”) having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: (F24I/S35T/Q179L/N275D/C277S/S308G/F317S).

SEQ ID NO: 46 is the amino acid sequence of the “A3” variant acetyl xylan esterase.

SEQ ID NO: 47 is the nucleic acid sequence encoding the N275D/C277S variant acetyl xylan esterase.

SEQ ID NO: 48 is the amino acid sequence of the N275D/C277S variant acetyl xylan esterase.

SEQ ID NO: 49 is the nucleic acid sequence encoding the C277S/F317S variant acetyl xylan esterase.

SEQ ID NO: 50 is the amino acid sequence of the C277S/F317S variant acetyl xylan esterase.

SEQ ID NO: 51 is the nucleic acid sequence encoding the S35T/C277S variant acetyl xylan esterase.

SEQ ID NO: 52 is the amino acid sequence of the S35T/C277S variant acetyl xylan esterase.

SEQ ID NO: 53 is the nucleic acid sequence encoding the Q179L/C277S variant acetyl xylan esterase.

SEQ ID NO: 54 is the amino acid sequence of the Q179L/C277S variant acetyl xylan esterase.

SEQ ID NO: 55 is the nucleic acid sequence encoding the variant acetyl xylan esterase 843H9 having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: (L8R/L125Q/Q176L/V183D/F247I/C277S/P292L).

SEQ ID NO: 56 is the amino acid sequence of the 843H9 variant acetyl xylan esterase.

SEQ ID NO: 57 is the nucleic acid sequence encoding the variant acetyl xylan esterase 843F12 having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: K77E/A266E/C277S.

SEQ ID NO: 58 is the amino acid sequence of the 843F12 variant acetyl xylan esterase.

SEQ ID NO: 59 is the nucleic acid sequence encoding the variant acetyl xylan esterase 843C12 having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: F27Y/I149V/A266V/C277S/I295T/N302S.

SEQ ID NO: 60 is the amino acid sequence of the 843C12 variant acetyl xylan esterase.

SEQ ID NO: 61 is the nucleic acid sequence encoding the variant acetyl xylan esterase 842H3 having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: L195Q/C277S.

SEQ ID NO: 62 is the amino acid sequence of the 842H3 variant acetyl xylan esterase.

SEQ ID NO: 63 is the nucleic acid sequence encoding the variant acetyl xylan esterase 841A7 having the following substitutions relative to the wild-type *Thermotoga maritima* acetyl xylan esterase amino acid sequence: Y110F/C277S.

SEQ ID NO: 64 is the amino acid sequence of the 841A7 variant acetyl xylan esterase.

SEQ ID NOs: 65-221, 271, and 368 are a non-limiting list of amino acid sequences of peptides having affinity for hair.

SEQ ID NO: 217-269 are the amino acid sequences of peptides having affinity for skin.

SEQ ID NOs: 270-271 are the amino acid sequences of peptides having affinity for nail.

SEQ ID NOs 272-382 are the amino acid sequences of peptides having affinity to an oral cavity surface. SEQ ID NOs: 272-291 and 312-382 have affinity for tooth pellicle. SEQ ID NOs 292-311 have affinity for tooth enamel.

SEQ ID NOs: 383-396 are the amino acid sequences of peptide linkers/spacers.

SEQ ID NO: 397 if the nucleic acid sequence of expression plasmid pLD001.

SEQ ID NO: 398 is the nucleic acid sequence of a sequencing primer.

SEQ ID NOs: 399-410 are the amino acid sequences of tooth enamel-binding and tooth pellicle-binding peptides from Example 2.

SEQ ID NO: 411 is the amino acid sequence of tooth-binding peptides DenP03 with a C-terminal lysine as shown in Table 4.

SEQ ID NOs: 412-422 are the amino acid sequence of tooth enamel-binding peptides and tooth pellicle-binding peptides with a C-terminal lysine as shown in Table 4.

SEQ ID NO: 423 is the amino acid sequence of peptide HC263.

SEQ ID NO: 424 is the amino acid sequence of *Thermotoga maritima* variant C277S also referred to in the present application as enzyme “EZ-1”.

SEQ ID NO: 425-430 and 437-467 and 479 are the amino acid sequences of various perhydrolase constructs as disclosed in Table 5 and/or Table 6.

SEQ ID NOS: 431-436 and 468-475 are the amino acid sequences of various targeting sequences disclosed in Example 4.

SEQ ID NO: 476 is the amino acid sequence of a *Thermotoga maritima* variant HTS-007-D5 having the following substitutions: C277T/R296P.

SEQ ID NO: 477 is the amino acid sequence of a *Pseudomonas fluorescens* esterase having perhydrolytic activity (U.S. Patent 7,384,787; "L29P" variant. Note that the numbering of the substitution is followed from the cited patent which did not include the initial methionine residue. SEQ ID NO: 477 comprises the L29P substitution at residue position number 30 as the initial methionine is included in the present sequence).

SEQ ID NO: 478 is the amino acid sequence of the wild type *Mycobacterium smegmatis* aryl esterase (U.S. Patent 7,754,460).

DETAILED DESCRIPTION OF THE INVENTION

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

As used herein, the articles "a", "an", and "the" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances (*i.e.*, occurrences) of the element or component. Therefore "a", "an", and "the" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

As used herein, the term "comprising" means the presence of the stated features, integers, steps, or components as referred to in the claims, but that it does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof. The term "comprising" is intended to include embodiments encompassed by the terms "consisting essentially of" and "consisting of". Similarly, the term "consisting essentially of" is intended to include embodiments encompassed by the term "consisting of".

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

Where present, all ranges are inclusive and combinable. For example, when a range of "1 to 5" is recited, the recited range should be construed as including ranges "1 to 4", "1 to 3", "1-2", "1-2 & 4-5", "1-3 & 5", and the like.

As used herein, "contacting" refers to placing a composition in contact with the target body surface for a period of time sufficient to achieve the desired result (target surface binding, peracid based effects, etc). In one embodiment, "contacting" may refer to placing a composition comprising (or capable of producing) an efficacious concentration of peracid in contact with a target body surface for a period of time sufficient to achieve the desired result. In another embodiment, "contacting" may also refer to the placing at least one component of a personal care composition, such as one or more of the reaction components used to enzymatic perhydrolysis, in contact with a target body surface. Contacting includes spraying, treating, immersing, flushing, pouring on or in, mixing, combining, painting, coating, applying, affixing to and otherwise communicating a peracid solution or a composition comprising an efficacious concentration of peracid, a solution or composition that forms an efficacious concentration of peracid or a component of the composition that forms an efficacious concentration of peracid with the body surface.

As used herein, the terms "substrate", "suitable substrate", and "carboxylic acid ester substrate" interchangeably refer specifically to:

(a) one or more esters having the structure

$[X]_mR_5$

wherein

X is an ester group of the formula $R_6C(O)O$;

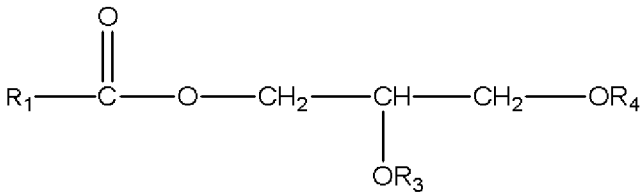
R_6 is a C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with a hydroxyl group or C1 to C4 alkoxy group, wherein R_6 optionally comprises one or more ether linkages where R_6 is C2 to C7;

R_5 is a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a cyclic five-membered heteroaromatic or six-membered cyclic aromatic or heteroaromatic moiety optionally substituted with a hydroxyl group; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group, and wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5,

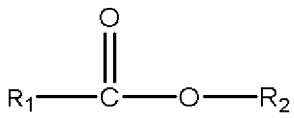
said one or more esters having solubility in water of at least 5 ppm at 25 °C; or

(b) one or more glycerides having the structure



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

(c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; or

(d) one or more acetylated monosaccharides, acetylated disaccharides, or acetylated polysaccharides; or

(e) any combination of (a) through (d).

As used herein, the term "peracid" is synonymous with peroxyacid, peroxycarboxylic acid, peroxy acid, percarboxylic acid and peroxoic acid.

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

As used herein, the term "monoacetin" is synonymous with glycerol monoacetate, glycerin monoacetate, and glyceryl monoacetate.

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

As used herein, the term "triacetin" is synonymous with glycerin triacetate; glycerol triacetate; glyceryl triacetate, 1,2,3-triacetoxypropane; 1,2,3-propanetriol triacetate and all other synonyms of CAS Registry Number 102-76-1.

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

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

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

As used herein, the term “monopropionin” is synonymous with glycerol monopropionate, glycerin monopropionate, and glyceryl monopropionate.

As used herein, the term “dipropionin” is synonymous with glycerol dipropionate and glyceryl dipropionate.

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

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

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

As used herein, the terms “monoesters” and “diesters” of 1,2-ethanediol; 1,2-propanediol; 1,3-propanediol; 1,2-butanediol; 1,3-butanediol; 2,3-butanediol; 1,4-butanediol; 1,2-pentanediol; 2,5-pentanediol; 1,5-pentandiol; 1,6-pentanediol; 1,2-hexanediol; 2,5-hexanediol; 1,6-hexanediol; and mixtures thereof, refer to said compounds comprising at least one ester group of the formula RC(O)O,

wherein R is a C1 to C7 linear hydrocarbyl moiety. In one embodiment, the carboxylic acid ester substrate is selected from the group consisting of propylene glycol diacetate (PGDA), ethylene glycol diacetate (EDGA), and mixtures thereof.

As used herein, the term "propylene glycol diacetate" is synonymous with 1,2-diacetoxypropane, propylene diacetate, 1,2-propanediol diacetate, and all other synonyms of CAS Registry Number 623-84-7.

As used herein, the term "ethylene glycol diacetate" is synonymous with 1,2-diacetoxyethane, ethylene diacetate, glycol diacetate, and all other synonyms of CAS Registry Number 111-55-7.

As used herein, the terms "suitable enzymatic reaction mixture", "components suitable for *in situ* generation of a peracid", "suitable reaction components", "suitable aqueous reaction mixture", "reaction mixture", and "peracid-generating components" refer to the materials and water in which the reactants and the perhydrolytic enzyme catalyst come into contact. The peracid-generating components will include at least enzyme having perhydrolytic activity, preferably wherein the perhydrolytic enzyme is at least one CE-7 perhydrolase (optionally in the form of a fusion protein targeted to a body surface), at least one suitable carboxylic acid ester substrate, a source of peroxygen, and water (aqueous solution comprising a source of peroxygen, for example, hydrogen peroxide). In one embodiment, any perhydrolytic enzyme not belonging to the CE-7 class of carbohydrate esterases will, by proviso, be used in the form of a fusion protein having at least one peptide component having affinity for a target surface, preferably an oral cavity surface.

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

which a carboxylic acid ester substrate (a peracid precursor) is combined with a source of hydrogen peroxide and water whereby the enzyme catalyst catalyzes the formation of peracid.

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

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

As used herein, the terms “enzyme catalyst” and “perhydrolase catalyst” refer to a catalyst comprising an enzyme having perhydrolysis activity and may be in the form of a whole microbial cell, permeabilized microbial cell(s), one or more cell components of a microbial cell extract, partially purified enzyme, or purified enzyme. The enzyme catalyst may also be chemically modified (such as by pegylation or by reaction with cross-linking reagents). The perhydrolase catalyst may also be immobilized on a soluble or insoluble support using methods well-known to those skilled in the art; see for example, Immobilization of Enzymes and Cells; Gordon F. Bickerstaff, Editor; Humana Press, Totowa, NJ, USA; 1997. In one embodiment, the perhydrolase catalyst may be immobilized non-covalently or covalently in or on an oral care strip (*e.g.*, a whitening strip) or dental tray. The immobilized enzyme may be coupled directly to the polymeric support and/or a component within the oral care strip or dental tray (*e.g.*, titanium dioxide, hydroxyapatite, an orally acceptable adhesive, polyethylene, polypropylene, etc.). In a further embodiment, the non-covalent immobilization to the strip or dental tray may be through the use of a peptidic binding domain having strong affinity for a material in or on the strip or tray (*e.g.*, a fusion protein comprising a perhydrolytic enzyme coupled through an optional peptide spacer to a peptidic binding domain). In another embodiment, the dental tray is deformable tray. In yet a further embodiment, the perhydrolase catalyst is immobilized in or on the deformable tray after the formation of the dental impression.

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

As used herein, the terms "cephalosporin C deacetylase" and "cephalosporin C acetyl hydrolase" refer to an enzyme (E.C. 3.1.1.41) that catalyzes the deacetylation of cephalosporins such as cephalosporin C and 7-aminocephalosporanic acid (Mitsushima *et al.*, (1995) *Appl. Env. Microbiol.* 61(6):2224-2229). The amino acid sequences of several cephalosporin C deacetylases having significant perhydrolytic activity are provided herein.

As used herein, the term "*Bacillus subtilis* ATCC® 31954™" refers to a bacterial cell deposited to the American Type Culture Collection (ATCC) having international depository accession number ATCC® 31954™. As described herein, an enzyme having significant perhydrolase activity from *B. subtilis* ATCC® 31954™ is provided as SEQ ID NO: 2 (see United States Patent Application Publication No. 2010-0041752).

As used herein, the term "*Thermotoga maritima* MSB8" refers to a bacterial cell reported to have acetyl xylan esterase activity (GENBANK® NP_227893.1; see U.S. Patent Application Publication No. 2008-0176299). The amino acid sequence of the enzyme having perhydrolase activity from *Thermotoga maritima* MSB8 is provided as SEQ ID NO: 16.

As used herein, an "isolated nucleic acid molecule", "isolated polynucleotide", and "isolated nucleic acid fragment" will 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.

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

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

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

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);

2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, and Trp.

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

As used herein, the terms "signature motif" and "diagnostic motif" refer to conserved structures shared among a family of enzymes having a defined activity. The signature motif can be used to define and/or identify the family of structurally-related enzymes having similar enzymatic activity for a defined family of substrates. The signature motif can be a single contiguous amino acid sequence or a collection of discontiguous, conserved motifs that together form the signature motif. Typically, the conserved motif(s) is represented by an amino acid sequence. In one embodiment, the perhydrolytic enzyme comprises a CE-7 carbohydrate esterase signature motif.

As used herein, the term "codon optimized", as it refers to genes or coding regions of nucleic acid molecules for transformation of various hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide for which the DNA codes.

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

As used herein, "gene" refers to a nucleic acid molecule that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different from that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

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

As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid molecule so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence, *i.e.*, the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

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

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

As used herein, the term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to, the GCG suite of programs (Wisconsin Package Version 9.0, Accelrys Software Corp., San Diego, CA), BLASTP,

BLASTN, BLASTX (Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990)), and DNASTAR (DNASTAR, Inc. 1228 S. Park St. Madison, WI 53715 USA), CLUSTALW (for example, version 1.83; Thompson *et al.*, *Nucleic Acids Research,* 22(22):4673-4680 (1994)), and the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Publisher: Plenum, New York, NY), Vector NTI (Informax, Bethesda, MD) and Sequencher v. 4.05. Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters set by the software manufacturer that originally load with the software when first initialized.

The term "body surface" refers to any surface of the human body that may serve as the target for a benefit agent, such as a peracid benefit agent. The present methods and compositions are directed to oral care applications and products. As such, the body surface comprises an oral cavity material/surface. In one embodiment, the oral cavity material comprises tooth enamel, tooth pellicle, soft tissues such as the cheeks, tongue, and gums, and oral cavity biofilms (*e.g.*, oral plaque).

As used herein, the term "biological contaminants" refers to one or more unwanted and/or pathogenic biological entities including, but not limited to, microorganisms, spores, viruses, prions, and mixtures thereof. In one embodiment, a process is provided to enzymatically produce an efficacious concentration of at least one peracid useful to reduce and/or eliminate the presence of the biological contaminants.

As used herein, the term "disinfect" refers to the process of destruction of or prevention of the growth of biological contaminants. As used herein, the term "disinfectant" refers to an agent that disinfects by destroying, neutralizing, or inhibiting the growth of biological contaminants, which may include biological contaminants within a human oral cavity, such as microorganisms associated

with dental caries, gingivitis, oral candidiasis, or periodontitis. As used herein, the term "disinfection" refers to the act or process of disinfecting. As used herein, the term “antiseptic” refers to a chemical agent that inhibits the growth of disease-carrying microorganisms. In one aspect, the biological contaminants are pathogenic microorganisms.

As used herein, the term “sanitary” means of or relating to the restoration or preservation of health, typically by removing, preventing or controlling an agent that may be injurious to health. As used herein, the term “sanitize” means to make sanitary. As used herein, the term “sanitizer” refers to a sanitizing agent. As used herein the term “sanitization” refers to the act or process of sanitizing.

As used herein, the term “biocide” refers to a chemical agent, typically broad spectrum, which inactivates or destroys microorganisms. A chemical agent that exhibits the ability to inactivate or destroy microorganisms is described as having “biocidal” activity. Peracids can have biocidal activity. Typical alternative biocides may include, for example, chlorine, chlorine dioxide, chloroisocyanurates, hypochlorites, ozone, acrolein, amines, chlorinated phenolics, copper salts, organo-sulphur compounds, and quaternary ammonium salts.

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

As used herein, “cleaning compositions” and “cleaning formulations” refer to compositions that find use in the removal of undesired compounds from teeth (mouthwashes, toothpastes, etc.). The term encompasses any

materials/compounds selected for the particular type of cleaning composition desired and the form of the product (*e.g.*, liquid, paste, gel, emulsion, granule, or spray composition), as long as the composition is compatible with the perhydrolase and other enzyme(s) used in the composition.

As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Oral care compositions that find use in conjunction with the perhydrolases of the present invention are well known in the art (See *e.g.*, U.S. Pat. Nos. 5,601,750; 6,379,653; and 5,989,526, all of which are incorporated herein by reference, in their entirety).

As used herein, "pharmaceutically-acceptable" means that drugs, medicaments and/or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and other animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

As used herein, "personal care products" means products used in the cleaning, bleaching and/or disinfecting of hair, skin, scalp, and teeth, including, but not limited to shampoos, body lotions, shower gels, topical moisturizers, toothpaste, toothgels, mouthwashes, mouthrinses, anti-plaque rinses, and/or other topical cleansers. In some particularly preferred embodiments, these products are utilized on humans, while in other embodiments, these products find use with non-human animals (*e.g.*, in veterinary applications).

As used herein, the terms "tooth whitening" and "tooth bleaching" are used interchangeably, to refer to improving the brightness (*e.g.*, whitening) of a tooth or teeth. It is intended that the term encompass any method suitable for whitening teeth, including the present invention, as well as chemical treatment, mild acid treatment, abrasive tooth whitening, and laser tooth whitening. In particularly preferred embodiments, the present invention provides a perhydrolase and perhydrolase-containing compositions suitable for whitening teeth.

As used in herein, “intrinsic stains” in teeth refer to the resulting color from chromogens within the enamel and underlying dentin. The intrinsic color of human teeth tends to become more yellow with aging, due to the thinning of the enamel and darkening of the underlying yellow dentin. Removal of intrinsic stain usually requires the use of peroxides or other oxidizing chemicals, which penetrate the enamel and decolorize the internal chromogens.

In contrast to intrinsic stains, “extrinsic stains” form on the surface of the teeth when exogenous chromogenic materials bind to the enamel, usually within the pellicle naturally coating the teeth. Most people accumulate some degree of unsightly extrinsic stains on their teeth over time. This staining process is promoted by such factors as: (1) the ingestion of tannin-containing foods and beverages such as coffee, tea, or red wine; (2) the use of tobacco products; and/or (3) exposure to certain cationic substances (e.g., tin, iron, and chlorhexidine). These substances tend to adhere to the enamel's hydroxyapatite structure, which leads to tooth discoloration and a concomitant reduction in tooth whiteness. Over a period of years, extrinsic stains may penetrate the enamel layer and result in intrinsic stains.

As used herein, the term “deodorize” means to eliminate or prevent offensive odor.

As used herein, the term “destain” or “destaining” refers to the process of removing a stain from an oral cavity surface. The stain(s) may be intrinsic stains, extrinsic stains, or a combination thereof.

As used herein, “enhanced performance” in a perhydrolase-containing composition is defined as increasing cleaning of bleach-sensitive stains compared to other compositions, as determined using standard methods in the dental art. In particular embodiments, the perhydrolase of the present invention provides enhanced performance in the oxidation and removal of colored stains. In further embodiments, the perhydrolase of the present invention provides enhanced performance in the removal and/or decolorization of stains.

As used herein, “effective amount of perhydrolase enzyme” refers to the quantity of perhydrolase enzyme necessary to achieve the enzymatic activity

required in the specific application. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or non-liquid (*e.g.*, emulsion) composition is required, and the like.

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

As used herein, the term "oligosaccharide" refers to compounds containing between 2 and at least 24 monosaccharide units linked by glycosidic linkages. The term "monosaccharide" refers to a compound of empirical formula $(CH_2O)_n$, where $n \geq 3$, the carbon skeleton is unbranched, each carbon atom except one contains a hydroxyl group, and the remaining carbon atom is an

aldehyde or ketone at carbon atom 1. The term "monosaccharide" also refers to intracellular cyclic hemiacetal or hemiketal forms.

As used herein, the term "excipient" refers to inactive substance used as a carrier for active ingredients in a formulation. The excipient may be used to stabilize the active ingredient in a formulation, such as the storage stability of the active ingredient. Excipients are also sometimes used to bulk up formulations that contain active ingredients. As described herein, the "active ingredient" may be an enzyme having perhydrolytic activity, a peracid produced by the perhydrolytic enzyme under suitable reaction conditions, or a combination thereof.

The term "substantially free of water" will refer to a concentration of water in a formulation that does not adversely impact the storage stability of the enzyme or an enzyme powder when present in the carboxylic acid ester. The carboxylic acid ester may contain a very low concentration of water, for example, triacetin typically has between 180 ppm and 300 ppm of water. In one embodiment, the perhydrolytic enzyme is stored in the carboxylic acid ester substrate that is substantially free of water. In a further embodiment, "substantially free of water" may mean less than 2000 ppm, preferably less than 1000 ppm, more preferably less than 500 ppm, and even more preferably less than 250 ppm of water in the formulation comprising the enzyme (or enzyme powder) and the carboxylic acid ester. In one embodiment, the perhydrolytic enzyme may be stored in an aqueous solution if the generation system is designed such that the enzyme is stable in the aqueous solution (for example, a solution that does not contain a significant concentration of a carboxylic acid ester substrate capable of being hydrolyzed by the enzyme during storage). In one embodiment, the perhydrolytic enzyme may be stored in a mixture comprising the carboxylic acid ester substrate that is substantially free of water and one or more buffers (*e.g.*, sodium and/or potassium salts of bicarbonate, citrate, acetate, phosphate, pyrophosphate, methylphosphonate, succinate, malate, fumarate, tartrate, and maleate).

Enzymes Having Perhydrolytic Activity

Enzymes having perhydrolytic activity may include some enzymes classified as lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations so long as the enzyme has perhydrolytic activity for one or more of the present substrates. Examples may include, but are not limited to perhydrolytic proteases (subtilisin Carlsberg variant; U.S. Patent 7,510,859), perhydrolytic aryl esterases (*Pseudomonas fluorescens*; SEQ ID NO: 477; U.S. Patent 7,384,787), the perhydrolytic aryl esterase/acyl transferase from *Mycobacterium smegmatis* (SEQ ID NOs: 460 and 478; U.S. Patent 7,754,460; WO2005/056782; and EP1689859 B1), and perhydrolase carbohydrate esterases. In a preferred aspect, the perhydrolytic carbohydrate esterase is a CE-7 carbohydrate esterase.

In one embodiment, suitable perhydrolases may include enzymes comprising an amino acid sequence having at least 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to any of the amino acid sequences reported herein with the proviso that non-CE-7 perhydrolases are limited to targeted perhydrolase applications (*i.e.*, perhydrolytic enzymes not belonging to the CE-7 carbohydrate esterase family are used in the form of a fusion protein comprising at least one peptidic targeting domain).

