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(54) **Title**: WATER-TRIGGERED ENZYME SUSPENSION

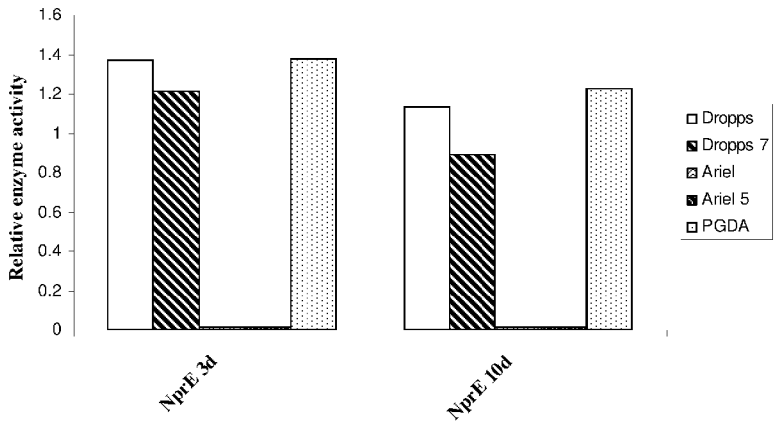

Figure 1

(57) **Abstract**: A water-triggered liquid enzyme suspension is provided. The suspension contains insoluble enzyme and a water miscible carrier liquid in a nonaqueous or low water composition. Catalytically active enzyme is released upon dilution with water.

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WATER-TRIGGERED ENZYME SUSPENSION

PRIORITY

[01] The present application claim priority to U.S. Provisional Application Serial No. 62/173,268, filed on June 9, 2015, which is hereby incorporated by reference in its entirety.

BACKGROUND

[01] Enzymes are supplied in both liquid and solid forms for incorporation within products used in a variety of consumer and industrial applications, including laundry and dish cleaning, personal care, textile treatment, pulp and paper production, leather production, food and beverage processing, starch processing, decontamination, oil and gas drilling, production of biofuels, and production (or modification) of biopolymers and other chemicals. Liquid products are in many cases preferred over powders, tablets, or other solid forms for several reasons, including solubility, convenience in handling (*e.g.*, dispensing, pouring, pumping or mixing), and compatibility with existing manufacturing processes, which are typically aqueous processes. For example, liquid enzyme concentrates are often added to liquid laundry detergent, dish detergent, or textile processing formulations. In manufacturing processes that utilize enzymes, such as starch liquefaction and textile bleaching, the storage and mix tanks, material transfer, and process operations are typically arranged to handle liquids that can be readily pumped or poured.

[02] However, liquid formulations have several disadvantages over solid formulations. Liquid enzyme formulations are typically aqueous, and enzymes are often biochemically less stable in aqueous liquids than in a dry state. In the aqueous state, undesirable reactions (*e.g.*, proteolysis, premature catalytic conversion of substrates, loss of cofactors, oxidation) often occur at unacceptable rates. Liquid formulations can also exhibit signs of physical instability, including the formation of precipitates, crystals, gels, or turbidity, during extended storage. Aqueous liquids are also more susceptible to microbial contamination than are dry materials. Finally, blending of two or more enzymes in a single liquid formulation is frequently not feasible due to incompatibilities, including the action of proteases on other enzymes, and precipitation or other undesirable physical interactions among the other components within the enzyme solutions.

[03] A number of strategies have been developed to improve the biochemical and physical stability of enzymes in aqueous liquids. In addition to storing enzymes at low temperatures, enzyme stabilizers are added to reduce activity or unfolding while keeping the enzyme soluble and physically stable. These stabilizers include buffers to adjust and maintain optimum pH, competitive or active site inhibitors, and addition of protective agents such as antioxidants and preferential exclusion agents. Preferential exclusion agents, such as sugars, sugar alcohols and other polyols, are excluded from the surface of the enzyme, reducing its tendency to unfold. At the same time, these protective agents are selected to maximize solubility of the enzyme as well as other background proteins, salts, and other species. Often proteases are removed or inactivated to minimize proteolysis. In short, the overall liquid formulation is optimized to simultaneously maximize biochemical stability of the enzyme and physical stability of the overall formulation.

[04] While enzyme stabilizers often enhance enzyme stability in aqueous liquids, they are frequently inadequate. This is the case with enzymes that are difficult to stabilize, such as a metalloprotease like thermolysin or Neutral Protease E (NprE), or in product formulations, particularly liquid cleaning compositions such as heavy duty liquid laundry detergents (HDLs), liquid dish gels, hard surface cleaners, and the like which contain chemicals such as surfactants, chelators, or bleaches. In particular, it is useful to incorporate anionic surfactants and builders or chelators in liquid cleaning compositions. Anionic surfactants provide a marked benefit in the emulsification and removal of oily and particulate soils, while builders or chelators improve the performance of these surfactants by sequestering divalent cations that would precipitate or otherwise reduce the effectiveness of these surfactants.

[05] However, anionic surfactants, such as linear alkylbenzene sulfonates (LAS) tend to destabilize enzymes by mechanisms such as unfolding, denaturation, and chelation of metal ion cofactors. To some extent, the destabilizing effects of LAS can be reduced by partially or completely substituting with more "enzyme friendly" anionic surfactants such as alcohol ethoxy sulfates (AES). However, the use of AES does not always provide satisfactory enzyme stability. Lalonde *et al.* ((1995) *J. Am. Oil Chem. Soc.*72:53-59) describe a strategy for stabilizing proteases using very high concentrations of LAS in substantially non-aqueous formulas to ensure stability. However, because the enzymes are not soluble under such conditions, the enzymes dispersions are kinetically unstable, leading to poor cleaning performance.

[06] Stabilization of enzymes is particularly challenging in liquid cleaning formulations, such as heavy duty liquid laundry detergents (HDLs), unit dose liquid laundry detergents, liquid dish gels, hard surface cleaners, and the like, in particular those which contain anionic surfactants and builders or other metal ion chelators, all of which can destabilize and inactivate enzymes by removing calcium, zinc, and other essential metal cofactors.

[07] Aside from present a challenge in terms of stability, enzymes are immunogenic molecules and can present problems relating to exposure and sensitization. In some cases, the maximum amount of enzymes that can be added to a liquid cleaning formulation is determined by exposure risk, as opposed to performance or economics.

[08] Accordingly, there is a need for a liquid enzyme suspension in which the enzyme remains stable and retains catalytic potential until use in an application in which enzyme activity is desired.

BRIEF SUMMARY OF THE INVENTION

[09] The invention provides water-triggered liquid enzyme suspensions, compositions containing the suspensions, and methods of using the suspensions. Aspects and embodiments of the invention are described in the following numbered paragraphs.

1. In one aspect, a low-water, water-triggered enzyme suspension is provided, comprising an organic carrier liquid in which one or more enzymes are substantially insoluble but capable of enzymatic activity when the suspension is diluted with at least one equal volume of water, the carrier liquid being liquid at room temperature, miscible with water, forming a single thermodynamic phase, and comprising either about 5-20% water by weight, or if anionic surfactants are present in the suspension, the amount of water plus 20% of the amount of anionic surfactants adds up to be between about 5 and 20% by weight.

2. In some embodiments of the water-triggered enzyme suspension of paragraph 1, the organic carrier liquid is selected from the group consisting of a nonionic surfactant, an anionic surfactant, an alcohol, a glycol, a polyglycol, an acetate ester, and mixtures, thereof.

3. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, the one or more enzymes are dissolved at less than 1 gram per liter in the carrier liquid for at least the first 30 days of storage at 25°C.

4. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, less than 20% of the one or more enzyme is dissolved within the carrier liquid phase.

5. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, upon dilution of the suspension with at least one volume of water the one or more enzymes exhibit at least about 50% of their original catalytic potential in less than 5 minutes at a preselected temperature.

6. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, the one or more enzymes are selected from the group consisting of acyl transferases, α-amylases, β-amylases, α-galactosidases, arabinosidases, aryl esterases, β-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-β-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, peroxygenases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, β-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, additional serine proteases, and combinations, thereof.

7. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, the one or more enzymes are provided in the form of particles or granules that include the enzymes embedded within a uniform matrix, or as part of a core surrounded by a coating.

8. In some embodiments of the water-triggered enzyme suspension of paragraph 7, the particles or granules have a density within 0.2 g/ml of the density of the liquid suspension.

9. In some embodiments of the water-triggered enzyme suspension of paragraph 7 or 8, the liquid suspension and the particles or granules all have a density between 1.0 and 1.2 g/ml.

10. In some embodiments of the water-triggered enzyme suspension of the preceding paragraphs, the water-triggered enzyme suspension is a laundry or dishwashing liquid composition.

11. In another aspect, a laundry or dishwashing liquid composition comprising the water-triggered enzyme suspension of any of paragraphs 1-10 is provided.

12. In another aspect, a method for stabilizing enzymes in a liquid composition is provided comprising suspending the enzymes in an organic carrier liquid in which the

enzymes are substantially insoluble but capable of enzymatic activity when the suspension is diluted with at least one equal volume of water, wherein the carrier liquid is liquid at room temperature, miscible with water, forms a single thermodynamic phase, and comprises either about 5-20% water by weight, or if anionic surfactants are present in the suspension, the amount of water plus 20% of the amount of anionic surfactants adds up to be between about 5 and 20% by weight.

13. In some embodiments of the method of paragraph 12, the organic carrier liquid is selected from the group consisting of a nonionic surfactant, an anionic surfactant, an alcohol, a glycol, a polyglycol, an acetate ester, and mixtures, thereof.

14. In some embodiments of the method of paragraph 12 or 13, the enzymes are dissolved at less than 1 gram per liter in the carrier liquid for at least the first 30 days of storage at 25°C.

15. In some embodiments of the method of paragraphs 12-14, less than 20% of the one or more enzyme is dissolved within the carrier liquid phase.

16. In some embodiments of the method of paragraphs 12-15, upon dilution of the suspension with at least one volume of water, the one or more enzymes exhibit at least about 50% of their original catalytic potential in less than 5 minutes at a preselected temperature.

17. In some embodiments of the method of paragraphs 12-16, the one or more enzymes are selected from the group consisting of acyl transferases, α-amylases, β-amylases, α-galactosidases, arabinosidases, aryl esterases, β-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-β-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, peroxygenases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, β-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, additional serine proteases, and combinations, thereof.

18. In some embodiments of the method of paragraphs 12-18, the one or more enzymes are provided in the form of particles or granules that include the enzymes embedded within a uniform matrix, or as part of a core surrounded by a coating.

19. In some embodiments of the method of paragraph 18, the particles or granules have a density within 0.2 g/ml of the density of the liquid suspension.

20. In some embodiments of the method of paragraphs 18 or 19, the liquid suspension and the particles or granules all have a density between 1.0 and 1.2 g/ml.

[10] These and other aspects and embodiments of the water-triggered liquid enzyme suspensions are described, below.

BRIEF DESCRIPTION OF THE DRAWINGS

[11] Figure 1: NprE stability in DROPPS® detergent, DROPPS® detergent with additional 7% water, ARIEL® detergent, ARIEL® detergent with additional 5% water and propylene glycol diacetate

[12] Figure 2: *Bacillus lentus* subtilisin stability in DROPPS® detergent, DROPPS® detergent with additional 7% water, ARIEL® detergent, ARIEL® detergent with additional 5% water and propylene glycol diacetate

[13] Figures 3A and 3B: Stability of *Cerrena unicolor* laccaseD in DROPPS® HDL

[14] Figure 4: Cleaning performance of NprE in DROPPS® detergent

[15] Figure 5: Cleaning performance of NprE in Alfonic detergent

[16] Figure 6: Stain removal performance of *Cerrena unicolor* laccaseD

[17] Figure 7 is a ternary diagram showing the final composition of stock HDLs. The three vertices of the triangle represent compositions which are 100% water, 100% anionic surfactant (linear alkylbenzene sulfonate [LAS] plus monoethanolamine salt [MEA]), and 100% "other" (alcohol ethoxylate [AE] plus triethanolamine [TEA]). Note that, for the sake of simplicity and visualization, the "anionic surfactant" and "other" components are not pure chemicals but chemical mixtures represented as pseudocomponents.

DETAILED DESCRIPTION

Introduction

[18] Described are water-triggered liquid enzyme suspensions that are storage stable and activated upon dilution with water in an aqueous application. The liquid enzyme suspensions are suitable as products, or for incorporation into other liquid products used in

applications involving dilution with water, for example, laundry detergents, dishwashing gel detergents, hard surface cleaners, and textile processing compositions. The water-triggered liquid enzyme suspensions include one or more enzymes in a carrier liquid within which the enzymes are suspended as particulate solids and within which the enzymes are substantially insoluble or undissolved.

Definitions

[19] 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. 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 they are used by those of skill in the art.

[20] It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[21] As used herein, the term "water soluble polymer" refers to a polymer that is soluble in water in in an amount of at least 5 mg/g at 25°C.

[22] As used herein, an "aqueous medium" or "aqueous solution" is a solution and/or suspension in which the solvent is primarily water (*i.e.*, the solvent is at least 50% water, at least 60% water, at least 70% water, at least 80% water, or even at least 90% water). The aqueous medium may include any number of dissolved or suspended components, including

but not limited to surfactants, salts, buffers, stabilizers, complexing agents, chelating agents, builders, metal ions, additional enzymes and substrates, and the like. Exemplary aqueous media are laundry and dishwashing wash liquors. Materials such as textiles, fabrics, dishes, kitchenware, and other materials may also be present in or in contact with the aqueous medium.

[23] As used herein, the term "low-water" indicates that a composition contains about 5% to 20% water (vol/vol).

[24] As used herein, the term "substantially non-aqueous" indicates that a composition contains about 2-5% water (vol/vol).

[25] As used herein, the term "non-aqueous" indicates that a composition contains less than about 2% water (vol/vol).

[26] As used herein, where a component is "provided in" a specified form (*e.g.*, non-aqueous, very low water, solid, and the like), this form refers to the final form as the component exists in the unit-dose package, not the form in which it may be added to another component that is then added to the unit-dose package.

[27] As used herein, the phrase "insufficient to substantially dissolve water-soluble packaging" means that a subject liquid does not dissolve more than 5% of a water-soluble material over a period of six months at room temperature (*i.e.*, 25°C).

[28] As used herein, the term "bounded" with reference to the contents of water-soluble packaging means the specified contents, whether liquid, solid, or a combination, thereof, are physically contained in a compartment, at least a portion of which is defined by water-soluble material. In some cases, the contents are fully bounded by water-soluble material, meaning that the entire compartment is defined by the water-soluble material, as in the case of a pouch made of water-soluble material. In some cases, the contents are only partially bounded by water-soluble material, meaning that only a portion of the compartment is defined by the water soluble material, and the remainder is defined by water-insoluble material, as in the case of a cup or dish covered by a lid made of water-soluble material.

[29] As used herein, the terms "suspended" and "dispersed" refer to the distribution of one component in another, for example, the distribution of a solid form of acyl substrate in water-soluble material.

[30] As used herein, "cold" water is water having a temperature between freezing and about 25°C.

[31] As used herein, "room temperature" is 25°C.

[32] As used herein, "warm" water is water having a temperature between about 26°C and about 37°C.

[33] As used herein, "hot" water is water having a temperature between about 37°C and boiling.

[34] As used herein, a "low" pH is a pH of less than about 7.

[35] As used herein, a "high" pH is a pH of greater than about 7.

[36] As used herein, the term "contacting," means bringing into physical contact, such as by placing a unit-dose package in an aqueous solution.

[37] As used herein, a "solid" form of a chemical component refers to a powder, crystals, granules, aggregates, paste or wax thereof.

[38] As used herein, a "liquid" form of a chemical component refers to a liquid, gel, or slurry.

[39] As used herein, the terms "purified" and "isolated" refer to the removal of contaminants from a sample and/or to a material (*e.g.*, a protein, nucleic acid, cell, etc.) that is removed from at least one component with which it is naturally associated. For example, these terms may refer to a material which is substantially or essentially free from components which normally accompany it as found in its native state, such as, for example, an intact biological system.

[40] As used herein, the term "spray drying" refers to a method of producing a dry powder from a liquid or slurry by rapidly drying with a hot gas, as known in the art and discussed for example in US Patent 5,423,997 and WO2008/088751A2.

[41] As used herein "d50" refers to the size of the particles measured where 50% are above or below the mid-point within the population measured.

[42] As used herein, the term "UFC Solids" refers to ultrafiltrate concentrate from a fermentor/bioreactor, and is synonymous with enzyme concentrate solids.

[43] As used herein, the term "bleaching" refers to the treatment of a material (*e.g.*, fabric, laundry, pulp, etc.) or surface for a sufficient length of time and under appropriate pH and temperature conditions to effect a brightening (*i.e.*, whitening) and/or cleaning of the material. Examples of chemicals suitable for bleaching include but are not limited to ClO_2, H_2O_2, peracids, NO_2, etc.

[44] As used herein, "cleaning compositions" and "cleaning formulations" refer to compositions that may be used for the removal of undesired compounds from items to be cleaned, such as fabric, dishes, contact lenses, other solid substrates, hair (shampoos), skin

(soaps and creams), teeth (mouthwashes, toothpastes) etc. The term encompasses any materials/compounds selected for the particular type of cleaning composition desired. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use.

[45] The terms further refer to any composition that is suited for cleaning, bleaching, disinfecting, and/or sterilizing any object and/or surface. It is intended that the terms include, but are not limited to detergent compositions (*e.g.*, laundry detergents and fine fabric detergents; hard surface cleaning formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; fabric fresheners; fabric softeners; and textile and laundry pre-spotters, as well as dish detergents).

[46] As used herein, the terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some preferred embodiments, the term is used in reference to laundering fabrics and/or garments (*e.g.*, "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (*e.g.*, "dishwashing detergents").

[47] As used herein, the term "nonionic surfactant" refers to a surfactant molecule with a non-electrically charged polar group.

