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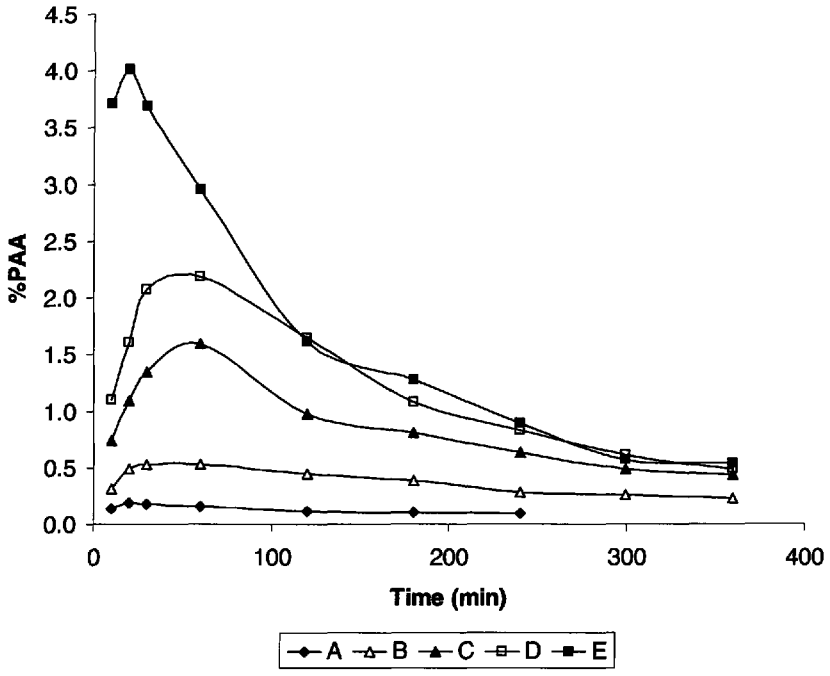
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(54) Title: MOLD TREATMENT

FIGURE 3



(57) Abstract: Provided herein is a method for treating a surface contaminated by mold, the method comprising the steps of: mixing a perhydrolase, an ester substrate, a source of hydrogen peroxide and water in suitable amounts to produce a mixture comprising at least 1% by weight peracetic acid; and applying the mixture to a surface contaminated by mold.

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MOLD TREATMENT

PRIORITY

The present application claims priority to U.S. Provisional Application Serial No. 61/296,675, filed on January 20, 2010, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to the field of mold remediation, for instance to the treatment of surfaces contaminated by mold. The invention is particularly applicable to the cleaning of surfaces in order to remove visible mold deposits, such as mold growing on or within building structures.

BACKGROUND OF THE INVENTION

Molds are a type of filamentous fungi which are commonly found on surfaces where moisture is present. Molds grow best in warm, damp, and humid conditions, and spread and reproduce by producing spores. Mold spores are small and lightweight, able to travel through air, and can survive harsh environmental conditions that do not support normal mold growth.

Mold growth requires a source of water, but also suitable substrate for growth. In particular, molds require a substrate capable of providing a source of nutrients. Many common building and decorating materials are capable of sustaining mold growth, provided that water is present. For instance, wood, concrete, bricks, wallpaper and carpets are typically capable of sustaining mold growth. Even where the substrate material itself does not directly provide a food source, dust and other organic matter deposited on or within the substrate can sustain mold growth. Porous or semi-porous materials can be particularly suitable for mold growth, in that they may provide a micro-environment which retains water and provides such a food source.

Thus mold growth can be a serious problem in damp or humid interior environments, for instance due to inadequate ventilation, plumbing problems or leaking walls or

roofs. Since mold spores are ubiquitious, molds can quickly colonise walls, ceilings and floors of domestic and commercial buildings which are affected by a moisture problem.

Mold growth can be unsightly and may damage building structures and particularly interior decoration. Widespread mold contamination in an internal environment is also commonly associated with a characteristic and undesirable odour. Moreover, molds may be associated with allergies and respiratory infections, and some molds produce toxins which are dangerous to humans. Accordingly, for economic and health reasons it is important to adequately remove mold growth from affected surfaces in buildings.

Various mold treatment products are available commercially. Typically these are based on agents such as sodium hypochlorite (*i.e.*, conventional "bleach"), alcohol, quaternary ammonium products, phenol, iodophors, gluteraldehyde or hydrogen peroxide, which can be effective at cleaning and killing fungal spores due to their high oxidation potential. Although many such products are suitable for use on some hard, non-porous surfaces, they may be much less effective on more porous materials such as wood, due to a lack of penetration into the material. A further problem is that even where the product is capable of effectively killing mold and mold spores, a dark stain may remain where the mold colony was previously present. Such stains can be very difficult to remove using existing products, which means that the visual impact of the product is unsatisfactory. This is particularly the case when the product is used to treat common building materials or surfaces, which are typically porous or semi-porous, since discoloration due to mold growth within the material is inadequately ameliorated.

Another problem with many known mold treatment products is that the use of caustic and hazardous ingredients makes them dangerous to handle and potentially corrosive when used on certain materials. In some cases these products can themselves leaves marks and stains which are as unsightly as the mold growth they are intended to treat.

Peracetic acid (PAA) is another compound which has been suggested as a general cleaning and decontamination/disinfection agent due to its high oxidizing potential. However, there are a number of limitations and dangers associated with traditional

means of shipping and storing PAA. In most cases, PAA is packaged at relatively high concentrations (*e.g.*, 5-35%) in an equilibrium solution to prevent the PAA from decomposing. At this high concentration, storage and transportation of PAA is problematic since it is particularly corrosive, explosive, dangerous and reactive.

Accordingly, enzymatic systems for generating peracetic acid have been proposed. An advantage of such enzymatic systems is that peracetic acid is only generated by enzymatic catalysis immediately prior to use. The product can be kept and transported in an inactive form which is much less hazardous, and without loss of potential activity over a prolonged storage time.

WO 2007/133263 and WO 2008/140988 disclose methods and compositions for general decontamination by the enzymatic generation of peracetic acid. These methods are highly effective for the killing of certain types of pathogen, and may be applied to a variety of different items. However, neither of these documents discloses a method which is specifically adapted to mold remediation, and in particular to the removal of visible mold contamination on the types of surfaces where mold growth is commonly found.

Accordingly, there is still a need for a method for safe and effective mold remediation, which is capable of both destroying mold growth and removing mold stains from a surface.

SUMMARY

In one aspect the present invention provides a method for treating a surface contaminated by mold, the method comprising the steps of mixing a perhydrolase, an ester substrate, a source of hydrogen peroxide and water in suitable amounts to produce a mixture comprising at least 1% by weight peracetic acid; and applying the mixture to a surface contaminated by mold.

In another aspect, the present invention provides use of a mixture for treating a mold-contaminated surface, wherein the mixture comprises a perhydrolase, water and at least 1% by weight peracetic acid.

In one embodiment, the mixture comprises an amount of peracetic acid selected from the group consisting of: at least 1.5% by weight, at least 2% by weight, and at least 2.5% by weight.

In another embodiment, the mixture comprises an amount of peracetic acid selected from the group consisting of: 1 to 10% by weight, 1.5 to 5% by weight, and 2 to 4% by weight.

In one embodiment, the mixture is applied to the surface 30 to 120 minutes after mixing.

The surface may be, for example, a semi-porous or porous surface. Preferably the surface is an interior or exterior surface of a building. In particular embodiments, the surface comprises wood, concrete, cement, metal, tile, grout, vinyl, roofing material, wall-paper, glass fiber film, a painted surface or building bricks.

In one embodiment, the surface comprises a visible mold contamination. Application of the mixture preferably reduces visibility of the mold contamination. Thus the mixture is preferably used to bleach a visible mold contamination on the surface.

In one embodiment, the perhydrolase has a perhydrolase to hydrolase ratio greater than 1:1, preferably when measured by a method for the determination of perhydrolysis to hydrolysis ratio described herein.

In some embodiments, the ester substrate is selected from the group consisting of: propylene glycol diacetate, ethylene glycol diacetate and triacetin.

In one embodiment, the source of hydrogen peroxide is sodium percarbonate.

Preferably the perhydrolase is at least 70% identical to SEQ ID NO: 1.

In some embodiments, the perhydrolase comprises one or more substitutions at one or more amino acid positions equivalent to position(s) in the sequence of SEQ ID NO: 1 selected from the group consisting of: M1, K3, R4, I5, L6, C7, D10, S11, L12, T13, W14, W16, G15, V17, P18, V19, D21, G22, A23, P24, T25, E26, R27, F28, A29, P30, D31, V32, R33, W34, T35, G36, L38, Q40, Q41, D45, L42, G43, A44, F46, E47, V48, I49, E50, E51, G52, L53, S54, A55, R56, T57, T58, N59, I60, D61, D62,

P63, T64, D65, P66, R67, L68, N69, G70, A71, S72, Y73, S76, C77, L78, A79, T80, L82, P83, L84, D85, L86, V87, N94, D95, T96, K97, Y99, F100, R101, R102, P104, L105, D106, I107, A108, L109, G110, M111, S112, V113, L114, V115, T116, Q117, V118, L119, T120, S121, A122, G124, V125, G126, T127, T128, Y129, P146, P148, W149, F150, I153, F154, I194, and F196, preferably wherein the perhydrolase enzyme comprises an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

In further embodiments, the perhydrolase comprises at least one amino acid substitution at amino acid positions equivalent to amino acid positions in SEQ ID NO: 1 selected from the group consisting of: L12C, L12Q, or L12G; T25S, T25G, or T25P; L53H, L53Q, L53G, or L53S; S54V, S54L, S54A, S54P, S54T, or S54R; A55G or A55T; R67T, R67Q, R67N, R67G, R67E, R67L, or R67F; K97R; V125S, V125G, V125R, V125A, or V125P; F154Y; F196G; preferably wherein the perhydrolase enzyme comprises an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

In further embodiments, the perhydrolase enzyme comprises a combination of amino acid substitutions at amino acid positions equivalent to amino acid positions in SEQ ID NO:1 selected from the group consisting of: L12I S54V; L12M S54T; L12T S54V; L12Q T25S S54V; L53H S54V; S54P V125R; S54V V125G; S54V F196G; S54V K97R V125G; or A55G R67T K97R V125G; preferably wherein the perhydrolase enzyme comprises an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

For example, the perhydrolase may have a V at position 54 corresponding to the sequence of SEQ ID NO: 1.

In one embodiment, step (i) of the method comprises (a) providing a composition comprising a perhydrolase, an ester substrate, and a source of hydrogen peroxide; and (b) adding the composition to water and mixing to produce the mixture.

Preferably the perhydrolase is in the form of granules. The granules may comprise, for example, from about 1% (w/w) to about 10% (w/w) of perhydrolase.

In one embodiment, the following amounts of perhydrolase, ester substrate, source of hydrogen peroxide and water are mixed in step (i): from about 8% by weight to about 12% by weight of sodium percarbonate, from about 8% to about 12% by weight of propylene glycol diacetate, from about 0.0001% to about 0.01% by weight perhydrolase enzyme and from about 75% to about 85% by weight of water.

Preferably the mixture is applied to the surface by spraying.

In particular embodiments, the mold comprises a visible filamentus fungus selected from the group consisting of: *Absidia* spp., *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Aureobasidium* spp., *Basidiobolus* spp., *Beauveria* spp., *Bipolaris* spp., *Blastomyces* spp., *Candila* spp., *Chaetomium* spp., *Chysosporium* spp., *Cladosporium* spp., *Cladophialophora* spp., *Conidiobolus* spp., *Coccidioides* spp., *Cryptococcus* spp., *Cunninghamella* spp., *Curvularia* spp., *Drechslera* spp., *Emmonsia* spp., *Epidermophyton* spp., *Exserohilum* spp., *Fonseceae* spp., *Fusarium* spp., *Histoplasma* spp., *Lecythophora* spp., *Madurella* spp., *Microsporum* spp., *Mucor* spp., *Paecilomyces* spp., *Paracoccidioides* spp., *Penicillium* spp., *Phialophora* spp., *Phoma* spp., *Rhinocladiella* spp., *Rhizomucor* spp., *Rhizopus* spp., *Scedosporium* spp., *Scopulariopsis* spp., *Scytalidium* spp., *Sporothrix* spp., *Stachybotrys* spp., *Stachy California* spp., *Trichoderma* spp., *Trichophyton* spp., *Wangiella* spp., and *Verticillium* spp.

Embodiments of the present invention provide a method particularly suited to the removal of mold growth from surfaces. In particular, it has been found that a mixture comprising at least 1% by weight peracetic acid produced by an enzymatic method is surprisingly effective for achieving both mold killing and mold stain bleaching of surfaces. The method is especially suited to removing visible mold growth from surfaces which are typically susceptible to mold contamination in buildings, such as walls, floors and ceilings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a graph of the percentage by weight of peracetic acid in a mixture of the present invention at various times following mixing of the ingredients.

Figure 2A shows the application of the mixture of the present invention by spraying onto wooden rafters contaminated with visible mold deposits.

Figure 2B shows wooden rafters before application of the mixture of the present invention.

Figure 2C shows wooden rafters 24 hours after application of the mixture of the present invention.

Figure 3 shows a graph of the percentage of peracetic acid by weight at various times after mixing, for mixtures comprising varying concentrations of ester substrate and hydrogen peroxide source.

Figure 4 shows a graph of peak peracetic acid concentration, in % by weight, against molar concentration of propylene glycol diacetate in mixtures according to the present invention.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. For example, Singleton and Sainsbury, Dictionary of Microbiology and Molecular Biology, 2d Ed., John Wiley and Sons, NY (1994); and Hale and Marham, The Harper Collins Dictionary of Biology, Harper Perennial, NY (1991) provide those of skill in the art with a general dictionaries of many of the terms used in the invention. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the Specification as a whole. Also, as used herein, the singular terms “a,” “an,” and “the” include the plural

reference unless the context clearly indicates otherwise. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context in which they are used by those of skill in the art.

As used herein, the term "enzyme" refers to any protein that catalyzes a chemical reaction. The catalytic function of an enzyme constitutes its "activity" or "enzymatic activity." An enzyme typically is classified according to the type of catalytic function it carries out, *e.g.*, hydrolysis of peptide bonds.

As used herein, the term "substrate" refers to a substance (*e.g.*, a chemical compound) on which an enzyme performs its catalytic activity to generate a product.