In another embodiment, the suitable perhydrolases may include enzymes comprising an amino acid sequence having at least 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO: 460, 477, and 478 with the proviso that non-CE-7 perhydrolases are limited to targeted perhydrolase applications (i.e., perhydrolytic enzymes not belonging to the CE-7 carbohydrate esterase family are used in the form of a fusion protein comprising at least one peptidic targeting domain). It is understood that percent identity comparisons and sequence alignments used to identify substantially similar perhydrolytic enzymes are conducted against the portion of fusion protein comprising the perhydrolytic enzyme (*i.e.*, targeting domains and linkers not included).

In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the S54V *Mycobacterium smegmatis* aryl esterase provided as SEQ ID NO: 460.

In one embodiment, the fusion protein comprises a perhydrolytic esterase from *Pseudomonas fluorescens*. In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the *Pseudomonas fluorescens* esterase provided as SEQ ID NO: 477.

In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 424, 425, 426, 427, 428, 429, 430, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 476, 477, 478, and 479.

In another embodiment, substantially similar perhydrolytic enzymes may include those encoded by polynucleotide sequences that hybridize under highly stringent hybridization conditions (0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by a final wash of 0.1X SSC, 0.1% SDS, 65°C) to the polynucleotide sequences encoding any of the present perhydrolytic enzymes with the proviso that non-CE-7 perhydrolases are limited to targeted perhydrolase applications (*i.e.*, perhydrolytic enzymes not belonging to the CE-7 carbohydrate esterase family are used in the form of a fusion protein comprising at least one peptidic targeting domain).

CE-7 Perhydrolases

In a preferred embodiment, the oral care compositions and method comprise enzymes having perhydrolytic activity that are structurally classified as members of the carbohydrate family esterase family 7 (CE-7 family) of enzymes (see Coutinho, P.M., Henrissat, B. "Carbohydrate-active enzymes: an integrated database approach" in Recent Advances in Carbohydrate Bioengineering, H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., (1999) The Royal Society of Chemistry, Cambridge, pp. 3-12.). The CE-7 family of enzymes has been

demonstrated to be particularly effective for producing peroxycarboxylic acids from a variety of carboxylic acid ester substrates when combined with a source of peroxygen (U.S. Patents 7,794,378; 7,951,566; 7,723,083; and 7,964,378 and U.S. Patent Application Publication Nos. 2008-0176299, 2010-0087529, 2011-0081693, and 2011-0236335 to DiCosimo *et al.*; each incorporated herein by reference).

Members of the CE-7 family include cephalosporin C deacetylases (CAHs; E.C. 3.1.1.41) and acetyl xylan esterases (AXEs; E.C. 3.1.1.72). Members of the CE-7 esterase family share a conserved signature motif (Vincent *et al.*, *J. Mol. Biol.*, 330:593-606 (2003)). Perhydrolases comprising the CE-7 signature motif ("CE-7 perhydrolases") and/or a substantially similar structure are suitable for use in the compositions and methods described herein. Means to identify substantially similar biological molecules are well known in the art (*e.g.*, sequence alignment protocols, nucleic acid hybridizations and/or the presence of a conserved signature motif). In one aspect, the perhydrolase includes an enzyme comprising the CE-7 signature motif and at least 20%, preferably at least 30%, more preferably at least 33%, more preferably at least 40%, more preferably at least 42%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, and most preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to one of the sequences provided herein.

As used herein, the phrase "enzyme is structurally classified as a CE-7 enzyme", "CE-7 perhydrolase" or "structurally classified as a carbohydrate esterase family 7 enzyme" will be used to refer to enzymes having perhydrolysis activity which are structurally classified as a CE-7 carbohydrate esterase. This family of enzymes can be defined by the presence of a signature motif (Vincent et al., *supra*). The signature motif for CE-7 esterases comprises three conserved motifs (residue position numbering relative to reference sequence SEQ ID NO: 2; the CE-7 perhydrolase from *B. subtilis* ATCC® 31954™):

a) Arg118-Gly119-Gln120;

b) Gly179-Xaa180-**Ser181**-Gln182-Gly183; and

c) **His298**-Glu299.

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

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

Leu267-Xaa268-**Asp269**.

The Xaa at amino acid residue position 268 is typically isoleucine, valine, or methionine. The fourth motif includes the aspartic acid residue (bold) belonging to the catalytic triad (**Ser181-Asp269-His298**).

The CE-7 perhydrolases may be in the form of fusion proteins having at least one peptidic component having affinity for at least one body surface. In one embodiment, all alignments used to determine if a targeted perhydrolase (fusion protein) comprises the CE-7 signature motif will be based on the amino acid sequence of the perhydrolytic enzyme without the peptidic component having the affinity for a body surface.

A number of well-known global alignment algorithms (*i.e.*, sequence analysis software) may be used to align two or more amino acid sequences representing enzymes having perhydrolase activity to determine if the enzyme is comprised of the present signature motif. The aligned sequence(s) are

compared to the reference sequence (SEQ ID NO: 2) to determine the existence of the signature motif. In one embodiment, a CLUSTAL alignment (such as CLUSTALW) using a reference amino acid sequence (as used herein the perhydrolase sequence (SEQ ID NO: 2) from the *Bacillus subtilis* ATCC® 31954™) is used to identify perhydrolases belonging to the CE-7 esterase family. The relative numbering of the conserved amino acid residues is based on the residue numbering of the reference amino acid sequence to account for small insertions or deletions (for example, typically five amino acids of less) within the aligned sequence.

Examples of other suitable algorithms that may be used to identify sequences comprising the present signature motif (when compared to the reference sequence) include, but are not limited to, Needleman and Wunsch (*J. Mol. Biol.* 48, 443-453 (1970); a global alignment tool) and Smith-Waterman (*J. Mol. Biol.* 147:195-197 (1981); a local alignment tool). In one embodiment, a Smith-Waterman alignment is implemented using default parameters. An example of suitable default parameters include the use of a BLOSUM62 scoring matrix with GAP open penalty = 10 and a GAP extension penalty = 0.5.

A comparison of the overall percent identity among perhydrolases indicates that enzymes having as little as 33% amino acid identity to SEQ ID NO: 2 (while retaining the signature motif) exhibit significant perhydrolase activity and are structurally classified as CE-7 carbohydrate esterases. In one embodiment, suitable perhydrolases include enzymes comprising the CE-7 signature motif and at least 20%, preferably at least 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO: 2.

Examples of suitable CE-7 carbohydrate esterases having perhydrolytic activity include, but are not limited to, enzymes having an amino acid sequence such as SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476. In one embodiment, the enzyme comprises an amino acid

sequence selected from the group consisting of 14, 16, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476.

As used herein, the term "CE-7 variant", "variant perhydrolase" or "variant" will refer to CE-7 perhydrolases having a genetic modification that results in at least one amino acid addition, deletion, and/or substitution when compared to the corresponding enzyme (typically the wild type enzyme) from which the variant was derived; so long as the CE-7 signature motif and the associated perhydrolytic activity are maintained. CE-7 variant perhydrolases may also be used in the present compositions and methods. Examples of CE-7 variants are provided as SEQ ID NOs: 27, 28, 29, 30, 31, 32, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476. In one embodiment, the variants may include SEQ ID NOs: 27, 28, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476.

The skilled artisan recognizes that substantially similar CE-7 perhydrolase sequences (retaining the signature motifs) may also be used in the present compositions and methods. In one embodiment, substantially similar sequences are defined by their ability to hybridize, under highly stringent conditions with the nucleic acid molecules associated with sequences exemplified herein. In another embodiment, sequence alignment algorithms may be used to define substantially similar enzymes based on the percent identity to the DNA or amino acid sequences provided herein.

As used herein, a nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single strand of the first molecule can anneal to the other molecule under appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J. and Russell, D., T. Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2001). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar molecules, such as homologous sequences from distantly related organisms, to highly similar molecules, such as genes that duplicate functional enzymes from closely related

organisms. Post-hybridization washes typically determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent hybridization conditions is 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by a final wash of 0.1X SSC, 0.1% SDS, 65°C.

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

As used herein, the term "percent identity" is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: Computational Molecular Biology (Lesk, A. M., ed.) Oxford University Press, NY (1988); Biocomputing: Informatics and Genome Projects (Smith, D. W., ed.) Academic Press, NY (1993); Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., eds.) Humana Press, NJ (1994); Sequence Analysis in Molecular Biology (von Heinje, G., ed.) Academic Press (1987); and Sequence Analysis Primer (Gribskov, M. and Devereux, J., eds.) Stockton Press, NY (1991). Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI), the AlignX program of Vector NTI v. 7.0 (Informax, Inc., Bethesda, MD), or the EMBOSS Open Software Suite (EMBL-EBI; Rice *et al.*, *Trends in Genetics* 16, (6):276-277 (2000)). Multiple alignment of the sequences can be performed using the CLUSTAL method (such as CLUSTALW; for example version 1.83) of alignment (Higgins and Sharp, *CABIOS*, 5:151-153 (1989); Higgins *et al.*, *Nucleic Acids Res.* 22:4673-4680 (1994); and Chenna *et al.*, *Nucleic Acids Res* 31 (13):3497-500 (2003)), available from the European Molecular Biology Laboratory via the European Bioinformatics Institute) with the default parameters. Suitable parameters for CLUSTALW protein alignments include GAP Existence penalty=15, GAP extension =0.2, matrix = Gonnet (*e.g.*, Gonnet250), protein ENDGAP = -1, protein GAPDIST=4, and KTUPLE=1. In one embodiment, a fast or slow alignment is used with the default settings where a slow alignment is preferred. Alternatively, the parameters using the CLUSTALW method (*e.g.*, version 1.83) may be modified to also use KTUPLE =1, GAP PENALTY=10, GAP

extension =1, matrix = BLOSUM (*e.g.*, BLOSUM64), WINDOW=5, and TOP DIAGONALS SAVED=5.

In one aspect, suitable isolated nucleic acid molecules encode a polypeptide having an amino acid sequence that is at least about 20%, preferably at least 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequences reported herein. In another aspect, suitable isolated nucleic acid molecules encode a polypeptide having an amino acid sequence that is at least about 20%, preferably at least 30%, 33%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequences reported herein; with the proviso that the polypeptide retains the CE-7 signature motif. Suitable nucleic acid molecules not only have the above homologies, but also typically encode a polypeptide having about 210 to 340 amino acids in length, about 300 to about 340 amino acids, preferably about 310 to about 330 amino acids, and most preferably about 318 to about 325 amino acids in length wherein each polypeptide is characterized as having perhydrolytic activity.

Targeted Perhydrolases

As used herein, the term "targeted perhydrolase" and "targeted enzyme having perhydrolytic activity" will refer to a fusion proteins comprising at least one perhydrolytic enzyme (wild type or variant thereof) fused/coupled to at least one peptidic component having affinity for a target surface, preferably a targeted body surface. The perhydrolytic enzyme within the targeted perhydrolase may be any perhydrolytic enzyme and may include lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations so long as the enzyme has perhydrolytic activity for one or more of the present substrates. Examples may include, but are not limited to perhydrolytic proteases (e.g., subtilisin variant; U.S. Patent 7,510,859), perhydrolytic esterases (e.g., *Pseudomonas fluorescens;* U.S. Patent 7,384,787; SEQ ID NO: 477), and perhydrolytic aryl esterases (e.g., *Mycobacterium smegmatis*; U.S. Patent

7,754,460; WO2005/056782; and EP1689859 B1; SEQ ID NOs: 460 [S54V variant] and 478 [wild type]).

In one embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the S54V *Mycobacterium smegmatis* aryl esterase provided as SEQ ID NO: 460.

In one embodiment, the fusion protein comprises a perhydrolytic esterase from *Pseudomonas fluorescens*. In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence with at least 95% identity to the *Pseudomonas fluorescens* esterase provided as SEQ ID NO: 477.

In another embodiment, the fusion protein comprises a perhydrolytic enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 424, 425, 426, 427, 428, 429, 430, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 476, 477, 478, and 479.

As used herein the terms “peptidic component”, “peptidic component having affinity for an oral cavity surface”, and “OCBD” will refer to component of the fusion protein that is not part of the perhydrolytic enzyme comprising at least one polymer of two or more amino acids joined by a peptide bond; wherein the component has affinity for the target oral cavity surface.

In one embodiment, the peptidic component having affinity for a body surface may be an antibody, an Fab antibody fragment, a single chain variable fragment (scFv) antibody, a *Camelidae* antibody (Muyldermans, S., *Rev. Mol. Biotechnol.* (2001) 74:277-302), a non-antibody scaffold display protein (Hosse *et al.*, *Prot. Sci.* (2006) 15(1): 14-27 and Binz, H. *et al.* (2005) *Nature Biotechnology* 23, 1257-1268 for a review of various scaffold-assisted approaches) or a single chain polypeptide lacking an immunoglobulin fold. In another aspect, the peptidic component having affinity for a body surface is a single chain peptide lacking an immunoglobulin fold (*i.e.*, a body surface-binding peptide or a body surface-binding domain comprising at least one body surface-binding peptide having affinity for an oral cavity surface). In a preferred embodiment, the peptidic

component is a single chain peptide comprising one or more body surface-binding peptides having affinity for an oral cavity surface.

The peptidic component having affinity for an oral cavity surface may be separated from the perhydrolytic enzyme by an optional peptide linker. Certain peptide linkers/spacers are from 1 to 100 or 1 to 50 amino acids in length. In some embodiments, the peptide spacers are about 1 to about 25, 3 to about 40, or 3 to about 30 amino acids in length. In other embodiments are spacers that are about 5 to about 20 amino acids in length. Multiple peptide linkers may be used. In one embodiment, at least one peptide linker is present and may be repeated up to 10 times.

Peptides previously identified as having affinity for one body surface may have affinity for an oral care surface as well. As such, the fusion peptide may comprise at least one previously reported to have affinity for another body surface, such as hair (SEQ ID NOs 65-221, 271, and 368); skin (SEQ ID NOs: 217-269); or nail (SEQ ID NOs: 270-271). In one embodiment, the fusion peptide comprises at least one oral cavity surface-binding peptide from the group comprising SEQ ID NOs: 272-382 and 399-422. In one embodiment, the fusion peptide comprises at least one oral cavity surface-binding peptide selected from the group consisting of SEQ ID NOs: 272-382, 399-410, and 412-422; wherein SEQ ID NOs 272-291 and 312-382 have affinity for tooth pellicle; SEQ ID NOs: 292-311 have affinity for tooth enamel; and SEQ ID NOs 399-410 and 412-422 have affinity for tooth enamel or pellicle. Some of the body surface-binding peptides may have strong affinity for more than one body surface, and as such, may be used to target perhydrolytic enzymes to different body surfaces. In another embodiment, the fusion peptide may include any body surface-binding peptide designed to have electrostatic attraction to the target body surface (e.g., a body surface-binding peptide engineered to electrostatically bind to the target body surface).

In another embodiment the target surface is a material that is part of the packaging and/or method of delivery to the oral cavity. The peptidic component is selected for it affinity to a material or materials in use such as polymers,

plastics and films. The targeted perhydrolase fusion protein design allows for the controlled delivery and removal of the perhydrolase from the user by maintaining it on a removable device such as, but not limited to, a mouth tray or strip.

Targeted CE-7 Perhydrolases

In a preferred embodiment, the "targeted perhydrolase" is a targeted CE-7 carbohydrate esterase having perhydrolytic activity. As used herein, the terms "targeted CE-7 perhydrolase" and "targeted CE-7 carbohydrate esterase" will refer to fusion proteins comprising at least one CE-7 perhydrolase (wild type or variant perhydrolase) fused/coupled to at least one peptidic component having affinity for a targeted surface, preferably a targeted body surface. The peptidic component having affinity for a body surface may be any of those describe above. In a preferred aspect, the peptidic component in a targeted CE-7 perhydrolase is a single chain peptide lacking an immunoglobulin fold (*i.e.*, a body surface-binding peptide or a body surface-binding domain comprising at least one body surface-binding peptide having affinity for an oral cavity surface). In a preferred embodiment, the peptidic component is a single chain peptide comprising one or more body surface-binding peptides having affinity for an oral cavity surface.

The peptidic component having affinity for an oral cavity surface may be separated from the CE-7 perhydrolase by an optional peptide linker. Certain peptide linkers/spacers are from 1 to 100 or 1 to 50 amino acids in length. In some embodiments, the peptide spacers are about 1 to about 25, 3 to about 40, or 3 to about 30 amino acids in length. In other embodiments are spacers that are about 5 to about 20 amino acids in length. Multiple peptide linkers may be used.

As such, examples of targeted CE-7 perhydrolases may include, but are not limited to, any of the CE-7 perhydrolases having an amino acid sequence selected from the group consisting of SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476 coupled to a peptidic component

having affinity for an oral cavity surface. In a preferred embodiment, examples of targeted perhydrolases may include, but are not limited to, any of CE-7 perhydrolases having an amino acid sequence selected from the group consisting of SEQ ID NOs 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476 coupled to one or more body surface-binding peptides having affinity for an oral cavity surface (optionally through a peptide spacer).

In another embodiment, targeted CE-7 perhydrolases may comprise peptides previously identified as having affinity for one body surface may have affinity for an oral care surface as well. As such, the fusion peptide may comprise at least one previously reported to have affinity for another body surface, such as hair (SEQ ID NOs 65-221, 271, and 368); skin (SEQ ID NOs: 217-269); or nail (SEQ ID NOs: 270-271). In one embodiment, the fusion peptide comprises at least one oral cavity surface-binding peptide from the group comprising SEQ ID NOs: 272-382 and 399-422. In one embodiment, the CE-7 perhydrolase fusion peptide comprises at least one oral cavity surface-binding peptide selected from the group consisting of SEQ ID NOs: 272-382, 399-410, and 412-422; wherein SEQ ID NOs 272-291 and 312-382 have affinity for tooth pellicle; SEQ ID NOs: 292-311 have affinity for tooth enamel; and SEQ ID NOs 399-410 and 412-422 have affinity for tooth enamel or pellicle. Some of the body surface-binding peptides may have strong affinity for more than one body surface, and as such, may be used to target perhydrolytic enzymes to different body surfaces. In another embodiment, the CE-7 perhydrolase fusion peptide may include any body surface-binding peptide designed to have electrostatic attraction to the target body surface (*e.g.*, a body surface-binding peptide engineered to electrostatically bind to the target body surface).

In another embodiment, the target surface is a material that is part of the packaging and or delivery to the oral cavity. The peptidic component is selected for it affinity to a material or materials in use such as polymers, plastics and films. The targeted CE-7 perhydrolase fusion protein design allows for the controlled

delivery and removal of the perhydrolase from the user by maintaining it on a removable device such as a mouth tray or strip.

Peptides Having Affinity for a Body Surface

Single chain peptides lacking an immunoglobulin fold that are capable of binding to an oral cavity surface are referred to as "oral cavity surface-binding peptides" (OCBP) and may include, for example, peptides that bind to a tooth surface (tooth-binding peptides), peptides having affinity for a soft tissue such as the gums, or peptides having affinity for an orally-acceptable material that is safe for use in the oral cavity. The tooth-binding peptides may include peptides having affinity for tooth enamel ("tooth enamel-binding peptides") and peptides having affinity for tooth pellicle ("tooth pellicle-binding peptides").

A non-limiting list of peptides having affinity for at least one body surface are provided herein including those having affinity for hair (hair-binding peptides having an amino acid sequence selected from the group consisting of SEQ ID NOs: 65-221, 271, and 368), skin (skin-binding peptides comprise an amino acid sequence selected from the group consisting of SEQ ID NOs: 217-269), and nail (nail-binding peptides comprise an amino acid sequence selected from the group consisting of SEQ ID NOs: 270-271). Examples of peptides having affinity for an oral cavity surface (oral cavity-binding peptides) comprise an amino acid sequence selected from the group consisting of SEQ ID NOs: 272-382 and 399-422. In a preferred aspect, the peptides having affinity for an oral cavity surface are selected from the group consisting of SEQ ID NOs: 272-382, 399-410, and 412-422; wherein SEQ ID NOs 272-291 and 312-382 have affinity for tooth pellicle; SEQ ID NOs: 292-311 have affinity for tooth enamel; and SEQ ID NOs 399-410 and 412-422 having affinity for tooth enamel or pellicle.

In one embodiment, a peptide that may also have affinity for an oral cavity surface may include one or more of SEQ ID NOs. 65-382, 399-410, and 412-422. Preferably, the peptides used in the present compositions and methods are selected from the group consisting of SEQ ID NOs: 272-382, 399-410, and 412-422. In another embodiment, oral cavity surface-binding peptides may include

skin-binding peptides for some surfaces with the oral cavity (*e.g.*, gums). In another embodiment, the fusion peptide may include any body surface-binding peptide designed to have electrostatic attraction to the target body surface (*e.g.*, a body surface-binding peptide engineered to electrostatically bind to the target body surface).

In another embodiment, the present compositions and methods comprise at least one oral cavity surface-binding peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs 399-410 and 412-422.

In some embodiments, oral cavity surface-binding domains are comprised of oral cavity surface-binding peptides that are up to about 60 amino acids in length. In one embodiment the oral cavity surface-binding peptides are 5 to 60 amino acids in length. In other embodiments a surface-binding peptides are 7 to 50 amino acids in length or 7 to 30 amino acids in length. In still other embodiments are those oral cavity surface-binding peptides that are 7 to 27 amino acids in length.

While fusion peptides comprising oral cavity surface-binding peptides are certain embodiments of the invention, in other embodiments of the invention, it may be advantageous to use multiple oral cavity surface-binding peptides. The inclusion of multiple, *i.e.*, two or more, oral cavity surface-binding peptides can provide a peptidic component that is, for example, even more durable than those binding elements including a single oral cavity surface-binding peptide. In some embodiments, the oral cavity surface-binding domains (that is, multiple, *i.e.*, two or more, oral cavity surface-binding peptides) includes from 2 to about 50 or 2 to about 25 oral cavity surface-binding peptides. Other embodiments include those oral cavity surface-binding domains including 2 to about 10 or 2 to 5 oral cavity surface-binding peptides.

Multiple binding elements (*i.e.*, oral cavity surface-binding peptides or oral cavity surface-binding domains) can be linked directly together or they can be linked together using peptide spacers. Certain peptide spacers/linkers are from 1 to 100 or 1 to 50 amino acids in length. In some embodiments, the peptide spacers are about 1 to about 25, 3 to about 40, or 3 to about 30 amino acids in

length. In other embodiments are spacers that are 1 to about 20 or about 5 to about 20 amino acids in length.

Oral cavity surface-binding domains, and the shorter oral cavity surface-binding peptides of which they are comprised, can be identified using any number of methods known to those skilled in the art, including, for example, any known biopanning techniques such as phage display, bacterial display, yeast display, ribosome display, mRNA display, and combinations thereof. Typically a random or substantially random (in the event bias exists) library of peptides is biopanned against the target body surface to identify peptides within the library having affinity for the target body surface.

The generation of random libraries of peptides is well known and may be accomplished by a variety of techniques including, bacterial display (Kemp, D.J.; *Proc. Natl. Acad. Sci. USA* 78(7):4520-4524 (1981), and Helfman *et al.*, *Proc. Natl. Acad. Sci. USA* 80(1):31-35, (1983)), yeast display (Chien *et al.*, *Proc Natl Acad Sci USA* 88(21):9578-82 (1991)), combinatorial solid phase peptide synthesis (U.S. Patent 5,449,754, U.S. Patent 5,480,971, U.S. Patent 5,585,275, U.S. Patent 5,639,603), and phage display technology (U.S. Patent 5,223,409, U.S. Patent 5,403,484, U.S. Patent 5,571,698, U.S. Patent 5,837,500); ribosome display (U.S. Patent 5,643,768; U.S. Patent 5,658,754; and U.S. Patent 7,074,557), and mRNA display technology (PROFUSION™; see U.S. Patent Nos. 6,258,558; 6,518,018; 6,281,344; 6,214,553; 6,261,804; 6,207,446; 6,846,655; 6,312,927; 6,602,685; 6,416,950; 6,429,300; 7,078,197; and 6,436,665).