[48] As used herein, the term "anionic surfactant" refers to a surfactant molecule with a negatively charged polar group at the pH of the composition or the application of use. Salts used to complex or neutralize the surfactant, *e.g.*, forming the monoethanolamine (MEA) salt of linear alkylbenzene sulfonate (LAS) are included I accounting herein for the mass or concentration of anionic surfactant.

[49] As used herein, the phrase "detergent stability" refers to the stability of a detergent composition. In some embodiments, the stability is assessed during the use of the detergent, while in other embodiments, the term refers to the stability of a detergent composition during storage.

[50] As used herein, the term "disinfecting" refers to the removal of contaminants from the surfaces, as well as the inhibition or killing of microbes on the surfaces of items. It is not intended that the present invention be limited to any particular surface, item, or contaminant(s) or microbes to be removed.

[51] As used herein the term "hard surface cleaning composition" refers to detergent compositions for cleaning hard surfaces such as floors, walls, tile, bath and kitchen fixtures, and the like.

[52] As used herein, "non-fabric cleaning compositions" encompass hard surface cleaning compositions, dishwashing compositions, personal care cleaning compositions (*e.g.*, oral cleaning compositions, denture cleaning compositions, personal cleansing compositions, etc.), and compositions suitable for use in the pulp and paper industry.

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

[54] As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length and any three-dimensional structure and single- or multi-stranded (*e.g.*, single-stranded, double-stranded, triple-helical, etc.), which contain deoxyribonucleotides, ribonucleotides, and/or analogs or modified forms of deoxyribonucleotides or ribonucleotides, including modified nucleotides or bases or their analogs. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present invention encompasses polynucleotides which encode a particular amino acid sequence. Any type of modified nucleotide or nucleotide analog may be used, so long as the polynucleotide retains the desired functionality under conditions of use, including modifications that increase nuclease resistance (*e.g.*, deoxy, 2'-O--Me, phosphorothioates, etc.). Labels may also be incorporated for purposes of detection or capture, for example, radioactive or nonradioactive labels or anchors, *e.g.*, biotin. The term polynucleotide also includes peptide nucleic acids (PNA). Polynucleotides may be naturally occurring or non-naturally occurring. The terms "polynucleotide" and "nucleic acid" and "oligonucleotide" are used herein interchangeably. Polynucleotides of the invention may contain RNA, DNA, or both, and/or modified forms and/or analogs thereof. A sequence of nucleotides may be interrupted by non-nucleotide components. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2

("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. Polynucleotides may be linear or circular or comprise a combination of linear and circular portions.

[55] As used herein, "polypeptide" refers to any composition comprised of amino acids and recognized as a protein by those of skill in the art. The conventional one-letter or three-letter code for amino acid residues is used herein. The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

[56] Related (and derivative) proteins encompass "variant" proteins. Variant proteins differ from a parent protein and/or from one another 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.

[57] In some embodiments, related proteins, such as 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.

[58] 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.

[59] As used herein, the term "a contaminated item" or "item in need of decontamination" refers to any item or thing in contact or associated with a contaminant and/or which needs to be decontaminated. It is not intended that the item be limited to any particular thing or type of item. For example, in some embodiments, the item is a hard surface, while in other embodiments, the item is an article of clothing. In yet additional

embodiments, the item is a textile. In yet further embodiments, the item is used in the medical and/or veterinary fields. In some preferred embodiments, the item is a surgical instrument. In further embodiments, the item is used in transportation (*e.g.*, roads, runways, railways, trains, cars, planes, ships, etc.). In further embodiments, the term is used in reference to food and/or feedstuffs, including but not limited to meat, meat by-products, fish, seafood, vegetables, fruits, dairy products, grains, baking products, silage, hays, forage, etc. Indeed, it is intended that the term encompass any item that is suitable for decontamination using the methods and compositions provided herein.

[60] As used herein, the term "decontamination" refers to the removal of substantially all or all contaminants from a contaminated item. In some preferred embodiments, decontamination encompasses disinfection, while in other embodiments, the term encompasses sterilization. However, it is not intended that the term be limited to these embodiments, as the term is intended to encompass the removal of inanimate contaminants, as well as microbial contamination. (*e.g.,* bacterial, fungal, viral, prions, etc.).

[61] As used herein, the term "disinfecting" refers to the removal of contaminants from the surfaces, as well as the inhibition or killing of microbes on the surfaces of items. It is not intended that the present invention be limited to any particular surface, item, or contaminant(s) or microbes to be removed.

[62] As used herein, the term "sterilizing" refers to the killing of all microbial organisms on a surface.

[63] As used herein, the term "sporicidal" refers to the killing of microbial spores, including but not limited to fungal and bacterial spores. The term encompasses compositions that are effective in preventing germination of spores, as well as those compositions that render spores completely non-viable.

[64] As used herein, the terms "bactericidal," "fungicidal," and "viricidal" refer to compositions that kill bacteria, fungi, and viruses, respectively. The term "microbiocidal" refers to compositions that inhibit the growth and/or replication of any microorganisms, including but not limited to bacteria, fungi, viruses, protozoa, rickettsia, etc.

[65] As used herein, the terms "bacteriostatic," "fungistatic," and "virostatic" refer to compositions that inhibit the growth and/or replication of bacteria, fungi, and viruses, respectively. The term "microbiostatic" refers to compositions that inhibit the growth and/or replication of any microorganisms, including but not limited to bacteria, fungi, viruses, protozoa, rickettsia, etc.

[66] The terms "recovered," "isolated," "purified," and "separated" as used herein refer to a material (*e.g.*, a protein, nucleic acid, or cell) that is removed from at least one component with which it is naturally associated. For example, these terms may refer to a material which is substantially or essentially free from components which normally accompany it as found in its native state, such as, for example, an intact biological system.

[67] The term "water-triggered" as used herein refers to an enzyme suspension that exhibits at least a 2-fold, often at least a 10-fold, more often at least a 100-fold increase in catalytic enzymatic activity when diluted with at least one equal volume of water.

[68] "Water miscible" as used herein refers to a liquid forming a single thermodynamic liquid phase or isotropic phase upon mixing with water, at a specified ratio of water to the liquid.

[69] A "suspension" or "dispersion" as used herein refers to a two phase system wherein a discontinuous solid phase is dispersed within a continuous liquid phase. The solid phase can consist of very fine particles or larger granules, and the particles or granules can have a wide variety of shapes, morphologies and structures. For example, the solids can be spray dried particles as small as 1 micron or core-shell granules between 100 and 1,000 microns in diameter.

[70] A "suspension aid" as used herein refers to a material added to a liquid composition to prevent or reduce sedimentation or floating of suspended particles. Suspension aids typically work by increasing either the viscosity or the yield stress of a carrier liquid. Fluids with a significant yield stress will flow only when stress is applied which is greater than the yield stress, and thus exhibit shear-thinning or thixotropic behavior. Effective suspension agents typically act by forming a reversible network of particles or fibers bridged by weak forces. Examples of suspending agents include, but are not limited to, xanthan gum and microfibrous cellulose, *e.g.*, CELLULON® (CP Kelco, San Diego, CA).

[71] The terms "immunogenicity," "immunogenenic," and related terms refers to the ability of an immunogen, *e.g.*, an α-amylase polypeptide, to initiate or perpetuate an immune reaction in an animal, thereby causing the animal to develop sensitivity to the immunogen, resulting in the need to avoid or reduce further contact with the immunogen.

[72] The term "less immunogenic" means a given composition has a reduced potential to initiate or perpetuate and immune response in a population of animals.

[73] The phrase "humans having contact with the detergent composition" refers to any number of workers at a detergent manufacturing site or consumers who are exposed to a

given detergent composition, including exposure to granules, liquids, and aerosols, such that they have a potential to develop an immune response to components of the composition.

[74]

°C	degrees Centigrade
AU	activity units
$CaCl_2$	calcium chloride
Cm	centimeter
cm^3	cubic centimeters
D(0.5)	median particle size where 50% of the particles are at or below the specified diameter
D(0.9)	median particle size where 90% of the particles are at or below the specified diameter
dH_2O or DI	deionized water
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
eq.	equivalents
ETOH	ethanol
g or gm	Grams (note, below)
H_2O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
kDa	kiloDalton
kg	kilograms
M	molar
melting temperature	melting temperature
MES	4-morpholineethanesulfonic acid
mg	milligrams
min	minute
mL and ml	milliliters
mm	millimeters
mM	millimolar
MW	molecular weight
MWCO	molecular weight cut-off
N	normal
Na_2SO_4	sodium sulfate
NaOH	sodium hydroxide
nm	nanometer
NOBS	nonanoyloxybenzenesulfonate
OD_x	optical density at x nanometers wavelength
PE	polyethylene
PEG	polyethyleneglycol
ppm	parts per million
PVA	poly(vinyl alcohol)
PVP	poly(vinylpyrrolidone)

RFU/sec	relative fluorescence units per second
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
SP1	spray 1
SP2	spray 2
SP3	spray 3
TiO_2	titanium dioxide
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
U	units
V	Volts
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
wt%	weight percent
µg	micrograms
µL and µl	microliters
µm	micrometer
µM	micromolar

Low-water, water-triggered enzyme suspensions enzyme suspensions

[75] The present liquid, low-water, water-triggered enzyme suspensions contain an organic carrier liquid in which one or more enzymes are substantially insoluble or undissolved by virtue of entrapment or encapsulation within a polymeric matrix or polymeric coating, but capable of enzymatic activity when the suspensions are diluted with at least one equal volume of water. The carrier liquid is an organic compound or a mixture of two or more organic compounds that is liquid at room temperature, miscible with water, contains at least 5% water by weight, and forms a single thermodynamic phase. Preferably, the low-water carrier liquid consists of water-miscible organic solvents and between 5-20% water by weight. The carrier liquid may also contain surfactants, solvents, builders and salts. The surfactants are preferably nonionic surfactants (such as alcohol ethoxylates) and anionic surfactants (such as linear alkylbenzene sulfonate). The solvents are preferably glycols (such as propylene glycol), glycerol, sugar alcohols (such as sorbitol), or organic acids (such as citric acid).

[76] If the carrier liquid contains anionic surfactants, then the total concentration of water plus 20% of the anionic surfactants should be between 5 and 20% by weight, i.e.

$$\% \text{ water} + \% \text{ anionics}/5 = 5 \text{ to } 20\%$$

[77] Low-water carrier liquids that contain modest concentrations of water and anionic surfactants can provide two simultaneous benefits. First, they have sufficient solvating

power to enable a homogeneous and thermodynamically miscible liquid phase to be produced. Second, they allow sufficiently low enzyme solubility to ensure excellent enzyme stability by projecting the majority of the enzyme from potentially harsh and denaturing conditions in the carrier liquid. Non-aqueous and substantially non-aqueous carrier liquids typically present challenges in formulating a homogenous liquid composition because they contain too little water, anionic surfactants, or other chemicals that promote miscibility and compatibility of detergent ingredients.

[78] In the present water-triggered enzyme suspensions, enzymes are dissolved at less than 1 gram per liter in the carrier liquid for at least the first 30 days of storage at 25°C, and less than 20% of the enzyme is dissolved within the carrier liquid phase. The enzyme are catalytically active upon dilution of the suspension with at least one volume of water and exhibit most of their original catalytic potential within minutes of dilution. In some embodiments, the enzymes exhibit at least about 50, 60, 70, 80, 90, 95% or essentially all of their original catalytic potential in less than 1, less than 2, less than 3, less than 4, or less than 5 minutes at a preselected temperature. For example, where a laundry detergent is selected for use in cold water or in hot water, the liquid enzyme suspension is formulated accordingly.

[79] Low concentrations of enzyme in solution within the carrier liquid can be effected by the inherent water solubility properties of the selected enzymes. Low dissolved concentration of enzyme within the carrier liquid can also be effected by formulating the solid enzyme within a polymeric matrix or within a polymeric coating. Suitable polymers are water soluble, but are insoluble in the low-water carrier liquids, examples include polyvinyl alcohols.

[80] In some embodiments, the non-aqueous carrier liquid is selected from a nonionic surfactant, an anionic surfactant, an alcohol, a glycol, a polyglycol, and an acetate ester, or a mixture, thereof. In some embodiments, the carrier liquid is an alcohol ethoxylate nonionic surfactant. In some embodiments, the carrier liquid is an anionic surfactant, for example a linear alkyl benzene sulfonate (LAS) or an alkyl ethoxy sulfate (AES) or a mixture thereof. In some embodiments, the carrier liquid is a short chain alcohol, for example, isopropyl alcohol. In some embodiments, the carrier liquid is a glycol, for example, an alkylene glycol such as hexylene glycol or propylene glycol. In some embodiments, the carrier liquid is a polyglycol, for example, a polyethylene glycol or polypropylene glycol, with a molecular weight of 200 to 100,000, or a copolymer thereof. In some embodiments, the carrier liquid

is an acetate ester, for example, propylene glycol diacetate. In some embodiments, the carrier liquid includes one or more of an alcohol ethoxylate nonionic surfactant (*e.g.*, an alcohol ethoxylate with a ten or twelve carbon chain and six ether groups), a LAS or AES anionic surfactant, isopropyl alcohol, hexylene glycol, and propylene glycol diacetate. In some embodiments, the carrier liquid includes both nonionic and anionic surfactants, for example, an alcohol ethoxylate nonionic surfactant and LAS and/or AES anionic surfactants.

Enzymes for use in water-triggered liquid enzyme suspensions

[81] The present water-triggered liquid enzyme suspensions can be used with a wide variety of enzymes, including acyl transferases, α-amylases, β-amylases, α-galactosidases, arabinosidases, aryl esterases, β-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-β-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, peroxygenases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, β-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, additional serine proteases, and combinations, thereof.

[82] Examples of suitable proteases include but are not limited to subtilisins, such as those derived from *Bacillus* (*e.g.,* subtilisin, lentus, amyloliquefaciens, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168), including variants as described in, *e.g.*, U.S. Pat. Nos. RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, all of which are incorporated herein by reference. Additional protease include trypsin (*e.g.*, of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270. In some embodiments the protease is one or more of MAXATASE®, MAXACAL™, MAXAPEM™, OPTICLEAN®, OPTIMASE®, PROPERASE®, PURAFECT®, PURAFECT® OXP, PURAMAX™, EXCELLASE™, and PURAFAST™ (Genencor); ALCALASE®, SAVINASE®, PRIMASE®, DURAZYM™, POLARZYME®, OVOZYME®, KANNASE®, LIQUANASE®, NEUTRASE®, RELASE® and ESPERASE® (Novozymes); BLAP™ and BLAP™ variants (Henkel Kommanditgesellschaft auf Aktien, Duesseldorf, Germany), and KAP *(B. alkalophilus*

subtilisin; Kao Corp., Tokyo, Japan). Additional proteases are described in WO95/23221, WO 92/21760, WO 09/149200, WO 09/149144, WO 09/149145, WO 11/072099, WO 10/056640, WO 10/056653, WO 11/140364, WO 12/151534, U.S. Pat. Publ. No. 2008/0090747, and U.S. Pat. Nos. 5,801,039, 5,340,735, 5,500,364, 5,855,625, US RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628.

[83] Suitable proteases include neutral metalloproteases including those described in WO 07/044993 and WO 09/058661. Other exemplary metalloproteases include nprE, the recombinant form of neutral metalloprotease expressed in *Bacillus subtilis* (see *e.g.*, WO 07/044993), and PMN, the purified neutral metalloprotease from *Bacillus amyloliquefacients*.

[84] Suitable lipases include, but are not limited to *Humicola lanuginosa* lipase (see *e.g.*, EP 258 068, and EP 305 216), *Rhizomucor miehei* lipase (See *e.g.*, EP 238 023), Candida lipase, such as *C. antarctica* lipase (*e.g.*, the *C. antarctica* lipase A or B; See *e.g.*, EP 214 761), Pseudomonas lipases such as *P. alcaligenes* lipase and *P. pseudoalcaligenes* lipase (See *e.g.*, EP 218 272), *P. cepacia* lipase (See *e.g.*, EP 331 376), *P. stutzeri* lipase (See *e.g.*, GB 1,372,034), *P. fluorescens* lipase, *Bacillus* lipase (*e.g.*, *B. subtilis* lipase (Dartois *et al.* (1993) *Biochem. Biophys. Acta* 1131:253-260); *B. stearothermophilus* lipase (see *e.g.*, JP 64/744992); and *B. pumilus* lipase (see *e.g.*, WO 91/16422)).

[85] Additional suitable lipases include *Penicillium camembertii* lipase (Yamaguchi *et al.* (1991) Gene 103:61-67), *Geotricum candidum* lipase (See, Schimada *et al.* (1989) *J. Biochem.* 106:383-388), and various Rhizopus lipases such as *R. delemar* lipase (Hass *et al.* (1991) *Gene* 109:117-113), a *R. niveus* lipase (Kugimiya *et al.* (1992) *Biosci. Biotech. Biochem.* 56:716-719) and *R. oryzae* lipase. Additional lipases are the cutinase derived from *Pseudomonas mendocina* (See, WO 88/09367), and the cutinase derived from *Fusarium solani pisi* (WO 90/09446). Various lipases are described in WO 11/111143, WO 10/065455, WO 11/084412, WO 10/107560, WO 11/084417, WO 11/084599, WO 11/150157, and WO 13/033318. In some embodiments the protease is one or more of M1 LIPASE™, LUMA FAST™, and LIPOMAX™ (Genencor); LIPEX®, LIPOLASE® and LIPOLASE® ULTRA (Novozymes); and LIPASE P™ "Amano" (Amano Pharmaceutical Co. Ltd., Japan).