A "perhydrolase" refers to an enzyme that is capable of catalyzing a perhydrolysis reaction that results in the production of peracids. In a preferred embodiment, a perhydrolase enzyme used in methods described herein exhibits a high perhydrolysis to hydrolysis ratio, *e.g.* a ratio of perhydrolysis to hydrolysis of at least about 1:1, 2:1, 5:1, 10:1, or greater, when measured by a method for the determination of perhydrolysis to hydrolysis ratio described herein. In some embodiments, the perhydrolase comprises, consists of, or consists essentially of the *Mycobacterium smegmatis* perhydrolase amino acid sequence set forth in SEQ ID NO: 1, or a variant or homologue thereof. In some embodiments, the perhydrolase enzyme comprises acyl transferase activity and catalyzes an aqueous acyl transfer reaction.

"Perhydrolysis" or "perhydrolyze" refers to an enzymatic reaction that produces peracids. In some embodiments, peracetic acid is produced by perhydrolysis of an ester substrate of the formula $CH_3C(=O)OR_1$, where R_1 is an organic moiety, in the presence of hydrogen peroxide (H_2O_2).

The phrase "source of hydrogen peroxide" includes hydrogen peroxide as well as the components of a system that can spontaneously or enzymatically produce hydrogen peroxide as a reaction product.

The phrase "perhydrolysis to hydrolysis ratio" refers to the ratio of the amount of enzymatically produced peracetic acid to the amount of enzymatically produced acetic acid by a perhydrolase enzyme from an ester substrate (*e.g.*, propylene glycol diacetate) under defined conditions and within a defined time. Perhydrolysis to hydrolysis ratio may be determined by a method as described below.

As used herein, the term "acyl" refers to an organic acid group, which is the residue of a carboxylic acid after removal of a hydroxyl (-OH) group (*e.g.*, ethanoyl chloride, CH_3CO-Cl, is the acyl chloride formed from ethanoic acid, CH_3CO-OH). The name of an individual acyl group is general formed by replacing the "-ic" of the acid by "-yl."

As used herein, the term "acylation" refers to a chemical transformation in which an acyl (RCO-) group is substituted into a molecule, generally for an active hydrogen of an -OH group.

As used herein, the term "transferase" refers to an enzyme that catalyzes the transfer of a functional group from one substrate to another substrate.

As used herein, the term "enzymatic conversion" refers to the modification of a substrate or intermediate to a product, by contacting the substrate or intermediate with an enzyme. In some embodiments, contact is made by directly exposing the substrate or intermediate to the appropriate enzyme. In other embodiments, contacting comprises exposing the substrate or intermediate to an organism that expresses and/or excretes the enzyme, and/or metabolizes the desired substrate and/or intermediate to the desired intermediate and/or end-product, respectively.

As used herein, "effective amount of enzyme" or "suitable amount of perhydrolase" refers to the quantity of enzyme (*i.e.*, perhydrolase) necessary to achieve the activity required in the specific application (*e.g.*, production of at least 1% by weight peracetic acid in the mixture used for application). Likewise, "suitable (or effective) amount of an ester substrate," "suitable (or effective) amount of a hydrogen peroxide source" and "suitable (or effective) amount of water" should be construed accordingly to refer to the required amounts necessary to achieve that result. Such effective amounts may be based on many factors, such as the particular enzyme variant used, the specific composition, the surface to be treated, and the like.

As used herein, the term "stability" in reference to a substance (*e.g.*, an enzyme) or composition refers to its ability to maintain a certain level of functional activity over a period of time under certain environmental conditions. Furthermore, the term "stability" can be used in a number of more specific contexts referring to the particular environmental condition that is of interest. For example, "thermal stability" as used herein refers to the ability of a substance or composition to maintain its function (*i.e.*, not degrade) at increased temperature. A substantial change in stability is evidenced by at least about a 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the functional activity being assayed, as compared to the activity present in the absence of the selected environmental conditions.

As used herein, the term "chemical stability" as used in reference to an enzyme refers to the stability of the enzyme in the presence of chemicals that adversely affect its activity. In some embodiments, such chemicals include, but are not limited to hydrogen peroxide, peracetic acid, anionic detergents, cationic detergents, non-ionic detergents, chelants, etc. However, it is not intended that the present invention be limited to any particular chemical stability level nor range of chemical stability.

As used herein, "pH stability" refers to the ability of a substance (*e.g.*, an enzyme) or composition to function at a particular pH. Stability at various pHs can be measured either by standard procedures known to those in the art and/or by the methods described herein. A substantial change in pH stability is evidenced by at least about 5% or greater increase or decrease (in most embodiments, it is preferably an increase) in the half-life of the functional activity, as compared to the activity at the optimum pH. It is not intended that the present invention be limited to any pH stability level nor pH range.

As used herein, "oxidative stability" refers to the ability of a substance (*e.g.*, an enzyme) or composition to function under oxidative conditions, *e.g.*, in the presence of an oxidizing chemical.

As used herein, "oxidizing chemical" refers to a chemical that has the capability of bleaching. The oxidizing chemical is present at an amount, pH and temperature

suitable for bleaching. The term includes, but is not limited to hydrogen peroxide and peracetic acid.

As used herein, the terms "purified" and "isolated" refer to the removal of contaminants from a sample. For example, a perhydrolase is purified by removal of contaminating proteins and other compounds within a solution or preparation that are not perhydrolases.

As used herein, the term "contaminant" refers to any substance which by its contact or association with another substance, material, or item makes it undesirable, impure, and/or unfit for use.

As used herein, the term "surface" refers to any solid material, typically on which a mold is growing.

As used herein, the term "mold" refers to any filamentous fungus.

As used herein, the term "a surface contaminated by mold" means that there is mold growth on or within the surface.

As used herein, "protein" refers to any composition comprised of amino acids and recognized as a protein by those of skill in the art. The terms "protein," "peptide" and polypeptide are used interchangeably herein. Wherein a peptide is a portion of a protein, those skilled in the art understand the use of the term in context. The terms "naturally-occurring," "wild-type" and "native" are used to refer to proteins found in nature. In some embodiments, the wild-type protein's sequence is the starting point of a protein engineering project.

"Variant" proteins differ from a parent protein, *e.g.*, a naturally-occurring or wild-type protein, by a small number of amino acid residues. In some embodiments, the number of different amino acid residues is any of about 1, 2, 3, 4, 5, 10, 20, 25, 30, 35, 40, 45, or 50. In some embodiments, variants differ by about 1 to about 10 amino acids from a parent protein.

In some embodiments, variant proteins comprise any of at least about 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or

99.5% amino acid sequence identity to the amino acid sequence of a parent protein, *e.g.*, a naturally-occurring or wild-type protein sequence.

In one embodiment, the parent enzyme is SEQ ID NO: 1.

Enzymatic production of peracetic acid in aqueous solution

The present invention relates in one aspect to a method for treating a surface contaminated by mold. In a first step, the method comprises mixing a perhydrolase, an ester substrate, a source of hydrogen peroxide and water in suitable amounts to produce a mixture comprising at least 1% by weight peracetic acid. In other words, this step involves the enzymatic production of a peracetic acid-containing solution.

Perhydrolase

One component of the mixture produced in the first step is a perhydrolase. The enzymes used in the present invention must have significant perhydrolase activity - *i.e.*, a catalytic activity that results in the formation of high amounts of peracetic acid suitable for applications such as cleaning, bleaching, and disinfecting. The use of enzymes with perhydrolase activity for peracetic acid generation is described in WO 2005/056782, WO 2007/133263 and WO 2008/140988, which are hereby incorporated by reference herein in their entirety.

Typically, enzymatic perhydrolase activity is accompanied by hydrolase activity. Indeed, as discussed below, many hydrolase enzymes also act as perhydrolases in the presence of hydrogen peroxide. In particularly preferred embodiments, the enzymes used in the present method have very high ratio of perhydrolase to hydrolase activity, and thereby produce a high ratio of peracid relative to acid products (*e.g.*, ratio of peracetic acid to acetic acid of at least about 1:1, 2:1, 5:1, 10:1, or greater), when measured by a method for the determination of perhydrolysis to hydrolysis ratio described herein. These high perhydrolysis to hydrolysis ratios of these distinct enzymes makes these enzymes suitable for use in the present method in which at least 1% peracetic acid is generated.

In one embodiment, the ratio of perhydrolysis (*i.e.* peracid formation) to hydrolysis (*i.e.*, acid formation) for an enzyme may be determined according to the following method.

Determination of Perhydrolysis to Hydrolysis Ratio

Preparation of Substrate

An ester substrate (*e.g.*, propylene glycol diacetate, ethyl acetate (EtOAc) or another water-soluble ester) is diluted in a desired buffer to a concentration of 10 mM of ester. Tributyrin or another water insoluble substrate is prepared by making substrate swatches. Polyester swatches are cut from non-dyed polyester fabric (Polycotton, PCW 22) using a 5/8-inch punch and placed in a 24-well microtiter plate (Costar, Cell Culture Plate). The insoluble ester is diluted to 1.03 M in hexane. Then, 10 L of the insoluble ester solution is adsorbed onto the polyester swatch.

Determination of Hydrolysis (GC Assay)

The hydrolytic assay described below is used to determine the amount of substrate hydrolysis.

In this assay, the assay solution is comprised of 50 mM potassium phosphate pH 7.5, 10 mM ester substrate, 29 mM hydrogen peroxide, and 20 mM potassium chloride in a total volume of 0.99 ml and an amount of enzyme that would generate 20 nmoles of acetic acid per minute at 25°C.

For measuring water insoluble ester hydrolysis, the reaction mixture is added to the insoluble ester fabric swatch. The swatch is prepared as described above ('Preparation of Substrate'). All the other conditions for the assay are the same except for exclusion of other ester substrates.

Hydrolytic activity is measured by monitoring the increase of acids generated by the enzyme from acyl donor substrates using gas chromatography coupled with flame ionization detection. The assay is conducted by first pipetting 50 µl of assay solution containing all the components except the enzyme into 200 mL of methanol (HPLC grade) to determine the amount of acid in the assay solution at time 0. Then, 10 µL of enzyme are added to the assay solution to a desired final concentration which

produces approximately 20 nanomoles of acid per minute. A timer is started and 50 µL aliquots are taken from the assay solution and added to 200 µL of methanol at various times, typically 2, 5, 10, 15, 25, 40, and 60 minutes, after addition of the enzyme.

These methanol-quenched samples are then injected into a gas chromatograph coupled with a flame ionization detector (Agilent 6890N) and analyzed for hydrolytic components, acetic, and butyric acids. Gas chromatography is conducted using a nitroterephthalic acid modified polyethylene glycol column (Zebron FFAP; with dimensions: 30 m long, 250 µm diameter, 250 nm film thickness). A 3 µL aliquot of sample is applied to the column by a splitless injection under constant a helium flow of 1.0 mL/minute. The inlet is maintained at a temperature of 250°C and is purged of any remaining sample components after 2 minutes. When analyzing acetic acid, the temperature of the column is maintained at 75°C for 1 minute after injection, increased 25°C/minute to 100°C, then increased 15°C/minute to 200°C.

When analyzing butyric acid, the temperature of the column is controlled as described above, except the temperature is additionally increased 25°C/minute to 225°C and held at 225°C for 1 minute. The flame ionization detector is maintained throughout the chromatography at 250°C and under constant hydrogen flow of 25 mL/minute, air flow of 200 mL/minute, and a combined column and makeup helium flow of 30 mL/minute. The amount of hydrolyzed acid in the sample is then determined by integrating the acid peak in the chromatogram for total ion counts and calculating the acid from the ion count using a standard curve generated under the above conditions for acetic and butyric acids at varying concentrations in the assay solution (without enzyme).

Determination of Perhydrolysis (OPD Assay)

The perhydrolytic activity assay described below is used to determine the amount of peracid formed in the reaction. In these assays, the solution comprises 50 mM potassium phosphate pH 7.5, 10 mM ester substrate, 29 mM hydrogen peroxide, 20 mM potassium chloride, and 10 mM O-phenylenediamine. When using water insoluble ester as the acyl donor, an ester adsorbed fabric swatch is used as the substrate, prepared as described above ('Preparation of Substrate').

Perhydrolytic activity is measured by monitoring the absorbance increase at 458 nm of oxidized O-phenylenediamine (OPD) by peracid generated with the enzyme. The perhydrolytic activity assay solution is prepared in the same manner as the hydrolytic activity assay solution, except that OPD is added to the assay solution to a final concentration of 10 mM. The OPD solution is prepared immediately before conducting the assay by dissolving 72 mg OPD (Sigma-Aldrich, dihydrochloride) in 19.94 mL of the same buffer and the pH is adjusted by slowly adding 60 µL of 13.5 M potassium hydroxide. The pH is measured and if needed, small quantities of potassium hydroxide are added to return the pH to the original pH of the buffer. Then, 495 µL of this OPD solution are added with the other assay components to a final assay volume of 0.990 mL. An assay quenching solution is also prepared by dissolving 36 mg OPD in 20 mL 100 mM citric acid and 70% ethanol.

The assay is typically conducted at 25°C. The assay is started by pipetting 100 µL of assay solution before the addition of the enzyme into 200 µl of quenching solution to determine the amount of perhydrolytic components and background absorbance in the assay solution at time 0. Then, 10 µL of enzyme are added to the assay solution to a desired final concentration which produced approximately 10 nanomoles of peracid per minute. A timer is started and 100 µL aliquots are taken from the assay solution and added to 200 µL of quenching solution at various times, typically 2, 5, 10, 15, 25, 40, and 60 minutes, after adding the enzyme. The quenched assay solutions are incubated for 30 minutes to allow any remaining peracid to oxidize the OPD. Then, 100 µL of each quenched assay solution is transferred to a 96-well microtiter plate (Costar) and the absorbance of the solution is measured at 458 nm by a spectrophotometric plate reader (Molecular Devices, SpectraMAX 250). The amount of peracid in each quenched sample is calculated using a standard curve generated under the above conditions with peracetic acid at varying concentrations in the assay solution (without enzyme).

Perhydrolysis/Hydrolysis ratio is determined according to the following formula:

Perhydrolysis/Hydrolysis ratio= Perhydrolysis measured in the Perhydrolysis assay / (Total acid detected in the hydrolysis assay - Perhydrolysis measured in the perhydrolysis assay)

In addition to having significant perhydrolase activity, the enzymes useful in the present method preferably exhibit relatively low activity in dry or substantially water free conditions. In some embodiments, the enzyme has less than about 1%, 0.5%, 0.2%, or less than about 0.1% activity (perhydrolase or hydrolase) when there is less than about 1% water by weight present in a composition comprising the perhydrolase, ester substrate and hydrogen peroxide source. In preferred embodiments, the enzyme exhibits less than about 0.1% activity when there is about 2% water by weight present or less.