Binding Affinity

The peptidic component having affinity for the oral cavity surface comprises a binding affinity for an oral cavity surface of 10^{-5} molar (M) or less. In certain embodiments, the peptidic component is one or more oral cavity surface-binding peptides and/or binding domain(s) having a binding affinity for human hair, skin, nail or oral cavity of 10^{-5} molar (M) or less. In some embodiments, the binding peptides or domains will have a binding affinity value

of 10^{-5} M or less in the presence of at least about 50 – 500 mM salt. The term "binding affinity" refers to the strength of the interaction of a binding peptide with its respective substrate, in this case, a human oral cavity surface (gums, teeth, etc). Binding affinity can be defined or measured in terms of the binding peptide's dissociation constant ("K_D"), or "MB_{50}."

"K_D" corresponds to the concentration of peptide at which the binding site on the target is half occupied, *i.e.*, when the concentration of target with peptide bound (bound target material) equals the concentration of target with no peptide bound. The smaller the dissociation constant, the more tightly the peptide is bound. For example, a peptide with a nanomolar (nM) dissociation constant binds more tightly than a peptide with a micromolar (μM) dissociation constant. Certain embodiments of the invention will have a K_D value of 10^{-5} or less.

"MB_{50}" refers to the concentration of the binding peptide that gives a signal that is 50% of the maximum signal obtained in an ELISA-based binding assay. See, *e.g.*, Example 3 of U.S. Patent Application Publication 2005/022683; hereby incorporated by reference. The MB_{50} provides an indication of the strength of the binding interaction or affinity of the components of the complex. The lower the value of MB_{50}, the stronger, *i.e.*, "better," the interaction of the peptide with its corresponding substrate. For example, a peptide with a nanomolar (nM) MB_{50} binds more tightly than a peptide with a micromolar (μM) MB_{50}. Certain embodiments of the invention will have a MB_{50} value of 10^{-5} M or less.

In some embodiments, the peptidic component having affinity for a oral cavity surface may have a binding affinity, as measured by K_D or MB_{50} values, of less than or equal to about 10^{-5} M, less than or equal to about 10^{-6} M, less than or equal to about 10^{-7} M, less than or equal to about 10^{-8} M, less than or equal to about 10^{-9} M, or less than or equal to about 10^{-10} M.

In some embodiments, the oral cavity surface-binding peptides and/or oral cavity surface-binding domains may have a binding affinity, as measured by K_D or MB_{50} values, of less than or equal to about 10^{-5} M, less than or equal to about 10^{-6} M, less than or equal to about 10^{-7} M, less than or equal to about 10^{-8} M, less than or equal to about 10^{-9} M, or less than or equal to about 10^{-10} M.

As used herein, the term "strong affinity" will refer to a binding affinity having a K_D or MB_{50} value of less than or equal to about 10^{-5} M, preferably less than or equal to about 10^{-6} M, more preferably less than or equal to about 10^{-7} M, more preferably less than or equal to about 10^{-8} M, less than or equal to about 10^{-9} M, or most preferably less than or equal to about 10^{-10} M.

Multicomponent Peroxycarboxylic acid Generation Systems

The design of systems and means for separating and combining multiple active components are known in the art and generally will depend upon the physical form of the individual reaction components. For example, multiple active fluids (liquid-liquid) systems typically use multi-chamber dispenser bottles or two-phase systems (*e.g.*, U.S. Patent Application Publication No. 2005/0139608; U.S. Patent 5,398,846; U.S. Patent 5,624,634; U.S. Patent 6,391,840; E.P. Patent 0807156B1; U.S. Patent Application. Pub. No. 2005/0008526; and PCT Publication No. WO 00/61713) 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 peroxycarboxylic acid may include, but are not limited to, those designed for one or more solid components or combinations of solid-liquid components, such as powders (*e.g.*, U.S. Patent 5,116,575), multi-layered tablets (*e.g.*, U.S. Patent 6,210,639), water dissolvable packets having multiple compartments (*e.g.*, U.S. Patent 6,995,125) and solid agglomerates that react upon the addition of water (*e.g.*, U.S. Patent 6,319,888).

In another embodiment, the carboxylic acid ester in the first component is selected from the group consisting of monoacetin, diacetin, triacetin, and combinations thereof. In another embodiment, the carboxylic acid ester in the first component is an acetylated saccharide. In another embodiment, the enzyme catalyst in the first component may be a particulate solid, a liquid or gel. In another embodiment, the first reaction component may be a solid tablet or powder

Peroxycarboxylic acids are quite reactive and generally decrease in concentration over time. This is especially true for commercial pre-formed peroxycarboxylic acid compositions that often lack long term stability. Aqueous solutions of pre-formed peroxycarboxylic acids may also present handling and/or shipping difficulties, especially when shipping large containers and/or highly concentrated peroxycarboxylic acid solutions over longer distances. Further, pre-formed peroxycarboxylic acid solutions may not be able to provide the desired concentration of peroxycarboxylic acid for a particular target application. As such, it is highly desirable to keep the various reaction components separated, especially for liquid formulations.

The use of multi-component peroxycarboxylic acid generation systems comprising two or more components that are combined to produce the desired peroxycarboxylic acid has been reported. The individual components should be safe to handle and stable for extended periods of time (*i.e.*, as measured by the concentration of peroxycarboxylic acid produced upon mixing). In one embodiment, the storage stability of a multi-component enzymatic peroxycarboxylic acid generation system may be measured in terms of enzyme catalyst stability.

Personal care products comprising a multi-component peroxycarboxylic acid generation formulation are provided herein that use an enzyme catalyst to rapidly produce an aqueous peracid solution having a desired peroxycarboxylic acid concentration. The mixing may occur immediately prior to use and/or at the site (*in situ*) of application. In one embodiment, the personal care product formulation will be comprised of at least two components that remain separated until use. Mixing of the components rapidly forms an aqueous peracid solution. Each component is designed so that the resulting aqueous peracid solution comprises an efficacious peracid concentration suitable for the intended end use. The composition of the individual components should be designed to (1) provide extended storage stability and/or (2) provide the ability to enhance formation of a suitable aqueous reaction formulation comprised of peroxycarboxylic acid.

The multi-component formulation may be comprised of at least two substantially liquid components. In one embodiment, the multi-component formulation may be a two-component formulation comprises a first liquid component and a second liquid component. The use of the terms "first" or "second" liquid component is relative provided that two different liquid components comprising the specified ingredients remain separated until use. At a minimum, the multi-component peroxycarboxylic acid formulation comprises (1) at least one enzyme catalyst having perhydrolysis activity, wherein said at least one enzyme is preferably classified as a CE-7 esterase, (2) a carboxylic acid ester substrate, and (3) a source of peroxygen and water wherein the formulation enzymatically produces the desired peracid upon combining the components.

The type and amount of the various ingredients used within two component formulation should to be carefully selected and balanced to provide (1) storage stability of each component, especially the perhydrolysis activity of the enzyme catalyst and (2) physical characteristics that enhance solubility and/or the ability to effectively form the desired aqueous peroxycarboxylic acid solution (*e.g.*, ingredients that enhance the solubility of the ester substrate in the aqueous reaction mixture and/or ingredients that modify the viscosity and/concentration of at least one of the liquid components [*i.e.*, at least one cosolvent that does not have a significant, adverse effect on the enzymatic perhydrolysis activity]).

Various methods to improve the performance and/or catalyst stability of enzymatic peracid generation systems have been disclosed. U.S. Patent Application Publication No. 2010-0048448 A1 describes the use of at least one cosolvent to enhance solubility and/or the mixing characteristics of certain ester substrates. The present personal care compositions and methods may also use a cosolvent. In one embodiment, the component comprising the carboxylic acid ester substrate and the perhydrolase catalyst comprises an organic solvent having a Log P value of less than about 2, wherein Log P is defined as the logarithm of the partition coefficient of a substance between octanol and water, expressed as $P = [solute]_{octanol}/[solute]_{water}$. Several cosolvents having a log P

value of 2 or less that do not have a significant adverse impact on enzyme activity are described. In another embodiment, the cosolvent is about 20 wt% to about 70 wt% within the reaction component comprising the carboxylic acid ester substrate and the enzyme. The reaction component comprising the carboxylic acid ester substrate and the enzyme may optionally comprise one or more buffers (*e.g.*, sodium and/or potassium salts of bicarbonate, citrate, acetate, phosphate, pyrophosphate, methylphosphonate, succinate, malate, fumarate, tartrate, and maleate).

U.S. Patent Application Publication No. 2010-0086534 A1 describes the use of a two component system wherein the first component comprises a formulation of a liquid carboxylic acid ester and solid enzyme powder; wherein said enzyme powder comprises a formulation of (a) at least one CE-7 esterase having perhydrolysis activity and (b) at least one oligosaccharide excipient; and the second component comprises water having a source of peroxygen and a hydrogen peroxide stabilizer. The present personal care compositions and methods may use a two-component formulation similar to the system described in US 2010-0086534 A1. As such, an oligosaccharide excipient may be used to help stabilize enzyme activity. In one embodiment, the oligosaccharide excipient may have a number average molecular weight of at least about 1250 and a weight average molecular weight of at least about 9000. In another embodiment, the oligosaccharide excipient has have a number average molecular weight of at least about 1700 and a weight average molecular weight of at least about 15000. In another embodiment, the oligosaccharide is maltodextrin.

U.S. Patent Application Publication No. 2010-0086535-A1 also describes a two component system wherein the first component comprises a formulation of a liquid carboxylic acid ester and solid enzyme powder, said formulation comprising (a) an enzyme powder comprising at least one CE-7 esterase having perhydrolysis activity and at least one oligosaccharide excipient and at least one surfactant; and (b) at least one buffer, where in a preferred embodiment the buffer is added as a separate (*i.e.* separate from the enzyme powder) insoluble component to the carboxylic acid ester substrate; and the second component

comprises water having a source of peroxygen and a hydrogen peroxide stabilizer. The present personal care compositions and methods may use a two component formulation similar to the system described in US 2010-0086535 A1. In one embodiment, the excipient may be an oligosaccharide excipient that has a number average molecular weight of at least about 1250 and a weight average molecular weight of at least about 9000. In another embodiment, the oligosaccharide excipient may have a number average molecular weight of at least about 1700 and a weight average molecular weight of at least about 15000. In another embodiment, the oligosaccharide is maltodextrin. In a further embodiment, the optional pH buffer is a bicarbonate buffer. In yet a further embodiment, the hydrogen peroxide stabilizer is TURPINAL® SL.

Enzyme Powders

In some embodiments, the personal care compositions may use an enzyme catalyst in form of a stabilized enzyme powder. Methods to make and stabilize formulations comprising an enzyme powder are described in U.S. Patent Application Publication Nos. 2010-0086534 and 2010-0086535.

In one embodiment, the enzyme may be in the enzyme powder in an amount in a range of from about 5 weight percent (wt%) to about 75 wt% based on the dry weight of the enzyme powder. A preferred weight percent range of the enzyme in the enzyme powder/spray-dried mixture is from about 10 wt% to 50 wt%, and a more preferred weight percent range of the enzyme in the enzyme powder/spray-dried mixture is from about 20 wt% to 33 wt%

In one embodiment, the enzyme powder may further comprise an excipient. In one aspect, the excipient is provided in an amount in a range of from about 95 wt% to about 25 wt% based on the dry weight of the enzyme powder. A preferred wt % range of excipient in the enzyme powder is from about 90 wt% to 50 wt%, and a more preferred wt % range of excipient in the enzyme powder is from about 80 wt% to 67 wt%.

In one embodiment, the excipient used to prepare an enzyme powder may be an oligosaccharide excipient. In one embodiment, the oligosaccharide

excipient has a number average molecular weight of at least about 1250 and a weight average molecular weight of at least about 9000. In some embodiments, the oligosaccharide excipient has a number average molecular weight of at least about 1700 and a weight average molecular weight of at least about 15000. Specific oligosaccharides may include, but are not limited to, maltodextrin, xylan, mannan, fucoidan, galactomannan, chitosan, raffinose, stachyose, pectin, insulin, levan, graminan, amylopectin, sucrose, lactulose, lactose, maltose, trehalose, cellobiose, nigerotriose, maltotriose, melezitose, maltotriulose, raffinose, kestose, and mixtures thereof. In a preferred embodiment, the oligosaccharide excipient is maltodextrin. Oligosaccharide-based excipients may also include, but are not limited to, water-soluble non-ionic cellulose ethers, such as hydroxymethyl-cellulose and hydroxypropylmethylcellulose, and mixtures thereof. In yet a further embodiment, the excipient may be selected from, but not limited to, one or more of the following compounds: trehalose, lactose, sucrose, mannitol, sorbitol, glucose, cellobiose, α-cyclodextrin, and carboxymethylcellulose.

The formulations may comprise at least one optional surfactant, where the presence of at least one surfactant is preferred. Surfactants may include, but are not limited to, ionic and nonionic surfactants or wetting agents, such as ethoxylated castor oil, polyglycolyzed glycerides, acetylated monoglycerides, sorbitan fatty acid esters, poloxamers, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene derivatives, monoglycerides or ethoxylated derivatives thereof, diglycerides or polyoxyethylene derivatives thereof, sodium docusate, sodium lauryl sulfate, cholic acid or derivatives thereof, lecithins, phospholipids, block copolymers of ethylene glycol and propylene glycol, and non-ionic organosilicones. Preferably, the surfactant is a polyoxyethylene sorbitan fatty acid ester, with polysorbate 80 being more preferred.

When the formulation comprises an enzyme powder, the surfactant used to prepare the powder may be present in an amount ranging from about 5 wt% to 0.1 wt% based on the weight of protein present in the enzyme powder, preferably from about 2 wt% to 0.5 wt% based on the weight of protein present in the enzyme powder.

The enzyme powder may additionally comprise one or more buffers (*e.g.*, sodium and/or potassium salts of bicarbonate, citrate, acetate, phosphate, pyrophosphate, methylphosphonate, succinate, malate, fumarate, tartrate, and maleate), and an enzyme stabilizer (*e.g.*, ethylenediaminetetraacetic acid, (1-hydroxyethylidene)bisphosphonic acid)).

Spray drying of the formulation to form the enzyme powder is carried out, for example, as described generally in Spray Drying Handbook, 5th ed., K. Masters, John Wiley & Sons, Inc., NY, N.Y. (1991), and in PCT Patent Publication Nos. WO 97/41833 and WO 96/32149 to Platz, R. *et al.*

In general spray drying consists of bringing together a highly dispersed liquid, and a sufficient volume of hot air to produce evaporation and drying of the liquid droplets. Typically the feed is sprayed into a current of warm filtered air that evaporates the solvent and conveys the dried product to a collector. The spent air is then exhausted with the solvent. Those skilled in the art will appreciate that several different types of apparatus may be used to provide the desired product. For example, commercial spray dryers manufactured. by Buchi Ltd. (Postfach, Switzerland) or GEA Niro Corp. (Copenhagen, Denmark) will effectively produce particles of desired size. It will further be appreciated that these spray dryers, and specifically their atomizers, may be modified or customized for specialized applications, such as the simultaneous spraying of two solutions using a double nozzle technique. More specifically, a water-in-oil emulsion can be atomized from one nozzle and a solution containing an anti-adherent such as mannitol can be co-atomized from a second nozzle. In other cases it may be desirable to push the feed solution though a custom designed nozzle using a high pressure liquid chromatography (HPLC) pump. Provided that microstructures comprising the correct morphology and/or composition are produced the choice of apparatus is not critical and would be apparent to the skilled artisan in view of the teachings herein.

The temperature of both the inlet and outlet of the gas used to dry the sprayed material is such that it does not cause degradation of the enzyme in the sprayed material. Such temperatures are typically determined experimentally,

although generally, the inlet temperature will range from about 50 °C to about 225 °C, while the outlet temperature will range from about 30 °C to about 150 °C. Preferred parameters include atomization pressures ranging from about 20-150 psi (0.14 MPa – 1.03 MPa), and preferably from about 30-40 to 100 psi (0.21-0.28 MPa to 0.69 MPa). Typically the atomization pressure employed will be one of the following (MPa) 0.14, 0.21, 0.28, 0.34, 0.41, 0.48, 0.55, 0.62, 0.69, 0.76, 0.83 or above.

In one embodiment, "substantially retains its enzymatic activity" is meant that the enzyme powder or a formulation of the enzyme powder in carboxylic acid ester retains at least about 75 percent of the enzyme activity of the enzyme in the enzyme powder or a formulation of the enzyme powder after an extended storage period at ambient temperature and/or after a short storage period at an elevated temperature (above ambient temperature) in a formulation comprised of a carboxylic acid ester and the enzyme powder as compared to the initial enzyme activity of the enzyme powder prior to the preparation of a formulation comprised of the carboxylic acid ester and the enzyme powder. The extended storage period is a period of time of from about one year to about two years at ambient temperature. In one embodiment, the short storage period at an elevated temperature is a period of time of from when the formulation comprised of a carboxylic acid ester and the enzyme powder is produced at 40 °C to about eight weeks at 40 °C. In another embodiment, the elevated temperature is in a range of from about 30 °C to about 52 °C. In a preferred embodiment, the elevated temperature is in a range of from about 30 °C to about 40 °C.

In some embodiments, the enzyme powder retains at least 75 percent of the enzyme activity after eight weeks storage at 40 °C in a formulation comprised of a carboxylic acid ester and the enzyme powder as compared to the initial enzyme activity of the enzyme powder prior to the preparation of a formulation comprised of the carboxylic acid ester and the enzyme powder at 40 °C. In other embodiments, the enzyme powder retains at least 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent of the enzyme activity of the at least one enzyme after eight weeks storage at 40 °C in

a formulation comprised of a carboxylic acid ester and the enzyme powder as compared to the initial enzyme activity of the enzyme powder prior to the preparation of a formulation comprised of the carboxylic acid ester and the enzyme powder at 40 °C. Preferably, perhydrolysis activity is measured as described in Examples 8-13 of U.S. Patent Application Publication No. 2010-0086510; but any method of measuring perhydrolysis activity may used.

A further improvement in enzyme activity over the stated periods of time can be achieved by adding a buffer having a buffering capacity in a pH range of from about 5.5 to about 9.5 to the formulation comprised of the carboxylic acid ester and the spray-dried enzyme powder as described in U.S. Patent Application Publication No. 2010-0086534. A suitable buffer may include, but is not limited to, sodium salt, potassium salt, or mixtures of sodium or potassium salts of bicarbonate, pyrophosphate, phosphate, methylphosphonate, citrate, acetate, malate, fumarate, tartrate maleate or succinate. Preferred buffers for use in the formulation comprised of the carboxylic acid ester and the spray-dried enzyme powder include the sodium salt, potassium salt, or mixtures of sodium or potassium salts of bicarbonate, pyrophosphate, phosphate, methylphosphonate, citrate, acetate, malate, fumarate, tartrate maleate or succinate.

In embodiments where a buffer may be present in the carboxylic acid ester and enzyme powder formulation, the buffer may be present in an amount in a range of from about 0.01 wt% to about 50 wt% based on the weight of carboxylic acid ester in the formulation comprised of carboxylic acid ester and enzyme powder. The buffer may be present in a more preferred range of from about 0.10 % to about 10 % based on the weight of carboxylic acid ester in the formulation comprised of carboxylic acid ester and enzyme powder. Further, in these embodiments, the comparison between perhydrolysis activity of the enzyme is determined as between an enzyme powder which retains at least 75 percent of the perhydrolysis activity of the at least one enzyme after eight weeks storage at 40 °C in a formulation comprised of a carboxylic acid ester, a buffer having a buffering capacity in a pH range of from about 5.5 to about 9.5, and the enzyme powder as compared to the initial perhydrolysis activity of the enzyme

powder prior to the preparation of a formulation comprised of the carboxylic acid ester, the buffer having a buffering capacity in a pH range of from about 5.5 to about 9.5, and the enzyme powder.

It is intended that the dried enzyme powder be stored as a formulation in the organic compound that is a substrate for the at least one enzyme, such as triacetin. In the absence of added hydrogen peroxide, triacetin is normally hydrolyzed in aqueous solution by a CE-7 carbohydrate esterase to produce diacetin and acetic acid, and the production of acetic acid results in a decrease in the pH of the reaction mixture. One requirement for long term storage stability of the enzyme in triacetin is that there is not a significant reaction of the triacetin with any water that might be present in the triacetin; the specification for water content in one commercial triacetin (supplied by Tessenderlo Group, Brussels, Belgium) is 0.03 wt% water (300 ppm). Any hydrolysis of triacetin that occurs during storage of the enzyme in triacetin would produce acetic acid, which could result in a decrease in activity or inactivation of the CE-7 perhydrolases; the perhydrolases are typically inactivated at or below a pH of 5.0 (see U.S. Patent Application Publication No. 2009-0005590 to DiCosimo, R., *et al.*). The excipient selected for use in the present application must provide stability of the enzyme in the organic substrate for the enzyme under conditions where acetic acid might be generated due to the presence of low concentrations of water in the formulation. The dried enzyme powder may be stored as a formulation in the organic compound that is a substrate for the at least one enzyme, where the formulation additionally comprises an excipient and one or more buffers (*e.g.*, sodium and/or potassium salts of bicarbonate, citrate, acetate, phosphate, pyrophosphate, methylphosphonate, succinate, malate, fumarate, tartrate, and maleate).

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

One or more enzymes having perhydrolytic activity may be used to generate an efficacious concentration of the desired peracid(s) in the present personal care compositions and methods. The desired peroxycarboxylic acid

may be prepared by reacting carboxylic acid esters with a source of peroxygen including, but not limited to, hydrogen peroxide, zinc peroxide, sodium peroxide, urea peroxide, calcium peroxide, sodium perborate, sodium percarbonate or complexes of hydrogen peroxide, in the presence of an enzyme catalyst having perhydrolysis activity.

The perhydrolytic enzyme within the targeted perhydrolase may be any perhydrolytic enzyme and may include lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations so long as the enzyme has perhydrolytic activity for one or more of the present substrates. Examples may include, but are not limited to perhydrolytic proteases (subtilisin variant; U.S. Patent 7,510,859), perhydrolytic esterases (*Pseudomonas fluorescens;* U.S. Patent 7,384,787; "L29P" variant SEQ ID NO: 477), and perhydrolytic aryl esterases (*Mycobacterium smegmatis*; U.S. Patent 7,754,460; WO2005/056782; and EP1689859 B1; SEQ ID NOs: 460 [S54V variant] and 478 [wild type]).

In one embodiment, the enzyme catalyst comprises at least one enzyme having perhydrolase activity, wherein said enzyme is structurally classified as a member of the CE-7 carbohydrate esterase family (CE-7; see Coutinho, P.M., and Henrissat, B., *supra*). In another embodiment, the perhydrolase catalyst is structurally classified as a cephalosporin C deacetylase. In another embodiment, the perhydrolase catalyst is structurally classified as an acetyl xylan esterase.

In one embodiment, the perhydrolase catalyst comprises an enzyme having perhydrolysis activity and a signature motif comprising:

a) an RGQ motif that aligns with amino acid residues 118-120 of SEQ ID NO: 2;
b) a GXSQG motif that aligns with amino acid residues 179-183 of SEQ ID NO: 2; and
c) an HE motif that aligns with amino acid residues 298-299 of SEQ ID NO: 2.

In a preferred embodiment, the alignment to reference SEQ ID NO: 2 is performed using CLUSTALW.

In a further embodiment, the CE-7 signature motif additional may comprise and additional (*i.e.*, fourth) motif defined as an LXD motif at amino acid residues 267-269 when aligned to reference sequence SEQ ID NO:2 using CLUSTALW.

In another embodiment, the perhydrolase catalyst comprises an enzyme having perhydrolase activity, said enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476.

In another embodiment, the perhydrolase catalyst comprises an enzyme having perhydrolase activity, said enzyme having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 424, 437, and 476 wherein said enzyme may have one or more additions, deletions, or substitutions so long as the signature motif is conserved and perhydrolase activity is retained.