[86] Suitable amylases include, but are not limited to those of bacterial or fungal origin, or even mammalian origin. Numerous suitable are described in W09510603, WO9526397, WO9623874, WO9623873, WO9741213, WO9919467, WO0060060, WO0029560,

WO9923211, WO9946399, WO0060058, WO0060059, WO9942567, WO0114532, WO02092797, WO0166712, WO0188107, WO0196537, WO0210355, WO9402597, WO0231124, WO9943793, WO9943794, WO2004113551, WO2005001064, WO2005003311, WO0164852, WO2006063594, WO2006066594, WO2006066596, WO2006012899, WO2008092919, WO2008000825, WO2005018336, WO2005066338, WO2009140504, WO2005019443, WO2010091221, WO2010088447, WO0134784, WO2006012902, WO2006031554, WO2006136161, WO2008101894, WO2010059413, WO2011098531, WO2011080352, WO2011080353, WO2011080354, WO2011082425, WO2011082429, WO2011076123, WO2011087836, WO2011076897, WO94183314, WO9535382, WO9909183, WO9826078, WO9902702, WO9743424, WO9929876, WO9100353, WO9605295, WO9630481, WO9710342, WO2008088493, WO2009149419, WO2009061381, WO2009100102, WO2010104675, WO2010117511, WO2010115021, WO2013184577, WO9418314, WO2008112459, WO2013063460, WO10115028, WO2009061380, WO2009100102, WO2014099523, WO2015077126A1, WO2013184577, WO2014164777, PCT/US12/70334, PCT/US13/74282, PCT/CN2013/077294, PCT/CN2013/077134, PCT/CN2013/077137, PCT/CN2013/077142, PCT/CN2012/087135, PCT/US12/62209, PCT/CN2013/084808, PCT/CN2013/084809, and PCT/US14/23458. Commercially available amylases include, but are not limited to one or more of DURAMYL®, TERMAMYL®, FUNGAMYL®, STAINZYME®, STAINZYME PLUS®, STAINZYME ULTRA®, and BAN™ (Novozymes), as well as POWERASE™, RAPIDASE® and MAXAMYL® P, PREFERENZ® S100, PREFERENZ® S110, and PREFERENZ® S1000 (Genencor).

[87] Suitable cellulases include but are not limited to those having color care benefits (see *e.g.*, EP 0 495 257). Examples include *Humicola insolens* cellulases (See *e.g.*, U.S. Pat. No. 4,435,307) and ommercially available cellulases such as CELLUZYME®, CAREZYME® (Novozymes), and KAC-500(B)™ (Kao Corporation). [NEED DuPont cellulase] In some embodiments, cellulases are incorporated as portions or fragments of mature wild-type or variant cellulases, wherein a portion of the N-terminus is deleted (See *e.g.*, U.S. Pat. No. 5,874,276). Additional suitable cellulases include those found in WO2005054475, WO2005056787, U.S. Pat. No. 7,449,318, and U.S. Pat. No. 7,833,773.

[88] Suitable mannanases are described in U.S. Pat. Nos. 6,566,114, 6,602,842, 5, 476, and 775, 6,440,991, and U.S. Patent Application Number 61/739267, all of which are

incorporated herein by reference). Commercially available include, but are not limited to MANNASTAR®, PURABRITE™, and MANNAWAY®.

[89] In some embodiments, peroxidases are used in combination with hydrogen peroxide or a source thereof (*e.g.*, a percarbonate, perborate or persulfate) in the compositions of the present teachings. In some alternative embodiments, oxidases are used in combination with oxygen. Both types of enzymes are used for "solution bleaching" (i.e., to prevent transfer of a textile dye from a dyed fabric to another fabric when the fabrics are washed together in a wash liquor), preferably together with an enhancing agent (See *e.g.*, WO 94/12621 and WO 95/01426). Suitable peroxidases/oxidases include, but are not limited to those of plant, bacterial or fungal origin. Chemically or genetically modified mutants are included in some embodiments.

[90] Suitable perhydrolases include the enzyme from *Mycobacterium smegmatis.* This enzyme, its enzymatic properties, its structure, and numerous variants and homologs, thereof, are described in detail in International Patent Application Publications WO 05/056782A and WO 08/063400A, and U.S. Patent Publications US2008145353 and US2007167344, which are incorporated by reference. In some embodiments, the *Mycobacterium smegmatis* perhydrolase, or homolog, includes the S54V substitution.

[91] Other suitable perhydrolases include members of the carbohydrate family esterase family 7 (CE-7 family) described in, *e.g.*, WO2007/070609 and U.S. Patent Application Publication Nos. 2008/0176299, 2008/176783, and 2009/0005590. Members of the CE-7 family include cephalosporin C deacetylases (CAHs; E.C. 3.1.1.41) and acetyl xylan esterases (AXEs; E.C. 3.1.1.72). Members of the CE-7 esterase family share a conserved signature motif (Vincent *et al.*, *J. Mol. Biol.*, 330:593-606 (2003)).

[92] Other suitable perhydrolase enzymes include those from *Sinorhizobium meliloti*, *Mesorhizobium loti*, *Moraxella bovis*, *Agrobacterium tumefaciens*, or Prosthecobacter *dejongeii* (WO2005056782), *Pseudomonas mendocina* (U.S. Patent No. 5,389,536), or *Pseudomonas putida* (U.S. Patent Nos. 5,030,240 and 5,108,457).

[93] The enzymes may be crystalized, precipitated, spray dried, lyophilized, and/or compressed and provided in dry form, or resuspended liquid form, thereof. The enzymes may be provided as an ultrafiltration concentrate. They may be purified to a preselected level.

Enzyme-containing particles for use in water-triggered liquid enzyme suspensions

[94] The enzymes in the suspension may be provided in the form of particles or granules that include the enzymes embedded within a uniform matrix, or as a coating applied to such particles. The nominal diameter and size distribution of the particles is not critical but can be tailored to suit manufacturing, performance, safety, and other requirements. Smaller particles generally have a higher payload to core weight ratio but are more readily aerosolized. Particles smaller than 10 μm, and especially smaller than 5 μm, should be avoided for respiratory tract safety reasons. Particles smaller than about 40 μm are not visible to the human eye. Larger particles, *e.g.*, greater than about 100 μm, 150 μm, or even 200 μm, are visible to the human eye and may be brightly colored such that they are prominently visible in the enzyme suspension. Exemplary size ranges are 50-100 μm, 50-150 μm, 100-150 μm, 100-200 μm, 150-250 μm, 200-250 μm, 200-300 μm, 250-300 μm, 300-350 μm, 300-400 μm, 350-500 μm, 400-550 μm, and the like. In some cases, the size distribution range is narrow, such that the particles are uniform in size. In some cases, the size distribution is not critical.

[95] The core of a particle is preferably made from non-toxic and biodegradable materials. The core may be structured as a matrix that includes enzymes, or enzymes may be coated onto a core that either includes or does not include enzymes. Enzyme may be coated onto the core using fluid bed and other known processes to produce an enzyme/active layer.

[96] Exemplary materials for making the core include salts, such as sodium sulfate, sugars, such as sucrose, and natural or synthetic polymers, such as starch, cellulose, polyvinyl alcohol (PVA), or polyethylene glycol (PEG). The enzyme can be added to cores or layers as an unpurified fermentation broth, optionally clarified by filtration or centrfiguation to remove cell debris and insoluble solids, and optionally purified to a desired level. Cores can include fillers, buffers, stabilizers, plasticizers, distintegrants and the like. The core materials preferably dissolve or disperse in water after activation of the enzyme suspension. The cores may dissolve or disperse almost immediately or preferably within 15 min, 10 min, 5 min, 3 min, 2 min, or even 1, min following activation.

[97] In some cases, it may be desirable to include a further barrier layer to further protect the enzyme and/or to impart a desired appearance to the particles. Barrier layers may include, *e.g.*, a polymer such as PVA, optionally with a polymer modifier, surfactants, whiteners, and dyes.

[98] The particles may have a density similar to that of the carrier liquid, such that they remain uniformly suspended in the carrier liquid without substantial settling. Most aqueous liquids have a density between 1.0 g/cm^3 and 1.3 g/cm^3, depending on the dissolved solutes, and the density of the particles should be within 0.3 g/cm^3, 0.2 g/cm^3, or even 0.1 g/cm^3 of the density of the liquid.

[99] The desired density of the particles depends on the relative size of the cores compared to the overall size of the particles. A larger core represents a larger portion of the overall particle, making its density more critical. A smaller core may represent only a small portion of the overall particle, making its density less critical. The desired density of the core can be selected based on Stoke's law for calculating the settling velocity of a particle in a viscous medium:

$$v_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\mu} g R^2$$

[100] In the equation, above, v_s is the particle's settling velocity (m/s), which is vertically downwards if $\rho_p > \rho_f$ and vertically upwards if $\rho_p < \rho_f$), g is gravitational acceleration (m/s^2), ρ_p is the mass density of the particle (kg/m^3), ρ_f is the mass density of the fluid (kg/m^3), μ is the dynamic viscosity (kg/m*s) of the water liquid in which the particle is suspended, and R is the particle radius.

[101] For a given liquid composition, the viscosity (μ) is held constant, so to maintain a constant settling viscosity the required density difference scales with the square of the particle radius or diameter and the other coefficients can be ignored since they cancel out of any ratio. An exemplary particle has a diameter of 250 μm and a radius of 125 μm. For this particle, the absolute value of the density difference between particle density (ρ_p) and fluid density (ρ_f), i.e., (ρ_p-ρ_f or $\Delta\rho_{pf}$) should be no more than 0.5 g/cm^3, so any particle that is larger or smaller than 250 μm diameter is acceptable as long as the settling rate (v_s) does not increase. With the liquid medium viscosity fixed, any particle will have the same v_s when:

$$(|\Delta\rho_{pf}| * D_p^2) = (0.5)*(250)^2$$

where D_p is the overall diameter of the particle. Such a particle will not settle (or rise) faster than v_s when for the maximum density the difference is given by:

$$|\Delta\rho_{pf}| < (0.5)*(250)^2 / D_p^2$$

or

$$|\Delta\rho_{pf}| \leq 31250/D_p^2$$

[102] Expressed in another way:

$$\rho_p \leq \rho_f + 31250/D_p^2, \text{ to avoid settling}$$
$$\rho_p \geq \rho_f - 31250/D_p^2, \text{ to avoid floating}$$

[103] Using the latter formula, the maximum density difference ($|\Delta\rho_{pf}|$) required as a function of overall particle diameters (D_p) can be calculated, as shown in Table 1:

[104]

Table 1. Maximum density differences for different overall particle diameters

D_p (μm)	$\|\Delta\rho_{pf}\|$ max (g/cm^3)
50	12.5
100	3.13
150	1.39
200	0.78
250	0.50
300	0.35
350	0.26
400	0.20
500	0.13
600	0.09
700	0.06
800	0.05
900	0.04
1000	0.03

[105] The above relationship can also be extended to define the constraints on the density of the core (ρ_c) within the overall particle (ρ_p). The density of the core can be related to the density of the overall particle according to the relationship:

$$\rho_c/\rho_p = (m_c/v_c)/(m_p/v_p)$$

where m_c and m_p represent the mass of the core and mass of the overall particle, respectively, and v_p and v_c represent the respective volumes of the overall particle and the core. Rearranging:

$$\rho_c = \rho_c * m_c/m_p * (v_p/v_p)$$

[106] Expressing the volumes in terms of diameters of the core (D_c) and particle (D_p) and representing the mass fraction of the core as x_c, we obtain:

$$\rho_c = \rho_p * x_c / (D_c/D_p)^{(1/3)}$$

or we can show the particle density in terms of core density:

$$\rho_p = \rho_c *(D_c/D_p)^{(1/3)}/ x_c$$

[107] Therefore, the maximum density difference between the core and the fluid can be given by substituting the above expression to get the maximum density difference between the core and the fluid ρ_c-ρ_f or $\Delta\rho_{cf}$:

$$|\rho_p - \rho_f| \leq 18750/D_p^2$$

$$|\rho_c *(D_c/D_p)^{(1/3)}/ x_c - \rho_f| \leq 18750/D_p^2$$

[108] Therefore:

$\rho_c \leq (\rho_f + 31250/D_p^2)* x_c / (D_c/D_p)^{(1/3)}$, to minimize settling
$\rho_c \geq (\rho_f - 31250/D_p^2)* x_c / (D_c/D_p)^{(1/3)}$, to minimize floating

[109] Where larger particles are used, core density is critical and low density materials are preferable. Where smaller particles are used, the core density is less critical and higher density materials, such as salts can be used. Low density materials include sugars (*e.g.*, sucrose and sorbitol, carbohydrates (*e.g.*, starch and glycogen), saturated fatty acids (*e.g.*, stearic acid, myristic acid, palmitic acid, and their derivatives, waxes (*e.g.*, polyethylene wax), polymers (*e.g.*, polyvinyl alcohol (PVA), partially-hydrolylzed polyvinyl alcohol (PHPVA), polyethylene glycol (PEG), polyethylene oxide (PEO), polyvinylpyrrolidone (PVP), hydroxypropylmethyl cellulose (HPMC), hydroxypropylmethylcellulose (HPMC), plasticized PVA, carboxymethyl cellulose (CMC), carboxymethyl dextran (CMD), diethylaminoethyl dextran (DEAED), ethylhydroxyethyl cellulose (EHEC), hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC), hydroxyethylmethyl cellose HEMC), hydroxypropyl dextran (HPD) methyl cellulose (MC), polypropylene glycol (PPG), polypropylene oxide (PPO), polyvinylsulfuric acid (PVSA), alginates, and glycerol sulfate, having a molecular weight such that the polymer is solid at room temperature), and combinations, thereof. Higher density materials include salts, such as sodium sulfate),

[110] The core may include fillers, buffers, stabilizers, plasticizers, distintegrants, extenders, lubricants, dyes, pigments, fragrances and the like, but all such components contribute to the density of the core, and must be selected accordingly. The core may includes enzymes or enzymes may be coated onto a core that either includes or does not include enzymes.

[111] The nominal diameter and size distribution of the particles is not critical but can be tailored to suit manufacturing, performance, safety, and other requirements. Smaller particles having an enzyme/active coating generally have a higher payload to core weight ratio but are more readily aerosolized. Particles smaller than 10 μm, and especially smaller than 5 μm, should be avoided for respiratory tract safety reasons. Particles smaller than about 40 μm are not visible to the human eye. Larger particles, *e.g.*, greater than about 100 μm, 150 μm, or even 200 μm, are visible to the human eye and may be brightly colored such that they are prominently visible in the enzyme suspension. Exemplary size ranges are 50-100 μm, 50-150 μm, 100-150 μm, 100-200 μm, 150-250 μm, 200-250 μm, 200-300 μm, 250-300 μm, 300-350 μm, 300-400 μm, 350-500 μm, 400-550 μm, and the like. In some cases, the size distribution range is narrow, such that the particles are uniform in size. In some cases, the size distribution is not critical.

[112] Preferably, the cores dissolve or disperse in water within 15 min, 10 min, 5 min, 3 min, 2 min, or even 1, min following the dilution of the low-water liquid composition with at least one volume of water.

[113] The overall density of the particles can be modified by the incorporation of density modifiers. Density modifiers can be included in the core, itself, or provided in a coating layer. Density modifiers can be included in the core, itself, or provided in an enzyme/active-layer or coating layer. An advantage of providing the density modifier in an enzyme/active-layer or coating layer is that a preselected core can be fine-tuned for use in a given low-water composition simply by varying the amount of density modifier in a subsequently-applied coating.

[114] Exemplary density modifiers are materials having a density of less than 1 g/cm^3, and include starch, cellulose fibers, diatomaceous earth, feather particles, zeolites (such as used for molecular sieving), flour, milled plant derived fragments such as corn cobs, soy grit, corn syrup solids, among other small-particle, highly-porous materials. Other acceptable density modifiers include perlite and fumed silica (particularly, fumed silica that has been treated so as to be hydrophobic). It has been found that perlite and starch are especially

useful for making roughly spherical low-density granules having a diameter of less than 700 μM via a fluidized-bed spray coating process. Other possible density modifiers include fly ash, borosilicate glass hollow spheres, fused glass hollowspheres, ceramic hollowspheres, plastic hollowspheres, hollow fibers (*e.g.*, DACRON® (DuPont)), low density forms of silicates (such as sodium aluminosilicates used as flow aids for powders), low density forms of silicon dioxide (such as those used as flow aids for powders), sawdust, and/or aerogel shards.

Additional ingredients

[115] In addition to the carrier liquid and enzymes, the present water-triggered liquid enzyme suspensions may further include one or more adjuncts that are not incompatible with the carrier liquid and enzymes. The effect of the adjuvants on the performance of the water-triggered liquid enzyme suspensions can be readily tested. Exemplary adjuvants include, but are not limited to, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, photoactivators, fluorescers, fabric conditioners, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, and pH control agents, surfactants, builders, dye transfer inhibiting agents, deposition aids, catalytic materials, bleach activators, bleach boosters, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal/anti-redeposition agents, brighteners, structure elasticizing agents, fabric softeners, hydrotropes, processing aids and/or pigments. Suitable examples of such other adjuncts and levels of use are found in U.S. Patent Nos. 5,576,282, 6,306,812, 6,326,348, 6,610,642, 6,605,458, 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101 all of which are incorporated herein by reference. Representative detergent formulations useful for the present invention include the detergent formulations found in WO2013063460, WO2003010266, WO2006002755, WO2006088535, and US20110263475, all of which are hereby incorporated by reference.

Preparation of enzyme suspensions

[116] The present water-triggered liquid enzyme suspensions may be prepared starting either with preformed particulate enzyme solids, with particles containing or coated with enzymes, or with an aqueous or non-aqueous enzyme-containing liquid. When starting with preformed particulate solids or particles, the solids or particles may be dispersed within one or more liquid components of the suspension (*e.g.*, carrier liquid or carrier liquid plus water) or the liquid component(s) may be mixed into the solids.

[117] In some embodiments, particulate solids are formed by direct addition of an aqueous enzyme solution or suspension to the carrier liquid. The enzyme may be added to the carrier liquid in such a way that the enzymes will precipitate, crystallize, or otherwise form insoluble solid particles in the carrier liquid. For this purpose, it is useful to choose as a carrier liquid a water miscible organic solvent known to effectively precipitate proteins, such as, for example, polyethylene glycols of various molecular weights, alkylene glycols such as propylene glycol or hexylene glycol, short-chain alcohols such as ethanol or isopropanol, and ketones such as acetone. The ratio of solvent or polymer precipitant to water and protein concentrations at which the enzymes becomes substantially insoluble will vary with the physical properties of the precipitant and the enzyme.