In some embodiments, the perhydrolase enzyme is naturally-occurring (*i.e.*, a perhydrolase enzyme encoded by a genome of a cell). In some embodiments, the perhydrolase enzyme comprises, consists of, or consists essentially of an amino acid sequence that is at least about 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence of a naturally-occurring perhydrolase enzyme.

In some embodiments, the perhydrolase enzyme has a perhydrolysis:hydrolysis ratio of at least 1:1. High perhydrolysis to hydrolysis ratios means that these enzymes produce higher yields of peracetic acid, making them more effective in generating decontamination solutions. A perhydrolase with a favorable perhydrolysis to hydrolysis ratio that makes it particularly well-suited for the present invention is the *M. smegmatis* perhydrolase, also referred to as *M. smegmatis* acyl transferase (MsAcT), and variants and homologues thereof.

For example, *M. smegmatis* perhydrolase when used to enzymatically convert PGDA and hydrogen peroxide from percarbonate generates PAA product in at least 5:1 excess over acetic acid. Because the *M. smegmatis* perhydrolase produces a high ratio of PAA to acetic acid, the pH of the resulting aqueous peracetic acid solution is able to remain at relatively low pH, less than about pH 10, about pH 9.0, about pH 8.5, about pH 8.0, or about pH 7.5. Such low pH PAA aqueous solutions are considered non-corrosive (or at least substantially less corrosive) to metals than typical commercial PAA solutions which contain high levels of acetic acid.

Additionally, the *M. smegmatis* perhydrolase is essentially inactive in the absence of water. The *M. smegmatis* perhydrolase enzyme requires a significant amount of water present (*i.e.*, relatively high degree of hydration) in order to exhibit perhydrolase or

hydrolase activity. Typically, *M. smegmatis* perhydrolase and it variants exhibit less than about 1%, 0.5%, 0.2%, or less than about 0.1% activity (perhydrolase or hydrolases) when there is less about 1% water by weight present in a composition that includes the enzyme.

The wild-type *M. smegmatis* perhydrolase is described in WO 2005/056782, which is hereby incorporated by reference herein in its entirety. WO 2007/133263, which also is incorporated by reference herein, discloses a number of *M. smegmatis* perhydrolase variants including S54V-MsAcT, and the use of the wild-type and variants to generate aqueous peracetic acid solution when the enzyme is added to water along with a hydrogen peroxide source and an ester substrate (*e.g.*, propylene glycol diacetate (PGDA)).

In some embodiments, the perhydrolase enzyme is a naturally occurring *M. smegmatis* perhydrolase enzyme. In some embodiments, a perhydrolase enzyme comprises, consists of, or consists essentially of the amino acid sequence set forth in SEQ ID NO:1 or a variant or homologue thereof. In some embodiments, a perhydrolase enzyme comprises, consists of, or consists essentially of an amino acid sequence that is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

The amino acid sequence of *M. smegmatis* perhydrolase is shown below:
MAKRILCFGDSLTWGWVPVEDGAPTERFAPDVRWTGVLAQQLGADFEVIEE
GLSARTTNIDDPTDPRLNGASYLPSCLATHLPLDLVIIMLGTNDTKAYFRRTPL
DIALGMSVLVTQVLTSAGGVGTTYPAPKVLVVSPPPLAPMPHPWFQLIFEGGE
QKTTELARVYSALASFMKVPFFDAGSVISTDGVDGIHFTEANNRDLGVALAE
QVRSLL (SEQ ID NO: 1)

The corresponding polynucleotide sequence encoding *M. smegmatis* perhydrolase is:
5'-
ATGGCCAAGCGAATTCTGTGTTTCGGTGATTCCCTGACCTGGGGCTGGGTC
CCCGTCGAAGACGGGGCACCCACCGAGCGGTTCGCCCCCGACGTGCGCTG
GACCGGTGTGCTGGCCCAGCAGCTCGGAGCGGACTTCGAGGTGATCGAGG
AGGGACTGAGCGCGCGCACCACCAACATCGACGACCCCACCGATCCGCG
GCTCAACGGCGCGAGCTACCTGCCGTCGTGCCTCGCGACGCACCTGCCGC

TCGACCTGGTGATCATCATGCTGGGCACCAACGACACCAAGGCCTACTTC
CGGCGCACCCCGCTCGACATCGCGCTGGGCATGTCGGTGCTCGTCACGCA
GGTGCTCACCAGCGCGGGCGGCGTCGGCACCACGTACCCGGCACCCAAGG
TGCTGGTGGTCTCGCCGCCACCGCTGGCGCCCATGCCGCACCCCTGGTTCC
AGTTGATCTTCGAGGGCGGCGAGCAGAAGACCACTGAGCTCGCCCGCGTG
TACAGCGCGCTCGCGTCGTTCATGAAGGTGCCGTTCTTCGACGCGGGTTCG
GTGATCAGCACCGACGGCGTCGACGGAATCCACTTCACCGAGGCCAACAA
TCGCGATCTCGGGGTGGCCCTCGCGGAACAGGTGCGGAGCCTGCTGTAA-
3' (SEQ ID NO: 2)

It is not intended that the method of the present invention be limited to including wild type *M. smegmatis* perhydrolase. In alternative embodiments, a perhydrolase can be used that is a homologue or engineered variant of the *M. smegmatis* perhydrolase. In further preferred embodiments, a monomeric hydrolase is engineered to produce a monomeric or multimeric enzyme that has better perhydrolase activity than the native monomeric enzyme. In some particularly preferred embodiments, the variant comprises the substitution S54V of *M. smegmatis* perhydrolase (referred to herein as "S54V-MsAcT" or the "S54V variant" or "variant S54V").

The amino acid sequence of variant S54V of *M. smegmatis* perhydrolase is shown in SEQ ID NO: 3 below (substitution underlined):

MAKRILCFGDSLTWGWVPVEDGAPTERFAPDVRWTGVLAQQLGADFEVIEE
GLVARTTNIDDPTDPRLNGASYLPSCLATHLPLDLVIIMLGTNDTKAYFRRTPL
DIALGMSVLVTQVLTSAGGVGTTYPAPKVLVVSPPPLAPMPHPWFQLIFEGGE
QKTTELARVYSALASFMKVPFFDAGSVISTDGVDGIHFTEANNRDLGVALAE
QVRSLL (SEQ ID NO: 3)

In other embodiments, the perhydrolase used in the method is one of the variant enzymes or homologues disclosed and described in WO 05/056782.

In some embodiments, the perhydrolase enzyme comprises one or more substitutions at one or more amino acid positions equivalent to position(s) in the *M. smegmatis* perhydrolase amino acid sequence set forth in SEQ ID NO: 1. In some embodiments, the perhydrolase enzyme comprises one or more substitutions at one or more amino acid positions equivalent to position(s) in the sequence of SEQ ID NO: 1 selected

from the group consisting of: M1, K3, R4, I5, L6, C7, D10, S11, L12, T13, W14, W16, G15, V17, P18, V19, D21, G22, A23, P24, T25, E26, R27, F28, A29, P30, D31, V32, R33, W34, T35, G36, L38, Q40, Q41, D45, L42, G43, A44, F46, E47, V48, I49, E50, E51, G52, L53, S54, A55, R56, T57, T58, N59, I60, D61, D62, P63, T64, D65, P66, R67, L68, N69, G70, A71, S72, Y73, S76, C77, L78, A79, T80, L82, P83, L84, D85, L86, V87, N94, D95, T96, K97, Y99, F100, R101, R102, P104, L105, D106, I107, A108, L109, G110, M111, S112, V113, L114, V115, T116, Q117, V118, L119, T120, S121, A122, G124, V125, G126, T127, T128, Y129, P146, P148, W149, F150, I153, F154, I194, and F196, preferably wherein the perhydrolase enzyme comprises an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

In some embodiments, the perhydrolase enzyme comprises one or more of the following substitutions at one or more amino acid positions equivalent to position(s) in the *M. smegmatis* perhydrolase amino acid sequence set forth in SEQ ID NO: 1 selected from the group consisting of: L12C, L12Q, or L12G; T25S, T25G, or T25P; L53H, L53Q, L53G, or L53S; S54V, S54L, S54A, S54P, S54T, or S54R; A55G or A55T; R67T, R67Q, R67N, R67G, R67E, R67L, or R67F; K97R; V125S, V125G, V125R, V125A, or V125P; F154Y; F196G; preferably wherein the perhydrolase enzyme comprises an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

As used herein, an amino acid substitution of the form "XnZ", where "X" and "Z" are each an amino acid in single letter code and "n" is an integer, refers to the replacement of amino acid "X" by amino acid "Z" at position "n" in the specified amino acid sequence.

In some embodiments, the perhydrolase enzyme comprises a combination of amino acid substitutions at amino acid positions equivalent to amino acid positions in the *M. smegmatis* perhydrolase amino acid sequence set forth in SEQ ID NO: 1 selected from the group consisting of: L12I S54V; L12M S54T; L12T S54V; L12Q T25S S54V; L53H S54V; S54P V125R; S54V V125G; S54V F196G; S54V K97R V125G; or A55G R67T K97R V125G; preferably wherein the perhydrolase enzyme comprises

an amino acid sequence which is at least about 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequence set forth in SEQ ID NO: 1.

Several methods are known in the art that are suitable for generating variants of known perhydrolases (*e.g.*, the *M. smegmatis* perhydrolase), including but not limited to site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches.

In some embodiments, perhydrolases useful with the present invention include engineered variants within the SGNH-hydrolase family of proteins. In some preferred embodiments, the engineered proteins comprise at least one or a combination of the following conserved residues: L6, W14, W34, L38, R56, D62, L74, L78, H81, P83, M90, K97, G110, L114, L135, F180, or G205.

In alternative embodiments, these engineered proteins comprise a GDSL motif, a GRTT motif and/or an ARTT motif, preferably a GDSL-GRTT and/or a GDSL-ARTT motif. A GDSL motif is commonly found in esterases such as the *M. smegmatis* perhydrolase. The GDSL sequence motif is typically present at the active site and near to the N-terminus of the protein. As described in WO 2005/056782, the GRTT or ARTT motifs are also conserved among many variants having perhydrolase activity. By "GDSL-GRTT motif" it is meant that the GDSL sequence is separated from a GRTT sequence by one or more amino acid residues in the protein. By "GDSL-ARTT motif" it is meant that the GDSL sequence is separated from an ARTT sequence by one or more amino acid residues in the protein.

In further embodiments, the enzymes are multimers, including but not limited to dimers, octamers, and tetramers. In yet additional preferred embodiments, the engineered proteins exhibit a perhydrolysis to hydrolysis ratio that is greater than about 1:1, 2:1, 5:1, or even 10:1.

The position of an amino acid residue of a perhydrolase is equivalent to the position of an amino acid residue of a reference sequence, if it corresponds to the position in either the primary and/or tertiary structure of the reference sequence, wherein primary structure is compared by any of the alignment programs described herein. In one

embodiment the reference sequence is a parent sequence. In a preferred embodiment, the reference sequence is SEQ ID NO: 1.

As mentioned above, the present method encompasses the use of perhydrolases having a degree of sequence identity or sequence homology with amino acid sequence(s) defined herein, *e.g.*, with known perhydrolase sequences. The present invention encompasses, in particular, peptides having a degree of sequence identity with SEQ ID NO: 1, defined above, or homologues thereof. Here, the term "homologue" means an entity having sequence identity with the subject amino acid sequences or the subject nucleotide sequences. Here, the term "homology" can be equated with "sequence identity".

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the perhydrolase enzyme.

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 50%, preferably at least 55%, such as at least 60%, for example at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99%, identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (*i.e.*, amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Sequence identity comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs use complex comparison algorithms to align two or more sequences that best reflect the evolutionary events that might have led to the difference(s) between the two or more sequences. Therefore, these algorithms operate with a scoring system rewarding alignment of identical or similar amino acids and penalising the insertion of gaps, gap extensions and alignment of non-similar amino acids. The scoring system of the comparison algorithms include:

i) assignment of a penalty score each time a gap is inserted (gap penalty score),

ii) assignment of a penalty score each time an existing gap is extended with an extra position (extension penalty score),

iii) assignment of high scores upon alignment of identical amino acids, and

iv) assignment of variable scores upon alignment of non-identical amino acids.

Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons.

The scores given for alignment of non-identical amino acids are assigned according to a scoring matrix also called a substitution matrix. The scores provided in such substitution matrices are reflecting the fact that the likelihood of one amino acid being substituted with another during evolution varies and depends on the physical/chemical nature of the amino acid to be substituted. For example, the likelihood of a polar amino acid being substituted with another polar amino acid is higher compared to being substituted with a hydrophobic amino acid. Therefore, the scoring matrix will assign the highest score for identical amino acids, lower score for non-identical but similar amino acids and even lower score for non-identical non-similar amino acids. The most frequently used scoring matrices are the PAM matrices (Dayhoff *et al.* (1978), Jones *et al.* (1992)), the BLOSUM matrices (Henikoff and Henikoff (1992)) and the Gonnet matrix (Gonnet *et al.* (1992)).

Suitable computer programs for carrying out such an alignment include, but are not limited to, Vector NTI (Invitrogen Corp.) and the ClustalV, ClustalW and ClustalW2 programs (Higgins DG & Sharp PM (1988), Higgins *et al.* (1992), Thompson *et al.* (1994), Larkin *et al.* (2007). A selection of different alignment tools are available from the ExPASy Proteomics server at www.expasy.org. Another example of software that can perform sequence alignment is BLAST (Basic Local Alignment Search Tool), which is available from the webpage of National Center for Biotechnology Information which can currently be found at

http://www.ncbi.nlm.nih.gov/ and which was firstly described in Altschul *et al.* (1990) *J. Mol. Biol.* **215**; 403-410.

Once the software has produced an alignment, it is possible to calculate % similarity and % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

In one embodiment, it is preferred to use the ClustalW software for performing sequence alignments. Preferably, alignment with ClustalW is performed with the following parameters for pairwise alignment:

Substitution matrix:	Gonnet 250
Gap open penalty:	20
Gap extension penalty:	0.2
Gap end penalty:	None

ClustalW2 is for example made available on the internet by the European Bioinformatics Institute at the EMBL-EBI webpage www.ebi.ac.uk under tools – sequence analysis – ClustalW2. Currently, the exact address of the ClustalW2 tool is www.ebi.ac.uk/Tools/clustalw2.