As described above, the CE-7 perhydrolase may be a fusion protein having a first portion comprising CE-7 perhydrolase and a second portion comprising a peptidic component having affinity for a target body surface such that the perhydrolase is "targeted" to the desired body surface. In one embodiment, any CE-7 perhydrolase (as defined by the presence of the CE-7 signature motifs) may be fused to any peptidic component/binding element capable of targeting the enzyme to a body surface. In one aspect, the peptidic component having affinity for an oral cavity surface may include antibodies, antibody fragments (F_{ab}), as well as single chain variable fragments (scFv; a fusion of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins), single domain camelid antibodies, scaffold display proteins, and single chain affinity peptides lacking immunoglobulin folds. The compositions comprising antibodies, antibodies fragments and other immunoglobulin-derived binding elements, as well as large scaffold display proteins, are often not economically viable. As such, and in a preferred aspect,

the peptidic component/binding element is a single chain affinity peptide lacking an immunoglobulin fold and/or immunoglobulin domain. Short single chain body surface-binding peptides may be empirically generated (*e.g.*, positively charged polypeptides targeted to negatively charged surfaces) or generated using biopanning against a target body surface. Methods to identify/obtain affinity peptides using any number of display techniques (e.g., phage display, yeast display, bacterial display, ribosome display, and mRNA display) are well known in the art. Individual oral cavity surface-binding peptides may be coupled together, via optional spacers/linkers, to form larger binding "domains" (also referred to herein as binding "hands") to enhance attachment/localization of the perhydrolytic enzyme to the target oral cavity surface.

The fusion proteins may also include one or more peptide linkers/spacers separating the CE-7 perhydrolase enzyme the oral cavity surface-binding domain and/or between different oral cavity surface-binding peptides (*e.g.*, when a plurality of oral cavity surface-binding peptides are coupled together to form a larger target oral cavity surface-binding domain). Multiple peptide linkers/spacers may be present and the number of linkers may be repeated up to 10 times. A non-limiting list of exemplary peptide spacers are provided by the amino acid sequences of SEQ ID NOs: 383-396 and those illustrated in Table 5.

Suitable peptides having affinity for an oral cavity surface are described herein, *supra*. Methods to identify additional oral cavity surface-binding peptides using any of the above "display" techniques are well known and can be used to identify additional oral cavity surface-binding peptides.

Suitable carboxylic acid ester substrates may include esters having the following formula:

(a) one or more esters having the structure

$$[X]_mR_5$$

wherein

X is an ester group of the formula $R_6C(O)O$;

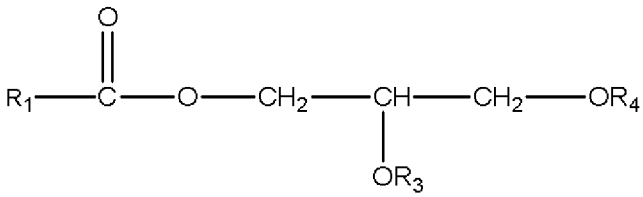
R_6 is a C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with a hydroxyl group or C1 to C4 alkoxy group, wherein R_6 optionally comprises one or more ether linkages where R_6 is C2 to C7;

R_5 is a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic moiety optionally substituted with a hydroxyl group; wherein each carbon atom in R_5 individually comprises no more than one hydroxyl group or no more than one ester group or carboxylic acid group, and wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5,

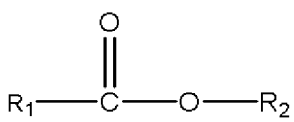
said one or more esters having solubility in water of at least 5 ppm at 25 °C; or

(b) one or more glycerides having the structure



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

(c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; or

(d) one or more acetylated monosaccharides, acetylated disaccharides, or acetylated polysaccharides; or

(e) any combination of (a) through (d).

Suitable substrates may also include one or more acylated saccharides selected from the group consisting of acylated mono-, di-, and polysaccharides. In another embodiment, the acylated saccharides are selected from the group consisting of acetylated xylan; fragments of acetylated xylan; acetylated xylose (such as xylose tetraacetate); acetylated glucose (such as α-D-glucose pentaacetate; β-D-glucose pentaacetate; 1-thio-β-D-glucose-2,3,4,6-tetraacetate); β-D-galactose pentaacetate; sorbitol hexaacetate; sucrose octaacetate; β-D-ribofuranose-1,2,3,5-tetraacetate; β-D-ribofuranose-1,2,3,4-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-D-glucal; β-D-xylofuranose tetraacetate, α-D-glucopyranose pentaacetate; β-D-glucopyranose-1,2,3,4-tetraacetate; β-D- glucopyranose-2,3,4, 6-tetraacetate; 2-acetamido-2-deoxy-1,3,4,6-tetracetyl-β-D-glucopyranose; 2-acetamido-2-deoxy-3,4,6-triacetyl-1-chloride-α-D-glucopyranose; α-D-mannopyranose pentaacetate, and acetylated cellulose. In a preferred embodiment, the acetylated saccharide is selected from the group consisting of β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-D-glucal; sucrose octaacetate; and acetylated cellulose.

In another embodiment, additional suitable substrates may also include 5-acetoxymethyl-2-furaldehyde; 3,4-diacetoxy-1-butene; 4-acetoxybenezoic acid; vanillin acetate; propylene glycol methyl ether acetate; methyl lactate; ethyl lactate; methyl glycolate; ethyl glycolate; methyl methoxyacetate; ethyl methoxyacetate; methyl 3-hydroxybutyrate; ethyl 3-hydroxybutyrate; and triethyl 2-acetyl citrate.

In another embodiment, suitable substrates are selected from the group consisting of: monoacetin; diacetin; triacetin; monopropionin; dipropionin; tripropionin; monobutyrin; dibutyrin; tributyrin; glucose pentaacetate; xylose tetraacetate; acetylated xylan; acetylated xylan fragments; β-D-ribofuranose-1,2,3,5-tetraacetate; tri-O-acetyl-D-galactal; tri-O-acetyl-D-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; 1,5-pentanediol; 1,6-pentanediol; 1,2-hexanediol; 2,5-hexanediol; 1,6-hexanediol; and mixtures thereof. In another embodiment, the substrate is a C1 to C6 polyol comprising one or more ester groups. In a preferred embodiment, one or more of the hydroxyl groups on the C1 to C6 polyol are substituted with one or more acetoxy groups (such as 1,3-propanediol diacetate; 1,2-propanediol diacetate; 1,4-butanediol diacetate; 1,5-pentanediol diacetate, etc.). In a further embodiment, the substrate is propylene glycol diacetate (PGDA), ethylene glycol diacetate (EGDA), or a mixture thereof.

In a further embodiment, suitable substrates are selected from the group consisting of monoacetin, diacetin, triacetin, monopropionin, dipropionin, tripropionin, monobutyrin, dibutyrin, and tributyrin. In yet another aspect, the substrate is selected from the group consisting of diacetin and triacetin. In a most preferred embodiment, the suitable substrate comprises triacetin.

In a preferred embodiment, the carboxylic acid ester is a liquid substrate selected from the group consisting of monoacetin, diacetin, triacetin, and combinations (*i.e.*, mixtures) thereof. The carboxylic acid ester is present in the reaction formulation at a concentration sufficient to produce the desired concentration of peroxycarboxylic acid upon enzyme-catalyzed perhydrolysis. The carboxylic acid ester need not be completely soluble in the reaction formulation, but has sufficient solubility to permit conversion of the ester by the perhydrolase catalyst to the corresponding peroxycarboxylic acid. The carboxylic acid ester is present in the reaction formulation at a concentration of 0.05 wt % to 40 wt % of the reaction formulation, preferably at a concentration of 0.1 wt % to

20 wt % of the reaction formulation, and more preferably at a concentration of 0.5 wt % to 10 wt % of the reaction formulation.

The peroxygen source may include, but is not limited to, hydrogen peroxide, hydrogen peroxide adducts (*e.g.*, urea-hydrogen peroxide adduct (carbamide peroxide)) perborate salts, percarbonate salts and peroxide salts. The concentration of peroxygen compound in the reaction formulation may range from 0.0033 wt % to about 50 wt %, preferably from 0.033 wt % to about 40 wt %, more preferably from 0.1 wt % to about 30 wt %.

The peroxygen source (*i.e.*, hydrogen peroxide) may also be generated enzymatically using enzyme capable of producing and effective amount of hydrogen peroxide. For example, various oxidases can be used in the present compositions and methods to produce an effective amount of hydrogen peroxide including, but not limited to glucose oxidase, lactose oxidases, carbohydrate oxidase, alcohol oxidase, ethylene glycol oxidase, glycerol oxidase, and amino acid oxidase.

Many perhydrolase catalysts (whole cells, permeabilized whole cells, and partially purified whole cell extracts) have been reported to have catalase activity (EC 1.11.1.6). Catalases catalyze the conversion of hydrogen peroxide into oxygen and water. In one aspect, the perhydrolysis catalyst lacks catalase activity. In another aspect, a catalase inhibitor may be added to the reaction formulation. One of skill in the art can adjust the concentration of catalase inhibitor as needed. The concentration of the catalase inhibitor typically ranges from 0.1 mM to about 1 M; preferably about 1 mM to about 50 mM; more preferably from about 1 mM to about 20 mM.

In another embodiment, the enzyme catalyst lacks significant catalase activity or may be engineered to decrease or eliminate catalase activity. The catalase activity in a host cell can be down-regulated or eliminated by disrupting expression of the gene(s) responsible for the catalase activity using well known techniques including, but not limited to, transposon mutagenesis, RNA antisense expression, targeted mutagenesis, and random mutagenesis. In a preferred embodiment, the gene(s) encoding the endogenous catalase activity are down-

regulated or disrupted (*i.e.*, knocked-out). As used herein, a "disrupted" gene is one where the activity and/or function of the protein encoded by the modified gene is no longer present. Means to disrupt a gene are well-known in the art and may include, but are not limited to, insertions, deletions, or mutations to the gene so long as the activity and/or function of the corresponding protein is no longer present. In a further preferred embodiment, the production host is an *E. coli* production host comprising a disrupted catalase gene selected from the group consisting of *kat*G and *kat*E (see U.S. Patent Application Publication No. 2008-0176299). In another embodiment, the production host is an *E. coli* strain comprising a down-regulation and/or disruption in both *kat*G and a *kat*E catalase genes.

The concentration of the catalyst in the aqueous reaction formulation depends on the specific catalytic activity of the catalyst, and is chosen to obtain the desired rate of reaction. The weight of catalyst in perhydrolysis reactions typically ranges from 0.0001 mg to 10 mg per mL of total reaction volume, preferably from 0.001 mg to 2.0 mg per mL. The catalyst may also be immobilized on a soluble or insoluble support using methods well-known to those skilled in the art; see for example, Immobilization of Enzymes and Cells; Gordon F. Bickerstaff, Editor; Humana Press, Totowa, NJ, USA; 1997. The use of immobilized catalysts permits the recovery and reuse of the catalyst in subsequent reactions. The enzyme catalyst may be in the form of whole microbial cells, permeabilized microbial cells, microbial cell extracts, partially-purified or purified enzymes, and mixtures thereof.

In one aspect, the concentration of peroxycarboxylic acid generated by the combination of chemical perhydrolysis and enzymatic perhydrolysis of the carboxylic acid ester is sufficient to provide an effective concentration of peroxycarboxylic acid for the chosen personal care application. In another aspect, the present methods provide combinations of enzymes and enzyme substrates to produce the desired effective concentration of peroxycarboxylic acid, where, in the absence of added enzyme, there is a significantly lower concentration of peroxycarboxylic acid 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 peroxycarboxylic acid generated to provide an effective concentration of peroxycarboxylic acid in the desired applications, and a significant increase in total peroxycarboxylic acid concentration is achieved by the addition of an appropriate perhydrolase catalyst to the reaction formulation.

The concentration of peroxycarboxylic acid generated (*e.g.* peracetic acid) by the perhydrolysis of at least one carboxylic acid ester is at least about 0.1 ppm, preferably at least 0.5 ppm, 1 ppm, 5 ppm, 10 ppm, 20 ppm, 100 ppm, 200 ppm, 300 ppm, 500 ppm, 700 ppm, 1000 ppm, 2000 ppm, 5000 ppm or 10,000 ppm of peracid within 10 minutes, preferably within 5 minutes, of initiating the perhydrolysis reaction. The product formulation comprising the peroxycarboxylic acid may be optionally diluted with water, or a solution predominantly comprised of water, to produce a formulation with the desired lower concentration of peroxycarboxylic acid base on the target application. Clearly one of skill in the art can adjust the reaction components and/or dilution amounts to achieve the desired peracid concentration for the chosen personal care product.

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, and most preferably in about 5 minutes or less. In other aspects, an oral cavity surface is contacted with the peroxycarboxylic acid formed in accordance with the processes described herein within 5 minutes of combining the reaction components. In one embodiment, the target oral cavity surface is contacted with the peroxycarboxylic acid produced with the processes described herein within about 5 minutes to about 168 hours of combining said reaction components, or within about 5 minutes to about 48 hours, or within about 5 minutes to 2 hours of combining said reaction components, or any such time interval therein.

The peracid formed in accordance with the processes describe herein is used in a personal care product/application wherein the peracid is contacted with

a target oral cavity surface to provide a peracid-based benefit to the oral cavity. In one embodiment, the process to produce a peracid for a target body surface is conducted *in situ*.

The temperature of the reaction may be chosen to control both the reaction rate and the stability of the enzyme catalyst activity. Clearly for certain personal care applications the temperature of the target body surface (*e.g.*, 37 ºC within the oral cavity) may be the temperature of the reaction. The temperature of the reaction may range from just above the freezing point of the reaction formulation (approximately 0 °C) to about 95 °C, with a preferred range of 5 °C to about 75 °C, and a more preferred range of reaction temperature of from about 5 °C to about 55 °C.

The pH of the final reaction formulation containing peroxycarboxylic acid is from about 2 to about 9, preferably from about 3 to about 8, more preferably from about 5 to about 8, even more preferably about 5.5 to about 8, and yet even more preferably about 6.0 to about 7.5. The pH of the reaction, and of the final reaction formulation, 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.

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

Single Step vs. Multi-Step Application Methods

Typically the minimum set of reaction components to enzymatically produce a peracid benefit agent will include (1) at least one enzyme having perhydrolytic activity as described herein, such as a CE-7 perhydrolase (optionally in the form of a targeted fusion protein), (2) at least one suitable carboyxlic acid ester substrate, and (3) a source of peroxygen.

The peracid-generating reaction components of the personal care composition may remain separated until use. In one embodiment, the peracid-generating components are combined and then contacted with the target body surface whereby the resulting peracid-based benefit agent provides a benefit to the body surface. The components may be combined and then contacted with the target body surface or may be combined on the targeted body surface. In one embodiment, the peracid-generating components are combined such that the peracid is produced *in situ*.

A multi-step application may also be used. One or two of the individual components of the peracid-generating system (*i.e.*, a sequential application on the body surface of at least one of the three basic reaction components) composition may be contacted with the oral cavity surface prior to applying the remaining components required for enzymatic peracid production. In one embodiment, the perhydrolytic enzyme is contacted with the oral cavity surface prior to contacting the oral cavity surface with the carboyxlic acid ester substrate and/or the source of peroxygen (i.e., a "two-step application"). In one embodiment, the enzyme having perhydrolytic activity is a targeted perhydrolase that is applied to the oral cavity surface prior to combining the remaining components necessary for enzymatic peracid production.

In a preferred embodiment, the enzyme having perhydrolytic activity is a "targeted CE-7 perhydrolase" (*i.e.*, CE-7 fusion protein) that is applied to the oral cavity surface prior to combining the remaining components necessary for enzymatic peracid production (*i.e.*, a two-step application method). The targeted perhydrolase is contacted with the oral cavity surface under suitable conditions to promote non-covalent bonding of the fusion protein to the oral cavity surface.

An optional rinsing step may be used to remove excess and/or unbound fusion protein prior to combining the remaining reaction components.

In a further embodiment, the perhydrolytic enzyme (optionally in the form of a fusion protein targeted to the oral cavity surface) and the carboxylic acid ester are applied to the target oral cavity surface prior to the addition of the source of peroxygen.

In a further embodiment, the perhydrolytic enzyme (optionally in the form of a fusion protein targeted to the oral cavity surface) and source of peroxygen (*e.g.*, an aqueous solution comprising hydrogen peroxide) are applied to the oral cavity surface prior to the addition of the carboxylic acid ester substrate.

In a further embodiment, the carboxylic acid ester substrate and the source of peroxygen (*e.g.*, an aqueous solution comprising hydrogen peroxide) are applied to the oral cavity surface prior to the addition of the perhydrolytic enzyme (optionally in the form of a fusion protein targeted to the oral cavity surface).

In yet another embodiment, any of the compositions or methods described herein can be incorporated into a kit for practicing the invention. The kits may comprise materials and reagents to facilitate enzymatic production of peracid. An exemplary kit comprises a substrate, a source of peroxygen, and an enzyme catalyst having perhydrolytic activity, wherein the enzyme catalyst can be optionally targeted to an oral cavity surface. Other kit components may include, without limitation, one or more of the following: sample tubes, solid supports, instruction material, and other solutions or other chemical reagents useful in enzymatically producing peracids, such as acceptable components or carriers.

Oral Care Compositions

Orally Acceptable Components/Carriers

The present compositions and method s may also include orally acceptable carriers as well as additional (*i.e.*, in addition to the peracid-based benefit agent) oral care benefit agents. As used herein, the term “oral care benefit agent” is a general term applying to a compound or substance that

provides a desired/beneficial effect or attribute to an oral surface. In one embodiment, benefit agents for oral surfaces may comprise (in addition to the peracid-based benefit agent) colorants including, but not limited to, white pigments such as titanium dioxide and white minerals such as hydroxyapatite or zircon. In another embodiment, oral care benefit agents may also include whitening agents and additional enzymes such as, for example, oxidases, peroxidases, proteases, lipases, glycosidases, esterases, and polysaccharide hydrolases. In another aspect, benefit agents may include anti-plaque agents, anti-stain agents, and antimicrobial agents. Antimicrobial agents may include, but are not limited to, antimicrobial peptides, magainins, cecropins, microbiocides, triclosan, chlorhexidine, cetylpyridinium chloride, quaternary ammonium compounds, chlorxylenol, chloroxyethanol, phthalic acid and its salts, thymol, and combinations thereof. Oral care benefit agents may also include anti-caries agents, such as sodium fluoride or sodium monofluorophosphate, and flavoring agents such as oil of wintergreen, peppermint, or spearmint, or methyl salicylate, eucalyptol, or vanillin. Oral care benefit agents may also include coolants, such as succinate-based coolant compounds, and salivating agents, to name a few. As is used herein, the term "salivating agent" refers to a material that promotes greater salivation in the user when present in the oral care composition. In one embodiment, the benefit agent is an orally-acceptable material approved for use in oral care products. In another embodiment, the orally-acceptable benefit agent is used to improve the cosmetic appearance of teeth.

A non-limiting list of components often used in an orally-acceptable carrier medium are described by White *et al.* in U.S. Patent No. 6,740,311; Lawler *et al.* in U.S. Patent No. 6,706,256; Fuglsang *et al.* in U.S. Patent No. 6,264,925; and Ibrahim *et al.* in U.S. Patent Application Publication No. 2005-0069501, each of which are incorporated herein by reference in their entirety. For example, the oral care composition may comprise one or more of the following: abrasives, surfactants, antioxidants, chelating agents, fluoride sources, thickening agents, buffering agents, solvents, humectants, carriers, bulking agents, anti-plaque

agents, anti-staining agents, antimicrobial agents, anti-caries agents, anti-inflammatory agents, desensitizing agents, sweetening agents, flavoring agents, breath-freshening agents, coolants, nutrients, and salivating agents.

It will be appreciated that the components in the mixture are chosen such that the oral care composition retains the ability to enzymatically product the desired peracid benefit agent. Suitable mixtures of oral care systems disclosed herein may be determined by one skilled in the art using routine experimentation. The total concentration of the oral care benefit agents with the oral care formulation may be about 0.001% to about 90% by weight relative to the total weight of the oral care composition.

The oral care compositions may include, but are not limited to, toothpaste, dental cream, tooth gel or tooth powder, mouth wash, breath freshener, and dental floss. Additional embodiments include the application of the reaction components in a paste or gel that is applied in the oral environment via a mouth tray. One or more of the reaction components can also be deposited first on a plastic strip that is adhered to the enamel to deliver one or more of the reaction components to generate the peracid benefit agent. In the case of the deposition of the perhydrolase fusion on a delivery device such as a strip, the perhydrolase fusion can be designed to include binding elements with affinity for the material of the strip to aid in the deposition and retention of the perhydrolase to the strip during use and removal of the device after use.

Peracid-Based Oral Care Products to Reduce Microbes Associated with Diseases of the Oral Cavity or Remove Unwanted Biofilm.

Peracid-based oral care products may be used to reduce oral cavity bacteria associated with dental caries (such as *Streptococcus mutans*), gingivitis, oral candidiasis, or periodontitis. The peracid-based oral care products may be used to reduce or remove oral biofilm(s).

In one embodiment, the use of an enzyme having perhydrolytic activity in an oral care product to produce an efficacious concentration of at least one

peracid is provided to bleach, whiten, disinfect, destain, deodorize or remove biofilm from an oral cavity surface.

In one embodiment, the enzyme having perhydrolytic activity is a targeted perhydrolase and may include lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations so long as the enzyme has perhydrolytic activity for one or more of the present substrates. Examples may include, but are not limited to perhydrolytic proteases (e.g., subtilisin variant; U.S. Patent 7,510,859), perhydrolytic esterases (e.g., *Pseudomonas fluorescens;* U.S. Patent 7,384,787; SEQ ID NO: 477), and perhydrolytic aryl esterases (e.g., *Mycobacterium smegmatis*; U.S. Patent 7,754,460; WO2005/056782; and EP1689859 B1; SEQ ID NOs: 460 [S54V variant] and 478 [wild type]).

In another embodiment, the use of a CE-7 carbohydrate esterase having perhydrolytic activity in an oral care product to produce an efficacious concentration of at least one peracid is provided to bleach, whiten, disinfect, destain, deodorize or remove biofilm from an oral cavity surface.

In another embodiment, the use the following peracid generation composition is also provided comprising:

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

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

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

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

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

1) esters having the structure

$$[X]_mR_5$$

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

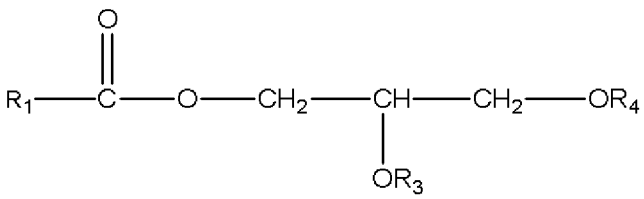
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

M is an integer ranging from 1 to the number of carbon atoms in R_5; and

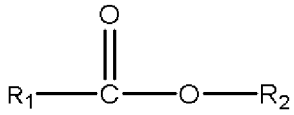
wherein said esters have a solubility in water of at least 5 ppm at 25 °C;

2) glycerides having the structure



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

3) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

c) a source of peroxygen;

whereby a peracid formed upon mixing (a), (b), and (c); wherein the peracid generation formulation is used for the treatment or prevention of dental caries, gingivitis, oral candidiasis, or periodontitis.

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

A variety of analytical methods can be used in the present methods to analyze the reactants and products including, but not limited to, titration, high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), capillary electrophoresis (CE), the analytical procedure described by U. Pinkernell *et al.*, (*Anal. Chem.*, 69(17):3623-3627 (1997)), and the 2,2'-azino-bis (3-ethylbenzothazoline)-6-sulfonate (ABTS) assay (U. Pinkernell *et. al. Analyst*, 122: 567-571 (1997) and Dinu *et. al. Adv. Funct. Mater.*, 20: 392-398 (2010)) as described in the present examples.