[118] The efficacy and yield of precipitation using such solvents will depend on the physical properties and solublility of the target enzymes, the concentration of water concentration, the presence of other components in the suspension, and conditions such as temperature, pH, and agitation conditions. When the enzyme is added as an aqueous solution, it may be important to limit the percentage of water in the mixture in order to minimize enzyme solubility.

[119] Losses of enzyme activity may be minimized by mixing the enzyme and solvent at colder temperatures, *e.g.*, about 0°C to about 10°C, in order to reduce the tendency of the enzymes to denature. After mixing is complete, the temperature of the suspension may be raised during subsequent storage.

[120] More detailed discussion of the factors that impact the yield of protein precipitation can be found in the extensive literature on protein precipitation by means of water-miscible organic solvents and water-soluble polymers (see, *e.g.*, Robert Scopes, Protein Purification: Principles and Practice, New York: Springer-Verlag, 1982, pp. 52-60). However, it should be kept in mind that in precipitation as normally practiced, the precipitated enzyme is typically removed from the precipitant solution by solid-liquid separation methods, such as

filtration or centrifugation, and the precipitated enzyme is resolubilized into an aqueous buffer for further processing or use. In the case of the present water-triggered liquid enzyme suspensions, the precipitating solvent remains as part of the stabilizing formula in order to minimize the solubility of the enzyme in the carrier liquid, and other agents such as solvents, surfactants, and suspension aids may be added to produce a useful product.

[121] The enzyme suspension may also be treated by various processes to reduce the diameter of the suspended particles, in order to reduce the tendency for the particles to sediment during storage. For example, the suspension may be homogenized or milled, or surfactants and/or wetting agents may be added. In some embodiments, sedimentation of particles is minimized by inclusion of one or more suspending agents in the suspension. Nonlimiting examples or such suspension agents include silica particles, natural gums (*e.g.*, xanthan gum), or microfibers, such as microbial cellulose, which provides a shear-thinning network that provides a nonzero yield stress when the suspension is at rest, but which readily flows upon pouring or mixing.

Compositions containing the liquid enzyme suspensions

[122] The water-triggered liquid enzyme suspensions described herein may be included in low water compositions, such as those used for cleaning, disinfection, decontamination, textile processing, feed, and food. The compositions may 5-20% water by weight. In some embodiments, the composition containing an enzyme suspension contains any of about 5-10%, 10-15%, or 15-20% water by weight.

[123] In various embodiments, less than about 0.01, 0.1, 0.3, 0.5, or 1 g/l of an enzyme of interest is soluble, and dissolved within 30 days at room temperature, in a composition containing an enzyme suspension, and the remainder of the enzyme is insoluble or not dissolved within 30 days at room temperature. Upon dilution of the composition containing an enzyme suspension with at least one equal volume of water, at least about 50, 60, 70, 80, 90, or 95% of the enzyme of interest becomes soluble and is catalytically active in the diluted composition.

[124] In some embodiments, the detergent composition contains water, one or more nonionic surfactant(s), and one or more anionic surfactant(s) at concentrations such that the sum of the water concentration plus one fifth of the anionic surfactant concentration is about 5% to about 20% by weight, with low solubility of the enzyme(s) in the composition, *e.g.*, solubility less than about 0.01, 0.1, 0.3, 0.5, or 1.0 g/l and/or less than about 10%, 15%, or

20% by weight of total enzyme present in soluble form. In one embodiment, the water content is about 5% to about 19% and the anionic surfactant concentration is about 1% to about 5% by weight. In one embodiment, the water content is about 5% to about 15% by weight and the anionic surfactant concentration is about 1% to about 25% by weight. In one embodiment, the water content is about 5% to about 10% by weight and the anionic surfactant concentration is about 1% to about 50% by weight. In one embodiment, the water content is about 5% to about 5% by weight and the anionic surfactant is about 5% to about 75% by weight.

[125] Enzyme(s) of interest are stable in a composition containing a water-triggered enzyme suspension as described herein (*i.e.*, are catalytically active upon dilution of the suspension with at least one volume of water) for at least 9 days at 37°C. In some embodiments, an enzyme of interest is stable in the composition containing an enzyme suspension, exhibiting at least about 50, 60, 70, 80, 90, 95% or essentially all of the initial catalytic potential upon dilution in water, after about 2 weeks, 1 month, 2 months, or 3 months or longer at 25°C. In some embodiments, an enzyme of interest is stable in the composition containing an enzyme suspension, exhibiting at least about 50, 60, 70, 80, 90, 95% or essentially all of the initial catalytic potential upon dilution in water, after about 2 weeks, 1 month, 2 months, or 3 months or longer at 37°C.

[126] Where the composition is a detergent composition, it may contain one or more surfactants, builders, bleaches, bleach precursors, enzyme stabilizers, complexing agents, chelating agents, foam regulators, corrosion inhibitors, anti-electrostatic agents, dyes, perfumes, bactericides, fungicides, and activators, and any of the additional ingredient listed, above for inclusion in the water-triggered liquid enzyme suspensions.

[127] In some embodiments, the detergent composition does not contain boron or borate. In some embodiments, the detergent contains a low (*e.g.*, submillimolar) level of calcium. In some embodiments, the detergent composition contains low (*e.g.*, submillimolar) levels of period IV metals, *e.g.*, K, Ca, Mn, Fe, Co, Ni, Cu, Zn.

[128] An advantage of the present water-triggered liquid enzyme suspensions is that they allow the use of greater amounts of enzymes in a given application without creating increased risk of sensitization as the result of immunoreactivity. This is an important consideration for, *e.g.*, workers in laundry detergent manufacturing facilities and consumers of laundry detergents. In some embodiments, the use of water-triggered liquid enzyme suspensions allows the inclusion of 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, or more enzymes

that would be acceptable in a comparable detergent composition that did not include the present water-triggered liquid enzyme suspensions.

Methods of use

[129] The water-triggered liquid enzyme suspensions described herein may be used in any application where enzymatic activity is desired. Activation requires adding at least one equal volume water to a water-triggered liquid composition, wherein at least about 50, 60, 70, 80, 90, or 95% of the enzyme is soluble and catalytically active in the diluted composition. In some embodiments, the present suspensions are added to low-water liquid laundry detergent composition containing about 5% to about 20% water (vol/vol), such as PUREX® ULTRAPACKS (Henkel), FINISH® QUANTUM (Reckitt Benckiser), CLOROX™ 2 PACKS (Clorox), OXICLEAN MAX FORCE POWER PAKS (Church & Dwight), TIDE® STAIN RELEASE, CASCADE® ACTIONPACS, TIDE® and ARIEL® PODS™ and GAIN FLINGS (Procter & Gamble), ALL™ MIGHTY PACS (Sun Products), KIRKLAND SIGNATURE™ ULTRACLEAN PACS™.

[130] In some embodiments, the application is cleaning and activation is performed in a bucket or other container, including a container to be cleaned. In the case of a laundry detergent composition, containing a water-triggered liquid enzyme suspension, activation is typically performed in a washing machine. In the case of a dishwashing detergent composition, containing a water-triggered liquid enzyme suspension, activation is typically performed in a dishwasher. In the case of a textile composition, containing a water-triggered liquid enzyme suspension, activation is typically performed in a suitable bath. In the case of a food, beverage, or feed, containing a water-triggered liquid enzyme suspension, activation is performed where needed to deliver active enzyme to the site of application.

[131] The following examples are intended to illustrate, but not limit, the water-triggered liquid enzyme suspensions.

EXAMPLES

Example 1

Assays

[132] The following assays were used in the examples described below. Any deviations from the protocols provided below are indicated in the examples.

A. 2-Aminobenzoyl-L-alanylglycyl-L-leucyl-L-alanino-4-nitrobenzylamide (Abz-AGLA-Nba) assay to determine Neutral metalloprotease activity (NprE)

[133] Equipment: Temperature controlled microplate mixer (Eppendorf Thermomixer), temperature controlled microplate fluorescence reader (Molecular Devices SpectraMax M5, Gemini EM), and clear-bottom 300 µL shallow 96-well microplates (Costar).

Reagents and Solutions: 4-Morpholineethanesulfonic acid (MES, Sigma, catalog # M-3671) buffer (52.6 mM MES/NaOH, 2.6 mM $CaCl_2$, 0.00526% (v/v) Tween-80, pH 6.5), 48 mM Abz-AGLA-Nba (Bachem catalog # H-6675) stock solution prepared in dimethylformamide (DMF) stored at room temperature shielded from light, assay solution (50 mM MES, 2.5 mM $CaCl_2$, 0.005% (v/v) Tween-80, 5% DMF, 2.4mM Abz-AGLA-Nba, pH 6.5), enzyme dilution buffer (50 mM MES, 2.5 mM $CaCl_2$, 0.005% (v/v) Tween-80, pH 6.5), substrate dilution buffer (50 mM MES, 2.5 mM $CaCl_2$, 0.005% (v/v) Tween-80, 5% DMF, pH 6.5). Enzyme stock solutions were diluted with enzyme dilution buffer to a concentration of approximately 1 ppm (1 ug/mL). *B. amyloliquefaciens* neutral metalloprotease expressed in *B. subtilis* (NprE protease) was diluted to concentrations below 6 ppm (6 µg/mL).

[134] Procedure: Each enzyme dilution was assayed in triplicate. 200 µL assay solution was added in a 96-well microplate and shielded from light. The assay was started by transferring 10 µL of the working enzyme solution to the assay microplate. The solutions were mixed vigorously for 15 seconds. The assay plate was immediately transferred to the microplate reader and fluorescence intensity measurements were recorded at excitation of 350 nm and emission of 415 nm to measure the proteolytic activity as the rate of appearance of the Abz-AG product. The reader was set to calculate the rate of RFU/sec (relative fluorescence units per second).

B. N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-p-nitroanilide (AAPF-pNA) assay to determine protease activity

[135] The following reagent solutions were used:

AAPF substrate stock:160 mM (i.e., 100 mg/mL) suc-AAPF-pNA dissolved in dimethylsulfoxide (DMSO), Stability buffer: 100 mM MES (pH 5.5) with 0.005% v/v

Tween 80 (may optionally include 10 mM $CaCl_2$), Activity buffer:100 mM Tris (pH 8.5 or 8.6) with 0.005% v/v Tween-80 (may optionally include 10 mM $CaCl_2$), Assay solution (substrate stock diluted 1:100 into activity buffe)r: 1.6 mM AAPF-pNA in 100 m1M Tris (pH 8.5 or 8.6).

[136] Procedure: An enzyme standard curve was prepared by making serial dilutions of purified subtilisin protease (0.5-10 ppm) in stability buffer. Test samples were prepared to achieve protease concentrations between 1-10ppm in stability buffer. Assay solution was prepared by diluting the substrate stock 1:100 with activity buffer. 200 µL of assay solution was added to each well of a 96-well plate.

[137] The assay was performed by adding 10 µl of diluted protease enzyme solution to each well of the assay solution plate. The solutions were mixed for 10 seconds, and the absorbance change was measured at 410 nm in a microplate reader at 25°C (set in kinetic mode, over 2 minutes). The subtilisin protease activity (AU = activity units) was calculated as mOD_{415}/min x dilution factor, where mOD_{410} refers to the optical density of the reaction product multiplied times 1000 as measured at 410 nm.

C. Megazyme (Ceralpha) assay for alpha amylase activity determination

[138] This assay is a modification of the Megazyme alpha amylase assay procedure (Ceralpha method) (ICC Standard No. 303) (Megazyme International Ireland). Entire contents of one vial of the substrate, [non-reducing end-blocked p-nitrophenyl maltoheptaoside (BPNPG7, 54.5 mg)] were dissolved in 10.0 mL of distilled water. 10 µL of the substrate solution was added to wells of a 96-well plate and the plate warmed up to 40°C for 5 minutes. Ten microliters of enzyme samples (diluted in 50 mM sodium malate, 50 mM sodium chloride, 2 mM calcium chloride 0.005% sodium azide buffer pH 5.4) were added per well. A standard curve was assayed alongside each sample set. The plate contents were mixed at 900 rpm at 40°C for exactly 10 minutes. After incubation, 150 µL of stopping buffer (20% (w/v) Trizma buffer ~pH 9) was added to each well. The solution was mixed at 900 rpm for 15 seconds and the end point absorbance was read at 400 nm.

D. Para-nitrophenyl butyrate (pNB) assay to determine aryl esterase activity

[139] Aryl esterase activity was measured by hydrolysis of *p*-nitrophenylbutyrate (Sigma, N9876, 4-Nitrophenyl butyrate) dissolved in DMSO (Sigma #154938). The reaction mixture was prepared by adding 40 µL of 100 mM pNB to 10 mL of assay buffer (0.1 M Tris-HCl pH 9.2). The background rate of hydrolysis was measured before the addition of enzyme at 405 nm. The reaction was initiated by the addition of 10 µL of diluted enzyme samples to

190 μL of the reaction mixture and the change in absorbance at 410nm was measured at room temperature.

E. SDS-PAGE for protein determination

[140] For quantitative protein determination, samples were analyzed by running the NuPAGE® Bis-Tris Electrophoresis System (Invitrogen, Carlsbad, CA). NuPAGE® Novex 4-12% Bis-Tris acrylamide gels (1 mm thickness, 8 cm x 8 cm, 15-well) were used with the NuPAGE® MES [2-(N-morpholino) ethane sulfonic acid] SDS Running Buffer pH 7.3-7.7. Samples were prepared according to the manufacturer's recommendation. Typical run conditions consisted of 200 V constant for 35 minutes. After electrophoresis, the gels were stained using the SimplyBlue™ Staining kit. Stained/destained gels were imaged using an Epson Perfection 3170 Photo scanner. Images were scanned using the Epson Scan version 2.65A in professional mode at 24-bit color and 300 dpi. Analysis of gel images were performed with ImageJ image processing toolkit. The relative intensity of each protein band was determined by measuring the integral area of each protein band, using this application. The integrated area consists of the area under the curve once the baseline on both sides is subtracted. Integrated areas for test samples and aliquots of known amount for each enzyme were determined, running all samples from same enzyme on one gel. The values obtained for the known protein samples were used to create a standard curve. A single parameter linear regression fit going through the origin (0) was generated and used to calculate the amount of enzyme (ppm) in each test sample, extrapolating the amount of protein corresponding to the signal (integrated area). Results are reported as ppm protein detected.

Example 2

Enzyme samples

[141] The following enzymes were used in stability and solubility studies described herein:

1. Subtilisin (*B. lentus* subtilisin (SEQ ID NO:2) or *B. amyloliquefaciens* subtilisin BPN′-Y217L; BPN′ Swissprot Accession Number P00782) (SEQ ID NO 3))
2. α-amylase (*Bacillus licheniformis* α-amylase, US2006014265, and WO028090395) (SEQ ID NO 4)
3. Metalloprotease (*B. amyloliquefaciens* NprE, WO08/153925) (SEQ ID NO 1)
4. Arylesterase *(Mycobacterium smegmatis* perhydrolase, S54V variant, WO/2005/056782, and US2008/145353A1) (SEQ ID NO 6)

[142] When ultrafiltration concentrate (UFC) was used, UFC of the fermentation broth of enzymes derived from the fermentation of *Bacillus subtilis* were prepared as described in WO0052150. Five percent aqueous volume of each enzyme was added into various detergent mixtures when preparing samples for stability and solubility studies.

Example 3

Stability of NprE

[143] In this example, experiments to test the stability of NprE enzyme in DROPPS® detergent (Cot'n Wash, Inc., Ardmore, PA), DROPPS® detergent with additional 7% water, ARIEL® detergent (Procter & Gamble, Inc., Cincinnati, OH), ARIEL® detergent with additional 5% water and propylene glycol diacetate (PGDA) were conducted. The water content of DROPPS® detergent was measured to be 4.9% and of ARIEL® 9.5%, using the Karl Fischer titration method. To prepare the enzyme granules, liquid enzyme concentrate was spray coated onto sand cores in a fluid bed granulator. The activity stability of these granules was evaluated after storage at 37°C with 250 rpm continuous mixing for either 3 or 10 days. Samples (0.5 mL final volume) of the various stability conditions were prepared in 2 mL glass vials with rubber stoppers. At the end of each incubation period, the entire sample contents were transferred to a 15 mL conical polypropylene tube containing 9.5 mL of assay buffer for NprE assay described in Example 1, the solution was mixed thoroughly, and a sample was removed for enzyme activity determination, according to Abz-AGLA-Nba assay method described in example 1. Remaining enzyme activity was reported relative to the amount expected from by a mass balance over the spray coating operation. NprE enzyme activity was assayed using Abz-AGLA-Nba assay described in Example 1. The results are shown in Table 1 and Figure 1.

Table 1. Stability of NprE

	Relative activity 3 day	Relative activity 10 day
DROPPS®	1.4	1.1
DROPPS® 7% H2O	1.2	0.9
ARIEL®	0.0	0.0
ARIEL® 5% H2O	0.0	0.0
PGDA	1.4	1.2

Example 4

Stability of *Bacillus lentus* subtilisin

[144] In this example, experiments to test the stability of *Bacillus lentus* subtilisin enzyme in DROPPS® detergent, DROPPS® detergent with additional 7% water, ARIEL® detergent, ARIEL® detergent with additional 5% water and propylene glycol diacetate (PGDA) were conducted. The water content of DROPPS® detergent was measured to be 4.9% and of ARIEL® 9.5%, using the Karl Fischer titration method. To prepare the enzyme granules, liquid enzyme concentrate was spray coated onto sand cores in a fluid bed granulator. The activity stability of these granules was evaluated after storage at 37°C with 250 rpm continuous mixing for either 3 or 10 days. Samples (0.5mL final volume) of the various stability conditions were prepared in 2 mL glass vials with rubber stoppers. At the end of each incubation period, the entire sample contents were transferred to a 15 mL conical polypropylene tube containing 9.5 mL of assay buffer for protease assay described in Example 1, the solution was mixed thoroughly, and a sample was removed for enzyme activity determination, according to AAPF-pNA assay method described in Example 1. Remaining enzyme activity was reported relative to the amount expected from by a mass balance over the spray coating operation. *Bacillus lentus* subtilisin enzyme activity was assayed using the AAPF-pNA assay described in Example 1. The results are shown in Table 2 and Figure 2.