In another embodiment, it is preferred to use the program Align X in Vector NTI (Invitrogen) for performing sequence alignments. In one embodiment, Exp10 has been may be used with default settings:

Gap opening penalty: 10

Gap extension penalty: 0.05

Gapseparation penalty range: 8

Score matrix: blosum62mt2

Thus, the present invention also encompasses the use of variants, homologues and derivatives of any amino acid sequence of a protein or polypeptide as defined herein, particularly that of SEQ ID NO: 1 or that encoded by SEQ ID NO: 2.

The sequences, particularly those of SEQ ID NO: 1, may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

The present invention also encompasses conservative substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur, *i.e.*, like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-conservative substitution may also occur, *i.e.*, from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Conservative substitutions that may be made are, for example within the groups of basic amino acids (Arginine, Lysine and Histidine), acidic amino acids (glutamic acid and aspartic acid), aliphatic amino acids (Alanine, Valine, Leucine, Isoleucine), polar amino acids (Glutamine, Asparagine, Serine, Threonine), aromatic amino acids (Phenylalanine, Tryptophan and Tyrosine), hydroxyl amino acids (Serine, Threonine), large amino acids (Phenylalanine and Tryptophan) and small amino acids (Glycine, Alanine).

Replacements may also be made by unnatural amino acids include; alpha* and alpha-disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of

natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, ß-alanine*, L-α-amino butyric acid*, L-γ-amino butyric acid*, L-α-amino isobutyric acid*, L-ε-amino caproic acid#, 7-amino heptanoic acid*, L-methionine sulfone#*, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thioproline*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid # and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above (relating to homologous or non-conservative substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ *et al.* (1992), Horwell DC. (1995).

In some embodiments, one or more of the following residues of *M. smegmatis* perhydrolase are modified: Cys7, Asp10, Ser11, Leu12, Thr13, Trp14, Trp16, Pro24, Thr25, Leu53, Ser54, Ala55, Thr64, Asp65, Arg67, Cys77, Thr91, Asn94, Asp95, Tyr99, Val125, Pro138, Leu140, Pro146, Pro148, Trp149, Phe150, Ile153, Phe154, Thr159, Thr186, Ile192, Ile194, and Phe196. However, it is not intended that the present invention be limited to sequence that are modified at these positions. Indeed, it is intended that the present invention encompass various modifications and combinations of modifications.

In some embodiments, some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. The perhydrolase mutants of

the present invention include various mutants, including those encoded by nucleic acid that comprises a signal sequence. In some embodiments of perhydrolase mutants that are encoded by such a sequence are secreted by an expression host. In some further embodiments, the nucleic acid sequence comprises a homologue having a secretion signal.

Characterization of wild-type and mutant proteins is accomplished via any means suitable and is preferably based on the assessment of properties of interest. For example, pH and/or temperature, as well as detergent and /or oxidative stability is/are determined in some embodiments of the present invention. Indeed, it is contemplated that enzymes having various degrees of stability in one or more of these characteristics (pH, temperature, proteolytic stability, detergent stability, and/or oxidative stability) will find use. In still other embodiments, perhydrolases with low peracetic acid degradation activity are selected.

Although perhydrolase from *M. smegmatis* (MsAcT) is used in one preferred embodiment of the present invention, any perhydrolase enzyme obtained from any source which in the presence of hydrogen peroxide converts an ester substrate into mostly peracid acid may be used in the present invention. In preferred embodiments, the enzyme exhibits a perhydrolysis to hydrolysis ratio that is greater than about 1:1, 2:1, 5:1, or even 10:1, *e.g.*, when measured by the assay described above. Consequently, the ratio of peracid acid to acid product is greater than about 1:1, 2:1, 5:1, or even 10:1, and the pH of the resulting aqueous peracid acid solution is able to remain relatively low, *e.g.*, less than about pH 10, about pH 9.0, about pH 8.5, about pH 8.0, or about pH 7.5.

Various hydrolases have perhydrolase activity that makes them useful as enzymes in the present method. *M. smegmatis* perhydrolase is a SGNH_arylesterase-like enzyme belonging to the SGNH-hydrolase family of enzymes. This family is defined by having four conserved amino acids SGN and H in four blocks, similar to the blocks that describe the lipolytic family of enzymes (see, Upton and Buckley (1995) *Trends Biochem. Sci.* 20:178). Thus, hydrolases useful in the present invention include but are not limited to: SGNH-hydrolase, carboxylate ester hydrolase, thioester hydrolase, phosphate monoester hydrolase, and phosphate diester hydrolase which act on ester bonds; a thioether hydrolase which acts on ether bonds; and α-amino-acyl-peptide

hydrolase, peptidyl-amino acid hydrolase, acyl-amino acid hydrolase, dipeptide hydrolase, and peptidyl-peptide hydrolase which act on peptide bonds. Such hydrolase(s) find use alone or in combination with *M. smegmatis* perhydrolase or another perhydrolase. Preferable among them are SGNH-hydrolase, carboxylate ester hydrolase, and peptidyl-peptide hydrolase. Most preferred is SGNH-hydrolase, particularly an SGNH hydrolase comprising a GDSL motif, *e.g.*, a GDSL-GRTT and/or GDSL-ARTT motif.

Other suitable hydrolases include: (1) proteases belonging to the peptidyl-peptide hydrolase class (*e.g.*, pepsin, pepsin B, rennin, trypsin, chymotrypsin A, chymotrypsin B, elastase, enterokinase, cathepsin C, papain, chymopapain, ficin, thrombin, fibrinolysin, renin, subtilisin, aspergillopeptidase A, collagenase, clostridiopeptidase B, kallikrein, gastrisin, cathepsin D, bromelin, keratinase, chymotrypsin C, pepsin C, aspergillopeptidase B, urokinase, carboxypeptidase A and B, and aminopeptidase); (2) carboxylate ester hydrolase including carboxyl esterase, lipase, pectin esterase, and chlorophyllase; and (3) enzymes having high perhydrolysis to hydrolysis ratios. Especially effective among them are lipases, as well as esterases that exhibit high perhydrolysis to hydrolysis ratios, as well as protein engineered esterases, cutinases, and lipases, using the primary, secondary, tertiary, and/or quaternary structural features of the perhydrolases used in the present invention.

In one embodiment, the enzyme(s) is provided in the form of granules or a spray-dried coating of enzyme-containing crude extract. In other embodiments, the enzyme(s) used in the method is substantially purified and in a granular, spray-dried, lyophilized, or otherwise dry solid form. In some embodiments, enzyme granules are used which have been formulated so as to contain an enzyme protecting agent and a dissolution retardant material (*i.e.*, material that regulates the dissolution of granules during use).

The present invention can be employed in connection with any number of processes for making dry enzyme granules or dry formulations of hydrogen peroxide source compounds, such as ENZOGUARD® (Genencor International Inc., Rochester, N. Y.) described in U.S. Pat. No. 5,324,649, which is hereby incorporated by reference herein, or Savinase granules (Novo Nordisk, Denmark), among others. Other exemplary dry formulation processes for enzymes or other compounds which can be

used to make the compositions used herein include those disclosed in U.S. Pat. Nos. 4,106,991, 4,689,297, 5,814,501, 5,879,920, 6,248,706, 6,413,749, 6,534,466, or PCT publications WO 97/12958, WO 99/32613, WO 01/29170, and those techniques described in "Enzymes in Detergency," *ed.* Jan H. van Ee, *et al.*, Ch. 15, pp. 310-12 (Marcel Dekker, Inc., New York, N.Y. (1997)); each of which is hereby incorporated by reference herein.

In embodiments where the perhydrolase also has arylesterase activity (*e.g.*, where the perhydrolase is *M. smegmatis* perhydrolase), the amount of enzyme to be added to the composition and/or mixture may be determined with reference to a specific level of arylesterase activity. For instance, arylesterase activity may be determined by a colorimetric assay based on a 1-minute hydrolysis of p-nitrophenyl butyrate (pNB) substrate at 30°C. The rate of release of p-nitrophenyl (pNP) is monitored at 405 nm and is proportional to the enzyme activity. Activity is reported as µmole pNP-butyrate hydrolyzed/(min*cm^3)/g, where cm^3 = mL or U/g of arylesterase, which is determined from the Beers law activity equation. A composition or enzyme having an activity of 1 AEG/g means that 1 gram of the composition or enzyme hydrolyses 1 µmole pNP-butyrate per minute per ml at 30°C in the above assay.

Where the enzyme is provided in the form of granules and the perhydrolase has arylesterase activity, the perhydrolase is preferably added to the granules in an amount such that the arylesterase activity of the granules is between about 9,000 to about 13,000 AEG/g, for example, 10,000 to about 12,000 AEG/g, based on the activity per unit mass of the granules. The granules may contain about 1 to about 10 wt% active enzyme, for example, about 3 to about 6 wt%, more preferably about 4 to about 5 wt % active enzyme. Such granules may account for less than 0.1% of the weight (*e.g.* about 0.07% by weight) of a composition comprising the perhydrolase, hydrogen peroxide source and ester substrate; therefore, the composition may include about 0.0001% to about 0.1% by weight enzyme, *e.g.*, 0.001 to about 0.01 wt% active enzyme, for example, about 0.002 to about 0.005 wt% (*i.e.*, 20 to 50 ppm) active enzyme.

Other enzymes

Additionally, it is contemplated that the mixtures used in the present invention may comprise additional enzymes. It is contemplated that by using combinations of enzymes, there will be a concurrent reduction in the amount of chemicals needed. The additional enzymes include but are not limited to: microbial cell wall- degrading and glycoprotein-degrading enzymes, lysozyme, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, β-glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, endoglucanases, PNGases, amylases, etc., as well as mixtures thereof. In some embodiments, enzyme stabilizers may also be included in the composition.

It is not intended that the present invention be limited to any specific enzyme for the generation of hydrogen peroxide, as any enzyme that generates H_2O_2 with a suitable substrate finds use in the methods of the present invention. For example, lactate oxidases from *Lactobacillus* species which are known to create H_2O_2 from lactic acid and oxygen find use with the present invention. Preferably an enzyme that is used to generate H_2O_2 also generates acid, which reduces the pH of a basic solution to the pH range in which the peracetic acid is most effective in bleaching (*i.e.*, at or below the pKa).

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

In addition, oxidases find use in the present invention, including carbohydrate oxidases selected from the group consisting of aldose oxidase (IUPAC classification EC1.1.3.9), galactose oxidase (IUPAC classification EC1.1.3.9), cellobiose oxidase (IUPAC classification EC1.1.3.25), pyranose oxidase (IUPAC classification EC1.1.3.10), sorbose oxidase (IUPAC classification EC1.1.3.11) and/or hexose oxidase (IUPAC classification EC1.1.3.5), glucose oxidase (IUPAC classification EC1.1.3.4) and mixtures thereof. [106] Indeed, it is contemplated that any suitable

oxidase can be used in the present invention that follows the equation: Enzyme + reduced substrate -> oxidized substrate + H_2O_2.

Hydrogen Peroxide Source

In the present method, a hydrogen peroxide source is included in the mixture. In some embodiments, the hydrogen peroxide source is a solid compound that generates hydrogen peroxide upon addition to water. Such compounds include adducts of hydrogen peroxide with various inorganic or organic compounds, of which the most widely employed is sodium carbonate perhydrate, also referred to as sodium percarbonate.

Inorganic perhydrate salts are one preferred embodiment of hydrogen peroxide source. Examples of inorganic perhydrate salts include perborate, percarbonate, perphosphate, persulfate and persilicate salts. The inorganic perhydrate salts are normally the alkali metal salts.

Other hydrogen peroxide adducts useful in the methods of the present invention include adducts of hydrogen peroxide with zeolites, or urea hydrogen peroxide.

The hydrogen peroxide source compounds may be included as the crystalline and/or substantially pure solid without additional protection. For certain perhydrate salts however, the preferred executions of such granular compositions utilize a coated form of the material which provides better storage stability for the perhydrate salt in the granular product. Suitable coatings comprise inorganic salts such as alkali metal silicate, carbonate or borate salts or mixtures thereof, or organic materials such as waxes, oils, or fatty soaps.

In some embodiments, the hydrogen peroxide source may comprise an enzymatic hydrogen peroxide generation system. In one preferred embodiment, the enzymatic hydrogen peroxide generation system comprises an oxidase and its substrate. Suitable oxidase enzymes include, but are not limited to: glucose oxidase, sorbitol oxidase, hexose oxidase, choline oxidase, alcohol oxidase, glycerol oxidase, cholesterol oxidase, pyranose oxidase, carboxyalcohol oxidase, L-amino acid oxidase, glycine oxidase, pyruvate oxidase, glutamate oxidase, sarcosineoxidase, lysine oxidase,

lactate oxidase, vanillyl oxidase, glycolate oxidase, galactose oxidase, uricase, oxalate oxidase, and xanthine oxidase.

In some embodiments, the hydrogen-peroxide generating compounds and enzyme systems can be combined into the mixture in their substantially pure form. In alternative embodiments, they may be combined with other components such as surfactants, sequestrants, builders, pH regulators, buffers, stabilizers, processing additives or any other known components of washing compositions, sanitising compositions, disinfecting compositions or bleach additives. In one embodiment, the mixture comprises a detergent.

Other compounds and enzymatic systems useful as hydrogen peroxide sources in the stable compositions of the present invention include but are not limited those described in U.S. Patent Pub. No. 20080145353 A1, which is hereby incorporated by reference herein.

Ester Substrates

According to the present method, an ester substrate is used that upon enzymatic conversion results in peracetic acid. Thus, in some embodiments, the ester substrate is a stable ester of acetic acid. Thus, exemplary acetic ester substrates include but are not limited to: methyl acetate, ethyl acetate, propyl acetate, triacetin (1, 3-diacetyloxypropan-2-yl acetate), and propylene glycol diacetate (PGDA).

In a preferred embodiment, the ester substrate is a stable alcohol ester of acetic acid. Thus, in one preferred embodiment, the ester substrate is any poly-alcohol ester of acetic acid, *e.g.*, a poly-ol diester such as PGDA. In another preferred embodiment, the ester substrate is a diol-diester compound.

