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(54) Title: POLYPEPTIDES HAVING LIPASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) Abstract: The present invention relates to isolated polypeptides having lipase activity and polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides.

**POLYPEPTIDES HAVING LIPASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME****Reference to a Sequence Listing**

This application contains a Sequence Listing in computer readable form, which is incorporated 5 herein by reference.

**Background of the Invention****Field of the Invention**

The present invention relates to polypeptides having lipase activity and polynucleotides encoding 10 the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing and using the polypeptides.

**Description of the Related Art**

The present invention provides polypeptides having lipase activity and polynucleotides encoding 15 the polypeptides. The lipase disclosed in Glogauer et al. *Microbial Cell Factories* 2011, 10:54 shows an amino acid sequence identity of 91.8% with SEQ ID NO: 2.

**Summary of the Invention**

The present invention relates to isolated polypeptides having lipase activity selected from the group 20 consisting of:

(a) a polypeptide having at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency 25 conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i);

(c) a polypeptide encoded by a polynucleotide having at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of 30 SEQ ID NO: 1;

(d) a variant of the polypeptide of (a), (b) or (c) comprising a substitution, deletion, and/or insertion at one or more positions; and

(e) a fragment of the polypeptide of (a), (b), (c), or (d) that has lipase activity.

The present invention also relates to isolated polynucleotides encoding the polypeptides of the

5 present invention; nucleic acid constructs; recombinant expression vectors; recombinant host cells comprising the polynucleotides; and methods of producing the polypeptides.

The present invention also relates to compositions, methods of producing a composition, methods of cleaning a surface, and methods of hydrolyzing a lipid.

## 10 Definitions

Prior to describing the present compositions and methods in detail, the following terms are defined for clarity. Terms and abbreviations not defined should be accorded their ordinary meaning as used in the art.

Lipase: The terms "lipase", "lipase enzyme", "lipolytic enzyme", "lipid esterase", "lipolytic 15 polypeptide", and "lipolytic protein" refers to an enzyme in class EC3.1.1 as defined by Enzyme Nomenclature. It may have lipase activity that may include but are not limited to triacylglycerol lipase activity (EC3.1.1.3), monoglyceride lipase activity (EC3.1.1.23), phospholipase activity (several ECs), lysophospholipase activity, ferulic acid esterase activity, cutinase activity (EC3.1.1.74), sterol esterase activity (EC3.1.1.13) and/or wax-ester hydrolase activity (EC3.1.1.50).

20 For purposes of the present invention, lipase activity is determined according to the procedure described in the Examples. In one aspect, the variants of the present invention have at least 20%, e.g., at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or 100% of the lipase activity of the mature polypeptide of SEQ ID NO: 2.

25 Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

30 Catalytic domain: The term "catalytic domain" means the region of an enzyme containing the catalytic machinery of the enzyme.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, 5 before appearing as mature spliced mRNA.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic 10 DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control 15 sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide 20 encoding a polypeptide.

Expression: The term "expression" includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" means a linear or circular DNA molecule that 25 comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression.

Fragment: The term "fragment" means a polypeptide having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has lipase activity. In one aspect, a fragment contains at least 50%, at least 55%, at least 30 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the number of amino acids of the mature polypeptide.

High stringency conditions: The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200

micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, 5 transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring 10 substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., 15 multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

Low stringency conditions: The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 20 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 50°C.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal 25 truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 292 of SEQ ID NO: 2.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having lipase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 30 to 905 of SEQ ID NO: 1.

30 Medium stringency conditions: The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following

standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 55°C.

Medium-high stringency conditions: The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE,

5 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either

10 single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

15 Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is

determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The

20 European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

25 
$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later.

30 The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

Subsequence: The term “subsequence” means a polynucleotide having one or more (e.g., several) nucleotides absent from the 5' and/or 3' end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having lipase activity. In one aspect, a fragment contains at least

5 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the number of nucleotides encoding the mature polypeptide.

Variant: The term “variant” means a polypeptide having lipase activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (e.g., several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion

10 means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position.

Very high stringency conditions: The term “very high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following 15 standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 70°C.

Very low stringency conditions: The term “very low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following 20 standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C.

## **Detailed Description of the Invention**

### **Polypeptides Having Lipase Activity**

25 In an embodiment, the present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have lipase activity. In one aspect, the polypeptides differ by no more than 24 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 from the mature polypeptide of SEQ ID NO: 30 2.

A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or is a fragment thereof having lipase activity. In

another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another aspect, the polypeptide comprises or consists of amino acids 1 to 292 of SEQ ID NO: 2.

In another embodiment, the present invention relates to an isolated polypeptide having lipase activity encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i) (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the polypeptide of SEQ ID NO: 2 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having lipase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of a cell of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with  $^{32}\text{P}$ ,  $^{3}\text{H}$ ,  $^{35}\text{S}$ , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having lipase activity. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that hybridizes with SEQ ID NO: 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to (i) SEQ ID NO: 1; (ii) the mature polypeptide coding sequence of SEQ ID NO: 1; (iii) the full-length complement thereof; or (v) a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film or any other detection means known in the art.

In another embodiment, the present invention relates to an isolated polypeptide having lipase activity encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

In another embodiment, the present invention relates to variants of the mature polypeptide of SEQ

5 ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In another embodiment, the present invention relates to variants of the polypeptide of the invention comprising a substitution, deletion, and/or insertion at one or more (e.g., several) positions. In an embodiment, the number of amino acid substitutions, deletions and/or insertions introduced into the mature polypeptide of SEQ ID NO: 2 is not more than 24, e.g., 1, 2, 3, 4, 5, 6, 7, 10 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24. The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing 15 net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids

20 (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, 25 and Asp/Gly.

Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in a polypeptide can be identified according to procedures known in the art,

30 such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for lipase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction

can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992,

5 *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide. Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; 10 WO95/17413; or WO95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; US5223409; WO92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et* 15 *al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

The polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the 20 N-terminus or the C-terminus of a region of another polypeptide.

The polypeptide may be a fusion polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fusion polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in 25 the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s) and terminator. Fusion polypeptides may also be constructed using intein technology in which fusion polypeptides are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

30 A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13:

498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

## 5 Sources of Polypeptides Having Lipase Activity

A polypeptide having lipase activity of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been 10 inserted. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

The polypeptide may be a bacterial polypeptide. For example, the polypeptide may be a Gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces* polypeptide having lipase activity, or a Gram-negative bacterial polypeptide such as a 15 *Campylobacter*, *Cedecea*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Illyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, *Serratia*, or *Ureaplasma* polypeptide.

In one aspect, the polypeptide is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus latus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, 20 *Bacillus subtilis*, or *Bacillus thuringiensis* polypeptide.

In another aspect, the polypeptide is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* polypeptide.

In another aspect, the polypeptide is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* polypeptide.

25 In another aspect, the polypeptide is a *Cedecea daviseae*, *Cedecea lapegei*, *Cedecea* sp 001 (also known as *Cedecea* species 3), *Cedecea neteri* (formerly known as *Cedecea* species 4 or *Cedecea* species 002), *Cedecea* sp 012 (also known as *Cedecea* species 5), or *Cedecea* sp-16640 polypeptide.

In another aspect, the polypeptide is a *Serratia entomophila*, *Serratia ficaria*, *Serratia fonticola*, 30 *Serratia grimesii*, *Serratia liquefaciens*, *Serratia marcescens*, *Serratia odorifera*, *Serratia plymuthica*, *Serratia proteamaculans*, *Serratia quinivorans*, *Serratia rubidaea*, *Serratia symbiotica* polypeptide.

The polypeptide may be a fungal polypeptide. For example, the polypeptide may be a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*,  
5 *Chrysosporium*, *Claviceps*, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpe*, *Lentinula*, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*,  
10 *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Postrasia*, *Pseudoplectania*, *Pseudotrichonympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*,  
*Thermoascus*, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or  
*Xylaria* polypeptide.

In another aspect, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide.

15 In another aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*,  
20 *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola insolens*, *Humicola lanuginosa*,  
25 *Irpe lacteus*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Thielavia achromatic*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia australensis*, *Thielavia fimet*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*, *Thielavia setosa*, *Thielavia spedoronion*, *Thielavia subthermophila*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*,  
30 *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

- 5 The polypeptide may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) or DNA samples obtained directly from natural materials (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding the polypeptide may then be obtained by similarly screening a genomic
- 10 DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a polypeptide has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

## 15 **Polynucleotides**

The present invention also relates to isolated polynucleotides encoding a polypeptide of the present invention, as described herein.

The techniques used to isolate or clone a polynucleotide are known in the art and include isolation from genomic DNA or cDNA, or a combination thereof. The cloning of the polynucleotides from 20 genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used. The 25 polynucleotides may be cloned from a strain of *Cedecea*, or a related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the polynucleotide.

Modification of a polynucleotide encoding a polypeptide of the present invention may be necessary for synthesizing polypeptides substantially similar to the polypeptide. The term "substantially 30 similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific activity, thermostability, pH optimum, or the like. The variants may be constructed on the basis of the polynucleotide presented as the mature

polypeptide coding sequence of SEQ ID NO: 1, e.g., a subsequence thereof, and/or by introduction of nucleotide substitutions that do not result in a change in the amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino 5 acid sequence. For a general description of nucleotide substitution, see, e.g., Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

### Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide of the 10 present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

A polynucleotide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides 15 utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including 20 mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), 25 *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis* *cryIIIA* gene (Agaisse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli* *lac* operon, *E. coli* *trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, 30 *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*. Examples of tandem promoters are disclosed in WO99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO96/00787), *Fusarium venenatum* amyloglucosidase (WO00/56900), *Fusarium venenatum* Daria (WO00/56900), *Fusarium venenatum* Quinn (WO00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, 5 *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, 10 *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by 15 an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

20 In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful 25 promoters for yeast host cells are described by Romanos *et al.*, 1992, Yeast 8: 423-488.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator is operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any terminator that is functional in the host cell may be used in the present invention.

30 Preferred terminators for bacterial host cells are obtained from the genes for *Bacillus clausii* alkaline protease (*aprH*), *Bacillus licheniformis* alpha-amylase (*amyL*), and *Escherichia coli* ribosomal RNA (*rrnB*).

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

10 Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis* cryIIA gene (WO94/25612) and a *Bacillus subtilis* SP82 gene (Hue *et al.*, 1995, *Journal of Bacteriology* 177: 3465-3471).

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader is operably linked to the 5'-terminus of the polynucleotide 15 encoding the polypeptide. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces*

20 *cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal 25 to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

30 Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the 5 coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the 10 polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha- 15 amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase. 20

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

25 The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA 10 alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the 15 regulatory sequence.

### Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various 20 nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the 25 coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with 30 the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a

plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or 5 two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to 10 auxotrophs, and the like.

Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* *dal* genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin, or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to, *ADE2*, *HIS3*, *LEU2*, *LYS2*, *MET3*, *TRP1*, and *URA3*. Selectable markers for 15 use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae* *amdS* and 20 *pyrG* genes and a *Streptomyces hygroscopicus* *bar* gene.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by 25 homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which 30 have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMβ1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication,

10 ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO00/24883.

15 More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide,

20 can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

## 25 Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production of a polypeptide of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations

that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be any cell useful in the recombinant production of a polypeptide of the present invention, e.g., a prokaryote or a eukaryote.

5 The prokaryotic host cell may be any Gram-positive or Gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, and *Streptomyces*. Gram-negative bacteria include, but are not limited to, *Campylobacter*, *Cedecea*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Illyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*,  
10 *Serratia*, and *Ureaplasma*.

The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells.

15 The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp. *Zooepidemicus* cells.

The bacterial host cell may also be any *Streptomyces* cell including, but not limited to, *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* cells.  
20

The bacterial host cell may also be any *Cedecea* cell including, but not limited to, *Cedecea davisae*, *Cedecea lapegei*, *Cedecea* sp 001 (also known as *Cedecea* species 3), *Cedecea neteri* (formerly known as *Cedecea* species 4 or *Cedecea* species 002), *Cedecea* sp 012 (also known as *Cedecea* species 5), or *Cedecea* sp-16640 polypeptide.

25 The bacterial host cell may also be any *Serratia* cell including, but not limited to, *Serratia entomophila*, *Serratia ficaria*, *Serratia fonticola*, *Serratia grimesii*, *Serratia liquefaciens*, *Serratia marcescens*, *Serratia odorifera*, *Serratia plymuthica*, *Serratia proteamaculans*, *Serratia quinivorans*, *Serratia rubidaea*, *Serratia symbiotica* polypeptide.

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, e.g.,  
30 Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6:

742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be 5 effected by protoplast transformation, electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or conjugation (e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural 10 competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804), or conjugation (e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can 15 be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 20 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, 25 Passmore, and Davenport, editors, *Soc. App. Bacteriol. Symposium Series* No. 9, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell, such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces oviformis*, or *Yarrowia 30 lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose,

glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

5 The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

10 For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium 20 sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 30 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic

Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

### Methods of Production

5 The present invention also relates to methods of producing a polypeptide of the present invention, comprising (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is a *Cedecea* cell. In a more preferred aspect, the cell is a *Cedecea* sp-16640 cell.

10 The present invention also relates to methods of producing a polypeptide of the present invention, comprising (a) cultivating a recombinant host cell of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

The host cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, or 15 small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be 20 prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptide may be detected using methods known in the art that are specific for the 25 polypeptides. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited 30 to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptide may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size

exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

5 In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing the polypeptide is used as a source of the polypeptide.

## Plants

The present invention also relates to isolated plants, e.g., a transgenic plant, plant part, or plant cell, comprising a polynucleotide of the present invention so as to express and produce a polypeptide or domain in recoverable quantities. The polypeptide or domain may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the polypeptide or domain may be used as such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

10 15 The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g., wheat, oats, rye, barley, rice, sorghum, and maize (corn).

20 Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family *Brassicaceae*), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

25 Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g., epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, e.g., embryos, endosperms, aleurone and seed coats.

30 Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

The transgenic plant or plant cell expressing the polypeptide or domain may be constructed in accordance with methods known in the art. In short, the plant or plant cell is constructed by

incorporating one or more expression constructs encoding the polypeptide or domain into the plant host genome or chloroplast genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

The expression construct is conveniently a nucleic acid construct that comprises a polynucleotide encoding a polypeptide or domain operably linked with appropriate regulatory sequences required for expression of the polynucleotide in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying plant cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

5 The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences, is determined, for example, on the basis of when, where, and how the polypeptide or domain is desired to be expressed. For instance, the expression of the gene encoding a polypeptide or domain may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as 10 seeds or leaves. Regulatory sequences are, for example, described by Tague *et al.*, 1988, *Plant Physiology* 86: 506.

15 For constitutive expression, the 35S-CaMV, the maize ubiquitin 1, or the rice actin 1 promoter may be used (Franck *et al.*, 1980, *Cell* 21: 285-294; Christensen *et al.*, 1992, *Plant Mol. Biol.* 18: 675-689; Zhang *et al.*, 1991, *Plant Cell* 3: 1155-1165). Organ-specific promoters may be, for example, a 20 promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards and Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito *et al.*, 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu *et al.*, 1998, *Plant Cell Physiol.* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad *et al.*, 1998, *J. Plant Physiol.* 152: 708-711), a promoter from a seed oil body protein (Chen *et al.*, 1998, *Plant Cell Physiol.* 39: 935-941), the storage protein *napA* promoter from *Brassica napus*, or any other seed specific promoter known in the art, e.g., as described in WO91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcS* promoter from rice or tomato (Kyozuka *et al.*, 1993, *Plant Physiol.* 102: 991-1000), the chlorella virus adenine methyltransferase 25 gene promoter (Mitra and Higgins, 1994, *Plant Mol. Biol.* 26: 85-93), the *aldP* gene promoter from rice (Kagaya *et al.*, 1995, *Mol. Gen. Genet.* 248: 668-674), or a wound inducible promoter such as the potato *pin2* promoter (Xu *et al.*, 1993, *Plant Mol. Biol.* 22: 573-588). Likewise, the promoter may be induced by abiotic treatments such as temperature, drought, or alterations in salinity or induced

by exogenously applied substances that activate the promoter, e.g., ethanol, oestrogens, plant hormones such as ethylene, abscisic acid, and gibberellic acid, and heavy metals.