Table 2. Stability of *Bacillus lentus* subtilisin

	Relative activity 3 days	Relative activity 10 days
DROPPS®	1.1	0.8
DROPPS® 7% H_2O	1.0	0.8
ARIEL®	0.8	0.2
ARIEL® 5% H_2O	0.7	0.1
PGDA	1.1	0.9

Example 5

Stability of NprE in different non-aqueous solvents

[145] In this example, experiments were conducted to test the stability of NprE in a spectrum of non-aqueous solvents with varying amounts of water. The solvents evaluated were: a HDL detergent (DROPPS®), Glycol (Hexylene glycol), Non-ionic ethoxylate

surfactant (Alfonic 1012-6, C12E6), Alcohol (Isopropyl alcohol, IPA), and PGDA, with water added to achieve 0, 5, 10, 15, and 25% total water. Enzyme was added to the solvent mixtures either as a solid (enzyme coated granule described in Example 3) or as a liquid (the enzyme concentrate used to spray-coat the granules of Example 3, also described as UFC (ultrafiltration concentrate)). The enzyme-solvent samples were held at 37°C with 250 rpm continuous mixing for 9 days. Following incubation, NprE activity was measured as described in Example 1 for an aliquot of each reaction vessel. The values reported correspond to enzyme activity relative to the amount expected from a mass balance over the spray-coating operation. The results of this stability evaluation are shown in Tables 3A and 3B, panels A and B (day 9).

Table 3A. Stability of NprE added as a solid

A	**NprE granule**				
	(fraction remaining)				
%H_2O-->	**0**	**5**	**10**	**15**	**25**
DROPPS®		1.1	1.1	1.0	0.6
hexylene glycol	1.3	1.2	1.2	0.6	0.5
IPA	1.2	1.0	0.9	0.2	0.0
ALFONIC® 1012-6	1.5	1.1	1.1	1.1	0.5
PGDA	1.4	0.8	0.0	0.0	0.0
dry control	0.8	ND	ND	ND	ND

Table 3B. Stability of NprE added as a liquid

B	**NprE liquid**				
	(fraction remaining)				
%H_2O-->	**0**	**5**	**10**	**15**	**25**
DROPPS®		1.1	0.9	0.9	0.4
hexylene glycol		1.0	0.9	0.2	0.2
IPA		1.1	0.7	0.2	0.0
ALFONIC® 1012-6		1.1	1.1	1.2	0.7
PGDA		0.7	0.0	0.0	0.0

Example 6

Stability of *Bacillus lentus* subtilisin in different non-aqueous solvents

[146] In this example, experiments were conducted to test the stability of *Bacillus lentus* subtilisin in a non-aqueous solvent with varying amounts of water. The solvent evaluated was a HDL detergent (DROPPS®) with water added to achieve 0, 5, 10, 15, and 25% total water. Enzyme was added to the solvent mixtures as a solid (enzyme coated granule

described in Example 3). The enzyme-solvent samples were held at 37°C with 250 rpm continuous mixing for 9 days. Following incubation, *Bacillus lentus* subtilisin activity was measured using the AAPF-pNA assay as described in Example 1 for an aliquot of each reaction vessel. The values reported correspond to enzyme activity relative to the amount expected from a mass balance over the spray-coating operation. The results are shown in Table 4.

Table 4. Stability of *Bacillus lentus* subtilisin

	***Bacillus lentus* subtilisin granule**				
	(fraction remaining)				
%H_2O-->	**0**	**5**	**10**	**15**	**25**
DROPPS®	-	0.8	0.8	1.0	0.8

Example 7

Stability of laccase enzyme

[147] In this example, experiments were conducted to study the stability of *Cerrena unicolor* laccaseD enzyme in DROPPS® detergent. Liquid laccase concentrate was spray-coated onto starch cores in a fluid bed coating operation. These enzyme-coated granules were mixed with DROPPS® detergent and incubated overnight at room temperature. Separately, 50mg of various mediators: ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), methyl syringate (MS), PTP-10-(carboxypropyl-phenothiazine), syringamide (SA), or syringonitrile (SN) were dissolved in 1mL of DROPPS® detergent and also incubated overnight at room temperature. Following this overnight incubation, 20 µL of single mediator solutions was transferred to 6 wells of a 30 well test plate. Ten microliters of supernatant from the enzyme granule sample were added to wells in the 2nd and the 5th column of the test plate. Enzyme granules incubated overnight in DROPPS® detergent were transferred to wells in the 3rd and 6th column of the test plate. Water (150 µL) was added to wells in the 4th-6th column of the test plate. The test plates were mixed thoroughly and the wells were visually compared for laccase reaction following 10 minutes, 30 minutes and 17 hours and 2 weeks of incubation at room temperature. The extent of laccase reaction was observed visually and the plate was photographed.

[148] The results after the 2 week incubation are shown in Figure 3A. After the two week image was taken, 150 µL water was added to columns 1 through 3. The plate was mixed

thoroughly for 10 minutes and then another image was taken. These results are shown in Figure 3B.

Example 8

Stability of NprE in DROPPS® and ALFONIC® preparations

[149] In this example, experiments were conducted to test the stability of NprE in either DROPPS® detergent or non-ionic ethoxylate surfactant ALFONIC® 1012-6 (C12E6) with water added to produce 0, 5, 10, 20 and 40% total water. Enzyme was added to the solvent mixtures either as: a solid (enzyme coated granule of Example 3) or as a liquid concentrate (the enzyme concentrate used to spray-coat the granules of Example 3) in both cases homogenized to create small particles, or as a freeze-dried solid and homogenized to create small particles using an Omni International High Shear Homogenizer GLH-115. The samples were held at 37°C with 250 rpm continuous mixing for 9 days. Following incubation, NprE activity was measured as described in Example 1. The enzyme activity values reported are relative to the amount expected from a mass balance over the spray coating operation. The results are shown in Table 5, panels A and B.

Table 5A. Stability of NprE in DROPPS®

A	**DROPPS®**			
	(fraction remaining activity)			
%H2O -->	**5**	**10**	**20**	**40**
liquid concentrate	-	1.1	0.8	0.0
enzyme granule	1.3	1.3	1.0	0.3
lyophilized enzyme	1.2	0.6	0.7	0.1

Table 5B. Stability of NprE in ALFONIC®

B		**ALFONIC® 1012-6 Ethoxylate**			
		(fraction remaining activity)			
%H2O -->	**0**	**5**	**10**	**20**	**40**
liquid concentrate	-	1.1	0.9	0.7	0
enzyme granule	1.5	1.2	1.2	1.0	0
lyophilized enzyme	1.3	1.1	1.2	0.9	0

Example 9

Cleaning performance of NprE under various conditions

[150] In this example, experiments were conducted to evaluate cleaning of formulated versus freshly prepared NprE in various low-water non-ionic detergent formulas evaluated on AS-10 POM (POM= pigment, oil, and milk) and Warwick Scrubbed Grass stains, and compared to commercially available TIDE® 2X coldwater detergent (Active). Test conditions are described below. Cleaning was measured by reflectometry using Minolta Reflectometer with 50mm aperture.

[151] Stains included per leg/pot (internal replicates): 4X AS-10 POM, Lot 180**,** 4X Warwick Scrubbed Grass, pre-selected**,** 2X White Bleached Cotton EMPA 221, extra Ballast to make the fabric loading 40g/L.

[152] In 1 L Terg-o-tometer set-up: 0.942, 0.837, 0.626 mL/L DROPPS®, 0.945, 0.895, 0.795, 0.595 mL/L ALFONIC® 1012-6, 0.98 g/L TIDE® 2X Coldwater (Active), 6gpg Water Hardness, 5mM HEPES, Temperature: 90°F (32°C)

[153] Additions (1 L deionized water/pot): 0.916mL TIDE® 2X Coldwater (commercially available, Active), 400µL of 15,000gpg 3:1 Ca:Mg Water Hardness, 3.3 ml 1.5M HEPES

[154] Enzyme: NprE (UFC sample) 22.3 mg/mL, 1ppm final concentration added in all instances.

[155] Sixteen treatments (labeled A to P on table below) were run in duplicate (32 Terg pots, 6 runs) were set up: 4x swatches/pot x 32 pots = 128 swatches per stain. Conditions for treatment are provided in Table 6 and match the liquid concentrate samples described in Example 7 above.

Table 6. NprE sample preparation

Label	Sample preparation	Enzyme (µL)
A	TIDE® 2X Coldwater (Active)	-
B	DROPPS® 10% H_2O no Enzyme	-
C	DROPPS® 40% H_2O no Enzyme	-
D	ALFONIC® 5% H_2O no Enzyme	-
E	ALFONIC® 40% H_2O no Enzyme	-
F	DROPPS® 10% H_2O + NprE (fresh)	45 fresh
G	DROPPS® 40% H_2O + NprE (fresh)	45 fresh
H	DROPPS® 10% H_20 + NprE 9d	Pre-made
I	DROPPS® 20% H_20 + NprE 9d	Pre-made
J	DROPPS® 40% H_20 + NprE 9d	Pre-made
K	ALFONIC® 5% H_2O + NprE (fresh)	45 fresh
L	ALFONIC® 40% H_2O+ NprE (fresh)	45 fresh

Label	Sample preparation	Enzyme (µL)
M	ALFONIC® 5% H_2O + NprE 9d	Pre-made
N	ALFONIC® 10% H_2O + NprE 9d	Pre-made
O	ALFONIC® 20% H_2O + NprE 9d	Pre-made
P	ALFONIC® 40% H_2O + NprE 9d	Pre-made

[156] The assays were performed as follows:

1. Pre-read multi-stain swatch or 2X for individual stains and bleached cotton on top of Black Background.
2. Prepare solutions.
3. Add 1L deionized water and Water Hardness, buffer, mix.
4. Add detergent to Terg-pots.
5. Mix for 5 minutes to allow solution to reach wash temperature of 90°F.
6. Measure pH of the Tergotometer solution.
7. Add enzyme, mix, stop Tergotometer, add swatches, start Tergotometer simultaneously.
8. Mix at 100rpm for 15 minutes, measure pH.
9. Transfer swatches to 4L plastic beaker, rinse under running tap water and running deionized water (from below) for 5 minutes.
10. Place swatches in front-loading washer and run spin cycle, 1000rpm, 7min.
11. Dry using drying machine, on low heat, automatic dry.
12. Read center of soil 2X on swatches on top of Black Background and 2X White bleached cotton.

[157] The results are shown in Figure 4 (DROPPS® detergent) and Figure 5 (ALFONIC®) and in Tables 7 and 8 below.

Table 7. Cleaning performance of NprE under various conditions

		%SRI (dE) Averages	
Label	Treatment	Warwick Equest Scrubbed Grass	CFT C-10 Pigment Oil Milk Lot 180
A	TIDE® 2X CW Active	39.20%	32.10%
B	DROPPS® 10% H_2O no Enzyme	26.17%	8.77%
F	DROPPS® 10% H_2O + NprE (fresh)	35.88%	34.42%
H	DROPPS® 10% H_20 + NprE 9d	34.43%	30.65%
I	DROPPS® 20% H_20 +	32.15%	29.40%

		%SRI (dE) Averages	
Label	Treatment	Warwick Equest Scrubbed Grass	CFT C-10 Pigment Oil Milk Lot 180
	NprE 9d		
J	DROPPS® 40% H_20 + NprE 9d	27.03%	15.27%
C	DROPPS® 40% H_2O no Enzyme	23.38%	8.83%
G	DROPPS® 40% H_2O + NprE (fresh)	35.73%	32.40%

Table 8. Cleaning performance of NprE under various conditions

		%SRI (dE) Averages	
Label	Treatment	Warwick Equest Scrubbed Grass	CFT C-10 Pigment Oil Milk Lot 180
A	TIDE® 2X CW Active	39.20%	32.10%
D	ALFONIC® 5% H_2O no Enzyme	34.62%	9.21%
K	ALFONIC® 5% H_2O + NprE (fresh)	45.81%	35.89%
M	ALFONIC® 5% H_2O + NprE 9d	40.17%	30.62%
N	ALFONIC® 10% H_2O + NprE 9d	35.67%	32.06%
O	ALFONIC® 20% H_2O + NprE 9d	34.31%	30.23%
P	ALFONIC® 40% H_2O + NprE 9d	25.71%	11.93%
E	ALFONIC® 40% H_2O no Enzyme	32.00%	9.07%
L	ALFONIC® 40% H_2O+ NprE (fresh)	39.80%	35.75%

Example 10

Stain removal performance of *Cerrena unicolor* laccase D

[158] In this example, experiments were conducted to study the stain removal performance of *Cerrena unicolor* laccaseD in DROPPS® detergent after incubating enzyme in detergent at 25°C for 7 days with mediator PTP (10H-phenothiazine-10-propionic acid).

[159] Two sets of the following preparations were made per test conditions, and each preparation was incubated in a glass vial at 25°C for 7 days.

1. 200 µL of DROPPS® solution
2. 200 µL of DROPPS® solution containing 1.25 mg/mL PTP (10H-phenothiazine-10-propionic acid)
3. 200 µL of DROPPS® solution containing 1.25 mg/mL PTP + 20 mg of 10% laccase (granular, 6% payload) dissolved in water

[160] After 7 day incubation, 20 mM sodium acetate buffer (pH 6) was added to each vial and volume adjusted to 1.7 mL. Then, the contents of each vial were transferred to one well of a 12 well microtiter plate containing 5/8 inch fabric disks cut out from 100% cotton swatches stained with tomato soil (manufactured by CFT, code number C-S-20) The disks were incubated with the preparations for 1 hour at 60°C. A buffer-only condition was included and run after 7 day incubation, like other preparations.

[161] Following incubation, the treated disks were thoroughly rinsed three times with DI water, dried in the convection oven for 30 minutes, and then cooled down. Reflectometer readings of each fabric disk were taken before and after treatment using Chroma Meter CR-200 by Minolta and then total color difference (ΔE) was calculated according to the following formula:

[162] Total color difference $(\Delta E) = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)}$ (where ΔL, Δa, Δb, are differences in CIE L*, CIE a*, and CIE b* values respectively before and after treatment).

[163] Results are shown on Table 9 and in Figure 6.

Table 9. Cleaning performance of *Cerrena unicolor* laccase

	Color Difference Between Before & After Treatments							
	Delta L		Delta a		Delta b		Delta E	
Buffer	3.95	0.22	-1.55	0.10	-3.91	0.56	5.77	0.55
Dropps	5.45	0.21	-2.52	0.04	-9.28	0.89	11.05	0.84
Dropps-PTP	5.57	0.02	-2.62	0.05	-9.32	1.05	11.17	0.97
Dropps-PTP (+) Laccase	6.25	0.31	-3.18	0.03	-15.35	1.07	16.88	1.08

Example 11

Preparation of test HDLs

[164] The following reagent solutions were used: deionized water, linear alkylbenzenesulfonic acid (H-LAS) anionic surfactant solution (Stepan Bio-Soft S-101), alcohol ethoxylate (AE) nonionic surfactant (Sasol Alfonic 1412-7) (melted by heating to ~37°C), monoethanolamine (MEA), and triethanolamine (TEA).

[165] Procedure: H-LAS solution was neutralized with MEA to form MEA-LAS as per the manufacturer's instructions. This formed a thick paste. MEA-LAS was added to AE to produce stock solutions of the desired anionic: nonionic surfactant ratios. The mixtures were heated in the water bath (80-95°C) with periodic shaking or stirring to break up chunks of MES-LAS paste. Once the MEA-LAS completely dissolved, a 1% solution was titrated by adding small aliquots of MEA to obtain a pH between 8.0 and 9.0 at ~25°C. Deionized

water was added to the titrated solution to create solutions with the desired anionic: nonionic: water ratios. One part of TEA was added to 10 parts of the [anionic + surfactant + water] solution. This solution was heated and mixed thoroughly. Each HDL stock solution (Table 10) was equilibrated to 37°C. A ternary diagram showing the final composition of the HDL stock solutions is shown in Figure 7. In this diagram the TEA and nonionic surfactant were treated as a single pseudocomponent shown as "other (AE + TEA)."

Table 10. Composition and phase behavior of the HDL stock solutions

HDL stock	nonionic surfactant (AE)	anionic surfactant (LAS, MEA salt)	water	TEA	Phase behavior liquid (l) or gel (g)
1A	91%	0%	0%	9%	l
1B	82%	9%	0%	9%	l
1C	73%	18%	0%	9%	l
1D	64%	27%	0%	9%	l
1E	55%	36%	0%	9%	l
1F	45%	45%	0%	9%	l
1G	36%	55%	0%	9%	l
1H	27%	64%	0%	9%	l
1I	18%	73%	0%	9%	g
2A	82%	0%	9%	9%	l
2B	74%	8%	9%	9%	l
2C	65%	16%	9%	9%	l
2D	57%	25%	9%	9%	l
2E	49%	33%	9%	9%	l
2F	41%	41%	9%	9%	l
2G	33%	49%	9%	9%	l
3A	73%	0%	18%	9%	l
3B	65%	7%	18%	9%	l
3C	58%	15%	18%	9%	l
3D	51%	22%	18%	9%	l
3E	44%	29%	18%	9%	l
3F	36%	36%	18%	9%	l
3G	29%	44%	18%	9%	l
4A	64%	0%	27%	9%	l
4B	57%	6%	27%	9%	l
4C	51%	13%	27%	9%	l
4D	45%	19%	27%	9%	l
4E	38%	25%	27%	9%	l

HDL stock	nonionic surfactant (AE)	anionic surfactant (LAS, MEA salt)	water	TEA	Phase behavior liquid (l) or gel (g)
4F	32%	32%	27%	9%	l
4G	25%	38%	27%	9%	l
5A	55%	0%	36%	9%	g
5B	49%	5%	36%	9%	g
5C	44%	11%	36%	9%	g
5D	38%	16%	36%	9%	g
5E	33%	22%	36%	9%	g
5F	27%	27%	36%	9%	l
5G	22%	33%	36%	9%	l
6A	45%	0%	45%	9%	g
6B	41%	5%	45%	9%	g
6C	36%	9%	45%	9%	g
6D	32%	14%	45%	9%	g
6E	27%	18%	45%	9%	g
6F	23%	23%	45%	9%	g
6G	18%	27%	45%	9%	g

Example 12

Subtilisin stability in detergents

[166] In this example, experiments to test the stability of BPN' subtilisin enzyme in various detergents were conducted. 25 µL of subtilisin concentrate was added to 475 µL of HDL stock solutions 1A, 2B, 3C, 4D, 1B, 1C, 1D, 1E, and 1F (from Example 11), mixed using a pipet tip, and incubated at 37°C without agitation for 6 and 50 days. At the end of each incubation period, samples were centrifuged, forcing the enzyme precipitate to form pellets. The pellets were washed with 2 × 500 µL aliquots of nonionic surfactant (Alfonic 1012-6), resuspended in 1000 µL of 100 mM Tris (pH 8.6) with 0.005% v/v Tween-80 and 10 mM $CaCl_2$ and vortexed until it was completely dissolved. Subtilisin enzyme activity in the pellet and the supernatant was assayed using the AAPF-pNA assay described in Example 1, n=1 per timepoint. Stability results determined by measuring enzyme activity are shown in Table 11.