A wide range of ester substrates for perhydrolase enzymes (*e.g.*, acyl transferase) useful in the methods of the present invention are disclosed in WO 2005/056782, which is hereby incorporated by reference herein in its entirety.

The ester substrates useful in the present invention may be in either solid or liquid form. In one embodiment, the ester substrate should be dry or substantially free of water that can activate the enzyme component of the mixture. In preferred

embodiments, the ester substrate includes less about 5%, less than about 1%, less than about 0.5%, less than about 0.1%, or even a lower percentage by weight of water.

In some embodiments, the ester substrate is a stable liquid at room temperature, for example, propylene glycol diacetate (PGDA). In some embodiments, the ester substrate is a stable liquid, in which the enzyme and hydrogen peroxide source components do not dissolve. In preferred embodiments, the ester substrate is substantially free of water, thereby minimizing any activation of the enzyme and/or the hydrogen peroxide source in the composition before addition of water.

Water

The water used in the mixture should be relatively clean and free from microbes, debris, and contaminants, but need not be sterile. The presence of small amounts of salts and buffers is unlikely to significantly effect peracetic acid generation. Nonetheless, the ability to use water, rather than buffer, is an advantageous feature of some embodiments of the present method.

Stable all-in-one compositions

In one embodiment, the step of forming a peracetic acid-comprising mixture comprises mixing a composition with water to form the mixture. Thus the composition comprises a perhydrolase, an ester substrate, and a source of hydrogen peroxide. Preferably the composition is a stable composition as described in WO 2008/140988, *e.g.*, an "all-in-one composition" (or "three component mixture") comprising a perhydrolase, an ester substrate, and a source of hydrogen peroxide which is stable for at least 7 days and can be activated to produce peracetic acid on addition of water. The terms "all-in-one composition" and "three component mixture" are used interchangeably herein.

Thus in some embodiments, the perhydrolase, ester substrate, and hydrogen peroxide source are combined together, in a non-reactive composition(s), in a single container. The container may be a bottle, a sachette, a bag, or similar. The container may optionally dissolve in water, so long as the dissolved material does not interfere with the reaction. Containers materials suitable for holding dry and liquid chemical

reagents are well-known in the art. Exemplary containers and materials are described, *e.g.*, in WO 08/140988.

In some embodiments, the contents of the container are homogenous to the extent that the perhydrolase, ester substrate, and hydrogen peroxide source are uniformally distributed, or can be uniformaly distrubuted following shaking, stirring, inverting, or otherwise mixing of the contents. Where the contents of the container are homogenous, only a portion of the contents need be mixed with water, with the remainder of the contents being saved for future use. Where the contents of the container are not homogenous, the entire contents should be mixed with water to preserve the ratios of the components.

Regardless of whether the contents of the container are homogenous, the composition may be provided in a single-dose format, wherein the entire contents of the container are mixed with water to produce the mixture comprising at least 1% by weight peracetic acid, such as, at least 1.2% by weight, at least 1.5% by weight, at least at least 1.8% by weight, at least 2% by weight, at least 2.2% by weight, or at least 2.5% by weight peracetic acid. For example, the mixture may comprise 1 to 10% by weight, 1.2 to 10% by weight, 1.5 to 10% by weight, 1.8 to 10% by weight, 2 to 10% by weight, 2.2 to 10% by weight, 2.5 to 10% by weight, 1 to 5% by weight, 1.2 to 5% by weight, 1.5 to 5% by weight, 1.8 to 5% by weight, 2 to 5% by weight, 2.2 to 5% by weight, 2.5 to 5% by weight, 1 to 4% by weight, 1.2 to 4% by weight, 1.5 to 4% by weight, 1.8 to 4% by weight, 2 to 4% by weight, 2.2 to 4% by weight, 2.5 to 4% by weight, 1 to 3% by weight, 1.2 to 3% by weight, 1.5 to 3% by weight, 1.8 to 3% by weight, 2 to 3% by weight, 2.2 to 3% by weight, 2.5 to 3% by weight, or about 2.6% by weight peracetic acid.

Adjunct Materials and Additional Components

Additional components may be included in the mixture and composition described herein. It is understood that such adjuncts are in addition to perhydrolase, hydrogen peroxide source and ester substrate. The precise nature of these additional components, and levels of incorporation thereof, will depend on the physical form of the composition and the nature of the surface to be treated. Suitable adjunct materials include, but are not limited to, surfactants, builders, chelating agents, dye transfer

inhibiting agents, deposition aids, dispersants, corrosion inhibitors, additional enzymes, and enzyme stabilizers, catalytic materials, bleach activators, bleach boosters, preformed peracids, polymeric dispersing agents, clay soil removal/anti-redeposition agents, brighteners, suds suppressors, dyes, perfumes, structure elasticizing agents, carriers, hydrotropes, processing aids and/or pigments. In addition to the disclosure below, suitable examples of such other adjuncts and levels of use are found in U.S. Patent Nos. 5,576,282, 6,306,812, and 6,326,348, herein incorporated by reference.

In some embodiments, the mixture and/or composition may further comprise enzymes that remove any residual peracetic acid and/or H_2O_2 after treatment of the surface. Such enzymes include but are not limited to catalases and/or hydrolytic enzymes.

Importantly, the present invention provides means for effective cleaning, bleaching, and disinfecting over broad pH and temperature ranges. In some embodiments, the pH range utilized in this generation is 4-12. In some embodiments, the temperature range utilized is between about 5°C and about 90°C. Preferably the method is performed at room or ambient temperature, typically in the range 15°C to 25°C.

Producing peracetic acid

A feature of the present invention is that the components described above are mixed to form a mixture comprising at least 1% by weight peracetic acid.

In particular embodiments, the mixture comprises at least 1.2% by weight, at least 1.5% by weight, at least at least 1.8% by weight, at least 2% by weight, at least 2.2% by weight, or at least 2.5% by weight peracetic acid. For example, the mixture may comprise 1 to 10% by weight, 1.2 to 10% by weight, 1.5 to 10% by weight, 1.8 to 10% by weight, 2 to 10% by weight, 2.2 to 10% by weight, 2.5 to 10% by weight, 1 to 5% by weight, 1.2 to 5% by weight, 1.5 to 5% by weight, 1.8 to 5% by weight, 2 to 5% by weight, 2.2 to 5% by weight, 2.5 to 5% by weight, 1 to 4% by weight, 1.2 to 4% by weight, 1.5 to 4% by weight, 1.8 to 4% by weight, 2 to 4% by weight, 2.2 to 4% by weight, 2.5 to 4% by weight, 1 to 3% by weight, 1.2 to 3% by weight, 1.5 to 3% by weight, 1.8 to 3% by weight, 2 to 3% by weight, 2.2 to 3% by weight, 2.5 to 3% by weight, or about 2.6% by weight peracetic acid.

The amounts of peracetic acid in the mixture as defined above typically refer to the peak concentration of peracetic acid producing after mixing of the components. Following mixing, the concentration of peracetic acid typically rises due to enzymatic activity and then decays due to decomposition of the peracetic acid. Thus the peak concentration refers to a maximum proportion of peracetic acid which is obtained in the mixture within a short period following mixing, for instance within 12 hours, within 6 hours, within 4 hours or within 2 hours. Typically the peak concentration of peracetic acid may be obtained within 30 to 120 minutes of mixing, particularly at room temperature. Preferably the concentration of peracetic acid in the mixture is within one or more of the above ranges at the time the mixture is applied to the surface.

The timescale and amplitude of peracetic acid production may vary according to the nature and amount of each component, temperature and so forth. However, a skilled person can easily produce a mixture having the desired peak concentration of peracetic acid by selecting suitable amounts of each component, and appropriate reaction conditions such as temperature.

The concentration of peracetic acid in the mixture may be obtained using standard methods known in the art, for instance as described in Pinkernell *et al.* (1997) *Analyst*, 122:567-571. In this ABTS assay, 100 µl of the solution to be analyzed is added to 1 ml of 125 mM potassium citrate buffer, pH 5.0, containing 1.0 mM 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 50 µM KI, and allowed to incubate at room temperature for 3 minutes. The absorbance is measured at 420 nm in a diode array spectrophotometer and compared to a standard curve prepared using authentic standard. In order to determine the peak peracetic acid concentration, enzymatic reactions to form PAA are initiated and aliquots withdrawn from the reactions at different times.

Typically the amounts of hydrogen peroxide source and ester substrate required depend on the specific compounds used as well as the enzyme. Generally, the enzymatic generation of a peracetic acid requires an equimolar amount of hydrogen peroxide and the corresponding acetic acid ester substrate compound. Consequently, in one preferred embodiment the weight percentage of each of the hydrogen peroxide source and the substrate depends on the formula weight of the specific compounds

and is selected so that there are equimolar amounts of hydrogen peroxide and substrate.

In order to generate higher concentrations (*e.g.*, at least 1%, or at least 2% by weight) of peracetic acid in the mixture, it is generally desirable to increase the amount of perhydrolase, ester sustrate and/or hydrogen peroxide source (*e.g.*, sodium percarbonate) added to the mixture, and/or to reduce the proportion of water in the mixture. In particular, the concentration of hydrogen peroxide source added to the mixture may be increased and the overall proportion of water in the mixture may be decreased. For instance, in one embodiment at least 8% by weight, *e.g.*, 8 to 20%, 8 to 15% or 8 to 12% by weight of hydrogen peroxide source (*e.g.*, sodium percarbonate) is added to the mixture. In another embodiment, the mixture comprises less than 85% by weight water, *e.g.*, 70 to 85%, 75 to 85% or 80 to 85% by weight water.

Generally, enzyme should preferably be incorporated into the mixture in an amount of 0.00001 to 1 weight percent, more preferably 0.0001 to 0.01 weight %, and more preferably 0.0001 to 0.001 weight percent, *e.g.* about 0.0005 to about 0.0008 weight % (*i.e.*, 5 to 8 ppm). Where crude enzyme is used, the granules are preferably added to the stable composition in such an amount that the purified enzyme is 1 to 10 weight percent in the granules. Relatively smaller amounts by weight are used with increasing enzyme purity. The granules are used in an amount of 0.001 to 10 and preferably 0.005 to 0.5 weight percent. Ultimately, specific amounts of enzyme will depend to some extent on the particular enzyme's empirically determined activity for a particular peracetic acid application.

Thus in one embodiment, the following amounts of perhydrolase, ester substrate, source of hydrogen peroxide and water are added to the mixture: from about 8% by weight to about 12% by weight of sodium percarbonate, from about 8% to about 12% by weight of propylene glycol diacetate, from about 0.0001% to about 0.01 % by weight perhydrolase enzyme and from about 75% to about 85% by weight of water.

The components of the mixture, for instance in the form of a stable composition comprising perhydrolase, ester substrate and hydrogen peroxide source, may be added to an appropriate amount of water and stirred. Typically any type of water may be

used, including tap water. In another embodiment, distilled, sterilized and/or deionized water is used. In particular embodiments, the mixture may be mixed for at least about 30 minutes, 20 minutes, 15 minutes, 10 minutes, 5 minutes, or even fewer minutes. In some embodiments, the method of preparation comprises: (a) providing a composition comprising a perhydrolase, a hydrogen peroxide source; and an ester substrate; and (b) adding said composition to water and mixing, thereby generating an aqueous solution of at least about 1% peracetic acid by weight, and a pH less than about 9.0. In one embodiment, the composition and the water comprise no other ingredients capable of buffering the pH.

The mixing step may be performed, for example, at typical ambient temperatures of about 15°C to about 25°C, or about 20°C to about 22°C. In some applications, however, the method may be performed at lower or higher temperatures. Generally, the functional temperature range is dependent on the ability of the enzyme to maintain sufficient activity. Thus, in one embodiment, the present invention contemplates a method using wild-type or engineered enzymes with high thermal stability.

Applying the mixture to a mold-contaminated surface

Following the preparation of the mixture comprising at least 1% by weight peracetic acid, the mixture is applied to a surface contaminated by mold.

The surface is typically a type of surface which is susceptible to mold formation. Porous or semi-porous surfaces may be particularly susceptible to mold formation, and mold removal from such surfaces using known products may be ineffective. Thus in one embodiment, the surface is a semi-porous or porous surface. By "porous or semi-porous" it is meant that the surface has a relatively high porosity, *e.g.*, compared to a hard surface, such that air and/or moisture can penetrate into the surface to a significant extent. Porosity can be quantitated by determining the proportion of the total volume of a material which consists of void spaces, and may be represented a fraction between 0 and 1 or a percentage. Porosity may be determined using standard techniques, such as mercury intrusion porosimetry (see, *e.g.*, Wood Material Science and Engineering 3 (2008), pages 62–70). Preferably the porosity of the material comprising the surface is at least 0.1%, at least 1%, or at least 10%.

In a further embodiment, the surface comprises an interior or exterior surface of a building. In some embodiments, the surface may comprise a wall, floor or ceiling of a building. Thus the surface may be comprised of a building material, *e.g.* wood, concrete, cement, metal, tile, grout, vinyl, glass fiber, roofing material or brick. In some embodiments, the surface may comprise an interior decorative material or covering, such as wallpaper, plaster or paint.

In another embodiment, the surface is an outdoor surface, including those of garden materials and features. Examples of such surfaces include outdoor or garden furniture (*e.g.*, chairs, tables, sunshades and the like), decking, paving, patios, walkways, fences and other landscaping material.

By "mold" it is typically meant a filamentous fungus, preferably a visible filamentous fungus. Thus in one embodiment the surface comprises visible mold contamination. By "visible mold contamination" it is meant that mold growth or colonization of the surface is detectable with the naked eye, *i.e.*, mold colonies can be seen without the assistance of a microscope or other optical device. Application of the mixture typically reduces visibility of the mold infection, for instance such that the appearance of surface (to the naked eye) is improved compared to the untreated surface. Thus the mixtures of the present invention are preferably used not only to remove microscopic fungal contamination (*e.g.*, by mold spores), but to bleach mold stains and/or to restore the surface to an acceptable appearance.