Determination of Minimum Biocidal Concentration of Peroxycarboxylic acids

Certain personal care applications may be associated with the removal of unwanted microbes, such as those associated with body order, fungal infections, and the development of dental caries, to name a few. As such, one may want to measure the minimum biocidal concentration for the target personal care application. The method described by J. Gabrielson, *et al.* (*J. Microbiol. Methods* 50: 63-73 (2002)) can be employed for determination of the Minimum Biocidal Concentration (MBC) of peroxycarboxylic acids, or of hydrogen peroxide and enzyme substrates. The assay method is based on XTT reduction inhibition, where XTT ((2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium, inner salt, monosodium salt) is a redox dye that indicates microbial respiratory activity by a change in optical density (OD) measured at 490 nm or 450 nm. However, there are a variety of other methods available for testing the activity of disinfectants and antiseptics including, but not limited to, viable plate counts, direct microscopic counts, dry weight, turbidity measurements, absorbance, and bioluminescence (see, for example Brock, Semour S., Disinfection, Sterilization, and Preservation, 5th edition, Lippincott Williams & Wilkins, Philadelphia, PA, USA; 2001).

Recombinant Microbial Expression

The genes and gene products of the instant sequences may be produced in heterologous host cells, particularly in the cells of microbial hosts. Preferred heterologous host cells for expression of the instant genes and nucleic acid molecules are microbial hosts that can be found within the fungal or bacterial families and which grow over a wide range of temperature, pH values, and solvent tolerances. For example, it is contemplated that any of bacteria, yeast, and filamentous fungi may suitably host the expression of the present nucleic acid molecules. The perhydrolase may be expressed intracellularly, extracellularly, or a combination of both intracellularly and extracellularly, where extracellular expression renders recovery of the desired protein from a fermentation product more facile than methods for recovery of protein produced

by intracellular expression. Transcription, translation and the protein biosynthetic apparatus remain invariant relative to the cellular feedstock used to generate cellular biomass; functional genes will be expressed regardless. Examples of host strains include, but are not limited to, bacterial, fungal or yeast species such as *Aspergillus*, *Trichoderma*, *Saccharomyces*, *Pichia*, *Phaffia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Yarrowia*, *Salmonella*, *Bacillus*, *Acinetobacter*, *Zymomonas*, *Agrobacterium*, *Erythrobacter*, *Chlorobium*, *Chromatium*, *Flavobacterium*, *Cytophaga*, *Rhodobacter*, *Rhodococcus*, *Streptomyces*, *Brevibacterium*, *Corynebacteria*, *Mycobacterium*, *Deinococcus*, *Escherichia*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Sphingomonas*, *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylosinus*, *Methylomicrobium*, *Methylocystis*, *Alcaligenes*, *Synechocystis*, *Synechococcus*, *Anabaena*, *Thiobacillus*, *Methanobacterium*, *Klebsiella*, and *Myxococcus*. In one embodiment, bacterial host strains include *Escherichia*, *Bacillus*, *Kluyveromyces*, and *Pseudomonas*. In a preferred embodiment, the bacterial host cell is *Bacillus subtilis* or *Escherichia coli*.

Large-scale microbial growth and functional gene expression may use a wide range of simple or complex carbohydrates, organic acids and alcohols or saturated hydrocarbons, such as methane or carbon dioxide in the case of photosynthetic or chemoautotrophic hosts, the form and amount of nitrogen, phosphorous, sulfur, oxygen, carbon or any trace micronutrient including small inorganic ions. The regulation of growth rate may be affected by the addition, or not, of specific regulatory molecules to the culture and which are not typically considered nutrient or energy sources.

Vectors or cassettes useful for the transformation of suitable host cells are well known in the art. Typically the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are

derived from genes homologous to the transformed host cell and/or native to the production host, although such control regions need not be so derived.

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

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

Industrial Production

A variety of culture methodologies may be applied to produce the perhydrolase catalyst. For example, large-scale production of a specific gene product over-expressed from a recombinant microbial host may be produced by batch, fed-batch, and continuous culture methodologies. Batch and fed-batch culturing methods are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989) and Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227-234 (1992).

Commercial production of the desired perhydrolase catalyst may also be accomplished with a continuous culture. Continuous cultures are an open system where a defined culture media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high liquid phase density where cells are primarily in log phase growth. Alternatively, continuous

culture may be practiced with immobilized cells where carbon and nutrients are continuously added, and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials.

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

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

GENERAL METHODS

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 follow techniques to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the presently disclosed methods and examples.

All reagents and materials were obtained from DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), TCI America (Portland, OR), Roche Diagnostics Corporation (Indianapolis, IN), Thermo Scientific (Pierce Protein Research Products) (Rockford, IL) or Sigma/Aldrich Chemical Company (St. Louis, MO), unless otherwise specified.

The following abbreviations in the specification correspond to units of measure, techniques, properties, or compounds as follows: "sec" or "s" means second(s), "min" means minute(s), "h" or "hr" means hour(s), "μL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "ppm" means part(s) per million, "wt" means weight, "wt%" means weight percent, "g" means gram(s), "mg" means milligram(s), "μg" means microgram(s), "ng" means nanogram(s), "*g*" means gravity, "HPLC" means high performance liquid chromatography, "dd H_2O" means distilled and deionized water, "dcw" means dry cell weight, "ATCC" or "ATCC®" means the American Type Culture Collection (Manassas, VA), "U" means unit(s) of perhydrolase activity, "rpm" means revolution(s) per minute, "Tg" means glass transition temperature, and "EDTA" means ethylenediaminetetraacetic acid.

Expression Vector pLD001

Plasmid pLD001 (SEQ ID NO: 397) has been previous reported as a suitable expression vector for E. coli (see U.S. Patent Application Publication No. 2010-0158823 A1 to Wang *et al.*; incorporated herein by reference).

The vector pLD001 was derived from the commercially available vector pDEST17 (Invitrogen, Carlsbad, CA). It includes sequences derived from the commercially available vector pET31b (Novagen, Madison, WI) that encode a fragment of the enzyme ketosteroid isomerase (KSI). The KSI fragment was included as a fusion partner to promote partition of the peptides into insoluble inclusion bodies in *E. coli*. The KSI-encoding sequence from pET31b was modified using standard mutagenesis procedures (QuickChange II, Stratagene, La Jolla, CA) to include three additional Cys codons, in addition to the one Cys codon found in the wild type KSI sequence. In addition, all Asp codons in the coding sequence were replaced by Glu codons. The plasmid pLD001, given by SEQ ID NO: 397, was constructed using standard recombinant DNA methods, which are well known to those skilled in the art.

EXAMPLE 1

EFFECTIVENESS OF PERACETIC ACID AS A TOOTH BLEACHING AGENT

This example describes the use of peracetic acid to achieve a bleaching effect on model stained enamel surfaces. Bovine enamel incisors were obtained from SE Dental (Baton Rouge, LA). Teeth were sectioned and cut into enamel slabs approximately 7 mm on each side using a DREMEL® rotary saw (Robert Bosch Power Tool Corporation; Chicago, IL) with a diamond blade. The enamel slabs were cleaned and lightly polished to remove surface debris. The enamel was pretreated with a mixture of coffee and tea for 1-5 days in order to stain to a color similar to human stained teeth.

Each enamel block was hydrated in water for at least 1 hr prior to use. Color measurements for the substrate were obtained prior to exposure to test solutions. Solutions of peracetic acid were prepared from a 32% stock in 500 mM sodium phosphate buffer, pH 7.2. A solution of 2.5% H_2O_2 was also

prepared in the same buffer. Multiple enamel blocks were exposed to each solution for 1 min followed by additional exposures of 5 min, 10 min, 15 min and 30 min. For each treatment, a fresh solution of peracetic acid and hydrogen peroxide was prepared from the stock solutions. After each treatment the enamel blocks were rinsed with water and measured with a Konica-Minolta 2600d spectrophotometer. Whiteness index was determined for each sample as listed in Table 1 and 2.

Whiteness index (WI) is defined by the International Commission on Illumination (CIE) and described in ASTM method E313-05 and calculated for D65/10 incident light as:

$$WI = Y + 800*(0.3138-x) + 1700*(0.3310-y)$$

Where Y, x, and y are the luminance factor and the chromaticity coordinates respectively of the enamel substrate.

Table 1. Comparison of Peracetic Acid Bleaching to Hydrogen Peroxide on Stained Bovine Enamel.

Sample	**Whiteness Index**						**ΔWI**
	0 min	**1 min**	**6 min**	**16 min**	**31 min**	**61 min**	
Buffer	-135.1	-136.4	-131.6	-135.7	-129.2	-124.0	11.1
2.5% H_2O_2	-127.5	-127.2	-124.5	-118.4	-103.6	-84.9	42.6
0.5% PAA	-129.1	-111.6	-80.7	-56.5	-44.5	-38.3	90.8

Table 2. Comparison of Peracetic Acid Bleaching to Hydrogen Peroxide on Stained Bovine Enamel at Various Concentrations.

Sample	**Whiteness Index**						**ΔWI**
	0 min	**1 min**	**6 min**	**16 min**	**31 min**	**61 min**	
Buffer	-100.8	-98.6	-100.0	-97.1	-89.5	-84.0	16.8
2.5% H_2O_2	-93.8	-92.6	-86.4	-79.5	-68.3	-56.4	37.4
0.05% PAA	-103.4	-97.1	-93.1	-70.1	-59.0	-34.7	68.7

0.2% PAA	-90.6	-85.5	-67.0	-47.5	-31.7	-16.2	74.4
0.5% PAA	-97.5	-86.6	-64.1	-46.4	-32.7	-24.7	72.8
1% PAA	-102.8	-89.3		-36.0	-20.6	-5.1	97.7

The change in whiteness index to a more positive value indicated a whitening effect. Visual inspection of the samples also showed a perceptible whitening effect for peracetic acid treated samples compared to the buffer and hydrogen peroxide controls. This data demonstrates that peracetic acid is an effective bleaching agent and provides superior performance to hydrogen peroxide at lower concentrations.

EXAMPLE 2

SELECTION OF TOOTH ENAMEL AND PELLICLE BINDING PEPTIDES USING STANDARD BIOPANNING

The purpose of this Example was to identify phage peptides that bind tooth enamel and pellicle using standard phage display biopanning.

Bovine enamel incisors were obtained from SE Dental (Baton Rouge, LA). The teeth were cut to approx. 5 mm squares and polished to remove surface debris. Enamel blocks were sterilized before use. Enamel blocks were embedded in a well plate contained molding material so as to only expose the enamel surface in the well. Pellicle was formed on additional enamel blocks by mounting the blocks on wax mounting for incubation in the mouth for 30 min to form a pellicle coated surface. The pellicle coated enamel substrates were brushed with a 1:2 slurry of COLGATE® MAXFRESH® toothpaste (Colgate-Palmolive, New York, NY) and reincubated for an additional 30 min. A portion of the blocks were removed from the wax and embedded in a well plate while others were rebrushed before embedding in a well plate. The embedding process allowed for solution contact with only the enamel and pellicle-coated enamel surfaces.

The substrates were then incubated in blocking buffer for 1 hour at room temperature (~22 ºC; 1 mg/mL Bovine Serum Albumin in Phosphate Buffered

Saline pH 7.2 (Pierce BUPH™ #28372) with 0.1% TWEEN®20 (PBST), followed by 2 washes with PBST. Libraries of phage containing random peptide inserts (10^{11} pfu) from 15 to 20 amino acids in length were added to each well. The final binding solution contained 10^{11} pfu phages, 10% UV treated whole saliva and 1 mg/mL BSA in 0.1% TWEEN®20 (PBST). After 30 minutes of incubation at 37 °C with shaking at 50 rpm, unbound phage were removed by aspirating the liquid out of each well followed by 6 washes with 1.0 mL PBST.

The enamel blocks were then transferred to clean tube and 1 mL of elution buffer consisting of 1 mg/mL BSA in 0.2 M glycine-HCl, pH 2.2, was added to each well and incubated for 10 min to elute the bound phages. Then, 167 µL of neutralization buffer consisting of 1 M Tris-HCl, pH 9.1, was added to each well. The phage particles, which were in the elution buffer as well as on the enamel blocks, were amplified by incubating with 20 mL diluted *E. coli* ER2738 cells, from an overnight culture diluted 1:100 in LB medium, at 37 °C for 4.5 h. After this time, the cell culture was centrifuged for 2 min and the upper 15 mL of the supernatant was transferred to a fresh tube, 2.5 mL of PEG/NaCl (20% polyethylene glycol-800, 2.5 M sodium chloride) was added, and the phage was allowed to precipitate overnight at 4 °C. The precipitate was collected by centrifugation at 10,000 x *g* at 4 °C and the resulting pellet was resuspended in 1 mL of PBS. This was the first round of amplified stock. The amplified first round phage stock was then titered according to the standard protocol. For subsequent rounds of biopanning, more than 2 x 10^{11} pfu of phage stock from the previous round was used. Each additional round after the first also included an additional wash with human whole saliva (UV treated for 2 hours at room temperature), two washes with carbonate buffer pH 9.4 (Pierce BUPH™ Carbonate-Bicarbonate Buffer #28382), 2 washes with 50 mM phosphate buffer, pH 2.5 and followed by 2 washes with normal PBST.

After the 3rd round of biopanning and each subsequent round, 95 random single phage plaques were isolated and the single stranded phage genomic DNA was prepared using the Illustra Templiphi 500 Amplification Kit (GE Healthcare, Piscataway, NJ) and sequenced at the DuPont Sequencing Facility

using -96 gIII sequencing primer (5'-CCCTCATAGTTAGCGTAACG-3'; SEQ ID NO: 398). The displayed peptide is located immediately after the signal peptide of gene III. Based on the peptide sequences, 12 phage candidates were identified for further binding analysis as indicated in Table 3.

Table 3. Tooth Enamel-Binding and Pellicle-Binding Peptide Sequences.

Sequence ID	Sequence	SEQ ID NO:
P301	SNATMYNIQSHSHHQ	399
P302	QAAQVHMMQHSRPTT	400
P303	HDPYTMKSALRQSTS	401
P304	DLGTFPNRTLKMAAH	402
P305	DTIHPNKMKSPSSPL	403
P306	GSNNHLPSTVPRLTV	404
P307	SNPIPNFAHDLRHSKYNS	405
P308	TKPPRTPTANTSRPHHNF	406
P309	ANSGFPIWLQKYPWSEVQQE	407
P310	ATPRLTPEAHHKAGNWYAS	408
P311	ATPSQHRYGLMQNHAPNGIE	409
P312	GMGSEVLSQYPQAPVG	410

EXAMPLE 3

CHARACTERIZATION OF TOOTH-BINDING CANDIDATES ON ENAMEL

The purpose of this example is to confirm the binding of peptide compositions on enamel surfaces using synthetically produced peptides.

A total of 11 synthetic peptides were manufactured using sequences obtained from Table 3. Peptides were obtained from SynBioSci Corp. (Livermore, CA) with biotin labeled lysine at the C-terminus.

Enamel substrates were prepared as described in Example 2. Each substrate was incubated for 1 h at room temperature (~ 22 ºC) with 1 mL of blocking buffer, consisting of 1 mg/mL BSA in PBST (Pierce BUPH™ #28372

with 0.1% TWEEN® 20). The blocking buffer was removed by aspirating the liquid out of each well. The tube was rinsed 2 times with wash buffer consisting of PBST. The wells were filled with 500 µL of 20 µM peptide solution which was prepared by diluting in blocking buffer. The samples were incubated for 30 min with slow shaking at 37 °C. The non-binding peptide was removed by washing 6 times with PBST. Then, 500 µL of horseradish peroxidase/streptavidin conjugate (Pierce #22127), diluted 1:1000 in PBST, was added and incubated for 1 h at room temperature (~22 °C). The conjugate solution was removed and the enamel blocks were washed 4 times with PBST.

Each enamel substrate was removed from the well and washed again in a 15-mL test tube with 10 mL of PBST. Each enamel substrate was then mounted in a clean well plate with only the enamel surface exposed. 200 µL of a QUANTABLU™ Substrate Solution (Thermo-Fisher, Rockford, IL; #1856187) was added directly to each enamel block. The solution was incubated for 20 min at room temperature. 200 µL of QUANTABLU™ Stop Solution (Thermo Fisher) was added. After mixing, 200 µL of solution was transferred to a clean 96-well black microcentrifuge plate. The fluorescence of the plate was measured with 325 nm excitation and 420 nm emission with no cutoff wavelength using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The resulting fluorescence values are given in Table 4. The analysis of the 11 pellicle/enamel binding candidates was compared to a known binding peptide, DenP03. Each sequence was tested with three replicate enamel substrates.

Table 4. Synthetic Peptide ELISA Results on Bovine Enamel for Binding Candidates Obtained from Biopanning.

Peptide ID	Amino Acid Sequence	Avg Fluorescence 325/420	SEQ ID NO
No peptide	---	494.2	---
DenP03	TTYHYKNIYQESYQQRNPAVK(Biotin)	3448.3	411
DenP301	SNATMYNIQSHSHHQK(Biotin)	1098.4	412

DenP302	QAAQVHMMQHSRPTTK(Biotin)	608.6	413
DenP303	HDPYTMKSALRQSTSK(Biotin)	948.6	414
DenP304	DLGTFPNRTLKMAAHK(Biotin)	642.8	415
DenP305	DTIHPNKMKSPSSPLK(Biotin)	581.2	416
DenP306	GSNNHLPSTVPRLTVK(Biotin)	1300.0	417
DenP307	SNPIPNFAHDLRHSKYNSK(Biotin)	861.6	418
DenP308	TKPPRTPTANTSRPHHNFK(Biotin)	12302.6	419
DenP309	ANSGFPIWLQKYPWSEVQQEK(Biotin)	1729.5	420
DenP311	ATPSQHRYGLMQNHAPNGIEK(Biotin)	795.3	421
DenP312	GMGSEVLSQYPQAPVGK(Biotin)	2301.0	422

EXAMPLE 4

CONSTRUCTION OF PERHYDROLASE AND PERHYDROLASE FUSIONS

This example describes the design of an expression system for the production of perhydrolases targeted to enamel via enamel-binding sequences.

The genes encoding for fusions of an enzyme having perhydrolytic activity (a "perhydrolase") to enamel-binding domains were designed to have the polynucleotide sequence of the various enzymes listed in Table 5 fused at the 3'-end to the nucleotide sequence encoding various amino acid flexible linkers; each linker further fused to the enamel-binding domains or non-binding sequence controls as described in Table 6. The genes were codon-optimized for expression in *E. coli* and synthesized by DNA2.0 (Menlo Park, California). The coding sequences were cloned in plasmids behind the *T7* promoter (expression vector pLD001 (SEQ ID NO: 397)) or the pBAD promoter, between the *Nde*I and *Asc*I restriction sites yielding plasmids. To express the fusion protein, the plasmids were transferred in an appropriate expression host: *E. coli* strain BL21AI (Invitrogen, Carlsbad, California) for constructs under the *T7* promoter or in an AraBAD derivative of *E. coli* MG1655 for constructs under the pBAD promoter.

The non-targeted perhydrolase variants listed in Table 5 were cloned similarly. The preparation and recombinant expression of the *Thermotoga*

maritima variants has previously been reported by DiCosimo *et al.* in U.S. Patent Application Publication No. 2010-0087529; hereby incorporated by reference.

Additional CE-7 perhydrolases from *Lactococcus lactis* (an acetyl xylan esterase; SEQ ID NO: 40), *Mesorhizobium loti* (an acetyl xylan esterase; SEQ ID NO: 42), and *Bacillus pumilus* (an acetyl xylan esterase; SEQ ID NO: 10) were cloned in a similar fashion. The cloning and expression of the CE-7 perhydrolases from *Lactococcus lactis*, *Mesorhizobium loti*, and *Bacillus pumilus* have been previously reported by DiCosimo *et al.* in U.S. Patent Application Publication No. 2011-0081693 and U.S. Patent 7,951,566; each hereby incorporated by reference.

Perhydrolytic enzymes not belonging to the CE-7 family of perhydrolases were also cloned in a similar fashion. The *Mycobacterium smegmatis* aryl esterase ("ArE"; the wild type sequence is SEQ ID NO: 478; the S54V variant is provided as SEQ ID NO: 460) having perhydrolytic activity is described in in U.S. Patent 7,754,460. A *Pseudomonas fluorescens* esterase variant L29P ("Pfl"; SEQ ID NO: 477) having perhydrolytic activity is described in U.S. Patent 7,384,787.

Table 5. Description and sequences for perhydrolase constructs.

Enzyme ID	**Abbrev Description**	**Sequence** (SEQ ID NO:)
WT	*T. maritima* wild-type	MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESHLKTV EAYDVTFSGYRGQRIKGWLLVPKLEEEKLPCVVQYIGYNGGRGFPHDWLFWPS MGYICFVMDTRGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGILDPRTYYYRRV FTDAVRAVEAAASFPQVDQERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLC HFRRAVQLVDTHPYAEITNFLKTHRDKEEIVFRTLSYFDGVNFAARAKIPALFSVG LMDNICPPSTVFAAYNYYAGPKEIRIYPYNNHEGGGSFQAVEQVKFLKKLFEKG (SEQ ID NO: 16)
C277S (EZ-1)	*T. maritima* C277S	MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESHLKTV EAYDVTFSGYRGQRIKGWLLVPKLEEEKLPCVVQYIGYNGGRGFPHDWLFWPS MGYICFVMDTRGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGILDPRTYYYRRV FTDAVRAVEAAASFPQVDQERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLC HFRRAVQLVDTHPYAEITNFLKTHRDKEEIVFRTLSYFDGVNFAARAKIPALFSVG LMDNI**S**PPSTVFAAYNYYAGPKEIRIYPYNNHEGGGSFQAVEQVKFLKKLFEKG (SEQ ID NO: 424)
C277T (EZ-12)	*T. maritima* C277T	MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESHLKTV EAYDVTFSGYRGQRIKGWLLVPKLEEEKLPCVVQYIGYNGGRGFPHDWLFWPS

		MGYICFVMDTRGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGILDPRTYYYRRV FTDAVRAVEAAASFPQVDQERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLC HFRRAVQLVDTHPYAEITNFLKTHRDKEEIVFRTLSYFDGVNFAARAKIPALFSVG LMDNITPPSTVFAAYNYYAGPKEIRIYPYNNHEGGGSFQAVEQVKFLKKLFEKG (SEQ ID NO: 437)
HTS- 007-D5	*T. maritima* C277T/R296P	MAFFDLPLEELKKYRPERYEEKDFDEFWEETLAESEKFPLDPVFERMESHLKTV EAYDVTFSGYRGQRIKGWLLVPKLEEEKLPCVVQYIGYNGGRGFPHDWLFWPS MGYICFVMDTRGQGSGWLKGDTPDYPEGPVDPQYPGFMTRGILDPRTYYYRRV FTDAVRAVEAAASFPQVDQERIVIAGGSQGGGIALAVSALSKKAKALLCDVPFLC HFRRAVQLVDTHPYAEITNFLKTHRDKEEIVFRTLSYFDGVNFAARAKIPALFSVG LMDNITPPSTVFAAYNYYAGPKEIPIYPYNNHEGGGSFQAVEQVKFLKKLFEKG (SEQ ID NO: 476)
Bpu	*B. pumilus* wild- type	MQLFDLSLEELKKYKPKKTARPDFSDFWKKSLEELRQVEAEPTLESYDYPVKGV KVYRLTYQSFGHSKIEGFYAVPDQTGPHPALVRFHGYNASYDGGIHDIVNWALH GYATFGMLVRGQGGSEDTSVTPGGHALGWMTKGILSKDTYYYRGVYLDAVRAL EVIQSFPEVDEHRIGVIGGSQGGALAIAAAALSDIPKVVVADYPYLSNFERAVDVA LEQPYLEINSYFRRNSDPKVEEKAFETLSYFDLINLAGWVKQPTLMAIGLIDKITPP STVFAAYNHLETDKDLKVYRYFGHEFIPAFQTEKLSFLQKHLLLST (SEQ ID NO: 10)
Mlo	*M. loti* wild-type	MPFPDLIQPELGAYVSSVGMPDDFAQFWTSTIAEARQAGGEVSIVQAQTTLKAV

		QSFDVTFPGYGGHPIKGWLILPTHHKGRLPLVVQYIGYGGGRGLAHEQLHWAAS GFAYFRMDTRGQGSDWSVGETADPVGSTSSIPGFMTRGVLDKNDYYYRRLFTD AVRAIDALLGLDFVDPERIAVCGDSQGGGISLAVGGIDPRVKAVMPDVPFLCDFP RAVQTAVRDPYLEIVRFLAQHREKKAAVFETLNYFDCVNFARRSKAPALFSVALM DEVCPPSTVYGAFNAYAGEKTITEYEFNNHEGGQGYQERQQMTWLSRLFGVG (SEQ ID NO: 42)
Lla	*L. lactis* wild-type	MTKINNWQDYQGSSLKPEDFDKFWDEKINLVSNHQFEFELIEKNLSSKVVNFYHL WFTAIDGAKIHAQLIVPKNLKEKYPAILQFHGYHCDSGDWVDKIGIVAEGNVVLAL DCRGQGGLSQDNIQTMGMTMKGLIVRGIDEGYENLYYVRQFMDLITATKILSEFD FVDETNISAQGASQGGALAVACAALSPLIKKVTATYPFLSDYRKAYELGAEESAF EELPYWFQFKDPLHLREDWFFNQLEYIDIQNLAPRIKAEVIWILGGKDTVVPPITQ MAAYNKIQSKKSLYVLPEYGHEYLPKISDWLRENQ (SEQ ID NO: 40)
ArE	*M. smegmatis* S54V	MAKRILCFGDSLTWGWVPVEDGAPTERFAPDVRWTGVLAQQLGADFEVIEEGL VARTTNIDDPTDPRLNGASYLPSCLATHLPLDLVIIMLGTNDTKAYFRRTPLDIALG MSVLVTQVLTSAGGVGTTYPAPKVLVVSPPPLAPMPHPWFQLIFEGGEQKTTEL ARVYSALASFMKVPFFDAGSVISTDGVDGIHFTEANNRDLGVALAEQVRSLL (SEQ ID NO: 460)
Pfl	*P. fluorescens* L29P	MSTFVAKDGTQIYFKDWGSGKPVLFSHGWPLDADMWEYQMEYLSSRGYRTIAF DRRGFGRSDQPWTGNDYDTFADDIAQLIEHLDLKEVTLVGFSMGGGDVARYIAR

		HGSARVAGLVLLGAVTPLFGQKPDYPQGVPLDVFARFKTELLKDRAQFISDFNAP FYGINKGQVVSQGVQTQTLQIALLASLKATVDCVTAFAETDFRPDMAKIDVPTLVI HGDGDQIVPFETTGKVAAELIKGAELKVYKDAPHGFAVTHAQQLNEDLLAFLKR (SEQ ID NO: 477)

Table 6. Perhydrolase Constructs With Targeting Sequences Produced for Use in Oral Care.