A promoter enhancer element may also be used to achieve higher expression of a polypeptide or domain in the plant. For instance, the promoter enhancer element may be an intron that is placed

5 between the promoter and the polynucleotide encoding a polypeptide or domain. For instance, Xu *et al.*, 1993, *supra*, disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

10 The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation, and electroporation (Gasser *et al.*, 1990, *Science* 244: 1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto *et al.*, 1989, *Nature* 338: 274).

15 *Agrobacterium tumefaciens*-mediated gene transfer is a method for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Mol. Biol.* 19: 15-38) and for transforming monocots, although other transformation methods may be used for these plants. A method for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant*  
20 *J.* 2: 275-281; Shimamoto, 1994, *Curr. Opin. Biotechnol.* 5: 158-162; Vasil *et al.*, 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh *et al.*, 1993, *Plant Mol. Biol.* 21: 415-428. Additional transformation methods include those described in US6395966 and US7151204 (both of which are herein incorporated by reference in their entirety).

25 Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using, for example, co-transformation with two separate T-DNA constructs or site specific excision of the selection gene by a specific recombinase.

30 In addition to direct transformation of a particular plant genotype with a construct of the present invention, transgenic plants may be made by crossing a plant having the construct to a second plant lacking the construct. For example, a construct encoding a polypeptide or domain can be

introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the present invention encompasses not only a plant directly regenerated from cells which have been transformed in accordance with the present invention, but also the progeny of such plants. As used herein, progeny may refer to the offspring of any 5 generation of a parent plant prepared in accordance with the present invention. Such progeny may include a DNA construct prepared in accordance with the present invention. Crossing results in the introduction of a transgene into a plant line by cross pollinating a starting line with a donor plant line. Non-limiting examples of such steps are described in US7151204.

Plants may be generated through a process of backcross conversion. For example, plants include 10 plants referred to as a backcross converted genotype, line, inbred, or hybrid.

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite 15 germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is 20 minimized.

The present invention also relates to methods of producing a polypeptide or domain of the present invention comprising (a) cultivating a transgenic plant or a plant cell comprising a polynucleotide encoding the polypeptide or domain under conditions conducive for production of the polypeptide or domain; and (b) recovering the polypeptide or domain.

25

## Compositions

The non-limiting list of composition components illustrated hereinafter are suitable for use in the compositions and methods herein may be desirably incorporated in certain embodiments of the invention, e.g. to assist or enhance cleaning performance, for treatment of the substrate to be 30 cleaned, or to modify the aesthetics of the composition as is the case with perfumes, colorants, dyes or the like. The levels of any such components incorporated in any compositions are in addition to any materials previously recited for incorporation. The precise nature of these additional components, and levels of incorporation thereof, will depend on the physical form of the

composition and the nature of the cleaning operation for which it is to be used. Unless otherwise indicated the amounts in percentage is by weight of the composition (wt%). Suitable component materials include, but are not limited to, surfactants, builders, chelating agents, dye transfer inhibiting agents, dispersants, enzymes, and enzyme stabilizers, catalytic materials, bleach activators, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal/anti-redeposition agents, brighteners, suds suppressors, dyes, hueing dyes, perfumes, perfume delivery systems, structure elasticizing agents, fabric softeners, carriers, hydrotropes, processing aids, solvents and/or pigments. In addition to the disclosure below, suitable examples of such other components and levels of use are found in US5576282; 5 US6306812; and US6326348 hereby incorporated by reference.

10

Thus, in certain embodiments the invention do not contain one or more of the following adjuncts materials: surfactants, builders, chelating agents, dye transfer inhibiting agents, dispersants, additional enzymes, and enzyme stabilizers, catalytic materials, bleach activators, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil 15 removal/anti-redeposition agents, brighteners, suds suppressors, dyes, perfumes, perfume delivery systems, structure elasticizing agents, fabric softeners, carriers, hydrotropes, processing aids, solvents and/or pigments. However, when one or more components are present, such one or more components may be present as detailed below:

Surfactants - The compositions according to the present invention may comprise a surfactant or 20 surfactant system wherein the surfactant can be selected from nonionic surfactants, anionic surfactants, cationic surfactants, amphotolytic surfactants, zwitterionic surfactants, semi-polar nonionic surfactants and mixtures thereof. When present, surfactant is typically present at a level of from 0.1 to 60wt%, from 1 to 50wt% or from 5 to 40wt%.

Suitable anionic detergents include sulphate and sulphonate detergents. 25

Suitable sulphonate detergents include alkyl benzene sulphonate, in one aspect,  $C_{10-13}$  alkyl benzene sulphonate. Suitable alkyl benzene sulphonate (LAS) may be obtained, by sulphonating commercially available linear alkyl benzene (LAB); suitable LAB includes low 2-phenyl LAB, such as Isochem® or Petrelab®, other suitable LAB include high 2-phenyl LAB, such as Hyblene®. A suitable anionic detergent is alkyl benzene sulphonate that is obtained by 30 DETAL catalyzed process, although other synthesis routes, such as HF, may also be suitable. In one aspect a magnesium salt of LAS is used.

Suitable sulphate detergents include alkyl sulphate, in one aspect,  $C_{8-18}$  alkyl sulphate, or predominantly  $C_{12}$  alkyl sulphate.

Another suitable sulphate detergents surfactant is alkyl alkoxylated sulphate, in one aspect, alkyl ethoxylated sulphate, in one aspect, a C<sub>8-18</sub> alkyl alkoxylated sulphate, in another aspect, a C<sub>8-18</sub> alkyl ethoxylated sulphate, typically the alkyl alkoxylated sulphate has an average degree of alkoxylation of from 0.5 to 20, or from 0.5 to 10, typically the alkyl alkoxylated sulphate is a C<sub>8-18</sub> alkyl ethoxylated sulphate having an average degree of ethoxylation of from 0.5 to 10, from 0.5 to 7, from 0.5 to 5 or from 0.5 to 3.

The alkyl sulphate, alkyl alkoxylated sulphate and alkyl benzene sulphonates may be linear or branched, substituted or un-substituted.

The detergents surfactant may be a mid-chain branched detergents surfactant, in one aspect, a mid-chain branched anionic detergents surfactant, in one aspect, a mid-chain branched alkyl sulphate and/or a mid-chain branched alkyl benzene sulphonate, e.g. a mid-chain branched alkyl sulphate. In one aspect, the mid-chain branches are C<sub>1-4</sub> alkyl groups, typically methyl and/or ethyl groups.

Suitable non-ionic detergents surfactants are selected from the group consisting of: C<sub>8-C<sub>18</sub></sub> alkyl ethoxylates, such as, NEODOL®; C<sub>6-C<sub>12</sub></sub> alkyl phenol alkoxylates wherein the alkoxylate units may be ethyleneoxy units, propyleneoxy units or a mixture thereof; C<sub>12-C<sub>18</sub></sub> alcohol and C<sub>6-C<sub>12</sub></sub> alkyl phenol condensates with ethylene oxide/propylene oxide block polymers such as Pluronic®; C<sub>14-C<sub>22</sub></sub> mid-chain branched alcohols; C<sub>14-C<sub>22</sub></sub> mid-chain branched alkyl alkoxylates, typically having an average degree of alkoxylation of from 1 to 30; alkylpolysaccharides, in one aspect, alkylpolyglycosides; polyhydroxy fatty acid amides; ether capped poly(oxyalkylated) alcohol surfactants; and mixtures thereof.

Suitable non-ionic detergents surfactants include alkyl polyglucoside and/or an alkyl alkoxylated alcohol.

In one aspect, non-ionic detergents surfactants include alkyl alkoxylated alcohols, in one aspect C<sub>8-18</sub> alkyl alkoxylated alcohol, e.g. a C<sub>8-18</sub> alkyl ethoxylated alcohol, the alkyl alkoxylated alcohol may have an average degree of alkoxylation of from 1 to 50, from 1 to 30, from 1 to 20, or from 1 to 10. In one aspect, the alkyl alkoxylated alcohol may be a C<sub>8-18</sub> alkyl ethoxylated alcohol having an average degree of ethoxylation of from 1 to 10, from 1 to 7, more from 1 to 5 or from 3 to 7. The alkyl alkoxylated alcohol can be linear or branched, and substituted or un-substituted. Suitable nonionic surfactants include Lutensol®.

Suitable cationic detergents surfactants include alkyl pyridinium compounds, alkyl quaternary ammonium compounds, alkyl quaternary phosphonium compounds, alkyl ternary sulphonium compounds, and mixtures thereof.

Suitable cationic detergents surfactants are quaternary ammonium compounds having the general

formula:  $(R)(R_1)(R_2)(R_3)N^+ X^-$ , wherein, R is a linear or branched, substituted or unsubstituted C<sub>6-18</sub> alkyl or alkenyl moiety, R<sub>1</sub> and R<sub>2</sub> are independently selected from methyl or ethyl moieties, R<sub>3</sub> is a hydroxyl, hydroxymethyl or a hydroxyethyl moiety, X is an anion which provides charge neutrality, suitable anions include: halides, e.g. chloride; sulphate; and sulphonate. Suitable cationic detergents 5 surfactants are mono-C<sub>6-18</sub> alkyl mono-hydroxyethyl di-methyl quaternary ammonium chlorides. Highly suitable cationic detergents are mono-C<sub>8-10</sub> alkyl mono-hydroxyethyl di-methyl quaternary ammonium chloride, mono-C<sub>10-12</sub> alkyl mono-hydroxyethyl di-methyl quaternary ammonium chloride and mono-C<sub>10</sub> alkyl mono-hydroxyethyl di-methyl quaternary ammonium chloride.

10 Suitable amphoteric/zwitterionic surfactants include amine oxides and betaines. Amine-neutralized anionic surfactants - Anionic surfactants of the present invention and adjunct anionic cosurfactants, may exist in an acid form, and said acid form may be neutralized to form a surfactant salt which is desirable for use in the present detergent compositions. Typical agents for neutralization include the metal counterion base such as hydroxides, eg, NaOH or KOH. Further preferred agents for 15 neutralizing anionic surfactants of the present invention and adjunct anionic surfactants or cosurfactants in their acid forms include ammonia, amines, or alkanolamines. Alkanolamines are preferred. Suitable non-limiting examples including monoethanolamine, diethanolamine, triethanolamine, and other linear or branched alkanolamines known in the art; e.g., highly preferred alkanolamines include 2-amino-1-propanol, 1-aminopropanol, monoisopropanolamine, or 1-amino-20 3-propanol. Amine neutralization may be done to a full or partial extent, e.g. part of the anionic surfactant mix may be neutralized with sodium or potassium and part of the anionic surfactant mix may be neutralized with amines or alkanolamines.

Surfactant systems comprising mixtures of one or more anionic and in addition one or more nonionic surfactants optionally with an additional surfactant such as a cationic surfactant, may be 25 preferred. Preferred weight ratios of anionic to nonionic surfactant are at least 2:1, or at least 1:1 to 1:10.

Builders - The compositions of the present invention may comprise one or more builders, co-builders, builder systems or a mixture thereof. When a builder is used, the cleaning composition will typically comprise at least 1wt%, from 2 to 60wt% or from 5 to 10wt% builder. In a dish wash 30 cleaning composition, the level of builder is typically 40 to 65wt%, or 50 to 65wt%. The composition may be substantially free of builder; substantially free means "no deliberately added" zeolite and/or phosphate. Typical zeolite builders include zeolite A, zeolite P and zeolite MAP. A typical phosphate builder is sodium tri-polyphosphate.

The builder and/or co-builder may particularly be a chelating agent that forms water-soluble complexes

with Ca and Mg. Any builder and/or co-builder known in the art for use in detergents may be utilized. Non-limiting examples of builders include zeolites, diphosphates (pyrophosphates), triphosphates such as sodium triphosphate (STP or STPP), carbonates such as sodium carbonate, soluble silicates such as sodium metasilicate, layered silicates (e.g., SKS-6 from Hoechst), ethanolamines such as 2-aminoethan-1-ol (MEA), iminodiethanol (DEA) and 2,2',2"-nitrilotriethanol (TEA), and carboxymethylinulin (CMI), and combinations thereof.

The cleaning composition may include a co-builder alone, or in combination with a builder, e.g. a zeolite builder. Non-limiting examples of co-builders include homopolymers of polyacrylates or copolymers thereof, such as poly(acrylic acid) (PAA) or copoly(acrylic acid/maleic acid) (PAA/PMA).

Further non-limiting examples include citrate, chelators such as aminocarboxylates, aminopolycarboxylates and phosphonates, and alkyl- or alkenylsuccinic acid. Additional specific examples include 2,2',2"-nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), iminodisuccinic acid (IDS), ethylenediamine-N,N'-disuccinic acid (EDDS), methylglycinediacetic acid (MGDA), glutamic acid-N,N-diacetic acid (GLDA), 1-hydroxyethane-1,1-diylbis(phosphonic acid) (HEDP), ethylenediaminetetrakis(methylene)tetrakis(phosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylene)pentakis(phosphonic acid) (DTPMPA), N-(2-hydroxyethyl)iminodiacetic acid (EDG), aspartic acid-N-monoacetic acid (ASMA), aspartic acid- N,N-diacetic acid (ASDA), aspartic acid-N- monopropionic acid (ASMP) , iminodisuccinic acid (IDA), N- (2-sulfomethyl) aspartic acid (SMAS), N- (2-sulfoethyl) aspartic acid (SEAS), N- (2- sulfomethyl) glutamic acid (SMGL), N- (2- sulfoethyl) glutamic acid (SEGL), N- methyliminodiacetic acid (MIDA),  $\alpha$ -alanine- N,N-diacetic acid ( $\alpha$ -ALDA) , serine-N,N-diacetic acid (SEDA), isoserine-N,N-diacetic acid (ISDA), phenylalanine-N,N-diacetic acid (PHDA) , anthranilic acid- N ,N - diacetic acid (ANDA), sulfanilic acid- N, N-diacetic acid (SLDA) , taurine-N, N-diacetic acid (TUDA) and sulfomethyl-N,N-diacetic acid (SMDA), N-(hydroxyethyl)-ethylidenediaminetriacetate (HEDTA), diethanolglycine (DEG), Diethylenetriamine Penta (Methylene Phosphonic acid) (DTPMP), aminotris(methylenephosphonic acid) (ATMP), and combinations and salts thereof. Further exemplary builders and/or co-builders are described in, e.g., WO09/102854, US5977053.

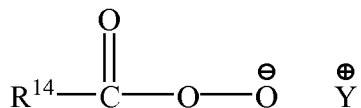
Chelating Agents and Crystal Growth Inhibitors - The compositions herein may contain a chelating agent and/or a crystal growth inhibitor. Suitable molecules include copper, iron and/or manganese chelating agents and mixtures thereof. Suitable molecules include DTPA (Diethylene triamine pentaacetic acid), HEDP (Hydroxyethane diphosphonic acid), DTPMP (Diethylene triamine penta(methylene phosphonic acid)), 1,2-Dihydroxybenzene-3,5-disulfonic acid disodium salt hydrate, ethylenediamine, diethylene triamine, ethylenediaminedisuccinic acid (EDDS), N-

hydroxyethylethylenediaminetri-acetic acid (HEDTA), triethylenetetraaminehexaacetic acid (TTHA), N-hydroxyethyliminodiacetic acid (HEIDA), dihydroxyethylglycine (DHEG), ethylenediaminetetrapropionic acid (EDTP), carboxymethyl inulin and 2-Phosphonobutane 1,2,4-tricarboxylic acid (Bayhibit® AM) and derivatives thereof. Typically the composition may comprise 5 from 0.005 to 15wt% or from 3.0 to 10wt% chelating agent or crystal growth inhibitor.