In particular embodiments, the surface may be contaminated by a mold comprising one or more of the following: *Absidia* spp., *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Aureobasidium* spp., *Basidiobolus* spp., *Basidomycetes* spp., *Beauveria* spp., *Bipolaris* spp., *Blastomyces* spp., *Candila* spp., *Chaetomium* spp., *Chysosporium* spp., *Cladosporium* spp., *Cladophialophora* spp., *Conidiobolus* spp., *Coccidioides* spp., *Cryptococcus* spp., *Cunninghamella* spp., *Curvularia* spp., *Drechslera* spp., *Emmonsia* spp., *Epidermophyton* spp., *Exserohilum* spp., *Fonseceae* spp., *Fusarium* spp., *Histoplasma* spp., *Lecythophora* spp., *Madurella* spp., *Microsporum* spp., *Mucor* spp., *Paecilomyces* spp., *Paracoccidioides* spp., *Penicillium* spp., *Phialophora* spp., *Phoma* spp., *Rhinocladiella* spp., *Rhizomucor* spp., *Rhizopus* spp., *Scedosporium* spp., *Scopulariopsis* spp., *Scytalidium* spp.,

Sporothrix spp., *Stachybotrys* spp., *Stachy California* spp., *Trichoderma* spp., *Trichophyton* spp., *Wangiella* spp., *Verticillium sp.*

In some embodiments, the method is used to treat a surface contaminated with a mold comprising *Aspergillus sp*, *Penecillium sp*, *Cladosporium sp*, *Acremonium sp.* or *Basidomycetes sp.*

In further embodiments, the surface may be contaminated with bacteria in addition to mold. Bacteria, *e.g.*, from sewage back-ups or floods, may also contribute to discoloration & odors on a surface contaminated with mold. Thus in one embodiment, the method is additionally used to bleach stains associated with bacterial growth.

The mixture may be applied to the surface in any manner, preferably by spraying. Apparatus suitable for spraying liquids is well-known in the art. Exemplary equipment includes hand and electric pump-driven sprayers. Spray equipment should be made of materials not likely to be affected by peracetic acid, at least for the time required to apply the mixture to a mold-contaminated surface. A sufficient amount of the mixture should be applied to thoroughly wet the contaminated area; however, a further excess of solution is not required. An advantage of the mixture used in the present method is that it is non-foaming and dries after spraying. Therefore the mixture is easy to apply and typically no vacuuming is required to remove residues after spraying. Appropriate personal protection should be worn to protect the eyes and skin from peracetic acid. A respirator should be worn to avoid breathing peracetic acid aerosols.

Preferably the mixture is applied to the surface at a time when peracetic acid concentration in the mixture is near its peak level, *e.g.*, when the mixture comprises at least 1%, at least 1.2%, at least 1.5%, at least 1.8%, at least 2%, at least 2.2%, at least 2.5% or at least 2.6% by weight peracetic acid. For example, the mixture may be applied when the mixture comprises from 1 to 10% by weight, 1.2 to 10% by weight, 1.5 to 10% by weight, 1.8 to 10% by weight, 2 to 10% by weight, 2.2 to 10% by weight, 2.5 to 10% by weight, 1 to 5% by weight, 1.2 to 5% by weight, 1.5 to 5% by weight, 1.8 to 5% by weight, 2 to 5% by weight, 2.2 to 5% by weight, 2.5 to 5% by weight, 1 to 4% by weight, 1.2 to 4% by weight, 1.5 to 4% by weight, 1.8 to 4% by

weight, 2 to 4% by weight, 2.2 to 4% by weight, 2.5 to 4% by weight, 1 to 3% by weight, 1.2 to 3% by weight, 1.5 to 3% by weight, 1.8 to 3% by weight, 2 to 3% by weight, 2.2 to 3% by weight, 2.5 to 3% by weight, or about 2.6% by weight peracetic acid. Further exemplary times for application of the mixture to the surface are 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 60 minutes, 65 minutes, 70 minutes, 75 minutes, 80 minutes, 85 minutes, 90 minutes, 95 minutes, 100 minutes, 105 minutes, 110 minutes, 115 minutes, and 120 minutes after mixing.

Depending on the specific type of surface and severity of mold contamination, the step of exposing the surface to the peracetic acid solution may be performed over a wide range of timescales. For example, in some embodiments exposure times as short as about 30 seconds, 1 minute, 5 minutes or 10 minutes may be sufficient. However, in other applications, it may be necessary to expose the item for considerably longer periods of time, such as about 30 minutes, 1 hour, 6 hours, 12 hours, 24 hours, or even longer, in order to achieve adequate level of decontamination.

Similarly, the temperature of the peracetic solution during the exposure step may be adjusted depending, for example, on the particular type of surface and severity of mold contamination. In a preferred embodiment, the exposure temperature is the ambient temperature at which the solution is prepared, *i.e.*, typically about 15-25°C. In other embodiments, higher temperatures may be used to facilitate the decontamination process. Generally, higher temperatures will accelerate the reactivity of the peracetic acid solution thereby accelerating the decontamination process. Thus, in some embodiments, the exposure step may be carried out with the peracetic acid solution at about 30°C, 37°C, 45°C, 50°C, 60°C, 75°C, 90°C, or even higher.

The present method produces exceptional results in terms of mold removal, including bleaching of mold stains on a surface. In particular, the method leaves a bright, clean surface, with virtually no evidence of the contaminants or stain resulting from the reaction components. For example, the system can be used to virtually eliminate all traces of dark, ugly, mold stains, even on porous surfaces, such as wood. In contrast, conventional methods of mold remediation often provide unsatisfactory mold stain removal and/or require post-decontamination cleaning to remove stains caused by the

action of the decontamination compositions on the surface being treated. In some cases, such stains and residues are as unsightly as the original contamination.

Typically the present method is capable of removing mold and mold stains from a surface, including semi-porous and porous surfaces, with a single application. However, in some embodiments, the mixture may be re-applied to the surface if required. In contrast to known methods with may require excessive manual scrubbing, according to the present method mold can typically be removed using little or no manual scrubbing.

Moreover, since peracetic acid is generated *in situ*, the components of the mixture and composition can be transported and stored safely. Since peracetic acid typically breaks down within about 24 hours of application, no dangerous residual chemicals remain on the treated surface.

As well as removing mold and mold stains, the present method typically kills mold spores, which may prevent mold regrowth. The method may additionally be suitable in some embodiments for killing bacteria and/or bacterial spores.

These and other aspects of the present method will be apparent to the skilled person from the present description. All references identified, herein, are hereby incorporated by reference.

EXAMPLES

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

EXAMPLE 1

Enzymatic mold remediation method using a stable "all-in-one" composition producing 2.6% peracetic acid

In this example, a single container, "all-in-one" composition is used to generate a mixture comprising peracetic acid for mold remediation. The composition is a single-container blend of perhydrolase granules with dry percarbonate (as a hydrogen

peroxide source) and liquid propylene glycol diacetate (PGDA) as a substrate. When mixed with an appropriate amount of water to produce a final solution, the three ingredients produce a pre-selected end concentration of peracetic acid in a pre-selected period of time.

Each 1 L container contains:

0.831 g (+/- 5%) perhydrolase granules (solid),

600 g (+/- 5%) propylene glycol diacetate (PGDA) liquid, and

585 g (+/- 5%) sodium percarbonate (solid).

The perhydrolase granules include the layers described in Table 5.

Table 5. Composition of perhydrolase granules

		Target (% w/w)	Min. (% w/w)	Max. (% w/w)
Core ASNP (66.0% w/w)	Sucrose	52.1	46.9	57.3
	Starch	12.6	11.3	13.9
	Polyvinyl alcohol	1.3	1.2	1.5
Enzyme layer (21.1% w/w)	Enzyme total solids	21.1	15	27
Pigment layer (12.9% w/w)	TiO_2	5.7	5.2	6.3
	Methyl cellulose	4.6	4.1	5.0
	Lutensol	1.1	1.0	1.2
	PEG600	1.6	1.4	1.7
Total		100		

The arylesterase activity of the granules is preferably between about 8,500 to about 10,400 AEG/g.

The particular *Mycobacterium smegmatis* perhydrolase used in this system includes the substitition S54V and is expressed in *Bacillus subtilis*. PGDA and sodium percarbonate are liquid and solid reagents, respectively, and may be obtained from a variety of chemical supply companies.

Stability

4-month stability testing of the three component mixture (*i.e.*, the all-in-one composition described above) demonstrated that it retained essentially 100% activity, based on its ability to make a preselected amount of peracid when mixed with water. Based on such data, the useful shelf life of the three component mixture is expected to be at least 6-months, or even a year or longer.

Time for peracetic acid generation

The generation of peracetic acid is initiated by adding the approximately 1 L (1.18 kg) of three-component mixture to 5.68 L (1.5 US gallons) of water, according to the "instructions for use" included with the system. The mixing is performed at room temperature (about 20°C).

The concentration of peracetic acid in the mixture is determined using the method as described in Pinkernell *et al.*, *Analyst*, 122:567-71 (1997). In this ABTS assay, 100 µl of the solution to be analyzed is added to 1 ml of 125 mM potassium citrate buffer, pH 5.0, containing 1.0 mM 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 50 µM KI, and allowed to incubate at room temperature for 3 minutes. The absorbance is measured at 420 nm in a diode array spectrophotometer and compared to a standard curve prepared using authentic standard. Aliquots are withdrawn from the mixture at different times and PAA concentration determined.

The peracetic acid generation curve is shown in Fig. 1.

The final solution is applied to surface to the mold contaminated surface when the level of peracetic acid in the solution is 2% or more by weight. As can be seen from Fig. 1, at ambient/room temperature, the time required to generate 2% peracetic acid is about 30 minutes. The peak amount of peracetic acid produced by the system is about 2.6% at about 60 minutes. Longer times result in a reduction of peracid levels,

although there remains 2% or more peracid until about 120 minutes following the addition of water.

Application

The solution is applied to the mold contaminated surface by spraying between 30 minutes and 120 minutes after mixing the three component mixture with water.

Results

A single application cleans and decontaminates most surfaces within about 24 hours, often overnight, with little or no scrubbing. The peracetic acid generated in the final solution breaks down within about 24 hours (again, often overnight), leaving no caustic residue. Following decontamination, the treated surface has a bright, clean appearance, in contrast to conventional treatments, which can stain the surface or leave visible evidence of the mold being treated.

EXAMPLE 2

Enzymatic mold remediation method using a stable "all-in-one" composition producing 2.0% peracetic acid

In this example, an aqueous solution comprising peracetic acid was prepared using an "all-in-one" composition as described in Example 1, except that the composition was added to 7.57 L (2 US gallons) of water, instead of 5.68 L (1.5 US gallons) as in Example 1. The peak concentration of peracetic acid in the composition was 2.0% by weight. The solution was applied by spraying to a wooden ceiling affected by mold contamination.

Figure 2A shows the application of the mixture by spraying onto wooden rafters contaminated with visible mold deposits. Figure 2B shows the rafters before application of the mixture, and Figure 2C shows the rafters 24 hours after application of the mixture. It can be seen from Figures 2B and 2C that application of the mixture removes all traces of mold contamination, without leaving any trace or stain of either the mold or the mixture itself, even on a porous surface such as wood.

A control mixture was also prepared in a similar manner to that described above, but wherein the peak concentration of peracetic acid in the mixture was 0.2% by weight. When this mixture was applied to a mold-contaminated surface, such as wooden rafters, it was substantially ineffective at removing mold growth and/or mold stains. Although the solution comprising 0.2% PAA may effectively kill bacteria and fungal spores on some surfaces, particularly hard surfaces, it showed little or no bleaching/cleaning effect, particularly on porous surfaces such as wood. This demonstrates that in order to achieve the visual cleaning & bleaching of the unsightly mold, higher concentrations of PAA (*e.g.*, at least 1% by weight) are required.

EXAMPLE 3

Enzymatic mold remediation using a stable "all-in-one" composition producing 2.0% peracetic acid

In this example, an aqueous solution comprising peracetic acid was prepared using an "all-in-one" composition as described in Example 2. The peak concentration of peracetic acid in the composition was 2.0% by weight. The solution was applied by spraying to wooden flooring areas affected by mold contamination.

The test site was a basement in a newly constructed two-story wood framed duplex which had sustained water intrusion due to ground water seepage. The basement was unattended which allowed the initiation and amplification of fungal and bacterial growth on the exposed floor joists and the underside of the sub-flooring on the first floor.

Methodology

General

Based on visual observations, three areas of sub-flooring with the heavy fungal contamination were chosen as sample locations. Sample locations were marked on an adjacent wall for later identification with an indelible black ink pen. One of the three locations was isolated using polyethylene sheeting to create a control area where a control mold remediation product was applied. Air samples (PCR, Total Spore, and

Viable spore) were collected first, then surface testing was conducted (*i.e.*, Swab, Tape Lift, and Contact Plate).

All samples collected, except the total spore samples, were placed in a cooler with ice packs and analysed.

Surface Testing

Tape Lift Sampling

Tape lift samples for mold were collected using a micro-tape sampling kit. The Micro-tape, held by the ends of the tape, is gently pressed on the surface to be sampled. The tape is then removed from the surface and placed onto a clean microscope slide, which is inserted into a slide box for transportation.

Swab Sampling

Swabs composed of polyurethane plastic or flexible wire and rayon were used to collect surface samples for mold and bacteria by this method. Swabs were contained in a sterile hermetically sealed collection and transport ampoule. Approximately one square inch of the sub-floor was swabbed for each sample.

Contact Plate Sampling

Viable surface samples for mold were collected using contact plates manufactured by Health Link. The contact plate is $25cm^2$ plastic dish contact plate filled with agar to give a convex surface. The plate was gently pressed to the exposed sub-floor in the test location, removed from the surface, covered and resealed.

Bulk Material Sampling

Bulk samples for mold were collected using a chisel and hammer to remove a thin layer of plywood approximately 1 square inch in size from the exposed sub-floor. Bulk samples were placed in clean plastic bags for transportation.

Air Testing

Viable Fungi

Air sampling for viable fungi was accomplished using a Biocassette impactor containing Malt Extract Agar (MEA) attached to a sampling pump calibrated to 28.5 L per minute (LPM). Sample collection times were 10 minutes.

Viable and Non-viable Fungi by PCR

Air sampling for twenty-three species of viable and non-viable fungi was accomplished using Polymerase Chain Reaction (PCR) a DNA based analytical method. Samples were collected using polycarbonate filter cassettes connected to an air sampling pump calibrated at 6 Liters per minute (LPM). Sample collection times were 300 minutes.

Viable and Non-viable by Spore Trap and Microscopic Analysis.