[167] In some cases, enzyme activity in the post-addition sample was different (oftentimes lower) from the enzyme activity expected from dilution of the input material. The causes for this initial recovery loss were not investigated.] The trend of sample stability was followed from the post-addition timepoint (<24h post HDL/enzyme mixing) over time, with sampling

at 6 days and 50 days to determine the level of enzyme stability in each of the evaluated HDL formulations.

Table 11. Time course stability study for subtilisin in various HDLs

Test HDL Compositions			Subtilisin activity (observed ppm)[1]					
including added enzyme[2]			insoluble portion (pellet)			soluble portion (supernatant)		
(% w/w)			*37°C incubation*			*37°C incubation*		
Stock HDL	Water	Anionic surfactant	*post addition*	*day 6*	*day 50*	*post addition*	*day 6*	*day 50*
1A	5%	0%	2395	2187	1439	ND[3]	ND	ND
2B	14%	8%	2248	2114	832	10	ND	ND
3C	23%	14%	1808	579	31	140	531	77
4D	32%	18%	597	10	ND	580	589	59
1B	5%	9%	2242	2092	1415	ND	ND	ND
1C	5%	17%	2206	2355	1674	3	ND	ND
1D	5%	26%	2145	2305	1605	18	ND	33
1E	5%	35%	1832	2050	1277	209	110	124
1F	5%	43%	2012	1865	988	241	261	109

Notes:
[1]The total amount of Subtilisin (enzyme activity) initially delivered to each test HDL was 3250 ppm
[2]One part aqueous Subtilisin concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 13

Amylase stability in detergents

[168] In this example, experiments to test the stability of alpha amylase enzyme in various detergents were conducted. 25 µL of amylase was added to 475 µL of HDL stock detergents 1A, 2B, 3C, 4D, 1B, 1C, 1D, 1E, and 1F (from Example 11), mixed using a pipet tip and incubated at 37°C without agitation for 12 and 52 days. At the end of each incubation period, samples were centrifuged, forcing the enzyme precipitate to form pellets. The pellets were washed with 2 × 500 µL aliquots of nonionic surfactant (Alfonic 1012-6), resuspended in assay buffer and amylase enzyme activity in the pellet and soluble portion (supernatant) was assayed using the Megazyme (Ceralpha) assay described in Example 1. Stability results determined by measuring enzyme activity are shown in Table 12.

[169] In some cases, enzyme activity in the post-addition sample was different from the enzyme activity measured for the input material. The causes for this initial recovery

difference were not investigated, but may be attributed to sampling volume or assay conditions. The trend of sample stability was followed from the post-addition timepoint (<24h post HDL/enzyme mixing) over time, with sampling at 12 days and 52 days to determine the level of enzyme stability in each of the evaluated HDL formulations.

Table 12. Time course stability study for alpha amylase in various HDLs

Test HDL Compositions			Alpha amylase activity (observed ppm)[1]					
including added enzyme[2]			insoluble portion (pellet)			soluble portion (supernatant)		
(% w/w)			*37°C incubation*			*37°C incubation*		
Stock HDL	Water	Anionic surfactant	*post addition*	*day 12*	*day 52*	*post addition*	*day 12*	*day 52*
1A	5%	0%	8671	10515	7802	28	10	ND[3]
2B	14%	8%	11894	12340	5927	12	10	ND
3C	23%	14%	12378	12155	5886	22	16	2
4D	32%	18%	9677	11979	5258	122	23	18
1B	5%	9%	12984	12139	5807	10	10	7
1C	5%	17%	4514	10881	5390	11	10	9
1D	5%	26%	12376	10904	5437	10	10	ND
1E	5%	35%	12935	11096	5262	12	10	ND
1F	5%	43%	13080	12828	3767	12	10	ND

Notes:
[1]The total amount of Alpha amylase initially delivered to each test HDL was 9555 ppm.
[2]One part aqueous Alpha amylase concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 14

Metalloprotease stability in detergents

[170] In this example, experiments to test the stability of metalloprotease in various detergents were conducted. 25 µL of metalloprotease was added to 475 µL of HDL stock detergents 1A, 2B, 3C, 4D, 1B, 1C, 1D, 1E, and 1F (from Example 11), mixed using a pipet tip and incubated at 37°C without agitation for 5, and 50 days. At the end of each incubation period, samples were centrifuged, forcing the enzyme precipitate to form pellets. The pellets were washed with 2 × 500 µL aliquots of nonionic surfactant (Alfonic 1012-6), resuspended in assay buffer and metalloprotease enzyme activity in the pellet and soluble portion (supernatant) was assayed using Abz-AGLA-Nba assay described in Example 1. Stability results determined by measuring enzyme activity are shown in Table 13.

[171] In some cases, enzyme activity in the post-addition sample was different (oftentimes lower) from the enzyme activity measured for the input material. The causes for this initial recovery loss were not investigated. The trend of sample stability was followed from the post-addition timepoint (<24h post HDL/enzyme mixing) over time, with sampling at 5 days and 50 days to determine the level of enzyme stability in each of the evaluated HDL formulations.

Table 13. Time course stability study for Metalloprotease in various HDLs

Test HDL Compositions			Metalloprotease activity (observed ppm)[1]					
including added enzyme[2]			insoluble portion (pellet)			soluble portion (supernatant)		
(% w/w)			*37°C incubation*			*37°C incubation*		
Stock HDL	Water	Anionic surfactant	*post addition*	*day 5*	*day 50*	*post addition*	*day 5*	*day 50*
1A	5%	0%	880	987	446	ND[3]	ND	ND
2B	14%	8%	869	881	305	9	ND	ND
3C	23%	14%	739	293	ND	198	ND	ND
4D	32%	18%	150	ND	ND	568	ND	ND
1B	5%	9%	952	905	322	ND	ND	ND
1C	5%	17%	937	919	498	ND	ND	ND
1D	5%	26%	747	839	709	37	3	ND
1E	5%	35%	884	702	451	30	ND	ND
1F	5%	43%	663	654	458	266	ND	ND

Notes:
[1]The total amount of Metalloprotease initially delivered to each test HDL was 1115 ppm.
[2]One part aqueous Metalloprotease concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 15

Aryl esterase stability in detergents

[172] In this example, experiments to test the stability of aryl esterase enzyme in various detergents were conducted. 25 µL of aryl esterase was added to 475 µL of HDL stock detergents 1A, 2B, 3C, 4D, 1B, 1C, 1D, 1E, and 1F (from Example 11), mixed using a pipet tip and incubated at 37°C without agitation for 13, and 51 days . At the end of each incubation period, samples were centrifuged, forcing the enzyme precipitate to form pellets. The pellets were washed with 2 × 500 µL aliquots of nonionic surfactant (Alfonic 1012-6),

resuspended in assay buffer and aryl esterase activity in the pellet and soluble portion (supernatant) was assayed using para-nitrophenyl butyrate (pNB) assay described in Example 1. Stability results determined by measuring enzyme activity are shown on Table 14.

[173] In some cases, enzyme activity in the post-addition sample was different from the enzyme activity measured for the input material. The causes for this initial recovery difference were not investigated, but may be attributed to sampling volume or assay conditions. The trend of sample stability was followed from post-addition timepoint (<24h post HDL/enzyme mixing) over time, with sampling at 13 days and 51 days to determine the level of enzyme stability in each of the evaluated HDL formulations.

Table 14. Time course stability study for aryl esterase in various HDLs

Test HDL Compositions including added enzyme[2] (% w/w)			Aryl esterase activity (observed ppm)[1] insoluble portion (pellet) *37°C incubation*			soluble portion (supernatant) *37°C incubation*		
Stock HDL	Water	Anionic surfactant	*post addition*	*day 13*	*day 51*	*post addition*	*day 13*	*day 51*
1A	5%	0%	1777	1364	1262	ND	ND	ND
2B	14%	8%	1767	1498	1412	ND	ND	ND
3C	23%	14%	1633	1323	1527	5	5	2
4D	32%	18%	1591	1013	1183	28	8	2
1B	5%	9%	1540	1385	1310	ND	ND	ND
1C	5%	17%	1602	1333	1099	3	3	2
1D	5%	26%	1529	1467	1268	7	8	2
1E	5%	35%	1519	1375	1346	13	12	9
1F	5%	43%	1653	962	1033	20	16	27

Notes:
[1]The total amount of Aryl esterase initially delivered to each test HDL was 1385 ppm.
[2]One part aqueous Aryl esterase concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 16

Subtilisin solubility in detergents

[174] Using the "post addition" samples, the solubility of BPN′ subtilisin enzyme in various test HDL compositions (from Example 11) was measured by SDS-PAGE. The

reconstituted solid phase enzyme (pellet) and solution phase enzyme (supernatant) were prepared as described in Example 12. These samples were inactivated by dilution into strong acid, i.e., by adding 200 µL of 1N HCl to 780 µL of water, then adding 20 µL of sample. Separately, standards containing known amounts of subtilisin were prepared. The solid phase samples, solution phase samples, and standards were then subjected to SDS-PAGE as described in Example 1C. Results are shown in Table 15 below.

Table 15. Solubility of subtilisin measured by SDS/PAGE

Test HDL Compositions including added enzyme[2] (% w/w)			Subtilisin protein (calculated ppm)[1]	
Stock HDL	Water	Anionic surfactant	insoluble portion (pellet)	soluble portion (supernatant)
1A	5%	0%	3629	ND[3]
2B	14%	8%	3280	ND
3C	23%	14%	2506	275
4D	32%	18%	409	614
1B	5%	9%	3695	ND
1C	5%	17%	3741	ND
1D	5%	26%	2705	ND
1E	5%	35%	3210	ND
1F	5%	43%	2960	141

Notes:
[1]The total amount of Subtilisin (enzyme activity) initially delivered to each test HDL was 3250 ppm
[2]One part aqueous Subtilisin concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 17

Amylase solubility in detergents

[175] Using the "post addition" samples, the solubility of amylase enzyme in various test HDL compositions (from Example 11) was measured by SDS-PAGE. The reconstituted solid phase enzyme (pellet) and solution phase enzyme (supernatant) were prepared as described in Example 13. These samples were inactivated by dilution into strong acid, i.e., by adding 200 µL of 1 N HCl to 780 µL of water, then adding 20 µL of sample. Separately, standards containing known amounts of amylase were prepared. The solid phase samples,

solution phase samples, and standards were then subjected to SDS-PAGE as described in Example 1C. Results are shown in Table 16 below.

Table 16. Solubility of alpha amylase measured by SDS/PAGE

Test HDL Compositions including added enzyme[2] (% w/w)			Alpha amylase protein (calculated ppm)[1]	
Stock HDL	Water	Anionic surfactant	insoluble portion (pellet)	soluble portion (supernatant)
1A	5%	0%	4911	ND[3]
2B	14%	8%	8210	ND
3C	23%	14%	8309	ND
4D	32%	18%	6451	ND
1B	5%	9%	10999	ND
1C	5%	17%	10995	ND
1D	5%	26%	11447	ND
1E	5%	35%	10965	ND
1F	5%	43%	11196	ND

Notes:
[1]The total amount of Alpha amylase (enzyme activity) initially delivered to each test HDL was 9555ppm
[2]One part aqueous Alpha amylase concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 18

Metalloprotease protease solubility in detergents

[176] Using the "post addition" samples, the solubility of metalloprotease enzyme in various test HDL compositions (from Example 11) was measured by SDS-PAGE. The reconstituted solid phase enzyme (pellet) and solution phase enzyme (supernatant) were prepared as described in Example 4. These samples were inactivated by dilution into strong acid, i.e., by adding 200 µL of 1N HCl to 780 µL of water, then adding 20 µL of sample. Separately, standards containing known amounts of metalloprotease were prepared. The solid phase samples, solution phase samples, and standards were then subjected to SDS-PAGE as described in Example 1. Results are shown in Table 17 below.

Table 17. Solubility Metalloprotease protease measured by SDS/PAGE

Test HDL Compositions including added enzyme[2] (% w/w)			Metalloprotease protein (calculated ppm)[1]	
Stock HDL	Water	Anionic surfactant	insoluble portion (pellet)	soluble portion (supernatant)
1A	5%	0%	901	ND[3]
2B	14%	8%	924	ND
3C	23%	14%	753	212
4D	32%	18%	205	515
1B	5%	9%	778	ND
1C	5%	17%	759	ND
1D	5%	26%	605	132
1E	5%	35%	747	ND
1F	5%	43%	570	100

Notes:
[1]The total amount of Metalloprotease (enzyme activity) initially delivered to each test HDL was 1115 ppm
[2]One part aqueous Metalloprotease concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%.
[3] ND: not detected

Example 19

Aryl esterase solubility in detergents

[177] Using the "post addition" samples, the solubility of aryl esterase enzyme in various test HDL compositions (from Example 11) was measured by SDS-PAGE. The reconstituted solid phase enzyme (pellet) and solution phase enzyme (supernatant) were prepared as described in Example 5. These samples were inactivated by dilution into strong acid, i.e., by adding 200 µL of 1 N HCl to 780 µL of water, then adding 20 µL of sample. Separately, standards containing known amounts of aryl esterase were prepared. The solid phase samples, solution phase samples, and standards were then subjected to SDS-PAGE as described in Example 1. Results are shown in Table 18 below.

Table 18. Solubility aryl esterase measured SDS/PAGE

Test HDL Compositions including added enzyme[2] (% w/w)			Aryl esterase protein (calculated ppm)[1]	
			insoluble portion (pellet)	soluble portion (supernatant)
Stock HDL	Water	Anionic surfactant		
1A	5%	0%	1484	ND[3]
2B	14%	8%	2059	ND
3C	23%	14%	1118	ND
4D	32%	18%	1100	ND
1B	5%	9%	1832	ND
1C	5%	17%	1895	ND
1D	5%	26%	1365	ND
1E	5%	35%	1292	ND
1F	5%	43%	1530	ND

Notes:
[1]The total amount of Aryl esterase (enzyme activity) initially delivered to each test HDL was 1385 ppm
[2]One part aqueous Aryl esterase concentrate was added to 19 parts HDL stock, increasing the total water content of each test HDL by approximately 5%
[3] ND: not detected

Example 20

Subtilisin stability in detergents containing oxygen bleach

[178] This example demonstrates subtilisin stability in low-water detergents containing sodium perborate oxygen bleach.

[179] For test HDLs 1F, 2F, and 4F (from Example 11), 15 parts powdered sodium perborate were added to 85 parts HDL stock solution and mixed to form a slurry. Around 25 milligrams of dried subtilisin concentrate was then mixed into 20 grams of each slurry, producing an expected subtilisin level of approximately 600 ppm. After thorough mixing, 12 x 1 g aliquots of the slurry were weighed into 50-mL centrifuge tubes. The tubes were placed in a 37°C incubator, and subtilisin activity was measured as a function of time.

[180] At each time interval, four of the tubes for each test HDL were removed from the incubator. Three of the tubes were reconstituted by diluting with 9 mL of water containing excess catalase (CAT HP® L5000, DuPont) (i.e., 3.4 g/L) and mixing thoroughly. The

fourth tube was reconstituted with pure water. After approximately 10 minutes of mixing/dissolution, the subtilisin activity in the reconstituted samples was determined using the method described above (Example 1B). Results are shown in Table 19 below.

[181] Hydrogen peroxide can oxidize subtilisin, dramatically reducing its preoteolytic activity (Stauffer and Etson, "The effect on subtilisin activity of oxidizing a methionine residue", Journal of Biological Chemistry, 1969, 5333-5338). Catalase is an enzyme that converts hydrogen peroxide to water and oxygen (see Biochemistry, 4th ed. L. Stryer, 1995). Thus, the presence of catalase during enzyme reconstitution prevented oxidation and inactivation of the enzyme during the mixing/dissolution. The high level of observed enzyme activity in the catalase-containing 1F sample showed that the enzyme remained active, even after it had been stored in the presence of a hydrogen peroxide source. The difference between the catalase-containing and catalase-free 1F samples shows that hydrogen peroxide was present in the dissolved detergent concentrate, i.e., that the sodium perborate hydrogen peroxide source remained viable even after storage.

Table 19. Subtilisin stability in the presence of bleach

	ppm subtilisin detected		
	after addition	**10 days**	**32 days**
1F[1]	669	514	389
1F - no catalase	129	192	168
2F[1]	378	19	NM
2F - no catalase	209	23	NM
4F[1]	157	ND	NM
4F - no catalase	99	ND	NM

[1]average of three measurements
ND means "not detected"
NM means "not measured"

Example 21

Subtilisin stability in detergents containing builders

[182] This example demonstrates subtilisin stability in low-water detergents containing builders.