Construct ID (SEQ ID NO:)	**Abbrev. Description**	**Targeting Sequence[a] of the Fusion Protein (SEQ ID NO:)**
EZ-1 (SEQ ID NO: 424)	C277S	N/A
EZ-2 (SEQ ID NO: 425)	C277S-link1-HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPEPEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAAG*SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-3 (SEQ ID NO: 426)	C277S-link2-H6	*GS*HHHHHH (SEQ ID NO:432)
EZ-4 (SEQ ID NO: 427)	C277S-link1-$(GK)_5$	*GPGSGGAGSPGSAGGPGS*GKGKGKGKGK (SEQ ID NO:433)
EZ-5 (SEQ ID NO: 428)	C277S-link1-$(GK)_5$-H6	*GPGSGGAGSPGSAGGPGS*GKGKGKGKGKHHHHHH (SEQ ID NO:434)
EZ-7 (SEQ ID NO: 429)	C277S-link1-DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*HHHHHH (SEQ ID NO:435)

EZ-9 (SEQ ID NO: 430)	C277S-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH (SEQ ID NO:436)
EZ-12 (SEQ ID NO: 437)	C277T	N/A
EZ-14 (SEQ ID NO: 438)	C277T-link1-DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*HHHHHH (SEQ ID NO:435)
EZ-15 (SEQ ID NO: 439)	C277T-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH (SEQ ID NO:436)
EZ-16 (SEQ ID NO: 440)	C277T-link1-HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPEPEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*GSGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-17 (SEQ ID NO:441)	C277T-link2-H6	*GS*HHHHHH (SEQ ID NO:432)
EZ-18 (SEQ ID NO:442)	C277T-link1-$(GK)_5$-H6	*GPGSGGAGSPGSAGGPGS*GKGKGKGKGKHHHHHH (SEQ ID NO:434)
EZ-19 (SEQ ID NO: 443)	C277S-EPEPE-link1-EPEPE-CXH201-H6	EPEPE*GPGSGGAGSPGSAGGPGS*EPEPEWKTKKILLSRTRRIMRQVVRSVMHKIWHHHHHH (SEQ ID NO: 468)

EZ-20 (SEQ ID NO: 444)	C277S-EPEPEPEPEPE-link1-CXH201-H6	EPEPEPEPEPE*GPGSGGAGSPGSAGGPGS*WKTKKILLSRTRRIMRQVVRSVMHKIWHHHHHH (SEQ ID NO: 469)
EZ-21 (SEQ ID NO:445)	C277S-EPEPE-link1-EPEPE-CXHG2-H6	EPEPE*GPGSGGAGSPGSAGGPGS*EPEPEPLWRRITKRKLVRPVATLMWYWFTSKRHHHHHH (SEQ ID NO: 470)
EZ-22 (SEQ ID NO: 446)	C277S-EPEPEPEPEPE-Link1-CXHG2-H6	EPEPEPEPEPE*GPGSGGAGSPGSAGGPGSP*LWRRITKRKLVRPVATLMWYWFTSKRHHHHHH (SEQ ID NO: 471)
EZ-23 (SEQ ID NO: 447)	C277S-EPEPE-Link1-EPEPE-CXH104-H6	EPEGPGSGGAGSPGSAGGPGSEPERMLSRILRMFVRILKRERLSQVRGLFVHHHHHH (SEQ ID NO: 472)
EZ-24 (SEQ ID NO: 448)	C277S-EPEPEPEPEPE-Link1-CXH104-H6	EPEPEPE*GPGSGGAGSPGSAGGPGS*RMLSRILRMFVRILKRERLSQVRGLFVHHHHHH (SEQ ID NO: 473)
EZ-25 (SEQ ID NO: 449)	C277S-EPEPE-Link1-EPEPE-CXHG102-H6	EPEPEPE*GPGSGGAGSPGSAGGPGS*EPEPEPELRFLARRFLKLRRARKWWNAWKVWVTRHHHHHH (SEQ ID NO: 474)
EZ-26 (SEQ ID NO: 450)	C277S-EPEPEPEPEPE-Link1-CXHG102-H6	EPEPEPEPEPEPEPE*GPGSGGAGSPGSAGGPGS*LRFLARRFLKLRRARKWWNAWKVWVTRHHHHHH

		(SEQ ID NO: 475)
EZ-27 (SEQ ID NO: 451)	Bpu-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH (SEQ ID NO:436)
EZ-28 (SEQ ID NO: 452)	Bpu-link1-HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-29 (SEQ ID NO: 453)	Bpu-link1-DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*H HHHHH (SEQ ID NO:435)
EZ-30 (SEQ ID NO: 454)	Mlo-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH (SEQ ID NO:436)
EZ-31 (SEQ ID NO: 455)	Mlo-link1-HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-32 (SEQ ID NO: 456)	Mlo-link1-DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*H HHHHH (SEQ ID NO:435)
EZ-33	Lla-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH

(SEQ ID NO: 457)		(SEQ ID NO:436)
EZ-34 (SEQ ID NO: 458)	Lla-link1- HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G* *SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-35 (SEQ ID NO: 459)	Lla-link1- DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*H HHHHH (SEQ ID NO:435)
EZ-36 (SEQ ID NO: 460)	*M. smegmatis* ArE S54V	N/A
EZ-37 (SEQ ID NO: 461)	ArE-link1-H6	*GPGSGGAGSPGSAGGPGS*HHHHHH (SEQ ID NO:436)
EZ-38 (SEQ ID NO: 462)	ArE-link1- HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G* *SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-39 (SEQ ID NO: 463)	ArE-link1-$(GK)_5$- H6	*GPGSGGAGSPGSAGGPGS*GKGKGKGKGKHHHHHH (SEQ ID NO:434)
EZ-40 (SEQ ID NO: 464)	ArE-link1- DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*H HHHHH

		(SEQ ID NO:435)
EZ-41 (SEQ ID NO: 465)	Pfl-link1-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G SGGGGSP*HHHHHH (SEQ ID NO:431)
EZ-42 (SEQ ID NO: 466)	Pfl-link1-$(GK)_5$-H6	*GPGSGGAGSPGSAGGPGS*GKGKGKGKGKHHHHHH (SEQ ID NO:434)
EZ-43 (SEQ ID NO: 467)	Pfl-link1-DenP308-H6	*GPGSGGAGSPGSAGGPGS*TKPPRTPTANTSRPHHNF*GSGGGGSP*H HHHHH (SEQ ID NO:435)
EZ-44 (SEQ ID NO: 479)	Pfl-link1-HC263-H6	*GPGSGGAGSPGSAGGPGS*PSAQSQLPDKHSGLHERAPQRYGPEPE PEPEPIPEPPKEAPVVIEKPKPKPKPKPKPPAHDHKNQKETHQRHAA*G SGGGGSP*HHHHHH (SEQ ID NO:431)

a = flexible linker(s) are italicized.

EXAMPLE 5

PRODUCTION OF THE FUSION PROTEINS

This example describes the expression and purification of perhydrolases with and without targeting sequences for binding to oral surfaces.

Strains were grown in 1 L of autoinduction medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 3 mM $MgSO_4$, 0.75% glycerol, 0.075% glucose and 0.05% arabinose) containing 50 mg/L spectinomycin at 37 ºC for 20 hrs under 200 rpm agitation. Production of the untargeted perhydrolase has been described previously in U.S. Patent Application Publication No. 2010-0087529 to DiCosimo *et al*. Production of the targeted perhydrolases followed a similar protocol. The cells were harvested by centrifugation at 8000 rpm and washed by resuspending the cell pellet in 20 mL of 50 mM potassium phosphate buffer, pH 7.1 containing 1 mM DTT. The solution was centrifuged again at 8000 rpm, the supernatant removed and the pellet redispersed again in the phosphate buffer containing DTT. The solution was then homogenized for 30 s to disperse the pellet (Brinkman Homogenizer model PCU11). The cells were then lysed by processing the solution through a French Press (SLM Instruments) at 13,000 psi (~89.6 MPa). The solution was processed through the press an additional two times to achieve complete lysis. The cell solution was then transferred to a conical tube and centrifuged at 8500 rpm for 5 min. For *T. maritima* constructs, the supernatant was removed and heated at 80 ºC for 30 min. The solution was centrifuged again and the supernatant was transferred to a clean vial.

For non-thermophilic enzymes no heat treatment was used to purify the enzymes away from contaminating cell components. Instead, the samples were purified using a His6 tag fused to the C-terminal end of the enzymes by metal chelation chromatography using Co-NTA agarose (HisPur Cobalt Resin, Thermo Scientific, product number: 89965). Typically, cell extracts were loaded onto a 5 to 10 mL column of Co-NTA agarose equilibrated with 4 volumes of equilibration buffer (10 mM Tris HCl pH 7.5, 10% glycerol, 1 mM imidazole and 150 mM NaCl). The amount of each extract loaded on the column was adjusted to

contain between 5 and 10 mg of perhydrolase fusion per mL of Co-NTA agarose beads. The resin was washed with two bed volumes of equilibration buffer and eluted with two volumes of elution buffer (10 mM Tris HCl pH 7.5, 10% glycerol, 150 mM imidazole, 500 mM NaCl). Fractions were collected and the presence of the full-length, purified proteins was confirmed by PAGE.

For production of constructs EZ-19- to EZ-26, after cell production, the cells were harvested by centrifugation at 8000 rpm and washed by resuspending the cell pellet in 20 mL of 50 mM potassium phosphate buffer, pH 7.2. The solution was centrifuged again at 8000 rpm, the supernatant removed and the pellet redispersed again in the phosphate buffer. The solution was then homogenized for 30 s to disperse the pellet (Brinkman Homogenizer model PCU11). The cells were then lysed by processing the solution through a French Press (SLM Instruments) at 13,000 psi (~89.6 MPa). The solution was processed through the press an additional two times to achieve complete lysis. The cell solution was then transferred to a conical tube and centrifuged at 8500 rpm for 5 min. The insoluble lysate pellets were dissolved in sarkosyl buffer (50 mM phosphate buffer at pH 7.2, 2% TRITON®-X100 and 1.5% sarkosyl) at 3 mL buffer per 50 mL cell lysate pellet. The solution was centrifuged and the supernatant was transferred to a new tube. The fusion proteins were purified by using a HisPur™ Cobalt Resin kit from Thermo Scientific (Rockford, IL).

The output of these production and purification protocols typically yielded 2-10 mg of protein per mL with a purity of the fusion perhydrolase between 90% and 75% of the protein as estimated by polyacrylamide gel electrophoresis (PAGE) analysis. Total protein was quantitated by the bicinchoninic acid (BCA) assay (Thermo Scientific) using a solution of Bovine Serum Albumin as a standard.

EXAMPLE 6

BINDING OF THE ENAMEL-TARGETED PERHYDROLASE FUSION TO HYDROXYAPATITE

This example describes the binding of the perhydrolase to hydroxyapatite particles. The hydroxyapatite is an effective mimic for enamel.

Perhydrolase enzymes listed in Table 6 were assessed for binding to hydroxyapatite. A dispersion of hydroxyapatite nanoparticles (Aldrich 677418) was made at 0.5% solids in 10 mM phosphate buffer at pH 7.2. Enzyme stock solution was added to the hydroxyapatite dispersion to a final concentration of 10 μM and incubated for 30 min in a microcentrifuge tube with gentle agitation. Each sample was centrifuged for 5 min at 10000 rpm. The supernatant was removed and additional buffer was added. The particles were resuspended and transferred to a new tube. The process was repeated two additional times. Samples of the original input of enzyme, the supernatant removed from the particles with each wash step and the final particles were prepared for SDS-PAGE analysis by combining 60 μL of sample, 20 μL of LDS buffer and 8 μL of reducing agent (Nu-PAGE). Samples were heated at 85 °C for 10 min. 25 μL of each sample was loaded on a 4-12% BisTris gel (Invitrogen) and run at 115 volts for 1.5 h. The gel was removed and stained with a 1:1 dilution of Simply Blue stain (Invitrogen) overnight and destained for 4 h in deionized water. The gel was analyzed and detection of enzyme in each fraction was estimated based on the presence of a band. The results of the analysis are provided in Table 7 with an indication of the relative strength of each band in the gel compared to the enzyme input (input = 1) to determine the strength of binding between the hydroxyapatite surface and each enzyme.

Table 7. Retention of Perhydrolase on Hydroxyapatite.

Enzyme	Input	Unbound	Wash1	Wash2	HAP Particles
EZ-1	1	0.98	0.01	0.0	0.01
EZ-2	1	0.5	0.0	0.0	0.5
EZ-3	1	0.8	0.05	0.05	0.1

EZ-4	1	0.5	0.05	0.05	0.4
EZ-5	1	0.2	0.05	0.05	0.7
EZ-7	1	0.5	0.01	0.0	0.5
EZ-9	1	0.9	0.01	0.00	0.05

The data in Table 7 demonstrates that the perhydrolase fusions with targeting sequences were retained on hydroxyapatite after washes whereas the untargeted perhydrolase was not.

EXAMPLE 7

QUANTITATION OF THE ENZYME PERHYDROLASE ACTIVITY IN SOLUTION AND BOUND TO HYDROXYAPATITE

This example describes the method for the detection and quantitation of the perhydrolase via its perhydrolase activity using triacetin and hydrogen peroxide to generate peracetic acid.

The detection of peracetic acid followed the method described in Pinkernell *et. al.* (*Analyst*, 1997, 122, 567) using colorimetric detection of 2,2'-azino-bis(3-ethylbenzothiazoline(-6-sulfonate (ABTS) oxidation by peracetic acid. Following the formation of peracetic acid with the addition of triacetin and hydrogen peroxide, 90 µL of solution was added to 10 µL of 0.1 M H_3PO_4 in a well plate. 50 µL of 1 M acetic acid, 50 µL of 0.5 g/L ABTS and 50 µL of 0.002 g/L of KI was added. The solution was allowed to develop for 5 min. The absorbance of the solution was measured at 405 nm using a microplate reader. The peracetic acid concentration was calculated based on a standard curve developed simultaneously using peracetic acid reagent solution.

The enzyme activity in solution was measured by making a solution of 0.625 µg/mL of each enzyme in 50 mM phosphate buffer, pH 7.2. 10 µL of enzyme solution was mixed with 90 µL buffer, 30 µL of 3% triacetin in water, 30 µL of 30 mM H_2O_2. The solution was incubated for 5 min. A 90 µL aliquot was removed for detection via ABTS oxidation as noted above. Results are listed in Table 8.

Table 8. Detection of peracetic acid with ABTS oxidation for enzyme with triacetin and hydrogen peroxide in solution

Enzyme ID	Avg Absorbance 405nm Background subtracted
No enzyme	0.000
EZ-1	1.830
EZ-2	1.577
EZ-3	1.751
EZ-5	1.658
EZ-7	1.619

The perhydrolase activity of the fusions once bound to HAP discs surfaces was determined with the same ABTS method. Hydroxyapatite discs (HiMed Inc, Old Bethpage, NY; 5 mm dia. X 1.8mm thick) were incubated in 20 µM enzyme solution (50 mM potassium phosphate buffer pH 7.2) for 60 min followed by 6 times of washes (50 mM potassium phosphate buffer pH 7.2). The discs with enzyme adsorbed were transferred to new wells and 200 µL of phosphate buffer (10 mM pH 7.2), 30 µL of 3% triacetin (final concentration of 0.346%) and 30 µL of 30 mM H_2O_2 (final concentration of 3.46 mM) were added. The solution was allowed to incubate at room temperature for 5 min. 90 µL of solution was pipetted to a new well containing 10 µL of 100 mM H_3PO_4. 50 µL of acetic acid, 50 µL KI, 50 µL ABTS were added as described above. The solution was developed for 5 min at room temperature and read at A405nm. Results are listed in Table 9.

Table 9. Peracetic Acid detection with ABTS with enzyme bound to hydroxyapatite

Enzyme	**Avg Absorbance at 5 min**	**Avg Absorbance Background subtract**	**PAA conc µM per disc**
No enzyme, No HAP	0.775	0	0
No enzyme control	0.840	0.065	1.2
EZ-1	0.901	0.126	3.5
EZ-5	2.154	1.379	49.7
EZ-7	1.45	1.450	23.5

This experiment demonstrates that EZ-5 and EZ-7 are active enzymes when bound to hydroxyapatite and produce peracetic acid with the addition of triacetin and hydrogen peroxide. The low value of peracetic acid detected with EZ-1 coincided with other observations that EZ-1 does not bind to hydroxyapatite (Example 6) and is not present on the surface to generate peracetic acid.

EXAMPLE 8

QUANTITATION OF THE ENZYME PERHYDROLASE ACTIVITY IN SOLUTION AND BOUND TO HYDROXYAPATITE FOR ADDITIONAL CE-7 PERHYDROLASE CONSTRUCTS

This example describes the perhydrolytic activities of the CE-7 perhydrolase and respective fusion proteins in solution and when bound to hydroxyapatite from *Thermotoga maritima*, *Bacillus pumilus* (Bpu), *Mesorhizobium loti* (Mlo) and *Lactobacillus lactis* (Lla) using triacetin and hydrogen peroxide to generate peracetic acid.

Perhydrolase enzymes listed in Table 6 were assessed for solution activities. The method to measure the perhydrolase in solution activity was described in Example 7 by making a 500 μL solution containing 0.5 μM of enzyme, 100 mM triacetin, 100 mM H_2O_2 in 100 mM phosphate buffer, pH 7.2. The solution was mixed and incubated for 10 min at 37 °C. A 10 μL aliquot was removed and diluted for detection as described in Example 7. Peracetic acid concentration was determined using a standard curve generated with a stock solution of peracetic acid (Aldrich). The experiment was also performed with no enzyme present as a control.

Table 10. Peracetic acid generated in solution for a 10 min reaction for various CE-7 perhydrolase fusions.

Enzyme ID	Description	PAA (ppm)
No enzyme	---	378.9
EZ-7	C277S-DenP308	4877.2
EZ-27	Bpu-H6	2332.9
EZ-28	Bpu-HC263	2512.7
EZ-29	Bpu- DenP308	2273.5
EZ-30	Mlo-H6	468.8
EZ-31	Mlo-HC263	434.9
EZ-32	Mlo- DenP308	497.7

EZ-33	Lla-H6	1062.5
EZ-34	Lla-HC263	786
EZ-35	Lla- DenP308	998

To assess binding of these constructs to hydroxyapatite, 33 mg of hydroxyapatite particles (Macro-prep Ceramic Hydroxyapatite TYPE I, 80 μm size BioRad, Hercules, CA), were washed with 10 mM phosphate buffer at pH 7.2. After removing the supernatant, enzyme stock solution was added to the hydroxyapatite dispersion to a final concentration of 10 μM and incubated for 30 min in a microcentrifuge tube with gentle agitation. Each sample was centrifuged for 1 min at 10000 rpm. The supernatant was removed and additional buffer was added. The particles were resuspended and transferred to a new tube. The process was repeated two additional times. The hydroxyapatite bound enzyme activity was measured by adding a 500 μl solution containing 100 mM triacetin, 100 mM H_2O_2 in 100 mM phosphate buffer, pH 7.2. The solution was incubated for 30 min, at 37ºC. An aliquot was removed and mixed with H_3PO_4 and properly diluted for detection via ABTS oxidation as described in Example 7. The experiments were performed at different day with no enzyme as control for each experiment. Results are listed in Table 11.

Table 11. Peracetic acid generation from hydroxyapatite bound CE-7 perhydrolase fusions.

Enzyme ID	**Description**	**Peracetic acid (ppm)**
No enzyme		118.8
EZ-9	C277S-H6	1885.1
EZ-27	Bpu-H6	634.9
EZ-30	Mlo-H6	658.5
EZ-33	Lla-H6	359.1
No enzyme		30
EZ-2	C277S-HC263	3666.4

EZ-28	Bpu-HC263	534.2
EZ-31	Mlo-HC263	241.4
EZ-34	Lla-HC263	242.2
No enzyme		113.9
EZ7	C277S-DenP308	4453.7
EZ-29	Bpu-DenP308	1299.8
EZ-32	Mlo-DenP308	419.3
EZ-35	Lla-DenP308	260

The experiments demonstrated that all CE-7 perhydrolase fusions from Bpu, Mlo and Llo enzyme showed significant perhydrolase activity in solution as well as after binding to hydroxyapatite surfaces compare to a no enzyme control. All three CE-7 perhydrolases fusions from *B. pumilus* have higher enzyme activity compared to the fusion enzymes from *M. loti* or *L. lactis*. Targeted EZ-29 from *B. pumilus* showed higher binding activity compared to the untargeted EZ-27.

EXAMPLE 9

QUANTITATION OF ENZYME ACTIVITY IN SOLUTION AND BOUND TO HYDROXYAPATITE FOR TARGETED C277S PERHYDROLASE FUSIONS WITH CXH PEPTIDES

This example describes the CE-7 C277S and fusions with CXH peptides and their perhydrolase activities using triacetin and hydrogen peroxide to generate peracetic acid in solution and when bound to hydroxyapatite. The hydroxyapatite is an effective mimic for enamel.