An Air-O-Cell spore trap sampler was used to collect air samples for total fungal spores. The spore trap is a particulate sampling device designed for the rapid collection and microscopic analysis of a wide range of airborne aerosols including fungal spores. Samples were collected using an air sampling pump calibrated to a flow rate of 15 LPM. Sample collection times were 5 minutes. All samples collected were submitted for analysis via light microscopy at 600X magnification Analytical results were reported as total fungal spores per cubic meter of air (count/m^3). Total counts include both viable and non-viable fungal spores. This technique can identify the type (*i.e.*, genera) of fungal spores present but cannot identify the species of spore present since samples cannot be cultured.

Discussion

General

The locations of the sampling areas were as follows:

Sample Location #1 -Northeast corner, 3 feet from the west wall

Sample Location #2 -West Side, 3 feet from the middle of the West wall

Sample Location #3 - Northwest corner, 2 feet from the west wall

A mixture according to the present invention comprising 2% peracetic acid was prepared and applied to Sample Locations #1 and #2 by spraying. Sample Location #3 was isolated from the rest of the test areas with polyethylene sheeting. A control mold remediation product was utilized in this area.

Surface Sampling

Tape Lift Samples

A total of six (6) tape lift samples were collected from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. All pre-application tape lifts analyzed showed the presence of significant fungal growth. After final application of the mixture of the invention two of the three sample locations had no fungal growth. One of the sample locations did have some minimal fungal growth but the fungus detected was not observed in the analysis of the pre-application testing.

Swab Samples - Mold

A total of six (6) swab samples for mold were collected from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. All pre-application swabs analyzed showed the presence of significant fungal growth with fungal concentrations ranging from 41,000 colony forming units per swab (CFU/Unit) to 1,100, 000 CFU/Unit. After the final application of the mixture of the invention, fungal concentrations ranged from 210 CFU/Unit to 200,000 CFU/Unit. Additionally, none of the fungal species identified in the post-application testing were present in the corresponding samples collected during the pre-application testing. The new fungal growth may have occurred during the time between the application of the mixture and the post-application testing.

Swab Samples - Bacteria

A total of six (6) swab samples for bacteria were collected from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the

mixture of the invention and three (3) were collected after application of the mixture. All pre-application swabs analyzed showed the presence of significant gram-negative bacteria contamination with concentrations ranging from 240,000 colony forming units per swab (CFU/Unit) to greater than (>) 3,000,000 CFU/Unit. Concentrations of gram-positive bacteria (*i.e.*, Actinomycetes) which were also detected during pre-application testing, ranged from 30,000 CFU/Unit to 120,000 CFU/Unit. After the final application of the mixture of the invention, gram-negative bacteria were not detected. Additionally, only 1 sample had detectable concentrations of gram-positive bacteria (*i.e.*, 30 CFU/Unit).

Contact Plate Samples

A total of six (6) samples were collected using the contact plates described above from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture.

Based on the Laboratory Analysis Reports all pre-application contact plate samples analyzed were overgrown with various species of *Aspergillus*, *Penecillium*, *Cladosporium*, and *Acremonium*. Overgrowth of *Basidomycetes* was also noted in one sample. After the final application of the mixture of the invention only limited fungal growth was observed on the contact plate samples collected. That is fungal concentrations ranged from 6 CFU/plate to 190 CFU/plate. Some of the post-application fungal growth appeared to new fungal growth which initiated and amplified during the time between the mixture application and the post-application testing.

Bulk Material Samples

A total of six (6) bulk samples were collected from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. Significant fungal growth was observed in the bulk samples collected prior to the application of the mixture. That is, fungal concentrations ranged from 1,280,000 CFU/Unit to 5,580, 000 CFU/Unit. After the final application of the mixture of the invention, fungal concentrations ranged from 45,200 CFU/Unit to 470,000 CFU/Unit.

After application of the mixture of the invention, fungal concentrations in the test areas #1 and #2 decreased significantly for species which were quantified during pre-application testing (*e.g. Aspergillus niger*, *Cladosporium cladosporioides*). In test area #3 the fungal concentrations were also significantly lower than the pre-application concentrations and no fungal species identified in the pre-application was observed in the post application testing.

Air Sampling

Viable Fungi

A total of six (6) air samples for viable fungi were collected using the Biocassettes described above. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. Analysis of the samples collected during the pre-application phase showed high levels of airborne fungi. In fact, the agar plates were overgrown with a *Penicillium* species. After the final application of the mixture of the invention the airborne fungal concentration was much lower than the pre-application concentrations. That is fungal concentrations ranged from 6 CFU/plate to 190 CFU/plate. Some of the post-application fungal growth appeared to new fungal which initiated and amplified during the time between the biocide application and the post-application testing.

Viable and Non-viable Fungi

A total of six (6) samples were collected using the contact plates described above from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. Based on the Laboratory Analysis Reports all pre-application contact plate samples analyzed were overgrown with various species of *Aspergillus*, *Penicillium*, *Cladosporium*, and *Acremonium*. Overgrowth of *Basidomycetes* was also noted in one sample. After the final application of the mixture of the invention only limited fungal growth was observed on the contact plate samples collected. That is fungal concentrations ranged from 6 CFU/plate to 190 CFU/plate. Some of the post-application fungal growth appeared to new fungal growth which initiated and amplified during the time between the biocide application and the post-application testing.

PCR

A total of six (6) samples were collected using the contact plates described from the sub-floor exposed in the basement. Three (3) of the samples were collected before the application of the mixture of the invention and three (3) were collected after application of the mixture. Based on the Laboratory Analysis Reports all pre-application contact plate samples analyzed were overgrown with various species of *Aspergillus*, *Penicillium*, *Cladosporium*, and *Acremonium*. Overgrowth of *Basidomycetes* was also noted in one sample. After the final application of the mixture of the invention only limited fungal growth was observed on the contact plate samples collected. That is fungal concentrations ranged from 6 CFU/plate to 190 CFU/plate. Some of the post-application fungal growth appeared to new fungal which initiated and amplified during the time between the biocide application and the post-application testing.

Conclusion

Based on the above results it is clear that the mixture of the invention effectively kills mold and inhibits initiation and amplification of mold growth. The mixture of the invention was at least as effective is this regard as the control mold remediation product.

EXAMPLE 4

Production of mixtures comprising varying concentrations of peracetic acid

In this example, mixtures were produced comprising perhydrolase granules, sodium percarbonate (as a hydrogen peroxide source), liquid propylene glycol diacetate (PGDA) as a substrate and water. The concentration of sodium percarbonate and PGDA added to each mixture was varied, in order to produce mixtures comprising varying concentrations of peracetic acid.

The following mixtures A to D, each having a composition as shown in the Table below, were produced.

	PGDA conc.	Mass PGDA	Sodium percarbonate	Mass sodium	Perhydrolase conc.	Mass perhydrolase	Volume water added

	(M)	added (g)	conc. (M)	percarbonate added (g)	(ppm)	granules added (mg)	(litres)
A	0.04	1.80	0.03	1.25	4	30	0.25
B	0.1	4.01	0.1	3.93	4	30	0.25
C	0.25	10.01	0.25	9.81	4	30	0.25
D	0.5	20.03	0.5	19.63	4	30	0.25
E	1	40.05	1	39.25	4	30	0.25

The concentration of peracetic acid in the mixtures at varying times following mixing was monitored using a method as described in Example 1. The time course of peracetic acid production in each of mixtures A to E, in terms of % peracetic acid by weight at times in minutes after mixing, is shown in Figure 3.

The following peak concentrations of peracetic acid in each mixture were obtained:

	Peak peracetic acid concentration (M)	Peak peracetic acid concentration (% by weight)
A	0.03	0.19
B	0.07	0.53
C	0.21	1.60
D	0.29	2.19
E	0.54	4.01

Figure 4 shows a graph of peak peracetic acid concentration, in % by weight, against the molar concentration of PGDA in the mixture. Each data point represents one of

the mixtures A to E, and thus the PGDA concentration is essentially the same as the sodium percarbonate concentration (in molar terms). In order to produce a mixture comprising a desired % peracetic acid by weight, using Figure 4 a suitable molar concentration of PGDA (and a corresponding molar concentration of sodium percarbonate) can be deduced.

Each of the mixtures A to E can be applied to a mold contaminated surface by spraying, as described in Examples 1 to 3 above.

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

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

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

CLAIMS

1. A method for treating a surface contaminated by mold, the method comprising the steps of:

i) mixing a perhydrolase, an ester substrate, a source of hydrogen peroxide and water in suitable amounts to produce a mixture comprising at least 1% by weight peracetic acid; and

ii) applying the mixture to a surface contaminated by mold.

2. A method according to claim 1, wherein the mixture comprises an amount of peracetic acid selected from the group consisting of: at least 1.5% by weight, at least 2% by weight, and at least 2.5% by weight.

3. A method according to claim 1 or claim 2, wherein the mixture comprises an amount of peracetic acid selected from the group consisting of: 1 to 10% by weight, 1.5 to 5% by weight, and 2 to 4% by weight.

4. A method according to any preceding claim, wherein the mixture is applied to the surface 30 to 120 minutes after mixing.

5. A method according to any preceding claim, wherein the surface is a semi-porous or porous surface.

6. A method according to any preceding claim, wherein the surface is an interior or exterior surface of a building.

7. A method according to any preceding claim, wherein the surface is selected from the group consisting of: wood, concrete, cement, metal, tile, grout, vinyl, roofing material, wall-paper, glass fiber film, a painted surface and building bricks.

8. A method according to any preceding claim, wherein the surface comprises a visible mold contamination.

9. A method according to any preceding claim, wherein application of the mixture reduces visibility of the mold contamination.

10. A method according to any preceding claim, wherein the perhydrolase has a perhydrolase to hydrolase ratio greater than 1:1.

11. A method according to any of preceding claim, wherein the ester substrate is selected from the group consisting of: propylene glycol diacetate, ethylene glycol diacetate and triacetin.

12. A method according to any preceding claim, wherein the source of hydrogen peroxide is sodium percarbonate.

13. A method according to any preceding claim, wherein the perhydrolase is at least 70% identical to SEQ ID NO: 1.

14. A method according to any preceding claim, wherein the perhydrolase comprises one or more substitutions at one or more amino acid positions equivalent to position(s) in the sequence of SEQ ID NO 1 selected from the group consisting of: M1, K3, R4, I5, L6, C7, D10, S11, L12, T13, W14, W16, G15, V17, P18, V19, D21, G22, A23, P24, T25, E26, R27, F28, A29, P30, D31, V32, R33, W34, T35, G36, L38, Q40, Q41, D45, L42, G43, A44, F46, E47, V48, I49, E50, E51, G52, L53, S54, A55, R56, T57, T58, N59, I60, D61, D62, P63, T64, D65, P66, R67, L68, N69, G70, A71, S72, Y73, S76, C77, L78, A79, T80, L82, P83, L84, D85, L86, V87, N94, D95, T96, K97, Y99, F100, R101, R102, P104, L105, D106, I107, A108, L109, G110, M111, S112, V113, L114, V115, T116, Q117, V118, L119, T120, S121, A122, G124, V125, G126, T127, T128, Y129, P146, P148, W149, F150, I153, F154, I194, and F196.

15. A method according to any preceding claim, wherein the perhydrolase comprises at least one amino acid substitution, at one or more amino acid positions equivalent to position(s) in SEQ ID NO: 1, selected from the group consisting of: L12C, L12Q, or L12G; T25S, T25G, or T25P; L53H, L53Q, L53G, or L53S; S54V, S54L, S54A, S54P, S54T, or S54R; A55G or A55T; R67T, R67Q, R67N, R67G,

R67E, R67L, or R67F; K97R; V125S, V125G, V125R, V125A, and V125P; F154Y; F196G.

16. A method according to any preceding claim, wherein the perhydrolase enzyme comprises a combination of amino acid substitutions, at amino acid positions equivalent to amino acid positions in SEQ ID NO: 1, selected from the group consisting of: L12I S54V; L12M S54T; L12T S54V; L12Q T25S S54V; L53H S54V; S54P V125R; S54V V125G; S54V F196G; S54V K97R V125G; and A55G R67T K97R V125G.

17. A method according to any preceding claim, wherein the perhydrolase has a V at position 54 corresponding to the sequence of SEQ ID NO: 1.

18. A method according to any preceding claim, wherein step (i) comprises:

(a) providing a composition comprising a perhydrolase, an ester substrate, and a source of hydrogen peroxide; and

(b) adding the composition to water and mixing to produce the mixture.

19. A method according to any of the preceding claims, wherein the perhydrolase is in the form of granules.

20. A method according to claim 19, wherein the granules comprise from about 1% (w/w) to about 30% (w/w) of perhydrolase.

21. A method according to any preceding claim, wherein the following amounts of perhydrolase, ester substrate, source of hydrogen peroxide and water are mixed in step i): from about 8% by weight to about 12% by weight of sodium percarbonate, from about 8% to about 12% by weight of propylene glycol diacetate, from about 0.0001% to about 0.01 % by weight perhydrolase enzyme and from about 75% to about 85% of water.

22. A method according to any preceding claim, wherein the mixture is applied to the surface by spraying.

23. A method according to any preceding claim, wherein the mold comprises a visible filamentus fungus selected from the group consisting of: *Absidia* spp., *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Aureobasidium* spp., *Basidiobolus* spp., *Beauveria* spp., *Bipolaris* spp., *Blastomyces* spp., *Candila* spp., *Chaetomium* spp., *Chysosporium* spp., *Cladosporium* spp., *Cladophialophora* spp., *Conidiobolus* spp., *Coccidioides* spp., *Cryptococcus* spp., *Cunninghamella* spp., *Curvularia* spp., *Drechslera* spp., *Emmonsia* spp., *Epidermophyton* spp., *Exserohilum* spp., *Fonseceae* spp., *Fusarium* spp., *Histoplasma* spp., *Lecythophora* spp., *Madurella* spp., *Microsporum* spp., *Mucor* spp., *Paecilomyces* spp., *Paracoccidioides* spp., *Penicillium* spp., *Phialophora* spp., *Phoma* spp., *Rhinocladiella* spp., *Rhizomucor* spp., *Rhizopus* spp., *Scedosporium* spp., *Scopulariopsis* spp., *Scytalidium* spp., *Sporothrix* spp., *Stachybotrys* spp., *Stachy California* spp., *Trichoderma* spp., *Trichophyton* spp., *Wangiella* spp., and *Verticillium sp.*

24. Use of a mixture for treating a mold-contaminated surface, wherein the mixture comprises a perhydrolase, water and at least 1% by weight peracetic acid.