Bleach Component - The bleach component suitable for incorporation in the methods and compositions of the invention comprise one or a mixture of more than one bleach component. Suitable bleach components include bleaching catalysts, photobleaches, bleach activators, hydrogen peroxide, sources of hydrogen peroxide, pre-formed peracids and mixtures thereof. In 10 general, when a bleach component is used, the compositions of the present invention may comprise from 0.00001 to 90wt%, 0.0001 to 50wt% or from 0.001 to 25wt%. Examples of suitable bleach components include:

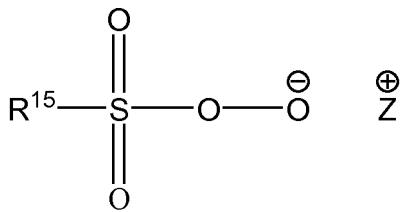
15 (1) Pre-formed peracids: Suitable preformed peracids include, but are not limited to, compounds selected from the group consisting of pre-formed peroxyacids or salts thereof, typically either a peroxycarboxylic acid or salt thereof, or a peroxy sulphonic acid or salt thereof.

The pre-formed peroxyacid or salt thereof is preferably a peroxycarboxylic acid or salt thereof, typically having a chemical structure corresponding to the following chemical formula:



wherein:  $\text{R}^{14}$  is selected from alkyl, aralkyl, cycloalkyl, aryl or heterocyclic groups; the  $\text{R}^{14}$  group can 20 be linear or branched, substituted or unsubstituted; and  $\text{Y}$  is any suitable counter-ion that achieves electric charge neutrality, preferably  $\text{Y}$  is selected from hydrogen, sodium or potassium. Preferably,  $\text{R}^{14}$  is a linear or branched, substituted or unsubstituted  $\text{C}_{6-9}$  alkyl. Preferably, the peroxyacid or salt thereof is selected from peroxyhexanoic acid, peroxyheptanoic acid, peroxyoctanoic acid, peroxynonanoic acid, peroxydecanoic acid, any salt thereof, or any combination thereof. 25 Particularly preferred peroxyacids are phthalimido-peroxy-alkanoic acids, in particular  $\epsilon$ -phthahlimido peroxy hexanoic acid (PAP). Preferably, the peroxyacid or salt thereof has a melting point in the range of from 30°C to 60°C.

The pre-formed peroxyacid or salt thereof can also be a peroxy sulphonic acid or salt thereof, typically having a chemical structure corresponding to the following chemical formula:



wherein:  $\text{R}^{15}$  is selected from alkyl, aralkyl, cycloalkyl, aryl or heterocyclic groups; the  $\text{R}^{15}$  group can be linear or branched, substituted or unsubstituted; and  $\text{Z}$  is any suitable counter-ion that achieves electric charge neutrality, preferably  $\text{Z}$  is selected from hydrogen, sodium or potassium. Preferably

5  $\text{R}^{15}$  is a linear or branched, substituted or unsubstituted  $\text{C}_{6-9}$  alkyl. Preferably such bleach components may be present in the compositions of the invention in an amount from 0.01 to 50wt% or from 0.1 to 20wt%.

(2) Sources of hydrogen peroxide include e.g., inorganic perhydrate salts, including alkali metal salts such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, 10 persulphate, perphosphate, persilicate salts and mixtures thereof. In one aspect of the invention the inorganic perhydrate salts such as those selected from the group consisting of sodium salts of perborate, percarbonate and mixtures thereof. When employed, inorganic perhydrate salts are typically present in amounts of 0.05 to 40wt% or 1 to 30wt% of the overall composition and are typically incorporated into such compositions as a crystalline solid that may be coated. Suitable 15 coatings include: inorganic salts such as alkali metal silicate, carbonate or borate salts or mixtures thereof, or organic materials such as water-soluble or dispersible polymers, waxes, oils or fatty soaps. Preferably such bleach components may be present in the compositions of the invention in an amount of 0.01 to 50wt% or 0.1 to 20wt%.

(3) Suitable bleach activators are those having  $\text{R}-(\text{C}=\text{O})-\text{L}$  wherein  $\text{R}$  is an alkyl group, optionally 20 branched, having, when the bleach activator is hydrophobic, from 6 to 14 carbon atoms, or from 8 to 12 carbon atoms and, when the bleach activator is hydrophilic, less than 6 carbon atoms or less than 4 carbon atoms; and  $\text{L}$  is leaving group. Examples of suitable leaving groups are benzoic acid and derivatives thereof - especially benzene sulphonate. Suitable bleach activators include 25 dodecanoyl oxybenzene sulphonate, decanoyl oxybenzene sulphonate, decanoyl oxybenzoic acid or salts thereof, 3,5,5-trimethyl hexanoyloxybenzene sulphonate, tetraacetyl ethylene diamine (TAED) and nonanoyloxybenzene sulphonate (NOBS). Suitable bleach activators are also disclosed in WO98/17767. While any suitable bleach activator may be employed, in one aspect of the invention the subject cleaning composition may comprise NOBS, TAED or mixtures thereof. When present, the peracid and/or bleach activator is generally present in the composition in an 30 amount of 0.1 to 60wt%, 0.5 to 40wt% or 0.6 to 10wt% based on the fabric and home care

composition. One or more hydrophobic peracids or precursors thereof may be used in combination with one or more hydrophilic peracid or precursor thereof. Preferably such bleach components may be present in the compositions of the invention in an amount of 0.01 to 50wt%, or 0.1 to 20wt%.

The amounts of hydrogen peroxide source and peracid or bleach activator may be selected such that the molar ratio of available oxygen (from the peroxide source) to peracid is from 1:1 to 35:1, or even 2:1 to 10:1.

(4) Diacyl peroxides – preferred diacyl peroxide bleaching species include those selected from diacyl peroxides of the general formula:  $R^1-C(O)-OO-(O)C-R^2$ , in which  $R^1$  represents a  $C_6-C_{18}$  alkyl, preferably  $C_6-C_{12}$  alkyl group containing a linear chain of at least 5 carbon atoms and

10 optionally containing one or more substituents (e.g.  $-N^+(CH_3)_3$ , -COOH or -CN) and/or one or more interrupting moieties (e.g. -CONH- or -CH=CH-) interpolated between adjacent carbon atoms of the alkyl radical, and  $R^2$  represents an aliphatic group compatible with a peroxide moiety, such that  $R^1$  and  $R^2$  together contain a total of 8 to 30 carbon atoms. In one preferred aspect  $R^1$  and  $R^2$  are linear unsubstituted  $C_6-C_{12}$  alkyl chains. Most preferably  $R^1$  and  $R^2$  are identical. Diacyl peroxides,

15 in which both  $R^1$  and  $R^2$  are  $C_6-C_{12}$  alkyl groups, are particularly preferred. Preferably, at least one of, most preferably only one of, the R groups ( $R_1$  or  $R_2$ ), does not contain branching or pendant rings in the alpha position, or preferably neither in the alpha nor beta positions or most preferably in none of the alpha or beta or gamma positions. In one further preferred embodiment the DAP may be asymmetric, such that preferably the hydrolysis of  $R_1$  acyl group is rapid to generate peracid,

20 but the hydrolysis of  $R_2$  acyl group is slow.

The tetraacyl peroxide bleaching species is preferably selected from tetraacyl peroxides of the general formula:  $R^3-C(O)-OO-C(O)-(CH_2)_n-C(O)-OO-C(O)-R^3$ , in which  $R^3$  represents a  $C_1-C_9$  alkyl, or  $C_3-C_7$ , group and n represents an integer from 2 to 12, or 4 to 10 inclusive.

Preferably, the diacyl and/or tetraacyl peroxide bleaching species is present in an amount sufficient to provide at least 0.5ppm, at least 10ppm, or at least 50ppm by weight of the wash liquor. In a preferred embodiment, the bleaching species is present in an amount sufficient to provide from 0.5 to 300ppm, from 30 to 150ppm by weight of the wash liquor.

Preferably the bleach component comprises a bleach catalyst (5 and 6).

(5) Preferred are organic (non-metal) bleach catalysts include bleach catalyst capable of accepting an oxygen atom from a peroxyacid and/or salt thereof, and transferring the oxygen atom to an oxidizable substrate. Suitable bleach catalysts include, but are not limited to: iminium cations and

polyions; iminium zwitterions; modified amines; modified amine oxides; N-sulphonyl imines; N-phosphonyl imines; N-acyl imines; thiadiazole dioxides; perfluoroimines; cyclic sugar ketones and mixtures thereof.

Suitable iminium cations and polyions include, but are not limited to, N-methyl-3,4-dihydroisoquinolinium tetrafluoroborate, prepared as described in *Tetrahedron* (1992), 49(2), 423-38 (see e.g. compound 4, p. 433); N-methyl-3,4-dihydroisoquinolinium p-toluene sulphonate, prepared as described in US5360569 (see e.g. Column 11, Example 1); and N-octyl-3,4-dihydroisoquinolinium p-toluene sulphonate, prepared as described in US5360568 (see e.g. Column 10, Example 3).

Suitable iminium zwitterions include, but are not limited to, N-(3-sulfopropyl)-3,4-dihydroisoquinolinium, inner salt, prepared as described in US5576282 (see e.g. Column 31, Example II); N-[2-(sulphooxy)dodecyl]-3,4-dihydroisoquinolinium, inner salt, prepared as described in US5817614 (see e.g. Column 32, Example V); 2-[3-[(2-ethylhexyl)oxy]-2-(sulphooxy)propyl]-3,4-dihydroisoquinolinium, inner salt, prepared as described in WO05/047264 (see e.g. page 18, Example 8), and 2-[3-[(2-butyloctyl)oxy]-2-(sulphooxy)propyl]-3,4-dihydroisoquinolinium, inner salt.

Suitable modified amine oxygen transfer catalysts include, but are not limited to, 1,2,3,4-tetrahydro-2-methyl-1-isoquinolinol, which can be made according to the procedures described in *Tetrahedron Letters* (1987), 28(48), 6061-6064. Suitable modified amine oxide oxygen transfer catalysts include, but are not limited to, sodium 1-hydroxy-N-oxy-N-[2-(sulphooxy)decyl]-1,2,3,4-tetrahydroisoquinoline.

Suitable N-sulphonyl imine oxygen transfer catalysts include, but are not limited to, 3-methyl-1,2-benzisothiazole 1,1-dioxide, prepared according to the procedure described in the *Journal of Organic Chemistry* (1990), 55(4), 1254-61.

Suitable N-phosphonyl imine oxygen transfer catalysts include, but are not limited to, [R-(E)]-N-[(2-chloro-5-nitrophenyl)methylene]-P-phenyl-P-(2,4,6-trimethylphenyl)- phosphinic amide, which can be made according to the procedures described in the *Journal of the Chemical Society, Chemical Communications* (1994), (22), 2569-70.

Suitable N-acyl imine oxygen transfer catalysts include, but are not limited to, [N(E)]-N-(phenylmethylene)acetamide, which can be made according to the procedures described in *Polish Journal of Chemistry* (2003), 77(5), 577-590.

Suitable thiadiazole dioxide oxygen transfer catalysts include but are not limited to, 3-methyl-4-phenyl-1,2,5-thiadiazole 1,1-dioxide, which can be made according to the procedures described in US5753599 (Column 9, Example 2).

Suitable perfluoroimine oxygen transfer catalysts include, but are not limited to, (Z)-2,2,3,3,4,4,4-heptafluoro-N-(nonafluorobutyl)butanimidoyl fluoride, which can be made according to the procedures described in *Tetrahedron Letters* (1994), 35(34), 6329-30.

Suitable cyclic sugar ketone oxygen transfer catalysts include, but are not limited to, 1,2:4,5-di-O-

5 isopropylidene-D-erythro-2,3-hexodiuro-2,6-pyranose as prepared in US6649085 (Column 12, Example 1).

The bleach catalyst may comprise an iminium and/or carbonyl functional group and is typically capable of forming an oxaziridinium and/or dioxirane functional group upon acceptance of an oxygen atom, especially upon acceptance of an oxygen atom from a peroxyacid and/or salt thereof.

10 The bleach catalyst may comprise an oxaziridinium functional group and/or is capable of forming an oxaziridinium functional group upon acceptance of an oxygen atom, especially upon acceptance of an oxygen atom from a peroxyacid and/or salt thereof. The bleach catalyst may comprise a cyclic iminium functional group, preferably wherein the cyclic moiety has a ring size of from five to eight atoms (including the nitrogen atom), preferably six atoms. The bleach catalyst may comprise an

15 aryliminium functional group, preferably a bi-cyclic aryliminium functional group, preferably a 3,4-dihydroisoquinolinium functional group. Typically, the imine functional group is a quaternary imine functional group and is typically capable of forming a quaternary oxaziridinium functional group upon acceptance of an oxygen atom, especially upon acceptance of an oxygen atom from a peroxyacid and/or salt thereof. In another aspect, the detergent composition comprises a bleach

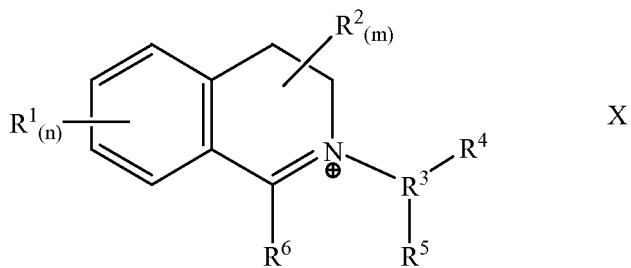
20 component having a  $\log P_{o/w}$  no greater than 0, no greater than -0.5, no greater than -1.0, no greater than -1.5, no greater than -2.0, no greater than -2.5, no greater than -3.0, or no greater than -3.5.

The method for determining  $\log P_{o/w}$  is described in more detail below.

Typically, the bleach ingredient is capable of generating a bleaching species having a  $X_{SO}$  of from 0.01 to 0.30, from 0.05 to 0.25, or from 0.10 to 0.20. The method for determining  $X_{SO}$  is described

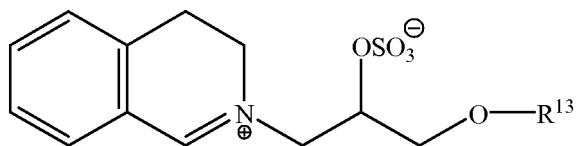
25 in more detail below. For example, bleaching ingredients having an isoquinolinium structure are capable of generating a bleaching species that has an oxaziridinium structure. In this example, the  $X_{SO}$  is that of the oxaziridinium bleaching species.