Perhydrolase enzymes EZ-19 to EZ-26 listed in Table 6 were assessed for enzyme activity in solution. The method to measure the perhydrolase in solution activity was described in Example 7. The enzyme activity in solution was measured by making a 500 μl sarkosyl buffer solution of 0.5 μM of each enzyme in 100 mM triacetin, 100 mM H_2O_2 and 10 mM phosphate buffer, pH 7.2. The solution was incubated for 10 min, at 37ºC. After the reaction was stopped by mixing with H_3PO_4, a 10 μL aliquot was removed for proper dilution, and then

detected via ABTS oxidation as described in Example 7. Results are listed in Table 12.

The same perhydrolase fusion enzymes were also assessed for binding to hydroxyapatite. Water hydrated hydroxyapatite discs (5 mm dia.x 1.8 mm thick, from HiMed Inc) were used. The discs were equilibrated in 10 mM phosphate buffer at pH 7.2 for 10 min. 200 μL of enzyme solution was added to the hydroxyapatite discs to a final concentration of 10 μM in 10 mM phosphate buffer and incubated for 30 min in a microcentrifuge tube with gentle agitation. The supernatant was removed and used for unbound enzyme activity assays. The discs were transferred to a new tube and rinsed with phosphate buffer. The process was repeated two additional times. The enzyme activity of the hydroxyapatite discs with bound enzymes was measured by adding a 500 μl solution containing 100 mM triacetin, 100 mM H_2O_2 and 100 mM phosphate buffer, pH 7.2 to the disc and incubating for 30 min, at 37°C. After the reaction was stopped by mixing with H_3PO_4, a 10 μL aliquot was removed for proper dilution, and then detected via ABTS oxidation as described in Example 7. The experiments were performed over several days with a no enzyme solution used as a control for each day. Results after subtraction of the no enzyme control are listed in Table 12.

Table 12. Peracetic acid generated from C277s and CXH perhydrolase fusions proteins in solution and when bound to hydroxyapatite discs.

Enzyme ID	Peracetic acid (ppm)		
	In solution	Exposed to Hydroxyapatite	
		Supernatant	Disc
EZ-7	5471	4864	2017
EZ-19	4088	1665	3136
EZ-20	2265	1229	2795
EZ-21	2764	1581	3109
EZ-22	2305	477	2495
EZ-23	2212	1712	4463
EZ-24	2951	719	3453
EZ-25	1703	1029	1791

EZ-26	2273	1636	1849

The experiments demonstrated that all the C277S variants with CXH targeting sequences are active and generate sufficient amounts of peracetic acid in solution and when bound to hydroxyapatite.

EXAMPLE 10
QUANTITATION OF THE ENZYME PERHYDROLASE ACTIVITY IN SOLUTION AND BOUND TO HYDROXYAPATITE FOR NON CE-7 PERHYDROLASE CONSTRUCTS

The purpose of this example is to demonstrate the use of targeted or untargeted aryl esterase enzyme variants from *M. smegmatis* and perhydrolase variants from *P. fluorescens* listed in Table 6 to generate peracetic acid in solution and when bound to hydroxyapatite.

For aryl esterase constructs, the enzyme activity in solution was measured by making a 1 mL solution of 0.5 μM of each enzyme, 100 mM triacetin, and 100 mM H_2O_2 in 100 mM phosphate buffer, pH 7.2. The solution was mixed and incubated for 30 min, at 37ºC. As described in Example 7, the reaction was stopped by removing a portion to H_3PO_4 and a 10 μL aliquot was removed and diluted for detection via ABTS oxidation.

For assessment of binding and activity on hydroxyapatite, the aryl esterase enzymes were exposed to hydroxyapatite particles as described in Example 8 using 33 mg of buffer washed HAP particles (Macro-prep Ceramic Hydroxyapatite TYPE I, 80 μm size BioRad, Hercules, CA) using a 10 μM solution in 10mM phosphate buffer. Following centrifugation and removal of the enzyme solution, the particles were rinsed with phosphate butter by centrifuging and removing the supernatant. A 200 μL solution containing 100 mM triacetin, 100 mM H_2O_2 and 100 mM phosphate buffer, pH 7.2 was added to the particles and incubated at 37ºC for 30 min. As described in Example 7, the reaction was stopped by removing a portion to H_3PO_4 and a 10 μL aliquot was removed

diluted for detection via ABTS oxidation. Results for both solution and surface bound assays are listed in Table 13.

Table 13. Solution and surface bound generation of peracetic acid for *M. smegmatis* aryl esterase samples

Enzyme ID	**Description (SEQ ID NO.)**	**Peracetic acid (ppm)**	
		In Solution	**On Hydroxyapatite**
No enzyme		158	323
EZ-36	ArE (SEQ ID NO: 460)	2697	452
EZ-37	ArE-H6 (SEQ ID NO: 461)	Not measured	927
EZ-39	ArE-$(GK)_5$H6 (SEQ ID NO: 463)	1366	3553
EZ-40	ArE-DenP308-H6 (SEQ ID NO: 464)	3605	2476

For *P. fluorescens* constructs, the solution activity was measured by mixing a 1mL solution containing 2 μM enzyme, 100 mM H_2O_2 in a 1 M sodium acetate buffer, pH 5.5. The solution was incubated at 37ºC for 30 min. As described in Example 7, the reaction was stopped by removing a portion to H_3PO_4 and a 10 μL aliquot was removed and diluted for detection via ABTS oxidation. For assessment of binding and activity on hydroxyapatite, the *P. fluorescens* enzymes were exposed to hydroxyapatite particles at 20 μM in 10 mM phosphate buffer as described in Example 8 using 100 mg of buffer washed HAP particles (Macro-prep Ceramic Hydroxyapatite TYPE I, 80 μm size BioRad, Hercules, CA). Following centrifugation and removal of the enzyme solution, the particles were rinsed with phosphate butter by centrifuging and removing the supernatant. A 200 μL solution containing 300 mM H_2O_2 in 1 M sodium acetate

buffer, pH 5.5 was added to the particles and incubated at 37°C for 10 min. As described in Example 7, the reaction was stopped by removing a portion to H_3PO_4 and a 10 µL aliquot was removed and diluted for detection via ABTS oxidation. Results for both solution and surface bound assays are listed in Table 14.

Table 14. Solution and surface bound generation of peracetic acid for *P. fluorescens* perhydrolase samples

Enzyme ID	**Description (SEQ ID NO.)**	**Peracetic acid (ppm)**	
		In Solution	**On Hydroxyapatite**
No enzyme		9	17
EZ-41	Pfl-link1-H6 (SEQ ID NO: 465)	Not measured	18
EZ-42	Pfl-link1-$(GK)_5$-H6 (SEQ ID NO: 466)	64	63
EZ-43	Pfl- link1-DenP308-H6 (SEQ ID NO: 467)	79	68
EZ-44	Pfl-link1-HC263-H6 (SEQ ID NO: 479)	58	97

These experiments demonstrate that perhydrolases from families beyond the CE-7 family are active in solution and when bound to hydroxyapatite for constructs including a targeting sequence.

EXAMPLE 11

QUANTITATION OF PERHYDROLYTIC ACTIVITY OF ENAMEL-TARGETED C277S AND C277T VARIANT PERHYDROLASE FUSIONS IN SOLUTION AND BOUND TO BOVINE ENAMEL

This example describes the binding of the perhydrolase fusion proteins to bovine enamel and measurement of enzyme activity in solution and when bound to bovine enamel.

Perhydrolase enzymes listed in Table 5 were assessed for enzyme activity in solution as described in Example 7. The enzyme activity in solution was measured by making a 500 μl solution of 0.5 μM of each enzyme in 100 mM triacetin, 100mM H_2O_2 and 10 mM phosphate buffer, pH 7.2. The solution was incubated for 10 min, at 37 ºC. After the reaction was stopped by mixing an aliquot with H_3PO_4, a 10 μL aliquot was removed for proper dilution, and then detected via ABTS oxidation as described in Example 7. Results are listed in Table 15.

The perhydrolase enzymes were also assessed for binding to bovine enamel substrates. Enamel substrates were prepared as described in Example 2 and 3. Each enamel block was hydrated in water for overnight at room temperature (~ 22 ºC). The enamel blocks were then equilibrated with 10 mM potassium phosphate buffer, pH7.2, for 10 min. The enamel substrates were rinsed 3 times the buffer. The enamel wells were filled with 500 μL of 10 μM enzyme solutions which was prepared by diluting in 10 mM phosphate buffer. The samples were incubated for 30 min with slow shaking at 37 ºC. The non-binding enzyme was removed by washing 4 times with phosphate buffer. Then, each enamel block was embedded inside a putty filled 24-well plate, with only the enamel top surface exposed. The perhydrolase activity from enamel bound enzyme was measured as described in Example 8. 100 μL reaction mixtures (100 mM phosphate buffer, pH7.2 and 100 mM H_2O_2 and 100 mM triacetin) was added on top of the enamel and incubated for 30 min at 37 ºC. A 90 μL aliquot was removed for detection via ABTS oxidation as noted in Example 8. Results

are listed in Table 15. Each sample data point represents an average of 3 independent enamel blocks.

Table 15. Peracetic acid generated in solution and when bound to a bovine enamel surface for *T. maritima* constructs.

Enzyme ID	**Description (SEQ ID NO:)**	**Peracetic acid (ppm)**	
		In Solution	**On Enamel**
No enzyme		114	570
EZ-1	C277S (SEQ ID NO: 424)	4270	628
EZ-2	C277S-HC263-H6 (SEQ ID NO: 425)	4761	2549
EZ-3	C277S-link2-H6 (SEQ ID NO: 426)	5187	733
EZ-5	C277S-$(GK)_5$H6 (SED ID NO: 428)	3956	1344
EZ-7	C277S-DenP308-H6 (SEQ ID NO: 429)	5519	1040
EZ-9	C277S-H6 (SEQ ID NO: 430)	5499	612
EZ-12	C277T (SEQ ID NO: 437)	4918	612
EZ-16	C277T-HC263-H6 (SEQ ID NO: 440)	6496	1832
EZ-17	C277T-link2-H6 (SEQ ID NO: 441)	5360	616
EZ-14	C277T-$(GK)_5$H6 (SEQ ID NO: 438)	4689	1418
EZ-18	C277T-DenP308-H6	5537	1158

	(SEQ ID NO: 442)		
EZ-15	C277T-H6 (SEQ ID NO: 439)	5921	631

This experiment demonstrates that fusions using C227S and C277T *from T. maritima* sequence variants are active in solution and when bound to bovine enamel, for constructs including a targeting sequence, and generate sufficient levels of peracetic acid to whiten teeth.

EXAMPLE 12

TOOTH BLEACHING EFFICACY USING PERHYDROLYTIC ENZYMES IN A ONE-STEP APPLICATION

The purpose of this example is to show the tooth bleaching effect of enzymatic generated peracetic acid in a one-step application and compare to the performance achieved with chemically derived peracetic acid. Two methods were developed to use a perhydrolytic enzyme (CE-7 perhydrolase) system to achieve the target level of tooth bleaching. The first method (referred to herein as the "one-step approach") comprises combining different amounts of at least one CE-7 perhydrolase with triacetin (an example of a suitable ester substrate) and hydrogen peroxide to generate peracetic acid using model stained enamel substrates. Bovine enamel incisors were prepared as indicated in Example 1. Stained bovine enamel blocks were hydrated in water at least 1 hr prior to use to stabilize the color of the substrate. The color for each enamel block was measured after hydration prior to the start of the experiment. Three enamel samples were treated for each solution type. The solutions used were a buffer only control, 2.5% H_2O_2, 1% peracetic acid, a perhydrolase composition including 10 µM EZ-1 (C277S; SEQ ID NO: 424), 100 mM triacetin and 250 mM H_2O_2 and a no enzyme control of 100 mM triacetin and 250 mM H_2O_2. All solutions were prepared in 500 mM sodium phosphate buffer, pH 7.2. The high buffer strength was necessary to maintain the 1% peracetic acid solution at neutral pH. All solutions were freshly prepared for each treatment point. The enzyme-triacetin-

H_2O_2 combination and triacetin- H_2O_2 combination was mixed immediately before enamel exposure. After each treatment step, each enamel block was rinsed with water and a color measurement was obtained. A total treatment time of 61 min was used with durations of exposure of 1 min, 5 min, 10 min, 15 min and 30 min. The concentration of peracetic acid in solution after the 1 min, 10 min and 30 min treatments was evaluated by colorimetric detection of ABTS oxidation. The results are provided in Table 16 and 17.

Table 16. Bleaching Efficacy of Perhydrolase System in a 1-step application on Stained Bovine Enamel

	Whiteness Index						
Sample	**0min**	**1min**	**6min**	**16min**	**31min**	**61min**	**ΔWI**
Buffer	-136.9	-134.9	-130.9	-130.4	-125.5	-121.2	15.6
H_2O_2	-149.3	-146.3	-142.5	-134.7	-125.2	-103.1	46.2
Triacetin/ H_2O_2	-144.1	-137.4	-138.2	-125.3	-111.0	-80.9	63.2
1% PAA	-145.7	-119.6	-73.4	-62.9	-59.0	-59.4	86.3
EZ-1/Triacetin/ H_2O_2	-141.2	-115.6	-54.0	-37.3	-32.4	-27.9	113.3

Determination of the peracetic acid (PAA) concentration in the reaction mixtures by a colorimetric method was performed according to the method described by Dinu *et. al.* (*supra*). A reagent solution of 1 mM 2, 2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonate (ABTS), 50 µM potassium iodide in 125 mM potassium citrate buffer at pH 5.0 was prepared. 25 µL of sample was mixed with 975 µL of this detection reagent and allowed to incubate for 5 min. The solution was analyzed for absorbance at 405 nm using a Microplate reader. A comparison of relative amounts of peracetic acid in solution was determined by comparing absorbance values directly.

Table 17. Assessment of peracetic acid concentration in solution at end of treatment time as indicated. Each sample is a 1:100 dilution into ABTS detection reagent measured at 405 nm.

	Absorbance 405nm		
Sample	**1min**	**10min**	**30min**
Buffer	0.055	0.059	0.055
H_2O_2	0.056	0.060	0.054
Triacetin/ H_2O_2	0.061	0.069	0.052
1% PAA	2.579	1.863	2.874
EZ-1/Triacetin/ H_2O_2	1.245	1.330	0.735

Data in Table 16 confirms that the enzyme-ester-peroxide composition is effective at whitening teeth. Data in Table 17 demonstrates that peracetic acid is produced from the combination of the EZ-1 (C277S; SEQ ID NO: 424) enzyme, triacetin and H_2O_2. Comparison of the absorbance data for the chemical peracetic acid also shows that surprisingly the enzymatic bleaching system shows better whitening performance with lower detectable peracetic acid. A low level of peracetic acid is also produced over time for the non-enzyme containing triacetin and H_2O_2 which was detected with a 1:10 dilution into the ABTS reagent (not included in Table). This results in detectable bleaching performance but at a much slower rate compared to the enzyme catalyzed production of a high level of peracetic acid.

EXAMPLE 13

TOOTH BLEACHING EFFICACY USING ENAMEL TARGETED PERHYDROLYTIC ENZYME IN A ONE-STEP APPLICATION

The purpose of this example is to show the tooth bleaching effect of enzymatic generated peracetic acid in a one-step application using a targeted CE-7 perhydrolase and compare to the performance achieved with chemically derived peracetic acid.

Bovine enamel incisors were prepared as indicated in Example 1. Stained bovine enamel blocks were hydrated in water at least 1 h prior to use to stabilize the color of the substrate. The color for each enamel block was measured after hydration prior to the start of the experiment. Two enamel samples were treated for each solution type. The solutions used were a buffer only control, 0.1% Peracetic Acid, a perhydrolase composition including 0.52 µM EZ-7 (C277S with enamel binding domain; SEQ ID NO: 429), 100 mM triacetin and 32.6 mM H_2O_2. All solutions were prepared in 100 mM sodium phosphate buffer, pH 7.2. All solutions were freshly prepared for each treatment point. The enzyme-triacetin-H_2O_2 combination was mixed immediately before enamel exposure. After each treatment step, each enamel block was rinsed with water and a color measurement was obtained. A treatment time of 30 min was repeated 4 times for each set of enamel substrates. The concentration of peracetic acid in solution after a 30 min treatment was evaluated by colorimetric detection of ABTS oxidation. The results are provided in Table 18 and 19.

Table 18. Bleaching Efficacy of a Targeted Perhydrolase in a 1-step application on Stained Bovine Enamel. Data is averaged from two substrates.

	Whiteness Index					
Sample	**0min**	**30min**	**60min**	**90min**	**120min**	**ΔWI**
Buffer	-133.5	-128.7	-123.4	-123.0	-127.8	5.8
0.1% PAA	-125.8	-101.9	-91.9	-80.2	-70.8	55.0

Table 19. Assessment of peracetic acid concentration in solution at end of treatment time as indicated. Each sample is a 1:10 dilution into ABTS detection reagent measured at 405 nm.

	Absorbance 405nm		[PAA] (ppm)
Sample	**Tooth1**	**Tooth2**	
Buffer	0.073	0.070	0
0.1% PAA	1.103	1.043	920
EZ-7/Triacetin/ H_2O_2	1.575	1.533	1350

Data in Table 18 confirms that the targeted enzyme-ester-peroxide composition is effective at whitening teeth in a 1-step process. Data in Table 19 demonstrates that peracetic acid is produced from the combination of the EZ-7 enzyme, triacetin and H_2O_2.

EXAMPLE 14

PERACETIC ACID PRODUCTION USING CE-7 PERHYDROLASES AGAINST A VARIETY OF ESTER SUBSTRATES

The purpose of this example was to demonstrate that a broad variety of CE-7 perhydrolases—including an enamel targeting perhydrolase—catalyze the formation of peracetic acid from a broad variety of esters under oral care specific conditions.

The variants of the *Thermotoga maritima* perhydrolase were cloned, recombinantly expressed and purified in a manner similar to that described in Example 4. The sequence changes in these perhydrolase variants are listed in Table 5.

Various ester substrates were tested against at least two or more of the perhydrolase variants listed in Table 5. With the exception of three esters that were custom synthesized, all other esters were procured from the Sigma-Aldrich

(St. Louis, MO), TCI America (Portland, OR), Alpha Aesar (Ward Hill, MA) or Tessendlo Company (Phoenix, AZ).

To test various enzymes against an ester substrate, it was convenient to run up to 4 reactions simultaneously by staggering the beginning of each individual reaction by 0.5 – 1 min. Each individual reaction was performed in a glass vessel (height: 76 mm; o.d.: 33 mm; i.d.: 24 mm) equipped with a magnetic stirring bar. Four reaction vessels were banded together and kept at constant temperature in a jacketed one-liter, stainless steel, tempering beaker (KGW IsoTherm, # TSS-G 1000W) with circulating water controlled by a Thermo NesLab recirculation bath (Model # RTE-7 Digital One). Buffer (6 mL, 100 mM sodium phosphate buffer, pH 7.2) was added to each reaction vessel and allowed to equilibrate to 37 °C. The ester substrate of interest was added to achieve 100 mM concentration. The reaction was initiated by the simultaneous addition of 40 ppm enzyme and 60 mM H_2O_2 (37 μL, 30% hydrogen peroxide solution). To follow the production of peracetic acid, 80 μL samples from each reaction were removed at specific intervals from 1 to 15 minutes. The sample was immediately quenched in a microfuge filter tube (NanoSep 30K VWR cat # 82031-354) containing a volume and concentration of phosphoric acid solution that was sufficient to stop the enzymatic reaction (by lowering the pH between 2 and 3) and to dilute the sample for convenient HPLC analyses. The acid-quenched samples were centrifuged for 5 minutes to remove any particulates. Once filtered, a Karst reaction and HPLC analyses for peracetic acid were immediately performed on each sample set using the method described previously in U.S. Patent 7,829,315 to DiCosimo *et al*. The maximum peracetic acid produced by each ester substrate is listed in Table 20.

Table 20. Maximum Peracetic Acid Produced from Neutral Conditions.[1]

	Peracetic Acid (PAA) (ppm)				
Ester Substrate (CAS#)	**No Enzyme**	**C277S** (SEQ ID NO: 424)	**C277T** (SEQ ID NO: 437)	**C277T/R296P** (SEQ ID NO: 476)	**Wild Type** (SEQ ID NO: 16)
1-thio-β-D glucose-2,3,4,6-tetraacetate (19879-84-6)	10	0	171	147	156
1,5-pentanediol diacetate (542-59-6)	11	335	484	364	435
Diethylene glycol diacetate (628-68-2)	17	469	653	530	424
Sorbitol hexaacetate (7208-47-1)	11	712	705	616	381
Sucrose octaacetate (126-14-7)	20	215	493	501	304
4-acetoxybenzoic acid (2345-34-8)	299	604	426	485	398
Vanillin acetate (881-68-5)	251	660	500	405	564
Propylene glycol methyl	22	173	197	181	177

ether acetate (108-65-5)					
2-acetamido-2-deoxy-3, 4, 6 triacetyl-1-chloride-α-D-glucopyranose (3068-34-6)	78	863	899	876	548
5-acetoxymethyl-2-furaldehyde (10551-58-3)	67	1185	1125	1136	778
Ethylene glycol diacetate (111-55-7)	51	1007	1059	1020	794
Propylene glycol diacetate (623-84-7)	17	1128	1245	1202	780
Diacetin (25395-31-7)	20	1013	1132	1087	1160
α-D-glucose pentaacetate (604-68-2)	406	2091	2542	1678	1188
β-D-glucose pentaacetate (604-69-3)	1124	2178	2531	1716	1158
1, 2, 3, 5-tetra-O-acetyl-ribofuranose (13035-61-5)	2246	3397	3472	3270	2828

1,2,3,4-tetra-O-acetyl-ribopyranose (4049-34-7)	2264	3525	3361	3503	2278
Tri-O-acetyl glucal (2873-29-2)	62	1047	1319	1034	729
Triacetin (102-76-1)	51	1977	2311	2291	937
β-D-galactose pentaacetate (4163-60-4)	176	2539	2546	1947	764
2-acetamido-2-deoxy-1,3,4,6-tetraacetyl-β-D-glucopyranose (7772-79-4)	2574	3374	3808	3790	2687
β-D-xylofuranose tetraacetate (CV Chem)[a]	1373	3354	3481	3158	2384
3,4-diacetoxy-1-butene (18085-02-4)	53	2281	2285	2478	1692
β-D-glucopyranose,1,2,3,4-tetraacetate	2102	2309	2225	2235	2129

(13100-46-4)					
2,3,4,6-tetraacetyl-β-D-glucopyranose (10343-06-3)[b]	1500	2820	3072	N/A	N/A
β-methyl xyloside triacetin (18531-01-6)[b]	412	3856	3635	N/A	N/A
1,3,4,6-tetra-O-acetyl-mannopyranose (18968-05-3)	1803	3593	3436	N/A	2361
α-D-mannopyranose pentaacetate (4163-65-9)	3659	4017	4212	3752	4023

TABLE NOTES: [1] [ester], 100 mM; [H_2O_2], 60 mM; [perhydrolase enzyme], 40 ppm with 95 mM phosphate, pH (7.2); [a]custom synthesis by CV-Chem (CiVenti Chem, Product #CV-3146; reference Number 121-RM-134); [b]Prepared following literature synthesis Robertson *et al.*, (1934)*J. Chem. Soc.*, 824-9. N/A = not tested.

These ester substrates were tested for peracetic acid production using a targeted C277S perhydrolase variant, EZ-7 under the same reaction conditions used to generate date for Table 20. Peracetic acid production using propylene glycol diacetate or sucrose octaacetate with the EZ-7 perhydrolase system is shown in Table 21. The data shown demonstrates that the alternative esters are effective substrates for targeted variants of perhydrolase as described in Example 5.

Table 21. Maximum peracetic acid produced by alternative substrates using targeted and untargeted CE-7 perhydrolase.