[183] For test HDL 1F (from Example 11), either 1 part sodium citrate powder was mixed with 9 parts HDL stock solution to form a slurry or 1 part of 25% diethylene triamine

pentaacetic acid (DTPA) was mixed with 49 parts HDL stock solution to form a homogeneous solution. Approximately 10ul of subtilisin ultra filtered concentrate was added to 500 µl of each sample to produce an expected subtilisin level of 500 ppm. Mixtures were prepared multiple times in microfuge tubes to measure subtilisin activity as a function of time. The tubes were placed in a 37°C incubator and subtilisin activity was measured as a function of time.

[184] At each time interval, one tube of each test HDL containing either sodium citrate or DTPA was removed from the incubator. Subtilisin activity was measured after adding and mixing 4.5 mL subtilisin buffer using the method described in Example 1B. Results are shown in Table 20 below.

Table 20. Subtilisin stability in the presence of builders

	ppm subtilisin detected		
	post addition	**10 days**	**30 days**
1F	345	245	179
1F + 10% sodium citrate	285	226	185
1F + 0.5% DTPA	376	259	101

Example 22

Test HDL dissolution kinetics in wash using subtilisin

[185] This example demonstrates the dissolution kinetics of a test HDL in a simulated wash cycle using subtilisin.

[186] For test HDL 1F (from Example 11), 1 part subtilisin ultrafiltered concentrate was added to 99 parts stock HDL solution to produce approximately 300 ppm of subtilisin. The mixture was prepared in a 50 ml falcon tube. Dissolution measurements were started when the HDL mixture was diluted 60 times with stability buffer (100 mM MES pH 5.5). Subtilisin activity was measured at varying time points to determine the dissolution kinetics. A control sample was prepared in stability buffer to determine the expected subtilisin concentration. Results are shown in Table 21 below.

Table 21. Dilution kinetics

	ppm subtilisin detected				
Buffer control	1 min	3 min	5 min	10 min	20 min
332	135	251	282	295	294

Example 23

Stability of enzyme blends in detergents

[187] This example demonstrates stability of enzyme blends in low-water detergents. For test HDLs 1A, 1F, 4D, 9A and 9E (from Example 11), approximately 10 µl of subtilisin and 2 µl of alpha amylase ultrafiltered concentrate was added to 500 µl of each HDL to produce an enzyme blend containing 500 ppm subtilisin and 500 ppm alpha amylase. Mixtures were prepared multiple times in microfuge tubes to measure subtilisin and amylase activity as a function of time. Control samples were enzyme blends prepared in buffer (50 mM sodium malate, 50 mM sodium chloride, 2 mM calcium chloride, 0.005% sodium azide, pH ~5.4). All tubes were placed in a 37°C incubator.

[188] At each time interval, one HDL sample containing the enzyme blend was removed from the incubator. Subtilisin activity was measured using the AAPF assay and amylase activity was measured using the Ceralpha assay after adding and mixing 4.5mL buffer (50 mM sodium malate, 50 mM sodium chloride, 2 mM calcium chloride, 0.005% sodium azide, pH ~5.4) as described in Example 1. Results are shown in Table 22 below.

Table 22. Stability in detergents

	ppm detected			
	subtilisin		amylase	
	post addition	60 days	post addition	60 days
Control	347	N/A	501	N/A
1A	295	102	476	20*
1F	242	214	331	322
4D	268	ND	428	247
9A	316	ND	539	502
9F	313	ND	536	327

ND = not detected

* = precipitate present

[189] Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to

those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[190] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference.

CLAIMS

What is claimed is:

1. A low-water, water-triggered enzyme suspension comprising an organic carrier liquid in which one or more enzymes are substantially insoluble but capable of enzymatic activity when the suspension is diluted with at least one equal volume of water, the carrier liquid being liquid at room temperature, miscible with water, forming a single thermodynamic phase, and comprising either about 5-20% water by weight, or if anionic surfactants are present in the suspension, the amount of water plus 20% of the amount of anionic surfactants adds up to be between about 5 and 20% by weight.

2. The water-triggered enzyme suspension of claim 1, wherein the organic carrier liquid is selected from the group consisting of a nonionic surfactant, an anionic surfactant, an alcohol, a glycol, a polyglycol, an acetate ester, and mixtures, thereof.

3. The water-triggered enzyme suspension of any of the preceding claims, wherein the one or more enzymes are dissolved at less than 1 gram per liter in the carrier liquid for at least the first 30 days of storage at 25°C.

4. The water-triggered enzyme suspension of any of the preceding claims, wherein less than 20% of the one or more enzyme is dissolved within the carrier liquid phase.

5. The water-triggered enzyme suspension of any of the preceding claims, wherein upon dilution of the suspension with at least one volume of water the one or more enzymes exhibit at least about 50% of their original catalytic potential in less than 5 minutes at a preselected temperature.

6. The water-triggered enzyme suspension of any of the preceding claims, wherein the one or more enzymes are selected from the group consisting of acyl transferases, α-amylases, β-amylases, α-galactosidases, arabinosidases, aryl esterases, β-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-β-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases,

glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, peroxygenases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, β-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, additional serine proteases, and combinations, thereof.

7. The water-triggered enzyme suspension of any of the preceding claims, wherein the one or more enzymes are provided in the form of particles or granules that include the enzymes embedded within a uniform matrix, or as part of a core surrounded by a coating.

8. The water-triggered enzyme suspension of claim 7, wherein the particles or granules have a density within 0.2 g/ml of the density of the liquid suspension.

9. The water-triggered enzyme suspension of claim 7 or 8, wherein the liquid suspension and the particles or granules all have a density between 1.0 and 1.2 g/ml.

10. The water-triggered enzyme suspension of any of the preceding claims, wherein the water-triggered enzyme suspension is a laundry or dishwashing liquid composition.

11. A laundry or dishwashing liquid composition comprising the water-triggered enzyme suspension of any of claims 1-10.

12. A method for stabilizing enzymes in a liquid composition comprising suspending the enzymes in an organic carrier liquid in which the enzymes are substantially insoluble but capable of enzymatic activity when the suspension is diluted with at least one equal volume of water, wherein the carrier liquid is liquid at room temperature, miscible with water, forms a single thermodynamic phase, and comprises either about 5-20% water by weight, or if anionic surfactants are present in the suspension, the amount of water plus 20% of the amount of anionic surfactants adds up to be between about 5 and 20% by weight.

13. The method of claim 12, wherein the organic carrier liquid is selected from the group consisting of a nonionic surfactant, an anionic surfactant, an alcohol, a glycol, a polyglycol, an acetate ester, and mixtures, thereof.

14. The method of claim 12 or 13, wherein the enzymes are dissolved at less than 1 gram per liter in the carrier liquid for at least the first 30 days of storage at 25°C.

15. The method of any of claims 12-14, wherein less than 20% of the one or more enzyme is dissolved within the carrier liquid phase.

16. The method of any of claims 12-15, wherein upon dilution of the suspension with at least one volume of water the one or more enzymes exhibit at least about 50% of their original catalytic potential in less than 5 minutes at a preselected temperature.

17. The method of any of claims 12-16, wherein the one or more enzymes are selected from the group consisting of acyl transferases, α-amylases, β-amylases, α-galactosidases, arabinosidases, aryl esterases, β-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-β-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, peroxygenases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, β-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, metalloproteases, additional serine proteases, and combinations, thereof.

18. The method of any of claims 12-18, wherein the one or more enzymes are provided in the form of particles or granules that include the enzymes embedded within a uniform matrix, or as part of a core surrounded by a coating.

19. The method of any of claim 18, wherein the particles or granules have a density within 0.2 g/ml of the density of the liquid suspension.

20. The method of any of claims 18 or 19, wherein the liquid suspension and the particles or granules all have a density between 1.0 and 1.2 g/ml.

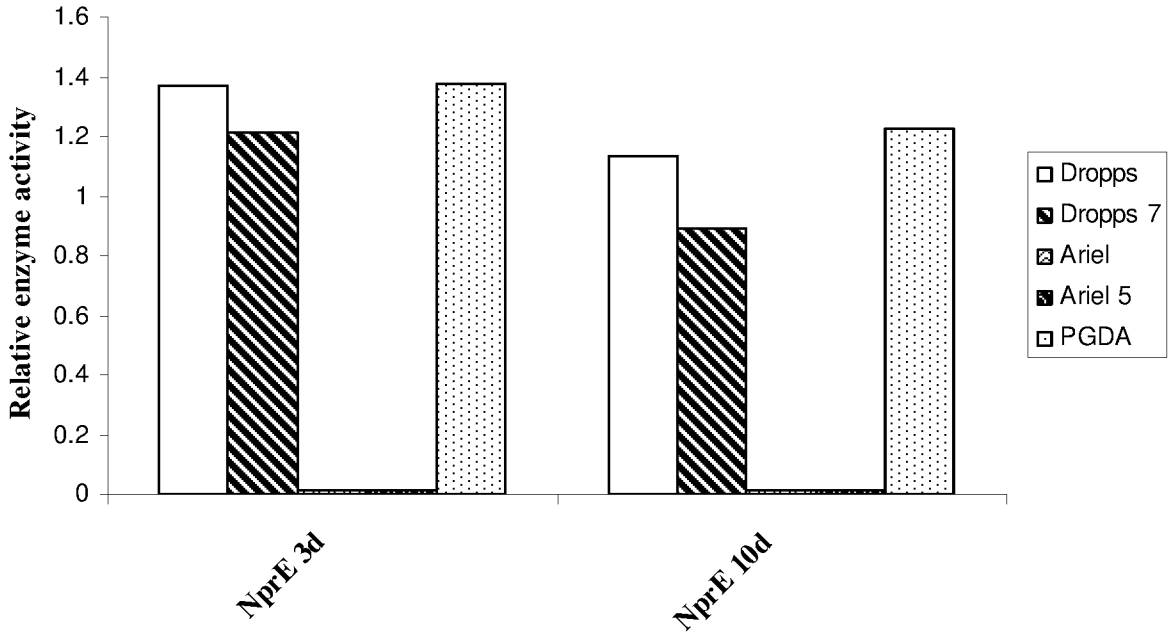


Figure 1

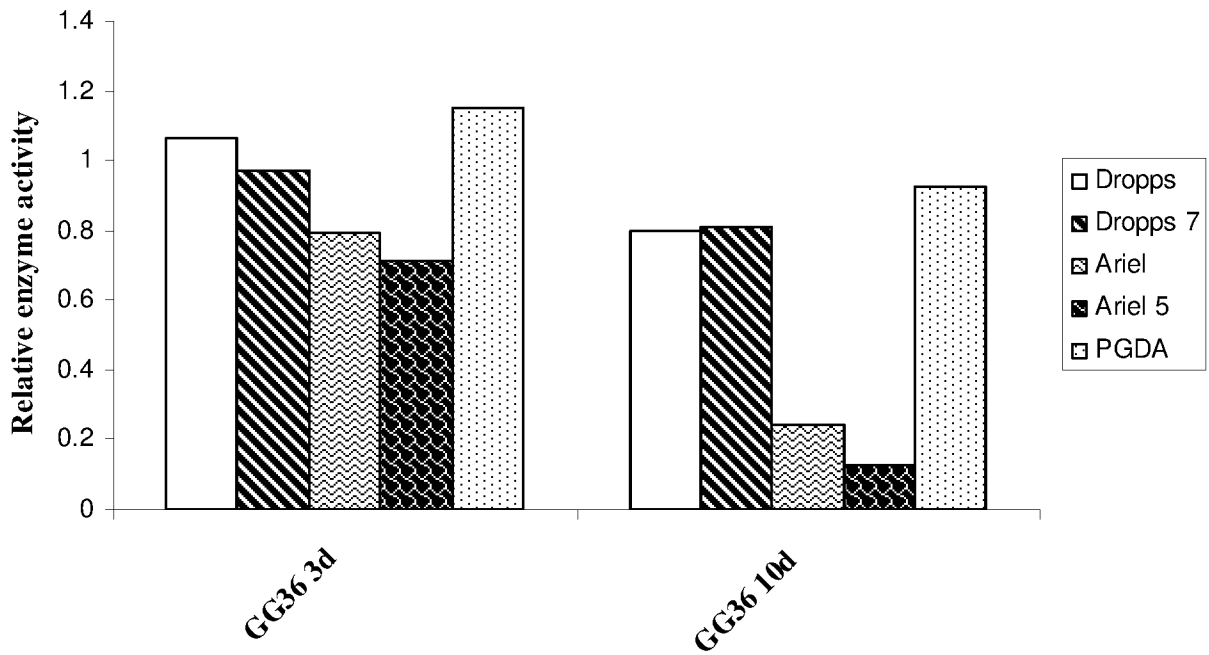
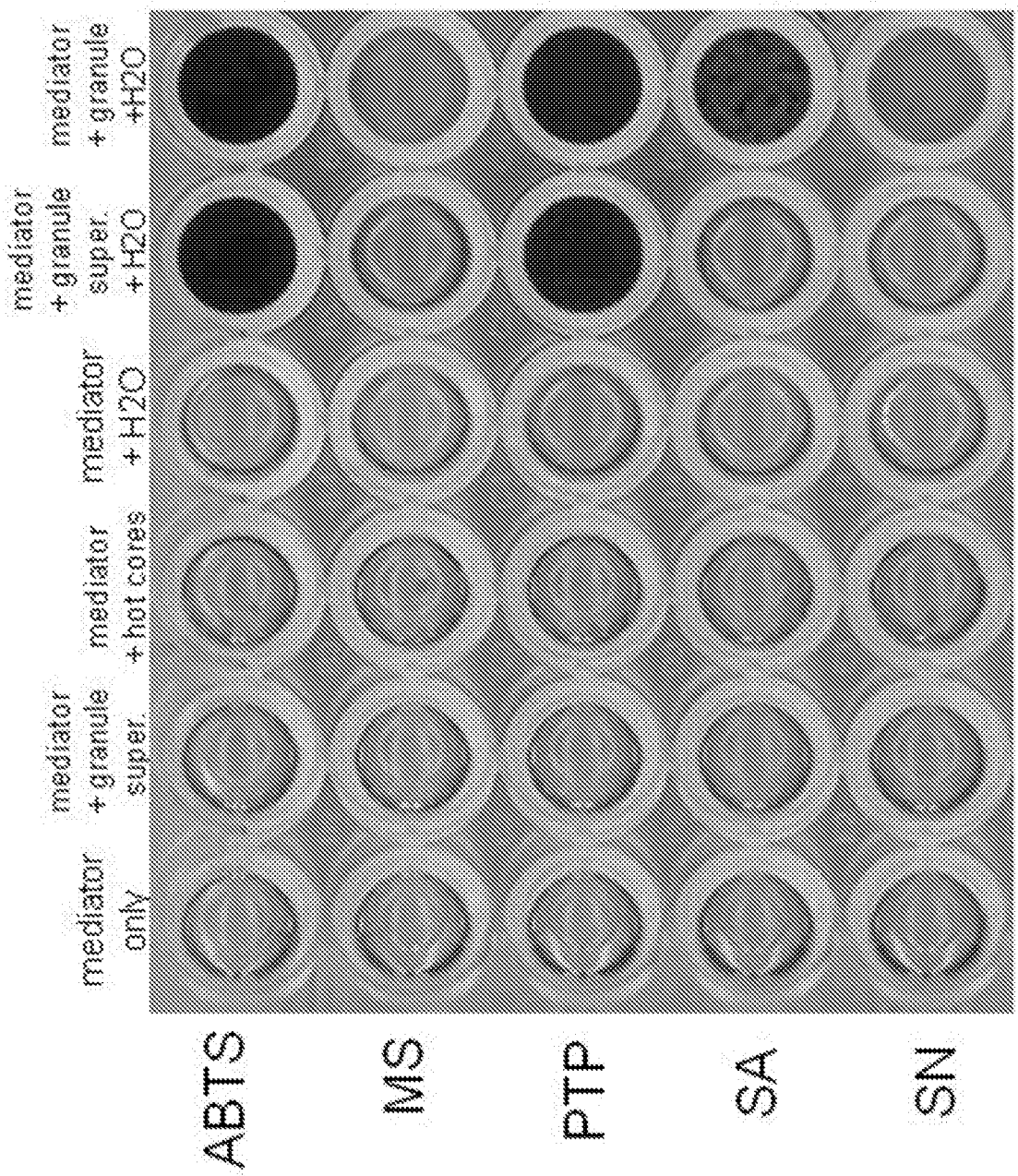


Figure 2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mediator + granule super.
mediator + hot cores
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ABTS
MS
PTP
SA
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Figure 3A