The bleach catalyst may have a chemical structure corresponding to the following chemical formula:



wherein: n and m are independently from 0 to 4, preferably n and m are both 0; each R<sup>1</sup> is independently selected from a substituted or unsubstituted radical selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, fused aryl, heterocyclic ring, fused heterocyclic ring, 5 nitro, halo, cyano, sulphonato, alkoxy, keto, carboxylic, and carboalkoxy radicals; and any two vicinal R<sup>1</sup> substituents may combine to form a fused aryl, fused carbocyclic or fused heterocyclic ring; each R<sup>2</sup> is independently selected from a substituted or unsubstituted radical independently selected from the group consisting of hydrogen, hydroxy, alkyl, cycloalkyl, alkaryl, aryl, aralkyl, alkynes, heterocyclic ring, alkoxy, arylcarbonyl groups, carboxyalkyl groups and amide groups; 10 any R<sup>2</sup> may be joined together with any other of R<sup>2</sup> to form part of a common ring; any geminal R<sup>2</sup> may combine to form a carbonyl; and any two R<sup>2</sup> may combine to form a substituted or unsubstituted fused unsaturated moiety; R<sup>3</sup> is a C<sub>1</sub> to C<sub>20</sub> substituted or unsubstituted alkyl; R<sup>4</sup> is hydrogen or the moiety Q<sub>t</sub>-A, wherein: Q is a branched or unbranched alkylene, t = 0 or 1 and A is an anionic group selected from the group consisting of OSO<sub>3</sub><sup>-</sup>, SO<sub>3</sub><sup>-</sup>, CO<sub>2</sub><sup>-</sup>, OCO<sub>2</sub><sup>-</sup>, OPO<sub>3</sub><sup>2-</sup>, OPO<sub>3</sub>H<sup>-</sup> 15 and OPO<sub>2</sub><sup>-</sup>; R<sup>5</sup> is hydrogen or the moiety -CR<sup>11</sup>R<sup>12</sup>-Y-G<sub>b</sub>-Y<sub>c</sub>-[(CR<sup>9</sup>R<sup>10</sup>)<sub>y</sub>-O]<sub>k</sub>-R<sup>8</sup>, wherein: each Y is independently selected from the group consisting of O, S, N-H, or N-R<sup>8</sup>; and each R<sup>8</sup> is independently selected from the group consisting of alkyl, aryl and heteroaryl, said moieties being substituted or unsubstituted, and whether substituted or unsubstituted said moieties having less 20 than 21 carbons; each G is independently selected from the group consisting of CO, SO<sub>2</sub>, SO, PO and PO<sub>2</sub>; R<sup>9</sup> and R<sup>10</sup> are independently selected from the group consisting of H and C<sub>1</sub>-C<sub>4</sub> alkyl; R<sup>11</sup> and R<sup>12</sup> are independently selected from the group consisting of H and alkyl, or when taken together may join to form a carbonyl; b = 0 or 1; c can = 0 or 1, but c must = 0 if b = 0; y is an integer from 1 to 6; k is an integer from 0 to 20; R<sup>6</sup> is H, or an alkyl, aryl or heteroaryl moiety; said 25 moieties being substituted or unsubstituted; and X, if present, is a suitable charge balancing counterion, preferably X is present when R<sup>4</sup> is hydrogen, suitable X, include but are not limited to: chloride, bromide, sulphate, methosulphate, sulphonate, p-toluenesulphonate, borontetraflouride and phosphate.

In one embodiment of the present invention, the bleach catalyst has a structure corresponding to general formula below:



wherein R<sup>13</sup> is a branched alkyl group containing from three to 24 carbon atoms (including the branching carbon atoms) or a linear alkyl group containing from one to 24 carbon atoms; preferably R<sup>13</sup> is a branched alkyl group containing from eight to 18 carbon atoms or linear alkyl group

5 containing from eight to eighteen carbon atoms; preferably R<sup>13</sup> is selected from the group consisting of 2-propylheptyl, 2-butyloctyl, 2-pentylnonyl, 2-hexyldecyl, n-dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, iso-nonyl, iso-decyl, iso-tridecyl and iso-pentadecyl; preferably R<sup>13</sup> is selected from the group consisting of 2-butyloctyl, 2-pentylnonyl, 2-hexyldecyl, iso-tridecyl and iso-pentadecyl.

10 The bleach component may comprise a source of peracid in addition to bleach catalyst, particularly organic bleach catalyst. The source of peracid may be selected from (a) pre-formed peracid; (b) percarbonate, perborate or persulfate salt (hydrogen peroxide source) preferably in combination with a bleach activator; and (c) perhydrolase enzyme and an ester for forming peracid in situ in the presence of water in a textile or hard surface treatment step.

15 When present, the peracid and/or bleach activator is generally present in the composition in an amount of from 0.1 to 60wt%, from 0.5 to 40wt% or from 0.6 to 10wt% based on the composition. One or more hydrophobic peracids or precursors thereof may be used in combination with one or more hydrophilic peracid or precursor thereof.

20 The amounts of hydrogen peroxide source and peracid or bleach activator may be selected such that the molar ratio of available oxygen (from the peroxide source) to peracid is from 1:1 to 35:1, or 2:1 to 10:1.

(6) Metal-containing Bleach Catalysts – The bleach component may be provided by a catalytic metal complex. One type of metal-containing bleach catalyst is a catalyst system comprising a transition metal cation of defined bleach catalytic activity, such as copper, iron, titanium, ruthenium, 25 tungsten, molybdenum, or manganese cations, an auxiliary metal cation having little or no bleach catalytic activity, such as zinc or aluminum cations, and a sequestrate having defined stability constants for the catalytic and auxiliary metal cations, particularly ethylenediaminetetraacetic acid, ethylenediaminetetra(methylenephosphonic acid) and water-soluble salts thereof. Such catalysts are disclosed in US4430243. Preferred catalysts are described in WO09/839406, US6218351 and 30 WO00/012667. Particularly preferred are transition metal catalyst or ligands therefore that are cross-bridged polydentate N-donor ligands.

If desired, the compositions herein can be catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art and include, e.g., the manganese-based catalysts disclosed in US5576282.

Cobalt bleach catalysts useful herein are known, and are described e.g. in US5597936;

5 US5595967. Such cobalt catalysts are readily prepared by known procedures, such as taught e.g. in US5597936 and US5595967.

Compositions herein may also suitably include a transition metal complex of ligands such as bispidones (US7501389) and/or macropolycyclic rigid ligands - abbreviated as "MRLs". As a practical matter, and not by way of limitation, the compositions and processes herein can be

10 adjusted to provide on the order of at least one part per hundred million of the active MRL species in the aqueous washing medium, and will typically provide from 0.005 to 25ppm, from 0.05 to 10ppm, or from 0.1 to 5ppm, of the MRL in the wash liquor.

Suitable transition-metals in the instant transition-metal bleach catalyst include e.g. manganese, iron and chromium. Suitable MRLs include 5,12-diethyl-1,5,8,12-tetraazabicyclo[6.6.2]hexadecane.

15 Suitable transition metal MRLs are readily prepared by known procedures, such as taught e.g. in US6225464 and WO00/32601.

(7) Photobleaches – suitable photobleaches include e.g. sulfonated zinc phthalocyanine sulfonated aluminium phthalocyanines, xanthene dyes and mixtures thereof. Preferred bleach components for use in the present compositions of the invention comprise a hydrogen peroxide source, bleach

20 activator and/or organic peroxyacid, optionally generated in situ by the reaction of a hydrogen peroxide source and bleach activator, in combination with a bleach catalyst. Preferred bleach components comprise bleach catalysts, preferably organic bleach catalysts, as described above.

Particularly preferred bleach components are the bleach catalysts in particular the organic bleach catalysts.

25 Fabric Hueing Agents - The composition may comprise a fabric hueing agent. Suitable fabric hueing agents include dyes, dye-clay conjugates, and pigments. Suitable dyes include small molecule dyes and polymeric dyes. Suitable small molecule dyes include small molecule dyes selected from the group consisting of dyes falling into the Color Index (C.I.) classifications of Direct Blue, Direct Red, Direct Violet, Acid Blue, Acid Red, Acid Violet, Basic Blue, Basic Violet and Basic

30 Red, or mixtures thereof.

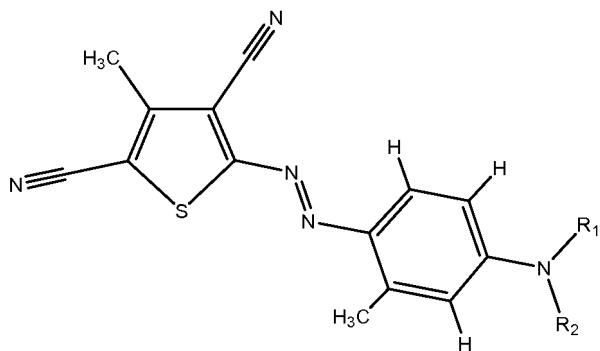
In another aspect, suitable small molecule dyes include small molecule dyes selected from the group consisting of Color Index (Society of Dyers and Colorists, Bradford, UK) numbers Direct Violet 9, Direct Violet 35, Direct Violet 48, Direct Violet 51, Direct Violet 66, Direct Violet 99, Direct

Blue 1, Direct Blue 71, Direct Blue 80, Direct Blue 279, Acid Red 17, Acid Red 73, Acid Red 88, Acid Red 150, Acid Violet 15, Acid Violet 17, Acid Violet 24, Acid Violet 43, Acid Red 52, Acid Violet 49, Acid Violet 50, Acid Blue 15, Acid Blue 17, Acid Blue 25, Acid Blue 29, Acid Blue 40, Acid Blue 45, Acid Blue 75, Acid Blue 80, Acid Blue 83, Acid Blue 90 and Acid Blue 113, Acid Black 1, Basic 5 Violet 1, Basic Violet 3, Basic Violet 4, Basic Violet 10, Basic Violet 35, Basic Blue 3, Basic Blue 16, Basic Blue 22, Basic Blue 47, Basic Blue 66, Basic Blue 75, Basic Blue 159 and mixtures thereof. In another aspect, suitable small molecule dyes include small molecule dyes selected from the group consisting of Color Index (Society of Dyers and Colorists, Bradford, UK) numbers Acid Violet 17, Acid Violet 43, Acid Red 52, Acid Red 73, Acid Red 88, Acid Red 150, Acid Blue 25, Acid Blue 10 29, Acid Blue 45, Acid Blue 113, Acid Black 1, Direct Blue 1, Direct Blue 71, Direct Violet 51 and mixtures thereof. In another aspect, suitable small molecule dyes include small molecule dyes selected from the group consisting of Color Index (Society of Dyers and Colorists, Bradford, UK) numbers Acid Violet 17, Direct Blue 71, Direct Violet 51, Direct Blue 1, Acid Red 88, Acid Red 150, Acid Blue 29, Acid Blue 113 or mixtures thereof.

15 Suitable polymeric dyes include polymeric dyes selected from the group consisting of polymers containing conjugated chromogens (dye-polymer conjugates) and polymers with chromogens co-polymerized into the backbone of the polymer and mixtures thereof.

In another aspect, suitable polymeric dyes include polymeric dyes selected from the group consisting of fabric-substantive colorants sold under the name of Liquitint® (Milliken), dye-polymer 20 conjugates formed from at least one reactive dye and a polymer selected from the group consisting of polymers comprising a moiety selected from the group consisting of a hydroxyl moiety, a primary amine moiety, a secondary amine moiety, a thiol moiety and mixtures thereof. In still another aspect, suitable polymeric dyes include polymeric dyes selected from the group consisting of Liquitint® Violet CT, carboxymethyl cellulose (CMC) conjugated with a reactive blue, reactive violet 25 or reactive red dye such as CMC conjugated with C.I. Reactive Blue 19, sold by Megazyme, Wicklow, Ireland under the product name AZO-CM-CELLULOSE, product code S-ACMC, alkoxylated triphenyl-methane polymeric colorants, alkoxylated thiophene polymeric colorants, and mixtures thereof.

Preferred hueing dyes include the whitening agents found in WO08/87497. These whitening 30 agents may be characterized by the following structure (I):



(I)

wherein  $R_1$  and  $R_2$  can independently be selected from:

a)  $[(CH_2CR'HO)_x(CH_2CR''HO)_yH]$

wherein  $R'$  is selected from the group consisting of H,  $CH_3$ ,  $CH_2O(CH_2CH_2O)_zH$ , and mixtures thereof; wherein  $R''$  is selected from the group consisting of H,  $CH_2O(CH_2CH_2O)_zH$ , and mixtures thereof; wherein  $x + y \leq 5$ ; wherein  $y \geq 1$ ; and wherein  $z = 0$  to 5;

b)  $R_1 =$  alkyl, aryl or aryl alkyl and  $R_2 = [(CH_2CR'HO)_x(CH_2CR''HO)_yH]$

wherein  $R'$  is selected from the group consisting of H,  $CH_3$ ,  $CH_2O(CH_2CH_2O)_zH$ , and mixtures thereof; wherein  $R''$  is selected from the group consisting of H,  $CH_2O(CH_2CH_2O)_zH$ , and mixtures thereof; wherein  $x + y \leq 10$ ; wherein  $y \geq 1$ ; and wherein  $z = 0$  to 5;

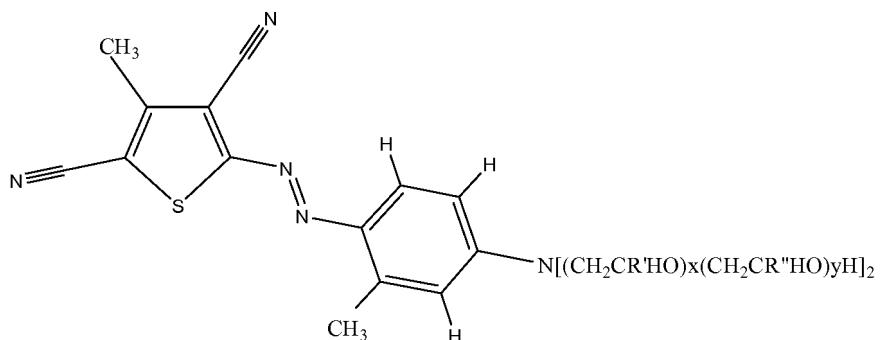
c)  $R_1 = [CH_2CH_2(OR_3)CH_2OR_4]$  and  $R_2 = [CH_2CH_2(O R_3)CH_2O R_4]$

wherein  $R_3$  is selected from the group consisting of H,  $(CH_2CH_2O)_zH$ , and mixtures thereof; and wherein  $z = 0$  to 10;

wherein  $R_4$  is selected from the group consisting of  $(C_{1-16})$ alkyl, aryl groups, and mixtures thereof; and

d) wherein  $R_1$  and  $R_2$  can independently be selected from the amino addition product of styrene oxide, glycidyl methyl ether, isobutyl glycidyl ether, isopropylglycidyl ether, t-butyl glycidyl ether, 2-ethylhexylglycidyl ether, and glycidylhexadecyl ether, followed by the addition of from 1 to 10 alkylene oxide units.

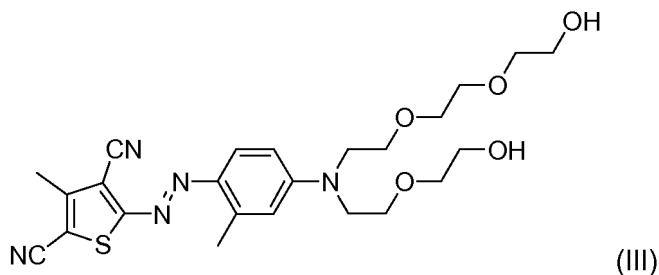
20 A preferred whitening agent of the present invention may be characterized by the following structure (II):



(II)

wherein R' is selected from the group consisting of H, CH<sub>3</sub>, CH<sub>2</sub>O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>z</sub>H, and mixtures thereof; wherein R" is selected from the group consisting of H, CH<sub>2</sub>O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>z</sub>H, and mixtures thereof; wherein x + y ≤ 5; wherein y ≥ 1; and wherein z = 0 to 5.

5 A further preferred whitening agent of the present invention may be characterized by the following structure (III):



(III)

typically comprising a mixture having a total of 5 EO groups. Suitable preferred molecules are those in Structure I having the following pendant groups in "part a" above.

10 TABLE 1

	R1		x	y	R2		x	y
	R'	R"			R'	R"		
a	H	H	3	1	H	H	0	1
b	H	H	2	1	H	H	1	1
c = b	H	H	1	1	H	H	2	1
d = a	H	H	0	1	H	H	3	1

Further whitening agents of use include those described in US2008/34511 (Unilever). A preferred

agent is "Violet 13".