	Peracetic Acid (ppm)	
Enzyme ID	**Propylene glycol diacetate**	**Sucrose octaacetate**
EZ-1	2165	2768
EZ-7	2109	2070

EXAMPLE 15

TOOTH BLEACHING USING CE-7 PERHYDROLASES AGAINST A VARIETY OF ESTER SUBSTRATES

The purpose of this example was to demonstrate tooth bleaching efficacy using a CE-7 perhydrolase that catalyzes the formation of peracetic acid from four esters under oral care relevant conditions.

For this example the C277S (EZ-1) variant of *Thermotoga maritima* perhydrolase was cloned, recombinantly expressed and purified as in Example 4. All of the substrates were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of triacetin (Tessendlo Company (Phoenix, AZ)).

The substrates triacetin (TA), α-D-glucose pentaacetate (GPA), sucrose octaacetate (SOC) and propylene glycol diacetate (PGDA) were used to demonstrate enzymatic mediated tooth whitening with EZ-1 (C277S; SEQ ID NO: 424). The optimal conditions used for the generation of peracetic acid with each substrate were derived from the studies in Example 14 and are listed below as follows:

40 ppm EZ-1, 100 mM Substrate, X mM H_2O_2, 95 mM phosphate, pH 7.2, where X varies depending on the identity of the substrate; 100 mM H_2O_2 (TA), 360 mM H_2O_2 (PGDA and SOC), 60 mM H_2O_2 (GPA).

Bovine enamel incisors were prepared as indicated in Example 1. The stained enamel blocks were placed in a 24 well plate with the dentin side facing down and hydrated overnight in phosphate buffer, pH 7.2. Prior to treatment, a color measurement of each tooth was performed using a Konica-Minolta 2600d spectrophotometer and whiteness index was calculated as specified in Example 1.

Each enzyme solution was prepared in a 1.5-mL microfuge tube (Eppendorf, #2243102-1) and 1 mL of the solution was immediately transferred to the 24 well plate containing the pre-hydrated teeth. The control samples consisted of 100 mM phosphate buffer, pH 7.2 and 9 % H_2O_2 in 100 mM citrate/phosphate buffer, pH 5.3. The teeth were incubated at room temperature for 30 minutes, removed, rinsed with 100 mM phosphate, pH 7.2 and a color measurement was taken. The teeth were placed back into the 24 well plate at which time fresh enzyme solutions were prepared and added to each well. This process was repeated for a total of 3 whitening treatments; 30 min per treatment (Table 22).

Table 22. Color Measurements of Coffee-Tea Stained Bovine Enamel Exposed to 40 ppm EZ-1, 100 mM Substrate, Varying H_2O_2[1], 95 mM Phosphate, pH 7.2.

Sample	**Whiteness Index**				**ΔWI**
	Treatment 0	**Treatment 1**	**Treatment 2**	**Treatment 3**	
Buffer[a]	-190.4	-183.3	-181.1	-179.0	11.4
9% H_2O_2[a]	-167.6	-129.6	-117.6	-115.2	52.4
TA	-189.9	-108.7	-86.7	-86.1	103.8
PGDA	-181.4	-108.0	-80.9	-80.1	101.3
SOC	-172.8	-135.2	-135.9	-128.8	44.0
GPA	-225.2	-128.7	-105.3	-95.9	129.3

Table notes: [1]H_2O_2 concentrations vary depending on substrate and are as follows: 100 mM H_2O_2 (TA), 360 mM H_2O_2 (PGDA and SOC), 60 mM H_2O_2 (GPA). [a]Control samples contained no enzyme.

As observed in Table 22, all of the enzymatically generated peracetic acid samples using TA, PGDA, SOC and GPA show a significant change in whiteness index as indicated by a shift to more positive values with each successive treatment. The change in whiteness index for the buffer control is marginal with a ΔWI of 11.4. These measurements also coincide with visual inspection of the teeth after each 30 minute treatment. This data demonstrates that enzymatically generated peracetic acid, using a variety of different substrates with EZ-1, is effective at whitening coffee-tea stained bovine teeth.

EXAMPLE 16

TOOTH BLEACHING EFFICACY USING PERHYDROLYTIC ENZYMES IN A TWO-STEP APPLICATION

The purpose of this example was to demonstrate the tooth bleaching efficacy of perhydrolases systems in a two-step application.

Bovine enamel incisors were prepared as indicated in Example 1. The stained enamel blocks were embedded in a 48 well plate to protect the dentin backside from solution exposure. A solution of 10 μM of each enzyme in 10 mM phosphate buffer, pH 7.2 was prepared and 500 μL of solution was incubated with each enamel substrate for 60 min. The enzyme solution was removed and each well was rinsed three times with an additional 500 μL of buffer. The enamel blocks were placed in a fresh well for the whitening process. A solution with a final concentration of 40 mM triacetin, 100 mM H_2O_2 in 50 mM phosphate pH 7.2 buffer was freshly prepared and 500 μL of solution was added to each enamel block. Over the course of 1 hr the enamel blocks were removed and a color measurement was obtained and then each was returned to the solution in the well plate. The whiteness index for each sample was monitored. The results are listed in Table 23.

Table 23. Color Measurements of Coffee-Tea Stained Bovine Enamel Exposed to a Variety of Perhydrolases and Triacetin/ H_2O_2 in 2 steps.

Enzyme	**Whiteness Index**						**ΔWI**
	0 min	**5 min**	**15 min**	**30 min**	**45 min**	**60 min**	
No	-108.2	-102.7	-104.0	-107.3	-98.3	-96.7	11.4

enzyme							
EZ-1	-152.7	-126.2	-138.9	-144.8	-107.3	-96.1	56.6
EZ-2	-154.5	-134.6	-97.3	-57.2	-35.8	-25.3	129.1
EZ-3	-159.0	-147.4	-161.5	-117.3	-104.7	-83.8	75.2
EZ-4	-158.9	-143.6	-149.5	-129.4	-106.5	-89.5	69.4
EZ-5	-135.8	-129.8	-97.4	-65.1	-49.9	-42.7	93.2

This example demonstrates that a surface bound perhydrolase can be used to whiten teeth by catalyzing the formation of peracetic acid at the surface of the enamel. The bleaching performance in this example correlates to the observed retention of each enzyme on hydroxyapatite. The untargeted EZ-1 (C277S; SEQ ID NO: 424) shows poor retention on hydroxyapatite and enamel and therefore low potential to achieve suitable whitening in a 2-step process. The addition of an effective targeting sequence to retain the enzyme on the enamel enables the production of peracetic acid in a 2-step application process.

EXAMPLE 17

TOOTH BLEACHING EFFICACY USING PERHYDROLYTIC ENZYMES IN A TWO-STEP APPLICATION

The purpose of this example was to demonstrate the tooth bleaching efficacy of targeted and untargeted perhydrolases systems in a two-step application.

Bovine enamel incisors were prepared as indicated in Example 1. The stained enamel blocks were embedded in SILLY PUTTY® (Crayola LLC, Easton, PA) filled well, a 24-well plate to protect the dentin backside from solution exposure. A solution of 20 µM of each enzyme in 10 mM phosphate buffer, pH 7.2 was prepared and 500 µL of the enzyme solution was added to each enamel blocks and incubated for 10 min at 37ºC. The enamel block was then rinsed three times with an additional 500 µL of buffer each time. The enamel blocks were transferred and embedded in a fresh putty well for the whitening process. A solution (200 µL) containing 100 mM phosphate buffer,

100 mM triacetin, 100 mM H_2O_2, pH 7.2 was added to the enamel and incubated for 10 min at 37°C. The enamel block was removed from the well using forceps, then rinsed and stored in a 1.5 mL of water filled well for color measurements. The 2-step process was repeated 5 times (50 min total). The results of color measurements of coffee-tea stained bovine enamel after the 2-step process were shown in Table 24. Each sample data point represents 2 repeats of independent enamel blocks.

For measuring the level of peracetic acid generated in the system, the ABTS method was used, 90 μL of the reaction mixtures was removed to a new well containing 10 μL stopping buffer (1.33 M H_3PO_4). The samples were diluted 1:100 with 100 mM phosphate buffer and added to ABTS detection reagent as noted in Example 8. Results are listed in Table 25. Each sample data point represents 2 repeats of independent enamel blocks.

Table 24. Color Measurements of Coffee-Tea Stained Bovine Enamel Exposed to a Variety of Perhydrolases and Triacetin/ H_2O_2 in 2 steps

Step 1	Step 2	Whiteness Index						
		Before	Rd1	Rd2	Rd3	Rd4	Rd5	ΔWI
Buffer	Buffer	-122.9	-117.9	-116.3	-126.7	-118.4	-128.6	-5.7
Buffer	Triacetin H_2O_2	-130.9	-150.4	-136.9	-134.4	-145.4	-141.9	-11.1
EZ1	Triacetin H_2O_2	-140.0	-129.5	-129.5	-129.2	-125.1	-122.1	17.9
EZ7	Triacetin H_2O_2	-120.8	-106.7	-96.0	-88.7	-85.7	-76.6	44.2

Table 25. Level of peracetic acid generation after each round of whitening process.

Step 1	Step 2	PAA (ppm) generated after each round of whitening				
		Rd1	Rd2	Rd3	Rd4	Rd5
Buffer	Buffer	1.2	0.5	8.7	1.3	2.0
Buffer	Triacetin/ H_2O_2	31.0	25.8	16.0	45.5	35.9
EZ1	Triacetin/ H_2O_2	33.2	41.0	30.2	39.3	47.1
EZ7	Triacetin/ H_2O_2	383.5	496.7	716.5	998.5	1224.5

This example demonstrates that a surface bound perhydrolase can be used to whiten teeth by catalyzing the formation of peracetic acid at the surface of the enamel. The bleaching performance in this example using bovine enamel correlates to the observed retention of each enzyme on hydroxyapatite. The untargeted EZ-1 (C277S) shows poor retention on hydroxyapatite and enamel and therefore low potential to achieve suitable whitening in a 2-step process. The addition of an effective targeting sequence to retain the enzyme on the enamel enables the production of peracetic acid in a 2-step application process.

CLAIMS

What is claimed is:

Claim 1. A method comprising:

1) providing a set of reaction components comprising:

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

i) esters having the structure

$$[X]_mR_5$$

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

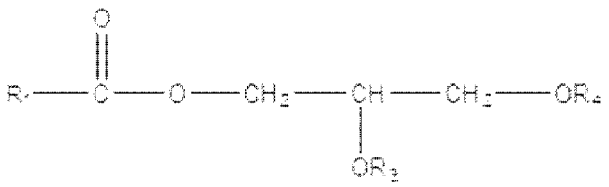
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

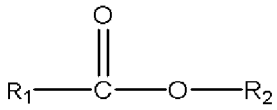
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



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

iii) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

b) a source of peroxygen; and

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

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

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

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

2) combining the reaction components of (1) under suitable reaction condition whereby at least one peracid is enzymatically produced; and

3) contacting an oral cavity surface with the at least one peracid whereby the oral cavity surface receives a peracid-based benefit selected from

the group consisting of bleaching, teeth whitening, disinfecting, destaining, deodorizing, decreasing or removing biofilm, and combinations thereof.

Claim 2. The method of claim 1 wherein the reaction components are combined in the oral cavity.

Claim 3. The method of claim 1 wherein the reaction components are combined outside of the oral cavity prior to contacting the oral cavity surface.

Claim 4. The method of claim 1 wherein the enzyme having perhydrolytic activity is present in the oral cavity prior to producing the peracid benefit agent.

Claim 5. A method comprising

1) providing a set of reaction components comprising:

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

i) esters having the structure

$[X]_mR_5$

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

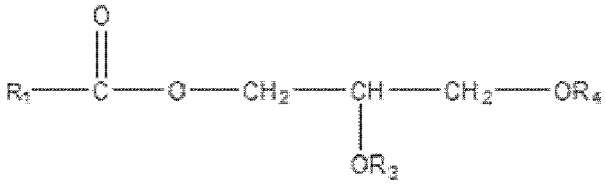
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

m is an integer ranging from 1 to the number of carbon atoms in R_5; and

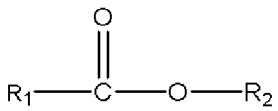
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

ii) glycerides having the structure



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

iii) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

b) a source of peroxygen; and

c) an enzyme catalyst having perhydrolytic activity, wherein said enzyme catalyst comprises a fusion protein having the following general structure:

$PAH-[L]_y-OCBD$

or

$OCBD-[L]_y-PAH$

wherein

PAH is the enzyme having perhydrolytic activity;
OCBD is a peptidic component having affinity for the oral cavity surface;
L is an optional peptide linker ranging from 1 to 100 amino acids in length; and
y is 0 or 1;

2) combining the reaction components of (1) under suitable reaction condition whereby at least one peracid is enzymatically produced; and
3) contacting an oral cavity surface with the at least one peracid whereby the oral cavity surface receives a peracid-based benefit selected from the group consisting of bleaching, teeth whitening, disinfecting, destaining, deodorizing, decreasing or removing biofilm, and combinations thereof.

Claim 6. The method of claim 5 wherein the enzymes having perhydrolytic activity are selected from the group of lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations thereof.

Claim 7. The method of claim 6 wherein the carbohydrate esterases are CE-7 carbohydrate esterases having a CE-7 signature motif that aligns with a

reference sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

1) an RGQ motif at positions corresponding to positions 118-120 of SEQ ID NO:2;

2) a GXSQG motif at positions corresponding to positions 179-183 of SEQ ID NO:2; and

3) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2.

Claim 8. The method of claim 5, claim 6 or claim 7 wherein the peptidic component having affinity for an oral cavity surface is an antibody, an F_{ab} antibody fragment, a single chain variable fragment (scFv) antibody, a *Camelidae* antibody, a scaffold display protein or a single chain polypeptide lacking an immunoglobulin fold.

Claim 9. The method of claim 6 wherein the peptidic component having affinity for an oral cavity surface is a single chain polypeptide lacking an immunoglobulin fold.

Claim 10. The method of claim 9 wherein the single chain polypeptide comprises at least one oral cavity surface-binding peptide ranging from 5 to 60 amino acids in length and having a K_D value or an MB_{50} value of 10^{-5} M or less for the oral cavity surface.

Claim 11. The method of claim 10 wherein the single chain polypeptide comprises 2 to 50 oral cavity surface-binding peptides wherein the oral cavity surface-binding peptides are independently and optionally separated by a polypeptide spacer ranging from 1 to 100 amino acids in length.

Claim 12. The method of claim 1 or claim 5 wherein the oral cavity surface is a tooth surface.

Claim 13. The method of claim 12 wherein the tooth surface comprises tooth enamel, tooth pellicle or a combination thereof.

Claim 14. The method of claim 10 wherein the oral cavity surface-binding peptide is a tooth enamel-binding peptide.

Claim 15. The method of claim 10 wherein the oral cavity surface-binding peptide is a tooth pellicle-binding peptide.

Claim 16. The method of claim 10 wherein the oral cavity surface-binding peptide is a skin-binding peptide.

Claim 17. The method of claim 1 or claim 5 where the peracid is produced at a concentration of 500 ppb to 10,000 ppm within 5 minutes of combining the set of reaction components.

Claim 18. The method of claim 17 wherein the peracid is contacted with the oral care surface for less than 1 hour.

Claim 19. The method of claim 1 or claim 5 wherein the peracid is peracetic acid.

Claim 20. The method of claim 19 wherein an efficacious concentration of peracetic acid is enzymatically produced and contacted with the oral cavity surface within 5 minutes of combining the reaction components.

Claim 21. The method of claim 1 or claim 5 wherein the substrate comprises triacetin.

Claim 22. A fusion protein comprising the following general structure:

$$PAH\text{-}[L]_y\text{-}OCBD$$

or

OCBD-[L]$_y$-PAH

wherein

1) PAH is an enzyme having perhydrolytic activity;

2) OCBD is a peptidic component having affinity for an oral cavity surface;

3) L is a peptide linker ranging from 1 to 100 amino acids in length; and

4) y is 0 or 1.

Claim 23. The fusion protein of claim 22 wherein the enzyme having perhydrolytic activity is selected from the group lipases, proteases, esterases, acyl transferases, aryl esterases, carbohydrate esterases, and combinations thereof.

Claim 24. The fusion protein of claim 23 wherein the enzyme is a CE-7 carbohydrate esterase having perhydrolytic activity; the PAH having a CE-7 signature motif that aligns with a reference sequence SEQ ID NO: 2 using CLUSTALW, said signature motif comprising:

1) an RGQ motif at positions corresponding to positions 118-120 of SEQ ID NO:2;

2) a GXSQG motif at positions corresponding to positions 179-183 of SEQ ID NO:2; and

3) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2.

Claim 25. The fusion protein of claim 23 wherein the enzyme having perhydrolytic activity is an aryl esterase from *Mycobacterium smegmatis*.

Claim 26. The fusion protein of claim 23, claim 24 or claim 25 wherein the peptidic component having affinity for an oral cavity surface is a single chain polypeptide lacking an immunoglobulin fold.

Claim 27. The fusion protein of claim 26 wherein the single chain polypeptide comprises at least one oral cavity surface-binding peptide ranging from 5 to 60 amino acids in length.

Claim 28. The fusion protein of claim 27 wherein said at least one oral cavity surface-binding peptide has a K_D value or an MB_{50} value of 10^{-5} M or less.

Claim 29. The fusion protein of claim 26 wherein the single chain polypeptide comprises 2 to 50 oral cavity surface-binding peptides, wherein the oral cavity surface-binding peptides are independently and optionally separated by a polypeptide spacer ranging from 1 to 100 amino acids in length.

Claim 30. The fusion protein of any of one of claims 22, 23, 24, and 25 wherein the peptidic component having affinity for an oral cavity surface comprises a length of no more than 200 amino acids.

Claim 31. An oral care product comprising:

1) an enzyme catalyst comprising the fusion protein of claim 23;

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

a) esters having the structure

$$[X]_mR_5$$

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

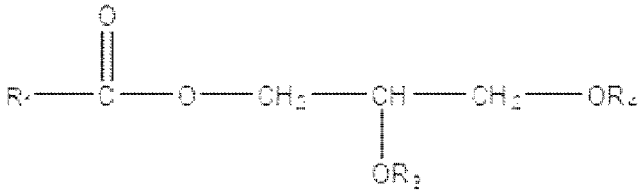
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

M is an integer ranging from 1 to the number of carbon atoms in R_5; and

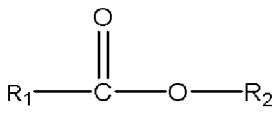
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

b) glycerides having the structure



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

c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

3) a source of peroxygen; and

4) an orally acceptable carrier medium.

Claim 32. An oral care product comprising:

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

a) an RGQ motif at positions corresponding to positions 118-120 of SEQ ID NO:2;

b) a GXSQG motif at positions corresponding to positions 179-183 of SEQ ID NO:2; and

c) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2;

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

a) esters having the structure

$$[X]_mR_5$$

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

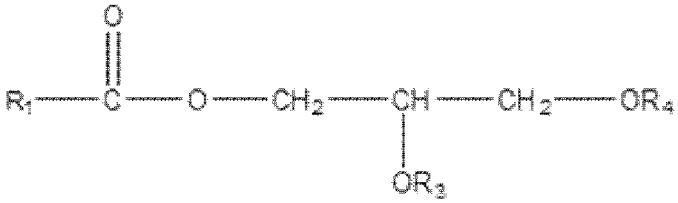
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

M is an integer ranging from 1 to the number of carbon atoms in R_5; and

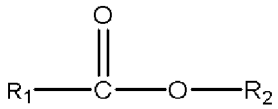
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

b) glycerides having the structure



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

c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

3) a source of peroxygen; and

4) an orally acceptable carrier medium.

Claim 33. The oral care product of claim 32 wherein the enzyme having perhydrolytic activity is the fusion protein of claim 22.

Claim 34. The oral care product of claim 33 wherein the second portion is a single chain peptide comprising at least one tooth enamel-binding or tooth pellicle-binding peptide.

Claim 35. The oral care product of claim 34 wherein the at least one tooth enamel-binding or tooth pellicle-binding peptides range from 5 to 60 amino acids in length.

Claim 36. The oral care product of claim 31, claim 32, claim 33, claim 34 or claim 35 wherein the oral care product is in the form of a powder, paste, gel, liquid, ointment, tablet, rinse or any combination thereof.

Claim 37. The oral care product of claim 36 wherein the oral care product is a toothpaste, a dental cream, a tooth gel, a tooth powder, a mouth wash, a breath freshener, a strip or a dental floss.

Claim 38. The oral care product of claim 36 wherein the oral care product is in the form of a whitening strip or dental tray.

Claim 39. The oral care product of claim 31 or claim 32 wherein the enzyme catalyst remains separated from the substrate, the source of peroxygen or both the substrate and the source of peroxygen prior to use of the oral care product.

Claim 40. An isolated polypeptide having affinity for an oral cavity surface having an amino acid sequence selected from the group consisting of SEQ ID NOs 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, and 422.

Claim 41. Use of a CE-7 carbohydrate esterase having perhydrolytic activity in an oral care product to produce an efficacious concentration of at least one

peracid to bleach, whiten, disinfect, destain, deodorize or remove biofilm from an oral cavity surface.

Claim 42. Use of a peracid generation composition comprising:

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

a) an RGQ motif at positions corresponding to positions 118-120 of SEQ ID NO:2;

b) a GXSQG motif at positions corresponding to positions 179-183 of SEQ ID NO:2; and

c) an HE motif at positions corresponding to positions 298-299 of SEQ ID NO:2; and

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

a) esters having the structure

$$[X]_mR_5$$

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

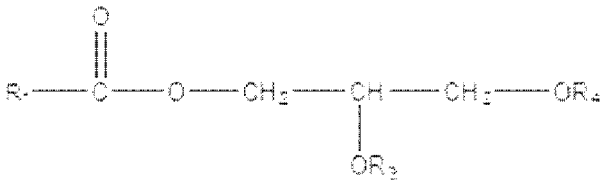
R_6 = C1 to C7 linear, branched or cyclic hydrocarbyl moiety, optionally substituted with hydroxyl groups or C1 to C4 alkoxy groups, wherein R_6 optionally comprises one or more ether linkages for R_6 = C2 to C7;

R_5 = a C1 to C6 linear, branched, or cyclic hydrocarbyl moiety or a five-membered cyclic heteroaromatic moiety or six-membered cyclic aromatic or heteroaromatic 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 or carboxylic acid group; wherein R_5 optionally comprises one or more ether linkages;

M is an integer ranging from 1 to the number of carbon atoms in R_5; and

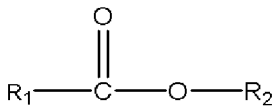
wherein said esters have solubility in water of at least 5 ppm at 25 °C;

b) glycerides having the structure



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

c) one or more esters of the formula



wherein R_1 is 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 is a C1 to C10 straight chain or branched chain alkyl, alkenyl, alkynyl, aryl, alkylaryl, alkylheteroaryl, heteroaryl, $(CH_2CH_2O)_n$, or $(CH_2CH(CH_3)\text{-}O)_nH$ and n is 1 to 10; and

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

3) a source of peroxygen;

whereby a peracid formed upon mixing (1), (2), and (3);

for the treatment or prevention of dental caries, gingivitis, oral candidiasis, or periodontitis.

Claim 43. The use of claim 41 wherein the enzyme having perhydrolytic activity is in the form of a fusion protein comprising at least one portion having affinity for an oral cavity surface.

Claim 44. Use of fusion protein comprising the following general structure in an oral cavity product:

$PAH-[L]_y-OCBD$

or

$OCBD-[L]_y-PAH$

wherein

1) PAH is an enzyme having perhydrolytic activity having an amino acid sequence with at least 95% amino acid identity to SEQ ID NO: 460;

2) OCBD is a peptidic component having affinity for an oral cavity surface;

3) L is a peptide linker ranging from 1 to 100 amino acids in length; and

4) y is 0 or 1.