25. Use according to claim 24, wherein the mixture is used to bleach a visible mold contamination on the surface.

26. Use according to claim 24 or claim 25, wherein the mixture is produced by mixing a perhydrolase, an ester substrate, a source of hydrogen peroxide and water.

FIGURE 1

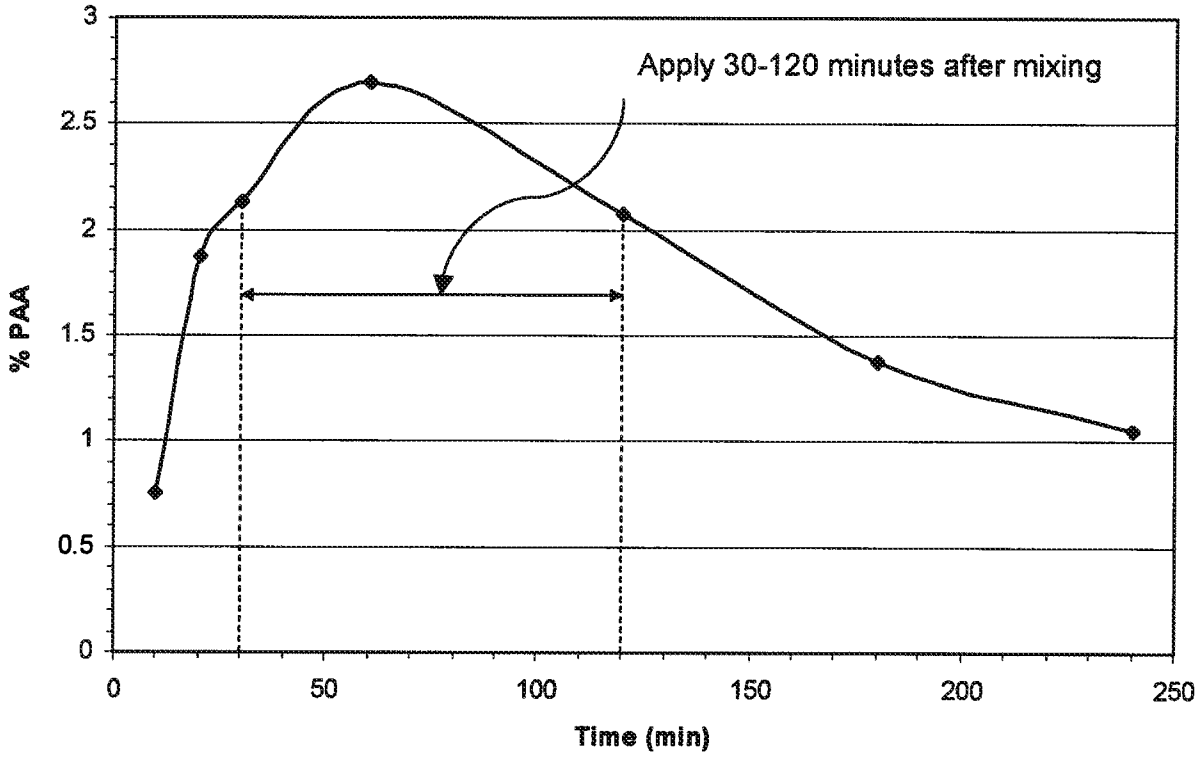
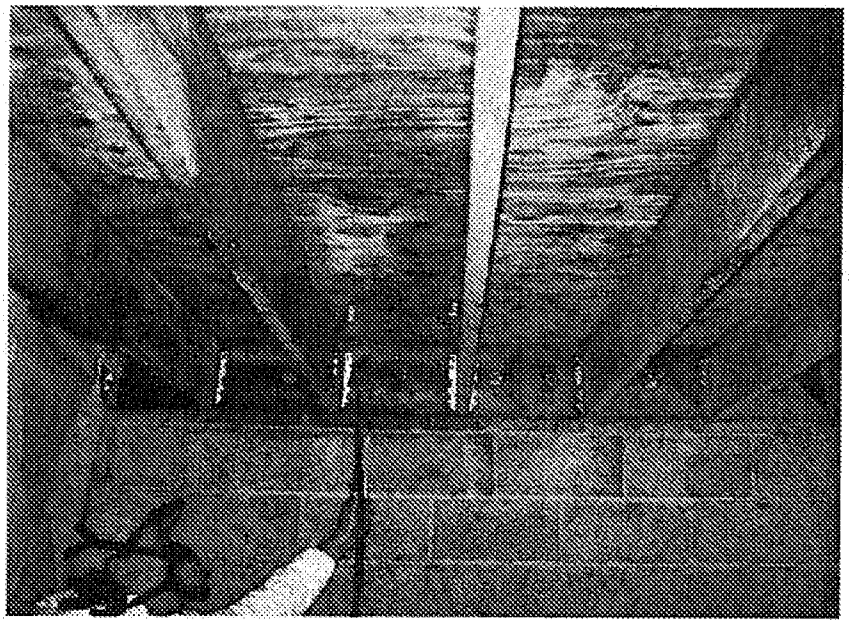


FIGURE 2A



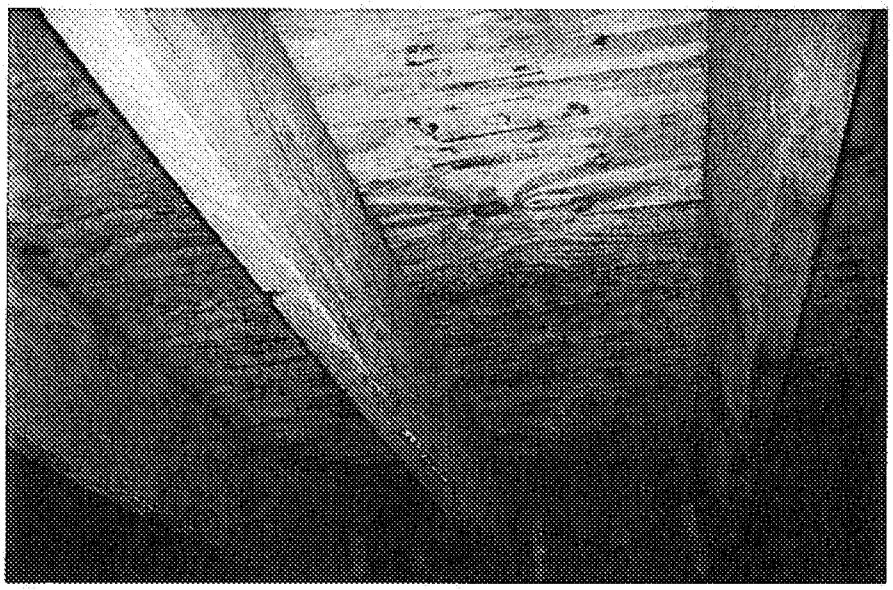


FIGURE 2B

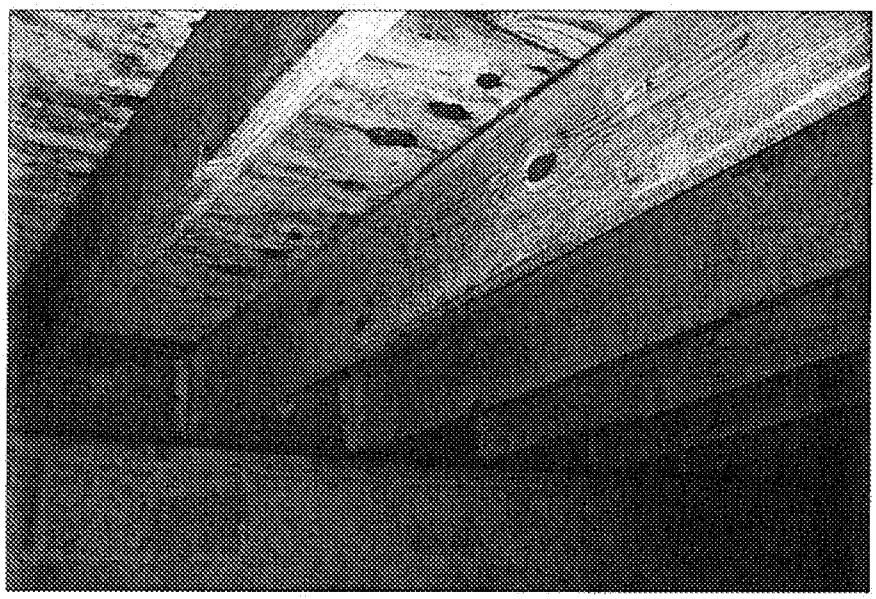


FIGURE 2C

FIGURE 3

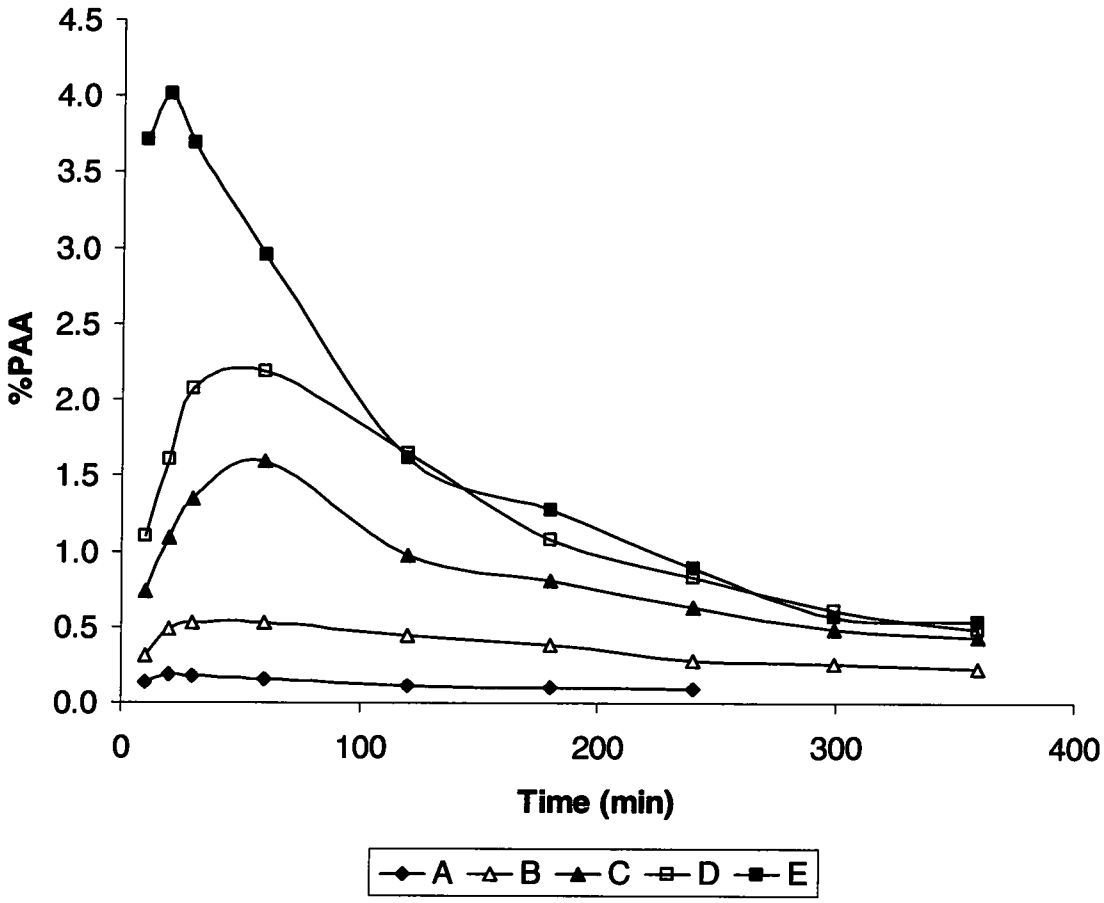
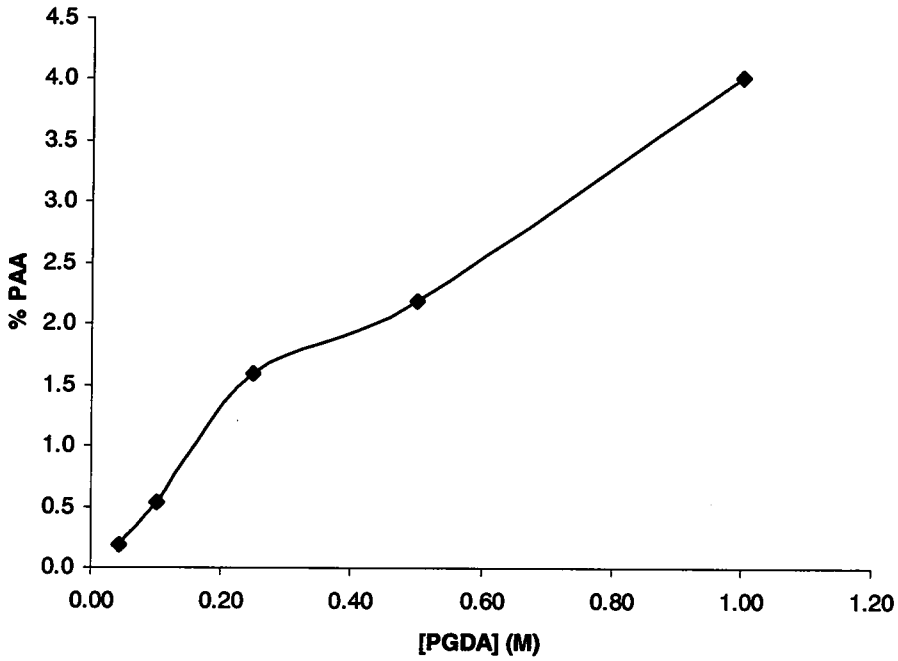


FIGURE 4



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/021646

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/00 C11D3/386 C12N9/16 A61L2/18 C12S9/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N C11D C12N A61L

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/067279 A1 (DU PONT [US]; DICOSIMO ROBERT [US]; GAVAGAN JOHN E [US]; PAYNE MARK [U) 28 May 2009 (2009-05-28) cited in the application	1,24
Y	paragraphs [0213], [0241]	1-26
Y	WO 2008/140988 A1 (DANISCO US INC GENENCOR DIV [US]; BARNETT CHRISTOPHER C [US]) 20 November 2008 (2008-11-20) cited in the application paragraph [0015]	1-26

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2011	18/05/2011

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/021646

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [ ] in the international application as filed

   [ ] together with the international application in electronic form

   [X] subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/021646

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