Suitable dye clay conjugates include dye clay conjugates selected from the group comprising at least one cationic/basic dye and a smectite clay, and mixtures thereof. In another aspect, suitable dye clay conjugates include dye clay conjugates selected from the group consisting of one cationic/basic dye selected from the group consisting of C.I. Basic Yellow 1 through 108, C.I. Basic Orange 1 through 69, C.I. Basic Red 1 through 118, C.I. Basic Violet 1 through 51, C.I. Basic Blue 1 through 164, C.I. Basic Green 1 through 14, C.I. Basic Brown 1 through 23, C.I. Basic Black 1 through 11, and a clay selected from the group consisting of Montmorillonite clay, Hectorite clay, Saponite clay and mixtures thereof. In still another aspect, suitable dye clay conjugates include dye clay conjugates selected from the group consisting of: Montmorillonite Basic Blue B7 C.I. 42595 conjugate, Montmorillonite Basic Blue B9 C.I. 52015 conjugate, Montmorillonite Basic Violet V3 C.I. 42555 conjugate, Montmorillonite Basic Green G1 C.I. 42040 conjugate, Montmorillonite Basic Red R1 C.I. 45160 conjugate, Montmorillonite C.I. Basic Black 2 conjugate, Hectorite Basic Blue B7 C.I. 42595 conjugate, Hectorite Basic Blue B9 C.I. 52015 conjugate, Hectorite Basic Violet V3 C.I. 42555 conjugate, Hectorite Basic Green G1 C.I. 42040 conjugate, Hectorite Basic Red R1 C.I. 45160 conjugate, Saponite Basic Blue B7 C.I. 42595 conjugate, Saponite Basic Blue B9 C.I. 52015 conjugate, Saponite Basic Violet V3 C.I. 42555 conjugate, Saponite Basic Green G1 C.I. 42040 conjugate, Saponite Basic Red R1 C.I. 45160 conjugate, Saponite C.I. Basic Black 2 conjugate and mixtures thereof.

20 Suitable pigments include pigments selected from the group consisting of flavanthrone, indanthrone, chlorinated indanthrone containing from 1 to 4 chlorine atoms, pyranthrone, dichloropyranthrone, monobromodichloropyranthrone, dibromodichloropyranthrone, tetrabromopyranthrone, perylene-3,4,9,10-tetracarboxylic acid diimide, wherein the imide groups may be unsubstituted or substituted by C1-C3 -alkyl or a phenyl or heterocyclic radical, and 25 wherein the phenyl and heterocyclic radicals may additionally carry substituents which do not confer solubility in water, anthrapyrimidinecarboxylic acid amides, violanthrone, isoviolanthrone, dioxazine pigments, copper phthalocyanine which may contain up to 2 chlorine atoms per molecule, polychloro-copper phthalocyanine or polybromochloro-copper phthalocyanine containing up to 14 bromine atoms per molecule and mixtures thereof.

30 In another aspect, suitable pigments include pigments selected from the group consisting of Ultramarine Blue (C.I. Pigment Blue 29), Ultramarine Violet (C.I. Pigment Violet 15) and mixtures thereof.

The aforementioned fabric hueing agents can be used in combination (any mixture of fabric hueing agents can be used). Suitable hueing agents are described in more detail in US7208459. Preferred

levels of dye in compositions of the invention are 0.00001 to 0.5wt%, or 0.0001 to 0.25wt%. The concentration of dyes preferred in water for the treatment and/or cleaning step is from 1ppb to 5ppm, 10ppb to 5ppm or 20ppb to 5ppm. In preferred compositions, the concentration of surfactant will be from 0.2 to 3g/l.

5 Encapsulates - The composition may comprise an encapsulate. In one aspect, an encapsulate comprising a core, a shell having an inner and outer surface, said shell encapsulating said core. In one aspect of said encapsulate, said core may comprise a material selected from the group consisting of perfumes; brighteners; dyes; insect repellants; silicones; waxes; flavors; vitamins; fabric softening agents; skin care agents in one aspect, paraffins; enzymes; anti-bacterial agents; 10 bleaches; sensates; and mixtures thereof; and said shell may comprise a material selected from the group consisting of polyethylenes; polyamides; polyvinylalcohols, optionally containing other co-monomers; polystyrenes; polyisoprenes; polycarbonates; polyesters; polyacrylates; aminoplasts, in one aspect said aminoplast may comprise a polyureas, polyurethane, and/or polyureaurethane, in one aspect said polyurea may comprise polyoxymethyleneurea and/or melamine formaldehyde; 15 polyolefins; polysaccharides, in one aspect said polysaccharide may comprise alginate and/or chitosan; gelatin; shellac; epoxy resins; vinyl polymers; water insoluble inorganics; silicone; and mixtures thereof.

In one aspect of said encapsulate, said core may comprise perfume.

In one aspect of said encapsulate, said shell may comprise melamine formaldehyde and/or cross 20 linked melamine formaldehyde.

In a one aspect, suitable encapsulates may comprise a core material and a shell, said shell at least partially surrounding said core material, is disclosed. At least 75%, 85% or 90% of said encapsulates may have a fracture strength of from 0.2 to 10 MPa, from 0.4 to 5MPa, from 0.6 to 3.5 MPa, or from 0.7 to 3MPa; and a benefit agent leakage of from 0 to 30%, from 0 to 20%, or from 0 25 to 5%.

In one aspect, at least 75%, 85% or 90% of said encapsulates may have a particle size from 1 to 80 microns, from 5 to 60 microns, from 10 to 50 microns, or from 15 to 40 microns.

In one aspect, at least 75%, 85% or 90% of said encapsulates may have a particle wall thickness from 30 to 250nm, from 80 to 180nm, or from 100 to 160nm.

30 In one aspect, said encapsulates' core material may comprise a material selected from the group consisting of a perfume raw material and/or optionally a material selected from the group consisting of vegetable oil, including neat and/or blended vegetable oils including castor oil, coconut oil,

cottonseed oil, grape oil, rapeseed, soybean oil, corn oil, palm oil, linseed oil, safflower oil, olive oil, peanut oil, coconut oil, palm kernel oil, castor oil, lemon oil and mixtures thereof; esters of vegetable oils, esters, including dibutyl adipate, dibutyl phthalate, butyl benzyl adipate, benzyl octyl adipate, tricresyl phosphate, trioctyl phosphate and mixtures thereof; straight or branched chain 5 hydrocarbons, including those straight or branched chain hydrocarbons having a boiling point of greater than about 80°C; partially hydrogenated terphenyls, dialkyl phthalates, alkyl biphenyls, including monoisopropylbiphenyl, alkylated naphthalene, including dipropylnaphthalene, petroleum spirits, including kerosene, mineral oil and mixtures thereof; aromatic solvents, including benzene, toluene and mixtures thereof; silicone oils; and mixtures thereof.

10 In one aspect, said encapsulates' wall material may comprise a suitable resin including the reaction product of an aldehyde and an amine, suitable aldehydes include, formaldehyde. Suitable amines include melamine, urea, benzoguanamine, glycoluril, and mixtures thereof. Suitable melamines include methylol melamine, methylated methylol melamine, imino melamine and mixtures thereof. Suitable ureas include dimethylol urea, methylated dimethylol urea, urea-resorcinol, and mixtures 15 thereof.

In one aspect, suitable formaldehyde scavengers may be employed with the encapsulates e.g. in a capsule slurry and/or added to a composition before, during or after the encapsulates are added to such composition. Suitable capsules may be made by the following teaching of US2008/0305982; and/or US2009/0247449.

20 In a preferred aspect the composition can also comprise a deposition aid, preferably consisting of the group comprising cationic or nonionic polymers. Suitable polymers include cationic starches, cationic hydroxyethylcellulose, polyvinylformaldehyde, locust bean gum, mannans, xyloglucans, tamarind gum, polyethyleneterephthalate and polymers containing dimethylaminoethyl methacrylate, optionally with one or monomers selected from the group comprising acrylic acid and acrylamide.

25 Perfumes - In one aspect the composition comprises a perfume that comprises one or more perfume raw materials selected from the group consisting of 1,1'-oxybis-2-propanol; 1,4-cyclohexanedicarboxylic acid, diethyl ester; (ethoxymethoxy)cyclododecane; 1,3-nanediol, monoacetate; (3-methylbutoxy)acetic acid, 2-propenyl ester; beta-methyl cyclododecaneethanol; 2-

30 methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol; oxacyclohexadecan-2-one; alpha-methyl-benzenemethanol acetate; trans-3-ethoxy-1,1,5-trimethylcyclohexane; 4-(1,1-dimethylethyl)cyclohexanol acetate; dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-b]furan; beta-methyl benzenopropanal; beta-methyl-3-(1-methylethyl)benzenopropanal; 4-phenyl-2-butanone; 2-methylbutanoic acid, ethyl ester; benzaldehyde; 2-methylbutanoic acid, 1-methylethyl ester;

dihydro-5-pentyl-2(3H)furanone; (2E)-1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-buten-1-one; dodecanal; undecanal; 2-ethyl- alpha, alpha-dimethylbenzenepropanal; decanal; alpha, alpha-dimethylbenzeneethanol acetate; 2-(phenylmethylene)octanal; 2-[[3-[4-(1,1-dimethylethyl)phenyl]-2-methylpropylidene]amino]benzoic acid, methyl ester; 1-(2,6,6-trimethyl-3-cyclohexen-1-yl)-2-buten-1-one; 2-pentylcyclopentanone; 3-oxo-2-pentyl cyclopentaneacetic acid, methyl ester; 4-hydroxy-3-methoxybenzaldehyde; 3-ethoxy-4-hydroxybenzaldehyde; 2-heptylcyclopentanone; 1-(4-methylphenyl)ethanone; (3E)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one; (3E)-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one; benzeneethanol; 2H-1-benzopyran-2-one; 4-methoxybenzaldehyde; 10-undecenal; propanoic acid, phenylmethyl ester; beta-10-methylbenzenepentanol; 1,1-diethoxy-3,7-dimethyl-2,6-octadiene; alpha, alpha-dimethylbenzeneethanol; (2E)-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-buten-1-one; acetic acid, phenylmethyl ester; cyclohexanepropanoic acid, 2-propenyl ester; hexanoic acid, 2-propenyl ester; 1,2-dimethoxy-4-(2-propenyl)benzene; 1,5-dimethyl-bicyclo[3.2.1]octan-8-one oxime; 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde; 3-buten-2-ol; 2-[[2,4(or 3,5)-dimethyl-3-cyclohexen-1-yl]methylene]amino]benzoic acid, methyl ester; 8-cyclohexadecen-1-one; methyl ionone; 2,6-dimethyl-7-octen-2-ol; 2-methoxy-4-(2-propenyl)phenol; (2E)-3,7-dimethyl-2,6-Octadien-1-ol; 2-hydroxy-Benzoic acid, (3Z)-3-hexenyl ester; 2-tridecenenitrile; 4-(2,2-dimethyl-6-methylenecyclohexyl)-3-methyl-3-buten-2-one; tetrahydro-4-methyl-2-(2-methyl-1-propenyl)-2H-pyran; Acetic acid, (2-methylbutoxy)-, 2-propenyl ester; Benzoic acid, 2-hydroxy-, 3-methylbutyl ester; 2-Buten-1-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (Z)-; Cyclopentanecarboxylic acid, 2-hexyl-3-oxo-, methyl ester; Benzenepropanal, 4-ethyl-.alpha.,alpha.-dimethyl-; 3-Cyclohexene-1-carboxaldehyde, 3-(4-hydroxy-4-methylpentyl)-; Ethanone, 1-(2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-1H-3a,7- methanoazulen-5-yl)-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]-; Undecanal, 2-methyl-2H-Pyran-2-one, 6-butyltetrahydro-; Benzenepropanal, 4-(1,1-dimethylethyl)-.alpha.-methyl-; 2(3H)-Furanone, 5-heptyldihydro-; Benzoic acid, 2-[(7-hydroxy-3,7-dimethyloctylidene)amino]-, methyl; Benzoic acid, 2-hydroxy-, phenylmethyl ester; Naphthalene, 2-methoxy-; 2-Cyclopenten-1-one, 2-hexyl-; 2(3H)-Furanone, 5-hexyldihydro-; Oxiranecarboxylic acid, 3-methyl-3-phenyl-, ethyl ester; 2-Oxabicyclo[2.2.2]octane, 1,3,3-trimethyl-; Benzenepentanol,.gamma.-methyl-; 3-Octanol, 3,7-dimethyl-; 3,7-dimethyl-2,6-octadienenitrile; 3,7-dimethyl-6-octen-1-ol; Terpineol acetate; 2-methyl-6-methylene-7-Octen-2-ol, dihydro derivative; 3a,4,5,6,7,7a-hexahydro-4,7-Methano-1H-inden-6-ol propanoate; 3-methyl-2-buten-1-ol acetate; (Z)-3-Hexen-1-ol acetate; 2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-2-buten-1-ol; 4-(octahydro-4,7-methano-5H-inden-5-ylidene)-butanal; 3-2,4-dimethyl-cyclohexene-1-carboxaldehyde; 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2- naphthalenyl)-ethanone; 2-hydroxy-benzoic acid, methyl ester; 2-hydroxy-benzoic acid, hexyl ester; 2-phenoxy-ethanol; 2-hydroxy-benzoic acid, pentyl ester; 2,3-

heptanedione; 2-hexen-1-ol; 6-Octen-2-ol, 2,6-dimethyl-; damascone (alpha, beta, gamma or delta or mixtures thereof), 4,7-Methano-1H-inden-6-ol, 3a,4,5,6,7,7a-hexahydro-, acetate; 9-Undecenal; 8-Undecenal; Isocyclocitral; Ethanone, 1-(1,2,3,5,6,7,8,8a-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)-; 3-Cyclohexene-1-carboxaldehyde, 3,5-dimethyl-; 3-Cyclohexene-1-carboxaldehyde, 5 2,4-dimethyl-; 1,6-Octadien-3-ol, 3,7-dimethyl-; 1,6-Octadien-3-ol, 3,7-dimethyl-, acetate; Lilial (p-t-Bucinal), and Cyclopentanone, 2-[2-(4-methyl-3-cyclohexen-1-yl)propyl]- and 1-methyl-4-(1-methylethenyl)cyclohexene and mixtures thereof.

In one aspect the composition may comprise an encapsulated perfume particle comprising either a water-soluble hydroxylic compound or melamine-formaldehyde or modified polyvinyl alcohol. In one 10 aspect the encapsulate comprises (a) an at least partially water-soluble solid matrix comprising one or more water-soluble hydroxylic compounds, preferably starch; and (b) a perfume oil encapsulated by the solid matrix.

In a further aspect the perfume may be pre-complexed with a polyamine, preferably a polyethylenimine so as to form a Schiff base.

15 Polymers - The composition may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

The composition may comprise one or more amphiphilic cleaning polymers such as the compound 20 having the following general structure: bis((C<sub>2</sub>H<sub>5</sub>O)(C<sub>2</sub>H<sub>4</sub>O)<sub>n</sub>)(CH<sub>3</sub>)-N<sup>+</sup>-C<sub>x</sub>H<sub>2x</sub>-N<sup>+</sup>-(CH<sub>3</sub>)-bis((C<sub>2</sub>H<sub>5</sub>O)(C<sub>2</sub>H<sub>4</sub>O)<sub>n</sub>), wherein n = from 20 to 30, and x = from 3 to 8, or sulphated or sulphonated variants thereof.

The composition may comprise amphiphilic alkoxyated grease cleaning polymers which have balanced hydrophilic and hydrophobic properties such that they remove grease particles from 25 fabrics and surfaces. Specific embodiments of the amphiphilic alkoxyated grease cleaning polymers of the present invention comprise a core structure and a plurality of alkoxyate groups attached to that core structure. These may comprise alkoxyated polyalkylenimines, preferably having an inner polyethylene oxide block and an outer polypropylene oxide block.

Alkoxyated polycarboxylates such as those prepared from polyacrylates are useful herein to 30 provide additional grease removal performance. Such materials are described in WO91/08281 and PCT90/01815. Chemically, these materials comprise polyacrylates having one ethoxy side-chain per every 7-8 acrylate units. The side-chains are of the formula -(CH<sub>2</sub>CH<sub>2</sub>O)<sub>m</sub> (CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub> wherein m is 2-3 and n is 6-12. The side-chains are ester-linked to the polyacrylate "backbone" to provide a

"comb" polymer type structure. The molecular weight can vary, but is typically in the range of 2000 to 50,000. Such alkoxylated polycarboxylates can comprise from 0.05wt% to 10wt% of the compositions herein.