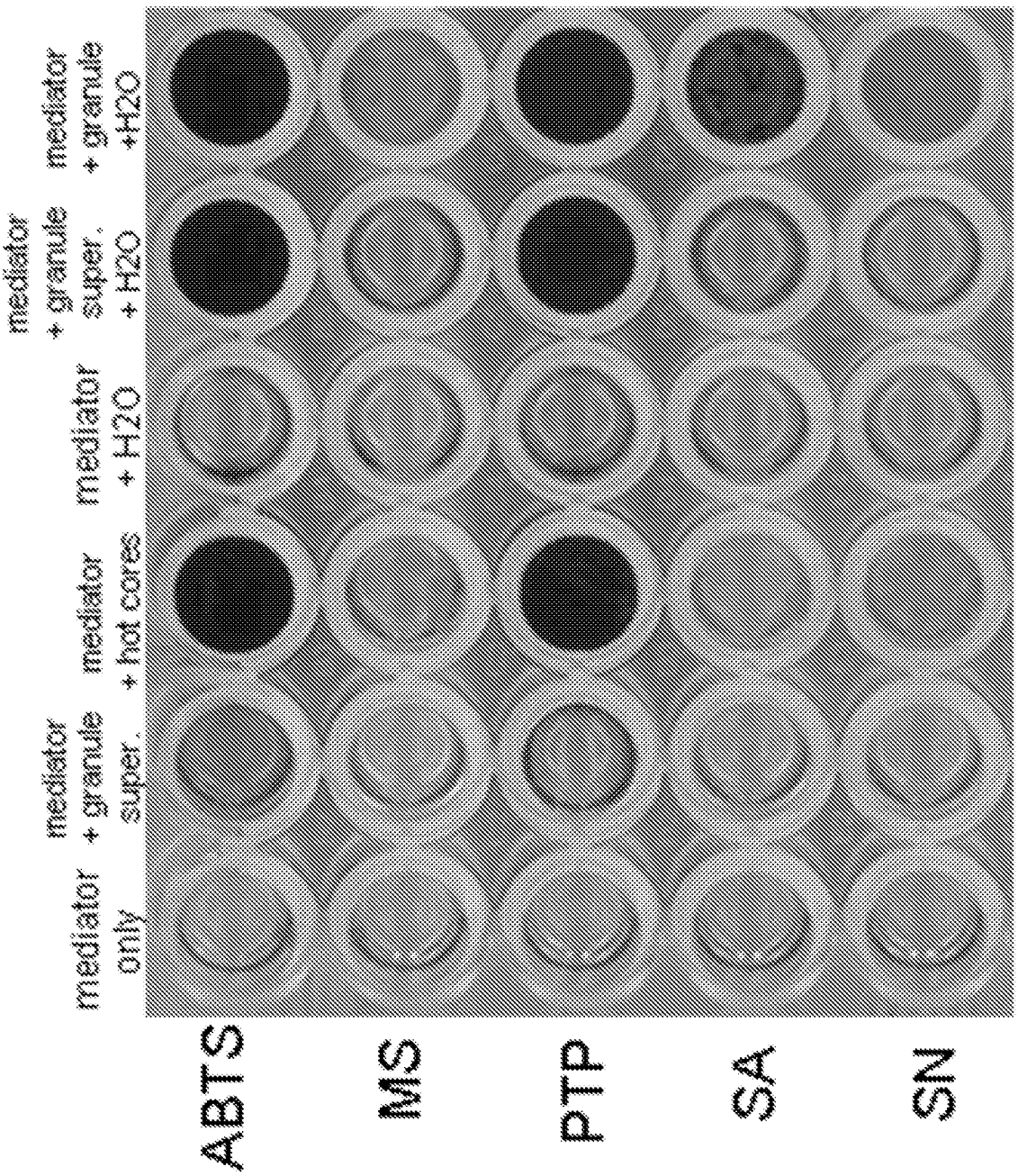
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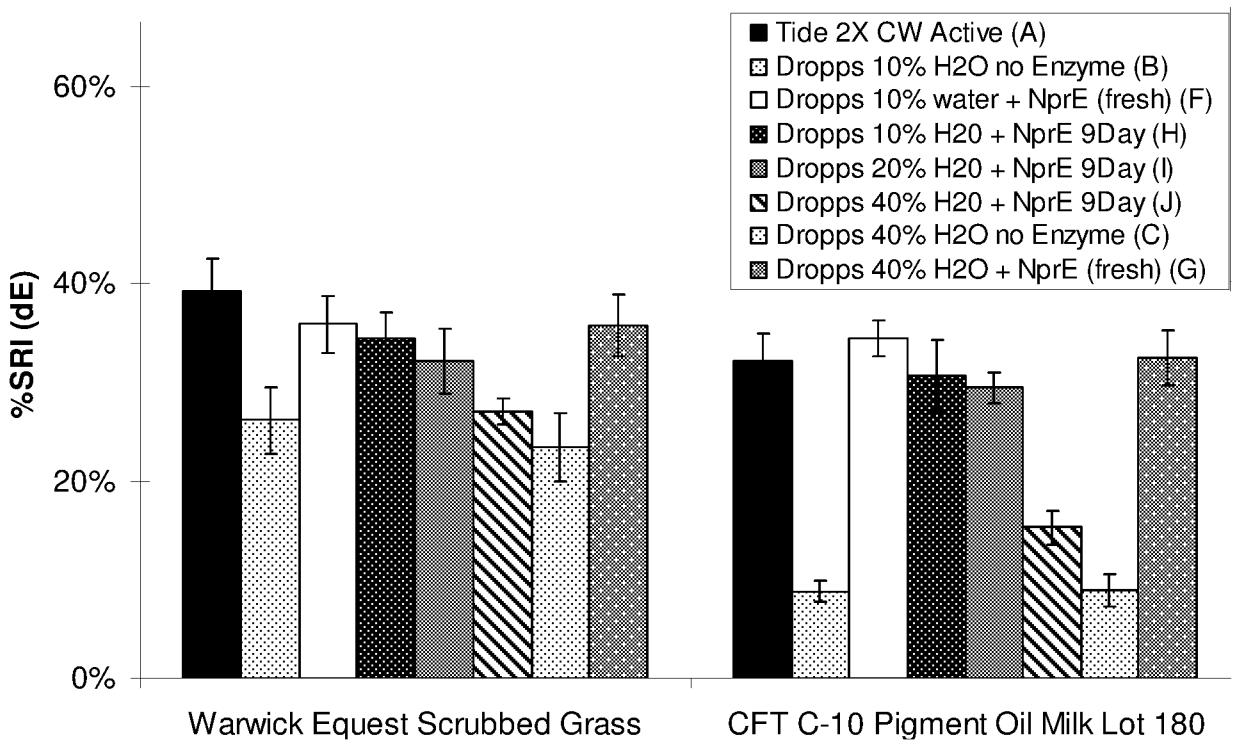
Figure 3B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Dropps 10% water + NprE (fresh) (F)
Dropps 10% H20 + NprE 9Day (H)
Dropps 20% H20 + NprE 9Day (I)
Dropps 40% H20 + NprE 9Day (J)
Dropps 40% H2O no Enzyme (C)
Dropps 40% H2O + NprE (fresh) (G)
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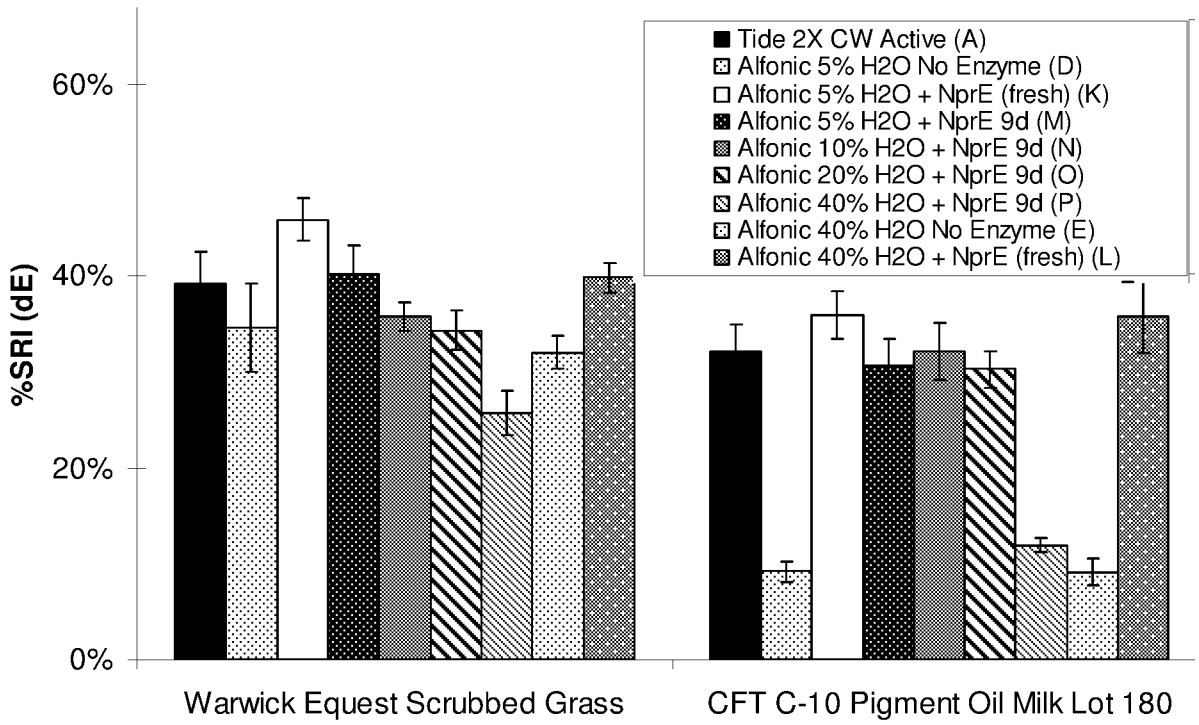
40%
20%
0%
Warwick Equest Scrubbed Grass
CFT C-10 Pigment Oil Milk Lot 180

Figure 4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Alfonic 5% H2O + NprE 9d (M)
Alfonic 10% H2O + NprE 9d (N)
Alfonic 20% H2O + NprE 9d (O)
Alfonic 40% H2O + NprE 9d (P)
Alfonic 40% H2O No Enzyme (E)
Alfonic 40% H2O + NprE (fresh) (L)
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20%
0%
Warwick Equest Scrubbed Grass
CFT C-10 Pigment Oil Milk Lot 180

Figure 5

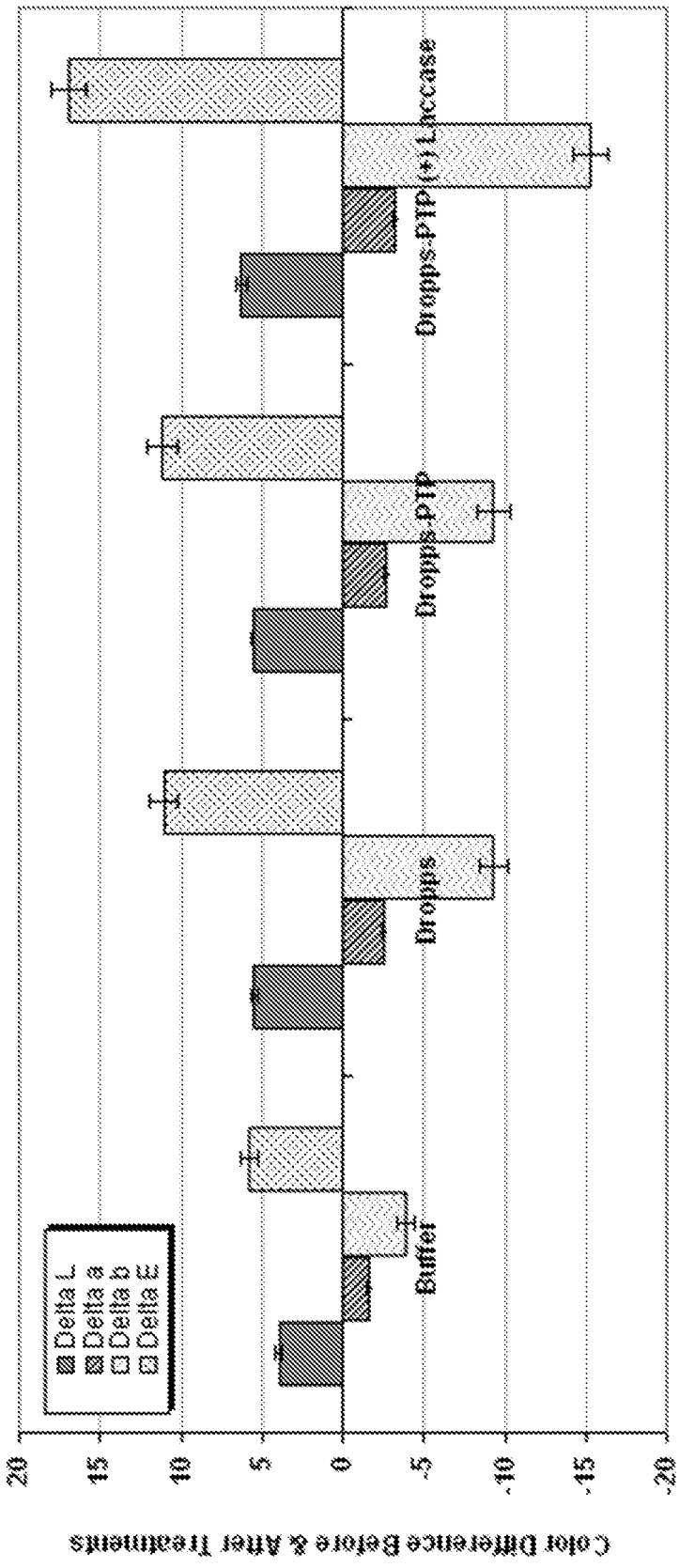
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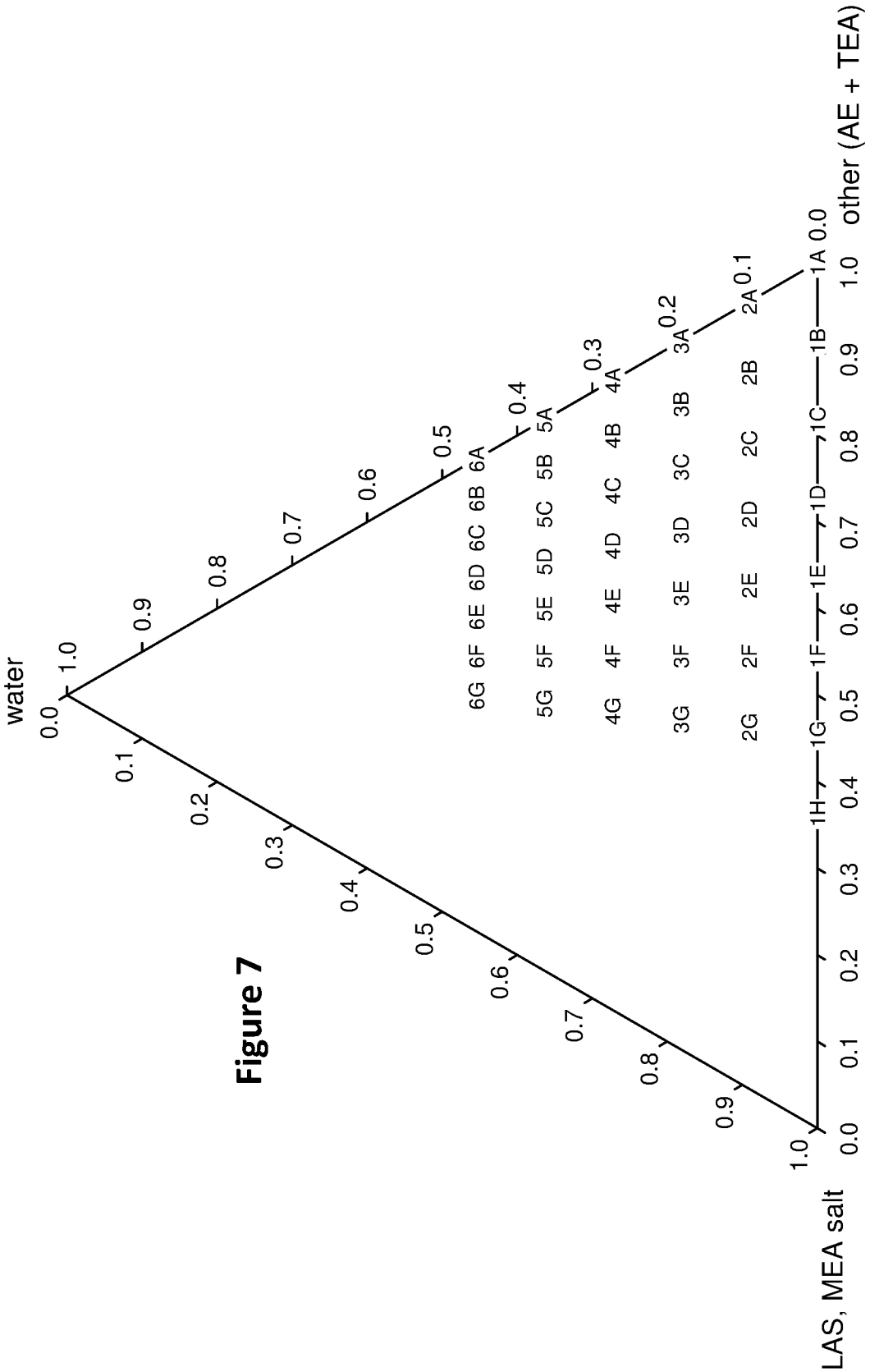
Figure 6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1.0
0.0
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3G 3F 3E 3D 3C 3B 3A
4G 4F 4E 4D 4C 4B 4A
5G 5F 5E 5D 5C 5B 5A
6G 6F 6E 6D 6C 6B 6A

Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/036589

A. CLASSIFICATION OF SUBJECT MATTER
INV. C11D3/386 C11D17/00
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)
C11D

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 practicable, 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	US 6 673 763 B1 (HANSEN OLE REGNAR [DK] ET AL) 6 January 2004 (2004-01-06) claims examples column 14, line 65 - column 16, line 9 column 2, line 6 - line 33 column 3, line 40 - column 8, line 63	1-20
X	EP 0 873 183 A1 (ALLIED COLLOIDS LTD [GB]; NOVO NORDISK AS [DK]) 28 October 1998 (1998-10-28)	1-7, 10-18
A	claims examples page 11, paragraph 112 page 20, paragraph 209 page 2, paragraph 1	8,9,19, 20

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent 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
5 September 2016	12/09/2016

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	Neys, Patricia

3

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/036589

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 350 250 A1 (DANISCO US INC [US]) 3 August 2011 (2011-08-03)	11
A	claims page 14, paragraphs 127, 129 - page 15, paragraph 134 page 11, paragraph 102 - paragraph 105	1-10, 12-20

X	DE 10 2004 018787 A1 (HENKEL KGAA [DE]) 10 November 2005 (2005-11-10)	11
A	claims page 2, paragraph 7	1-10, 12-20

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/036589

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		DE 69615582 T2	11-07-2002
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		JP 5837822 B2	24-12-2015
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		PT 2350250 E	22-05-2014
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		WO 2010062745 A1	03-06-2010
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