The isoprenoid-derived surfactants of the present invention, and their mixtures with other 5 cosurfactants and other adjunct ingredients, are particularly suited to be used with an amphilic graft co-polymer, preferably the amphilic graft co-polymer comprises (i) polyethyelene glycol backbone; and (ii) and at least one pendant moiety selected from polyvinyl acetate, polyvinyl alcohol and mixtures thereof. A preferred amphilic graft co-polymer is Sokalan HP22, supplied from BASF. Suitable polymers include random graft copolymers, preferably a polyvinyl acetate grafted 10 polyethylene oxide copolymer having a polyethylene oxide backbone and multiple polyvinyl acetate side chains. The molecular weight of the polyethylene oxide backbone is preferably 6000 and the weight ratio of the polyethylene oxide to polyvinyl acetate is 40 to 60 and no more than 1 grafting point per 50 ethylene oxide units.

Carboxylate polymer - The composition of the present invention may also include one or more 15 carboxylate polymers such as a maleate/acrylate random copolymer or polyacrylate homopolymer. In one aspect, the carboxylate polymer is a polyacrylate homopolymer having a molecular weight of from 4,000 to 9,000Da, or from 6,000 to 9,000Da.

Soil release polymer - The composition of the present invention may also include one or more soil release polymers having a structure as defined by one of the following structures (I), (II) or (III):

20 (I)  $-\left[(OCHR^1-CHR^2)_a-O-OC-Ar-CO\right]_d$   
 (II)  $-\left[(OCHR^3-CHR^4)_b-O-OC-sAr-CO\right]_e$   
 (III)  $-\left[(OCHR^5-CHR^6)_c-OR^7\right]_f$

wherein:

a, b and c are from 1 to 200;

25 d, e and f are from 1 to 50;

Ar is a 1,4-substituted phenylene;

sAr is 1,3-substituted phenylene substituted in position 5 with  $SO_3Me$ ;

Me is Li, K, Mg/2, Ca/2, Al/3, ammonium, mono-, di-, tri-, or tetraalkylammonium wherein the alkyl groups are  $C_1-C_{18}$  alkyl or  $C_2-C_{10}$  hydroxyalkyl, or mixtures thereof;

30  $R^1, R^2, R^3, R^4, R^5$  and  $R^6$  are independently selected from H or  $C_1-C_{18}$ n- or iso-alkyl; and

$R^7$  is a linear or branched  $C_1-C_{18}$  alkyl, or a linear or branched  $C_2-C_{30}$  alkenyl, or a cycloalkyl group

with 5 to 9 carbon atoms, or a C<sub>8</sub>-C<sub>30</sub> aryl group, or a C<sub>6</sub>-C<sub>30</sub> arylalkyl group.

Suitable soil release polymers are polyester soil release polymers such as Repel-o-tex polymers, including Repel-o-tex, SF-2 and SRP6 supplied by Rhodia. Other suitable soil release polymers include Texcare polymers, including Texcare SRA100, SRA300, SRN100, SRN170, SRN240,

5 SRN300 and SRN325 supplied by Clariant. Other suitable soil release polymers are Marloquest polymers, such as Marloquest SL supplied by Sasol.

Cellulosic polymer - The composition of the present invention may also include one or more cellulosic polymers including those selected from alkyl cellulose, alkyl alkoxyalkyl cellulose, carboxyalkyl cellulose, alkyl carboxyalkyl cellulose. In one aspect, the cellulosic polymers are

10 selected from the group comprising carboxymethyl cellulose, methyl cellulose, methyl hydroxyethyl cellulose, methyl carboxymethyl cellulose, and mixtures thereof. In one aspect, the carboxymethyl cellulose has a degree of carboxymethyl substitution from 0.5 to 0.9 and a molecular weight from 100,000 to 300,000Da.

Enzymes - The composition may comprise one or more enzymes which provide cleaning

15 performance and/or fabric care benefits. Examples of suitable enzymes include, but are not limited to, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, mannanases, pectate lyases, keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases,  $\beta$ -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and amylases, or mixtures 20 thereof. A typical combination is an enzyme cocktail that may comprise e.g. a protease and lipase in conjunction with amylase. When present in a composition, the aforementioned additional enzymes may be present at levels from 0.00001 to 2wt%, from 0.0001 to 1wt% or from 0.001 to 0.5wt% enzyme protein by weight of the composition.

In one aspect preferred enzymes would include a protease. Suitable proteases include

25 metalloproteases and serine proteases, including neutral or alkaline microbial serine proteases, such as subtilisins (EC3.4.21.62). Suitable proteases include those of animal, vegetable or microbial origin. In one aspect, such suitable protease may be of microbial origin. The suitable proteases include chemically or genetically modified mutants of the aforementioned suitable proteases. In one aspect, the suitable protease may be a serine protease, such as an alkaline 30 microbial protease or/and a trypsin-type protease. Examples of suitable neutral or alkaline proteases include:

(a) subtilisins (EC3.4.21.62), including those derived from *Bacillus*, such as *Bacillus latus*, *B. alkalophilus*, *B. subtilis*, *B. amyloliquefaciens*, *Bacillus pumilus* and *Bacillus gibsonii* described in

US6312936; US5679630; US4760025; US7262042 and WO09/021867.

(b) trypsin-type or chymotrypsin-type proteases, such as trypsin (e.g., of porcine or bovine origin), including the Fusarium protease described in WO89/06270 and the chymotrypsin proteases derived from Cellumonas described in WO05/052161 and WO05/052146.

5 (c) metalloproteases, including those derived from *Bacillus amyloliquefaciens* described in WO07/044993.

Preferred proteases include those derived from *Bacillus gibsonii* or *Bacillus Lentus*.

Suitable commercially available protease enzymes include those sold under the trade names Alcalase®, Savinase®, Primase®, Durazym®, Polarzyme®, Kannase®, Liquanase®, Liquanase

10 Ultra®, Savinase Ultra®, Ovozyme®, Neutrerase®, Everlase® and Esperase® by Novozymes, those sold under the tradename Maxatase®, Maxacal®, Maxapem®, Properase®, Purafect®, Purafect Prime®, Purafect Ox®, FN3®, FN4®, Excellase®, Purafect OXP®, Opticlean® and Optimase® by Danisco, those available from Henkel/ Kemira, namely BLAP (sequence shown in Figure 29 of US5352604 with the following mutations S99D+S101R+S103A+V104I+G159S, hereinafter referred 15 to as BLAP), BLAP R (BLAP +S3T+V4I+V199M+V205I+L217D), BLAP X (BLAP +S3T+V4I+V205I) and BLAP F49 (BLAP +S3T+V4I+A194P+V199M+V205I+L217D) - all from Henkel/Kemira; and KAP (*Bacillus alkalophilus subtilisin* with mutations A230V+S256G+S259N) from Kao.

Suitable alpha-amylases include those of bacterial or fungal origin. Chemically or genetically modified mutants (variants) are included. A preferred alkaline alpha-amylase is derived from a

20 strain of *Bacillus*, such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or other *Bacillus* sp., such as *Bacillus* sp. NCIB12289, NCIB12512, NCIB12513, DSM9375 (US7153818) DSM12368, DSMZ no.12649, KSM AP1378 (WO97/00324), KSM K36 or KSM K38 (EP1022334). Preferred amylases include:

(a) the variants described in WO94/02597, WO94/18314, WO96/23874 and WO97/43424, 25 especially the variants with substitutions in one or more of the following positions versus the enzyme listed as SEQ ID No. 2 in WO96/23874: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

(b) the variants described in US5856164, WO99/23211, WO96/23873, WO00/60060 & WO06/002643, especially the variants with one or more substitutions in the following positions 30 versus the AA560 enzyme listed as SEQ ID No. 12 in WO06/002643: 26, 30, 33, 82, 37, 106, 118, 128, 133, 149, 150, 160, 178, 182, 186, 193, 203, 214, 231, 256, 257, 258, 269, 270, 272, 283, 295, 296, 298, 299, 303, 304, 305, 311, 314, 315, 318, 319, 339, 345, 361, 378, 383, 419, 421, 437, 441, 444, 445, 446, 447, 450, 461, 471, 482, 484, preferably that also contain the deletions of

D183\* and G184\*.

(c) variants exhibiting at least 90% identity with SEQ ID No. 4 in WO06/002643, the wild-type enzyme from *Bacillus* SP722, especially variants with deletions in the 183 and 184 positions and variants described in WO00/60060, which is incorporated herein by reference.

5 (d) variants exhibiting at least 95% identity with the wild-type enzyme from *Bacillus* sp.707 (SEQ ID NO:7 in US6093562), especially those comprising one or more of the following mutations M202, M208, S255, R172, and/or M261. Preferably said amylase comprises one or more of M202L, M202V, M202S, M202T, M202I, M202Q, M202W, S255N and/or R172Q. Particularly preferred are those comprising the mutations M202L or M202T.

10 (e) variants described in WO09/149130, preferably those exhibiting at least 90% identity with SEQ ID NO: 1 or SEQ ID NO:2 in WO09/149130, the wild-type enzyme from *Geobacillus Stearothermophilus* or a truncated version thereof.

Suitable commercially available alpha-amylases include DURAMYL®, LIQUEZYME®, TERMAMYL®, TERMAMYL ULTRA®, NATALASE®, SUPRAMYL®, STAINZYME®, STAINZYME 15 PLUS®, FUNGAMYL® and BAN® (Novozymes), KEMZYM® AT 9000 Biozym Biotech Trading GmbH Wehlistrasse 27b A-1200 Wien Austria, RAPIDASE®, PURASTAR®, ENZYSIZE®, OPTISIZE HT PLUS®, POWERASE® and PURASTAR OXAM® (Danisco) and KAM® (Kao). In one aspect, suitable amylases include NATALASE®, STAINZYME® and STAINZYME PLUS® and mixtures thereof.

20 In one aspect, such enzymes may be selected from the group consisting of: lipases, including "first cycle lipases" such as those described in US6939702 and US2009/0217464. In one aspect, the lipase is a first-wash lipase, preferably a variant of the wild-type lipase from *Thermomyces lanuginosus* comprising one or more of the T231R and N233R mutations. The wild-type sequence is the 269 amino acids (amino acids 23–291) of the Swissprot accession number Swiss-Prot 25 O59952 (derived from *Thermomyces lanuginosus* (*Humicola lanuginosa*)). Preferred lipases would include those sold under the tradenames Lipex® and Lipolex®.

In one aspect, other preferred enzymes include microbial-derived endoglucanases exhibiting endo-beta-1,4-glucanase activity (EC3.2.1.4), including a bacterial polypeptide endogenous to a member of the genus *Bacillus* which has a sequence of at least 90%, 94%, 97% or 99% identity to the 30 amino acid sequence SEQ ID NO:2 in US7141403 and mixtures thereof. Suitable endoglucanases are sold under the tradenames Celluclean® and Whitezyme® (Novozymes).

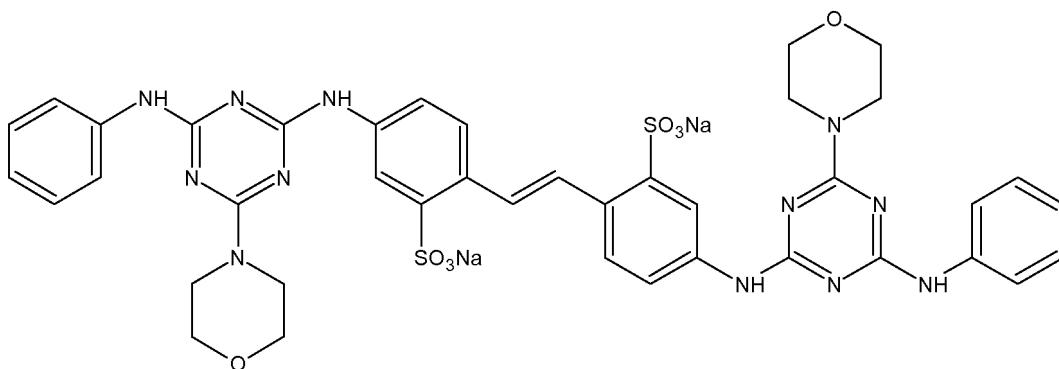
Other preferred enzymes include pectate lyases sold under the tradenames Pectawash®, Pectaway®, Xpect® and mannanases sold under the tradenames Mannaway® (Novozymes), and

Purabrite® (Danisco).

Dye Transfer Inhibiting Agents - The compositions of the present invention may also include one or more dye transfer inhibiting agents. Suitable polymeric dye transfer inhibiting agents include, but are not limited to, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinyloxazolidones and polyvinylimidazoles or mixtures thereof. When present in a composition, the dye transfer inhibiting agents may be present at levels from 0.0001% to 10%, from 0.01% to 5% or from 0.1% to 3% by weight of the composition.

Brighteners - The compositions of the present invention can also contain additional components that may tint articles being cleaned, such as fluorescent brighteners.

10 The composition may comprise C.I. fluorescent brightener 260 in alpha-crystalline form having the following structure:



In one aspect, the brightener is a cold water soluble brightener, such as the C.I. fluorescent brightener 260 in alpha-crystalline form. In one aspect the brightener is predominantly in alpha-crystalline form, which means that typically at least 50wt%, at least 75wt%, at least 90wt%, at least 99wt%, or even substantially all, of the C.I. fluorescent brightener 260 is in alpha-crystalline form.

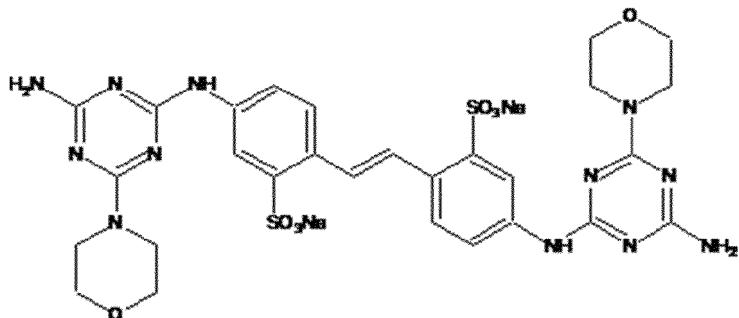
The brightener is typically in micronized particulate form, having a weight average primary particle size of from 3 to 30 micrometers, from 3 micrometers to 20 micrometers, or from 3 to 10 micrometers.

20 The composition may comprise C.I. fluorescent brightener 260 in beta-crystalline form, and the weight ratio of: (i) C.I. fluorescent brightener 260 in alpha-crystalline form, to (ii) C.I. fluorescent brightener 260 in beta-crystalline form may be at least 0.1, or at least 0.6. BE680847 relates to a process for making C.I fluorescent brightener 260 in alpha-crystalline form.

Commercial optical brighteners which may be useful in the present invention can be classified into subgroups, which include, but are not necessarily limited to, derivatives of stilbene, pyrazoline, coumarin, carboxylic acid, methinecyanines, dibenzothiophene-5,5-dioxide, azoles, 5- and 6-

membered-ring heterocycles, and other miscellaneous agents. Examples of such brighteners are disclosed in "The Production and Application of Fluorescent Brightening Agents", M. Zahradnik, Published by John Wiley & Sons, New York (1982). Specific nonlimiting examples of optical brighteners which are useful in the present compositions are those identified in US4790856 and 5 US3646015.

A further suitable brightener has the structure below:



Suitable fluorescent brightener levels include lower levels of from 0.01wt%, from 0.05wt%, from 0.1wt% or from 0.2wt% to upper levels of 0.5wt% or 0.75wt%.

10 In one aspect the brightener may be loaded onto a clay to form a particle. Silicate salts - The compositions of the present invention can also contain silicate salts, such as sodium or potassium silicate. The composition may comprise of from 0wt% to less than 10wt% silicate salt, to 9wt%, or to 8wt%, or to 7wt%, or to 6wt%, or to 5wt%, or to 4wt%, or to 3wt%, or even to 2wt%, and from above 0wt%, or from 0.5wt%, or from 1wt% silicate salt. A suitable silicate salt is sodium silicate.

15 Dispersants - The compositions of the present invention can also contain dispersants. Suitable water-soluble organic materials include the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated from each other by not more than two carbon atoms.

Enzyme Stabilizers - Enzymes for use in compositions can be stabilized by various techniques. 20 The enzymes employed herein can be stabilized by the presence of water-soluble sources of calcium and/or magnesium ions in the finished fabric and home care compositions that provide such ions to the enzymes. In case of aqueous compositions comprising protease, a reversible protease inhibitor, such as a boron compound including borate, 4-formyl phenylboronic acid, phenylboronic acid and derivatives thereof, or compounds such as calcium formate, sodium formate and 1,2-propane diol can be added to further improve stability.

Solvents – Suitable solvents include water and other solvents such as lipophilic fluids. Examples of suitable lipophilic fluids include siloxanes, other silicones, hydrocarbons, glycol ethers, glycerine

derivatives such as glycerine ethers, perfluorinated amines, perfluorinated and hydrofluoroether solvents, low-volatility nonfluorinated organic solvents, diol solvents, other environmentally-friendly solvents and mixtures thereof.

Structurant/Thickeners - Structured liquids can either be internally structured, whereby the structure

5 is formed by primary ingredients (e.g. surfactant material) and/or externally structured by providing a three dimensional matrix structure using secondary ingredients (e.g. polymers, clay and/or silicate material). The composition may comprise a structurant, from 0.01 to 5wt%, or from 0.1 to 2.0wt%. The structurant is typically selected from the group consisting of diglycerides and triglycerides, ethylene glycol distearate, microcrystalline cellulose, cellulose-based materials, microfiber 10 cellulose, hydrophobically modified alkali-swellable emulsions such as Polygel W30 (3VSigma), biopolymers, xanthan gum, gellan gum, and mixtures thereof. A suitable structurant includes hydrogenated castor oil, and non-ethoxylated derivatives thereof. A suitable structurant is disclosed in US6855680. Such structurants have a thread-like structuring system having a range of aspect ratios. Other suitable structurants and the processes for making them are described in 15 WO10/034736.

Conditioning Agents - The composition of the present invention may include a high melting point fatty compound.

20 The high melting point fatty compound useful herein has a melting point of 25°C or higher, and is selected from the group consisting of fatty alcohols, fatty acids, fatty alcohol derivatives, fatty acid derivatives, and mixtures thereof. Such compounds of low melting point are not intended to be included in this section. Non-limiting examples of the high melting point compounds are found in International Cosmetic Ingredient Dictionary, Fifth Edition, 1993, and 25 CTFA Cosmetic Ingredient Handbook, Second Edition, 1992.

The high melting point fatty compound is included in the composition at a level of from 0.1 to 40wt%, from 1 to 30wt%, from 1.5 to 16wt%, from 1.5 to 8wt% in view of providing improved 25 conditioning benefits such as slippery feel during the application to wet hair, softness and moisturized feel on dry hair.

The compositions of the present invention may contain a cationic polymer. Concentrations of the cationic polymer in the composition typically range from 0.05 to 3wt%, from 0.075 to 2.0wt%, or from 0.1 to 1.0wt%.

30 Suitable cationic polymers will have cationic charge densities of at least 0.5 meq/gm, at least 0.9 meq/gm, at least 1.2 meq/gm, at least 1.5 meq/gm, or less than 7 meq/gm, and less than 5 meq/gm, at the pH of intended use of the composition, which pH will generally range from pH 3 to pH 9, or between pH 4 and pH 8. Herein, "cationic charge density" of a polymer refers to the ratio of the number of positive charges on the polymer to the molecular weight of the

polymer. The average molecular weight of such suitable cationic polymers will generally be between 10,000 and 10 million, between 50,000 and 5 million, or between 100,000 and 3 million.

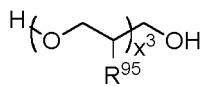
Suitable cationic polymers for use in the compositions of the present invention contain cationic nitrogen-containing moieties such as quaternary ammonium or cationic protonated amino moieties.

5 Any anionic counterions can be used in association with the cationic polymers so long as the polymers remain soluble in water, in the composition, or in a coacervate phase of the composition, and so long as the counterions are physically and chemically compatible with the essential components of the composition or do not otherwise unduly impair composition performance, stability or aesthetics. Nonlimiting examples of such counterions include halides (e.g., chloride, 10 fluoride, bromide, iodide), sulfate and methylsulfate.

Nonlimiting examples of such polymers are described in the CTFA Cosmetic Ingredient Dictionary, 3rd edition, edited by Estrin, Crosley, and Haynes, (The Cosmetic, Toiletry, and Fragrance Association, Inc., Washington, D.C. (1982)).

15 Other suitable cationic polymers for use in the composition include polysaccharide polymers, cationic guar gum derivatives, quaternary nitrogen-containing cellulose ethers, synthetic polymers, copolymers of etherified cellulose, guar and starch. When used, the cationic polymers herein are either soluble in the composition or are soluble in a complex coacervate phase in the composition formed by the cationic polymer and the anionic, amphoteric and/or zwitterionic surfactant component described hereinbefore. Complex coacervates of the cationic polymer can also be 20 formed with other charged materials in the composition. Suitable cationic polymers are described in US3962418; US3958581; and US2007/0207109.

The composition of the present invention may include a nonionic polymer as a conditioning agent. Polyalkylene glycols having a molecular weight of more than 1000 are useful herein. Useful are those having the following general formula:



wherein  $\text{R}^{95}$  is selected from the group consisting of H, methyl, and mixtures thereof. Conditioning agents, and in particular silicones, may be included in the composition. The conditioning agents useful in the compositions of the present invention typically comprise a water insoluble, water dispersible, non-volatile, liquid that forms emulsified, liquid particles. Suitable conditioning agents 30 for use in the composition are those conditioning agents characterized generally as silicones (e.g., silicone oils, cationic silicones, silicone gums, high refractive silicones, and silicone resins), organic conditioning oils (e.g., hydrocarbon oils, polyolefins, and fatty esters) or combinations thereof, or

those conditioning agents which otherwise form liquid, dispersed particles in the aqueous surfactant matrix herein. Such conditioning agents should be physically and chemically compatible with the essential components of the composition, and should not otherwise unduly impair composition stability, aesthetics or performance.

5 The concentration of the conditioning agent in the composition should be sufficient to provide the desired conditioning benefits. Such concentration can vary with the conditioning agent, the conditioning performance desired, the average size of the conditioning agent particles, the type and concentration of other components, and other like factors.

The concentration of the silicone conditioning agent typically ranges from 0.01 to 10wt%. Non-  
10 limiting examples of suitable silicone conditioning agents, and optional suspending agents for the silicone, are described in U.S. Reissue Pat. No. 34,584; US5104646; US5106609; US4152416; US2826551; US3964500; US4364837; US6607717; US6482969; US5807956; US5981681; US6207782; US7465439; US7041767; US7217777; US2007/0286837A1; US2005/0048549A1; US2007/0041929A1; GB849433; DE10036533, which are all incorporated herein by reference;  
15 Chemistry and Technology of Silicones, New York: Academic Press (1968); General Electric Silicone Rubber Product Data Sheets SE 30, SE 33, SE 54 and SE 76; Silicon Compounds, Petrarch Systems, Inc. (1984); and in Encyclopedia of Polymer Science and Engineering, vol. 15, 2d ed., pp 204-308, John Wiley & Sons, Inc. (1989).

The compositions of the present invention may also comprise from 0.05 to 3wt% of at least one  
20 organic conditioning oil as the conditioning agent, either alone or in combination with other conditioning agents, such as the silicones (described herein). Suitable conditioning oils include hydrocarbon oils, polyolefins, and fatty esters. Also suitable for use in the compositions herein are the conditioning agents described in US5674478 and US5750122 or in US4529586; US4507280; US4663158; US4197865; US4217914; US4381919; and US4422853.

25 Hygiene and malodour – The compositions of the present invention may also comprise one or more of zinc ricinoleate, thymol, quaternary ammonium salts such as Bardac®, polyethylenimines (such as Lupasol® from BASF) and zinc complexes thereof, silver and silver compounds, especially those designed to slowly release Ag<sup>+</sup> or nano-silver dispersions.

Probiotics – The compositions may comprise probiotics such as those described in WO09/043709.

30 Suds Boosters - If high sudsing is desired, suds boosters such as the C<sub>10</sub>-C<sub>16</sub> alkanolamides or C<sub>10</sub>-C<sub>14</sub> alkyl sulphates can be incorporated into the compositions, typically at 1 to 10wt% levels. The C<sub>10</sub>-C<sub>14</sub> monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and

sultaines noted above is also advantageous. If desired, water-soluble magnesium and/or calcium salts such as MgCl<sub>2</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub>, CaSO<sub>4</sub> and the like, can be added at levels of, typically, 0.1 to 2wt%, to provide additional suds and to enhance grease removal performance.

Suds Suppressors - Compounds for reducing or suppressing the formation of suds can be incorporated into the compositions of the present invention. Suds suppression can be of particular importance in the so-called "high concentration cleaning process" as described in US4489455 and US4489574, and in front-loading -style washing machines. A wide variety of materials may be used as suds suppressors, and suds suppressors are well known to those skilled in the art. See e.g. Kirk Othmer Encyclopedia of Chemical Technology, Third Edition, Volume 7, pages 430-447 (John Wiley & Sons, Inc., 1979). Examples of suds supressors include monocarboxylic fatty acid and soluble salts therein, high molecular weight hydrocarbons such as paraffin, fatty acid esters (e.g., fatty acid triglycerides), fatty acid esters of monovalent alcohols, aliphatic C<sub>18</sub>-C<sub>40</sub> ketones (e.g., stearone), N-alkylated amino triazines, waxy hydrocarbons preferably having a melting point below about 100°C, silicone suds suppressors, and secondary alcohols. Suds supressors are described in US2954347; US4265779; US4265779; US3455839; US3933672; US4652392; US4978471; US4983316; US5288431; US4639489; US4749740; US4798679; US4075118; EP89307851.9; EP150872; and DOS 2,124,526.

For any detergent compositions to be used in automatic laundry washing machines, suds should not form to the extent that they overflow the washing machine. Suds suppressors, when utilized, are preferably present in a "suds suppressing amount". By "suds suppressing amount" is meant that the formulator of the composition can select an amount of this suds controlling agent that will sufficiently control the suds to result in a low-sudsing laundry detergent for use in automatic laundry washing machines.

The compositions herein will generally comprise from 0 to 10wt% of suds suppressor. When utilized as suds suppressors, monocarboxylic fatty acids, and salts therein, will be present typically in amounts up to 5wt%. Preferably, from 0.5 to 3wt% of fatty monocarboxylate suds suppressor is utilized. Silicone suds suppressors are typically utilized in amounts up to 2.0wt%, although higher amounts may be used. Monostearyl phosphate suds suppressors are generally utilized in amounts ranging from 0.1 to 2wt%. Hydrocarbon suds suppressors are typically utilized in amounts ranging from 0.01 to 5.0wt%, although higher levels can be used. The alcohol suds suppressors are typically used at 0.2 to 3wt%.

The compositions herein may have a cleaning activity over a broad range of pH. In certain embodiments the compositions have cleaning activity from pH 4 to pH 11.5. In other embodiments, the compositions are active from pH 8 to pH 10.

The compositions herein may have cleaning activity over a wide range of temperatures, e.g., from 10°C or lower to 90°C. Preferably the temperature will be below 50°C or 40°C or even 30°C. In certain embodiments, the optimum temperature range for the compositions is from 10°C to 20°C, from 15°C to 25°C, from 15°C to 30°C, from 20°C to 30°C, from 25°C to 35°C, from 30°C to 40°C, 5 from 35°C to 45°C, or from 40°C to 50°C.

The compositions herein may further be stabilized by the addition of divalent cations such as calcium (Ca<sup>2+</sup>), magnesium (Mg<sup>2+</sup>) and iron-II (Fe<sup>2+</sup>) or any combination thereof.

In some aspects the invention relates to a composition, wherein the composition is more effective in hydrolyzing lipid substrates compared to an equivalent composition comprising *Thermomyces lanuginosus* lipase (Lipolase™) in place of Lipr139 lipase. In other aspects the composition 10 comprises Lipex™ in place of Lipr139 lipase.

### **Form of the composition**

The cleaning compositions described herein are advantageously employed for example, in laundry 15 applications, hard surface cleaning, dishwashing applications, as well as cosmetic applications such as dentures, teeth, hair and skin. In addition, due to the unique advantages of increased effectiveness in lower temperature solutions, the Lipr139 enzyme described herein are ideally suited for laundry applications. The compositions of the invention are in particular solid or liquid 20 cleaning and/or treatment compositions. In one aspect the invention relates to a cleaning composition, wherein the form of the composition is selected from the group consisting of a liquid, a gel, a soap bar, a powder, a granulated solid, a tablet, or any combination thereof. In another aspect of the invention, the composition has a single or a multi-compartment unit dose form.

Water-Soluble Film – The compositions of the present invention may also be encapsulated within a water-soluble film. Preferred film materials are preferably polymeric materials. The film material can 25 e.g. be obtained by casting, blow-moulding, extrusion or blown extrusion of the polymeric material, as known in the art. Preferred polymers, copolymers or derivatives thereof suitable for use as pouch material are selected from polyvinyl alcohols, polyvinyl pyrrolidone, polyalkylene oxides, acrylamide, acrylic acid, cellulose, cellulose ethers, cellulose esters, cellulose amides, polyvinyl acetates, polycarboxylic acids and salts, polyaminoacids or peptides, polyamides, polyacrylamide, 30 copolymers of maleic/acrylic acids, polysaccharides including starch and gelatine, natural gums such as xanthum and carragum. More preferred polymers are selected from polyacrylates and water-soluble acrylate copolymers, methylcellulose, carboxymethylcellulose sodium, dextrin, ethylcellulose, hydroxyethyl cellulose, hydroxypropyl methylcellulose, maltodextrin,

polymethacrylates, and most preferably selected from polyvinyl alcohols, polyvinyl alcohol copolymers and hydroxypropyl methyl cellulose (HPMC), and combinations thereof. Preferably, the level of polymer in the pouch material, e.g. a PVA polymer, is at least 60wt%. The polymer can have any weight average molecular weight, preferably from about 1.000 to 1.000.000, from about 5 10.000 to 300.000, from about 20.000 to 150.000. Mixtures of polymers can also be used as the pouch material.

Naturally, different film material and/or films of different thickness may be employed in making the compartments of the present invention. A benefit in selecting different films is that the resulting compartments may exhibit different solubility or release characteristics.

10 Preferred film materials are PVA films known under the MonoSol trade reference M8630, M8900, H8779 and those described in US6166117 and US6787512 and PVA films of corresponding solubility and deformability characteristics.

15 The film material herein can also comprise one or more additive ingredients. For example, it can be beneficial to add plasticisers, e.g. glycerol, ethylene glycol, diethyleneglycol, propylene glycol, sorbitol and mixtures thereof. Other additives include functional detergent additives to be delivered to the wash water, e.g. organic polymeric dispersants, etc.

### Processes of Making the Compositions

20 The compositions of the present invention can be formulated into any suitable form and prepared by any process chosen by the formulator, non-limiting examples of which are described in Applicants' examples and in US4990280; US20030087791A1; US20030087790A1; US20050003983A1; US20040048764A1; US4762636; US6291412; US20050227891A1; EP1070115A2; US5879584; US5691297; US5574005; US5569645; US5565422; US5516448; US5489392; US5486303 all of which are incorporated herein by reference. The compositions of 25 the invention or prepared according to the invention comprise cleaning and/or treatment composition including, but not limited to, compositions for treating fabrics, hard surfaces and any other surfaces in the area of fabric and home care, including: air care including air fresheners and scent delivery systems, car care, dishwashing, fabric conditioning (including softening and/or freshening), laundry detergency, laundry and rinse additive and/or care, hard surface cleaning 30 and/or treatment including floor and toilet bowl cleaners, granular or powder-form all-purpose or "heavy-duty" washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type;

machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; car or carpet shampoos, bathroom cleaners including toilet bowl cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types, substrate-laden compositions such as dryer added sheets. Preferred are compositions and 5 methods for cleaning and/or treating textiles and/or hard surfaces, most preferably textiles. The compositions are preferably compositions used in a pre-treatment step or main wash step of a washing process, most preferably for use in textile washing step.

As used herein, the term "fabric and/or hard surface cleaning and/or treatment composition" is a subset of cleaning and treatment compositions that includes, unless otherwise indicated, granular 10 or powder-form all-purpose or "heavy-duty" washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting 15 agents, car or carpet shampoos, bathroom cleaners including toilet bowl cleaners; fabric conditioning compositions including softening and/or freshening that may be in liquid, solid and/or dryer sheet form; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types, substrate-laden compositions such as dryer added sheets. All of such compositions which are applicable may be in standard, concentrated or even highly concentrated form even to 20 the extent that such compositions may in certain aspect be non-aqueous.

### **Method of Use**

The present invention includes a method for cleaning any surface including treating a textile or a hard surface or other surfaces in the field of fabric and/or home care. In a preferred aspect of the 25 invention, the method comprises the step of contacting the surface to be treated in a pre-treatment step or main wash step of a washing process, most preferably for use in a textile washing step or alternatively for use in dishwashing including both manual as well as automated/mechanical dishwashing. In one embodiment of the invention the lipase variant and other components are added sequentially into the method for cleaning and/or treating the surface. Alternatively, the lipase 30 variant and other components are added simultaneously.

As used herein, washing includes but is not limited to, scrubbing, and mechanical agitation. Washing may be conducted with a foam composition as described in WO08/101958 and/or by applying alternating pressure (pressure/vaccum) as an addition or as an alternative to scrubbing

and mechanical agitation. Drying of such surfaces or fabrics may be accomplished by any one of the common means employed either in domestic or industrial settings. The cleaning compositions of the present invention are ideally suited for use in laundry as well as dishwashing applications. Accordingly, the present invention includes a method for cleaning an object including but not 5 limiting to fabric, tableware, cutlery and kitchenware. The method comprises the steps of contacting the object to be cleaned with a said cleaning composition comprising at least one embodiment of Applicants' cleaning composition, cleaning additive or mixture thereof. The fabric may comprise most any fabric capable of being laundered in normal consumer or institutional use conditions. The solution may have a pH from 8 to 10.5. The compositions may be employed at 10 concentrations from 500 to 15.000ppm in solution. The water temperatures typically range from 5°C to 90°C. The water to fabric ratio is typically from 1:1 to 30:1.

In one aspect the invention relates to a method of using Lipr139 for producing a cleaning composition. In one aspect the invention relates to use of the cleaning composition for cleaning an object.

15 In one aspect the invention relates to a method of stabilizing a cleaning composition, comprising adding a lipase with at least 75% identity to SEQ ID NO: 2 to the composition wherein the stability of the composition is greater than the stability of an equivalent composition comprising *Thermomyces lanuginosus* lipase in place of the lipase with at least 75% identity to SEQ ID NO: 2, preferably wherein stability is measured in a final cleaning composition and/or in a final wash 20 medium.

In one aspect the invention relates to a method of producing the composition, comprising adding a lipase with at least 75% identity to SEQ ID NO: 2, and a surfactant. In one aspect the invention relates to a method for cleaning a surface, comprising contacting a lipid stain present on the surface to be cleaned with the cleaning composition. In one aspect the invention relates to a 25 method for hydrolyzing a lipid present in a soil and/or a stain on a surface, comprising contacting the soil and/or the stain with the cleaning composition.

## Examples

The present invention is further described by the following examples that should not be construed 30 as limiting the scope of the invention.

## Media and Solutions

Unless otherwise indicated the materials are of reagent grade. The commercially available enzymes Lipolase™ and Lipex™ were obtained from Novozymes A/S.

5

### Example 1: Identification, cloning and expression

Chromosomal DNA from Cedecea sp-16640 was isolated by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). 5ug of chromosomal DNA were sent for Illumina sequencing at FASTERIS SA, Switzerland. The genome sequences were analyzed for open reading frames encoding lipolytic 10 enzymes and the Lipr139 lipase was identified.

The nucleotide sequence of the Lipr139 synthetic gene set forth as SEQ ID NO: 1 was synthesized by Gene Art (GENEART AG BioPark, Josef-Engert-Str. 11, 93053, Regensburg, Germany). The gene sequence encoding Lipr139 was verified by Sanger sequencing. The synthetic gene was subcloned using SacI and MluI restriction sites into Bacillus expression vector as described in 15 WO12/025577. Transformants were selected on LB plates supplemented with 6ug/ml chloramphenicol.

The recombinant Bacillus subtilis clone containing the integrated expression construct was selected and cultivated on a rotary shaking table in 500ml baffled Erlenmeyer flasks each containing 10ml casein-based media supplemented with 34mg/l chloramphenicol. The clone was cultivated for 5 20 days at 26°C. The enzyme containing supernatants were harvested and the enzyme purified as described below.

### Example 2: Purification

The fermentation supernatant was filtered through PES Bottle top filter with a 0.22um cut-off 25 (Cat.no. 567-0020, Thermo Fisher Scientific, Kastrupsvej 90, 4000 Roskilde, Denmark) adjusted to 1M NaCl and applied to HIC/affinity chromatography on a 50ml Decylamine agarose column (Acetyleret Decylaminagarose, Cat.no. CS76, UpFront Cromatography A/S, Lersø Parkalle 42, 2100 Copenhagen Ø, Denmark). The column was washed with 3 column volumes (CV) Buffer A (50mM Borate; 1M NaCl; pH 9) and the protein was subsequently eluted with 3 CV 100% Buffer B 30 (50mM Borate; 30% isopropanol; pH 9). Elution of the protein was monitored at 280nm. Fractions with high 280nm absorbance were analysed on SDS-Page on their Lipr139 content.

Fractions of pure protein were pooled, diluted with MilliQ H<sub>2</sub>O until conductivity was below 3mS/cm and then loaded onto a 50ml Q-sepharose fast flow column (Cat. No.17-0510-01 GE Healthcare Europe GmbH, Park Allé 295, 2605 Brondby, Denmark). The column was washed with 3 CV Buffer A (50mM Borate; pH 9) and the protein was subsequently eluted with Buffer B (50mM Borate; 1M NaCl; pH 9) on a gradient from 0%-100%. Elution of the protein was monitored at 280nm and the majority of the protein eluted at around 50% BufferB.

The fractions were analysed on SDS-Page and pure fractions were pooled. The purified protein had an estimated MW of 30kD and a purity >95%.

The amino acid sequence of the mature Lipr139 lipase as set forth in SEQ ID NO: 2 was verified by 10 In-gel digest.

### **Example 3: Hydrolytic activity on triglycerides**

#### Plate assay

The hydrolytic activity of the lipase on various substrates was investigated using the following agar 15 plates: Olive oil plates (1% Olive oil; 1% Litex Agarose HSH 1000; 1mM CaCl<sub>2</sub>; 50mM Hepes pH 8.0), Tributyrin plates (1.25% tributyrin; 1.65% gum Arabic; 1% Litex Agarose HSH 1000; 50mM Hepes; 0.008% brilliant green; pH 7.0) and Lecithin plates (1% Phosphatidylcholin; 1% Litex Agarose HSH 1000; 50 mM Acetate; 1 mM CaCl<sub>2</sub>; pH 5.5).

The hydrolytic activity at various pH was assessed on Olive oil plates with the following pHs: pH 7, 20 pH 8, pH 9 and pH 10 (1% Olive oil; 1% Litex Agarose HSH 1000; 1mM CaCl<sub>2</sub>; 50mM Hepes (pH 7 & 8) or 50mM Borate (pH 9 & 10)).

20ul aliquots of the purified enzyme, buffer (negative control) and Lipolase™ and Lipex™ (positive controls) were each distributed into punched holes with a diameter of 3mm and incubated for 24 hours at 20°C. The plates were subsequently examined for the presence or absence of a clearing 25 zone around the holes. Hydrolytic activity was indicated by a clearing zone around the holes. The negative control did not give rise to a clearing zone.

Lipr139 showed hydrolytic activity on all three substrates tested. On Olive oil plates Lipr139 showed comparable hydrolytic activity with Lipolase™ and a higher activity than Lipex™. On Tributyrin plates Lipr139 showed a comparable activity with Lipolase™ and Lipex™. On Lecithin plates 30 Lipr139 showed an increased activity in comparison with both Lipolase™ and Lipex™.

Lipr139 showed hydrolytic activity at pH 8, pH 9 and pH 10 and maximum activity at pH 9.

**Example 4: Hydrolytic activity on fatty acids****pNP assay**

The hydrolytic activity of Lipr139 on various fatty acids was investigated in a kinetic assay using p-nitrophenyl acyl esters as substrate. The 100mM stock solutions in DMSO of the substrates: p-Nitrophenyl butyrate (C3), p-Nitrophenyl caproate (C6), p-Nitrophenyl caprate (C10), p-Nitrophenyl laurate (C12) and p-Nitrophenyl palmitate (C16) (all from Sigma-Aldrich Danmark A/S, Kirkebjerg Allé 84, 2605 Brøndby; Cat.no.: C3:N-9876, C6: N-0502, C10: N-0252, C12: N-2002, C16: N-2752) were diluted to a final concentration of 1mM into assay buffer (50mM Tris; pH 7.7; 0.4% TritonX-100).

Lipr139 and the controls: Lipolase™ and Lipex™ (in 50mM Hepes; pH 8.0; 10ppm TritonX-100; +/- 20mM CaCl<sub>2</sub>) were added to the substrate solution in the following final concentrations: 0.01 mg/ml; 5x10<sup>-3</sup> mg/ml; 2.5x10<sup>-4</sup> mg/ml; and 1.25x10<sup>-4</sup> mg/ml in 96-well NUNC plates (Cat. No:260836, Kamstrupvej 90, DK-4000, Roskilde). Release of p-nitrophenol by hydrolysis of p-nitrophenyl acyl was monitored at 405nm for 5 minutes in 10 second intervals on a Spectra max 190 (Molecular Devices GmbH, Bismarckring 39, 88400 Biberach an der Riss, GERMANY).

The hydrolytic activity of Lipr139 for all substrates measured was significantly higher in the presence of 2mM CaCl<sub>2</sub> in comparison with the activity measured in the absence of CaCl<sub>2</sub>.

In the presence of CaCl<sub>2</sub> Lipr139 showed a lower (40-50%) hydrolytic activity than Lipolase™ and Lipex™ for all chain-lengths tested. The maximum activity for Lipr139 was measured for pNP-caprate (C10).

In the absence of 2mM CaCl<sub>2</sub> the Lipr 139 showed its highest activity with pNP-caprate (C10) for which the activity was higher than the controls Lipex™ and Lipolase™. For the other substrates the activity was comparable to Lipolase™ and Lipex™.

25

**Example 5: Stability**

The thermostability of the lipases was determined by Differential Scanning Calorimetry (DSC) using a VP Differential Scanning Calorimeter (MicroCal Inc., Piscataway, NJ, USA). The thermal denaturation temperature, T<sub>d</sub> (°C), was taken as the top of denaturation peak (major endothermic peak) in thermograms (Cp vs. T) obtained after heating enzyme solutions in selected buffers at a constant programmed heating rate of 90 K/hr. Sample- and reference-solutions (approximately 0.5 ml) were loaded into the calorimeter (reference: buffer without enzyme) and thermally pre-

equilibrated for 10 minutes at 25°C prior to DSC scan from 25°C to 110°C. Denaturation temperatures were determined with an accuracy of approximately +/- 1°C.

At pH 8 (50 mM HEPES) the thermal denaturation temperature was measured to 73 °C.

5 **Example 6: Relative wash performance**

In order to assess the wash performance in laundry washing experiments were performed, using the Automatic Mechanical Stress Assay (AMSA). The AMSA plate has a number of slots for test solutions and a lid firmly squeezing the laundry sample, the textile to be washed against all the slot openings. During the washing time, the plate, test solutions, textile and lid were vigorously shaken 10 to bring the test solution in contact with the textile and apply mechanical stress in a regular, periodic oscillating manner. For further description see WO02/42740 especially the paragraph "Special method embodiments" at page 23-24.

The laundry experiments were conducted in glycine buffers at different pH and in Compositions with different surfactant level and with different pH. The experimental conditions are specified 15 below.

**TABLE 2**

Compositions/buffers	50 mM glycine buffer pH 8 50 mM glycine buffer pH 9 50 mM glycine buffer pH 10 3.3 g/L Composition 0% surfactant, 50 mM glycine buffer pH 8 3.3 g/L Composition 0% surfactant, 50 mM glycine buffer pH 9 3.3 g/L Composition 0% surfactant, 50 mM glycine buffer pH 10 3.3 g/L Composition 10% surfactant, 50 mM glycine buffer pH 8 3.3 g/L Composition 10% surfactant, 50 mM glycine buffer pH 9 3.3 g/L Composition 10% surfactant, 50 mM glycine buffer pH 10 3.3 g/L Composition 20% surfactant, 50 mM glycine buffer pH 8 3.3 g/L Composition 20% surfactant, 50 mM glycine buffer pH 9 3.3 g/L Composition 20% surfactant, 50 mM glycine buffer pH 10 3.3 g/L Composition 60% surfactant, 50 mM glycine buffer pH 8 3.3 g/L Composition 60% surfactant, 50 mM glycine buffer pH 9 3.3 g/L Composition 60% surfactant, 50 mM glycine buffer pH 10 3.3 g/L Composition 100% surfactant, 50 mM glycine buffer pH 8
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Test solution volume	160 microliter
Wash time	15 minutes
Temperature	25°C
Water hardness	15°dH
Lipase dosage	0 ppm & 1 ppm
Test material	Cream turmeric stain according to WO06/125437

TABLE 3

Composition (wt%)	Total surfactant comprised	0%	10%	20%	60%	100%
		0%	10%	20%	60%	100%
linear alkylbenzenesulfonic acid (LAS) (Marlon AS3)	0	1.3	2.6	7.8	13	
sodium alkyl(C12)ether sulfate (AEOS) (STEOL CS-370 E)	0	1	2	6	10	
alcohol ethoxylate (AEO) (Bio-Soft N25-7)	0	1.1	2.2	6.6	11	
coco soap (Radiacid 631)	2.75	2.75	2.75	2.75	2.75	
soy soap (Edenor SJ)	2.75	2.75	2.75	2.75	2.75	
sodium hydroxide	0	0.2	0.4	1.2	2	
ethanol	3	3	3	3	3	
propane-1,2-diol (MPG)	6	6	6	6	6	
glycerol	2	2	2	2	2	
triethanolamine (TEA)	3	3	3	3	3	
sodium formate	1	1	1	1	1	
sodium citrate	2	2	2	2	2	
Diethylenetriaminepentakis (methyleneephosphonic acid) (DTMPA)	0.2	0.2	0.2	0.2	0.2	
polycarboxylate polymer (PCA) (Sokalan CP-5)	0.2	0.2	0.2	0.2	0.2	

Water up to	100	100	100	100	100
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Final adjustments to the specified pH were done with NaOH or citric acid. Water hardness was adjusted to 15°dH by addition of CaCl<sub>2</sub> and MgCl<sub>2</sub> (Ca<sup>2+</sup>:Mg<sup>2+</sup> = 4:1) to the test system.

After washing the textiles were flushed in tap water and excess water was removed from the textiles using filter paper and immediately thereafter the textiles were dried at 85°C for 5 min.

5 The wash performance was measured as the color change of the washed soiled textile. The soil was cream mixed with turmeric. Turmeric contains the colorant curcumin, which function as a pH indicator by having pH dependent color change. Lipase activity leads to release of free fatty acids from the cream acylglycerides and this leads to pH decrease and thereby color change of the curcumin pH indicator. Lipase wash performance can therefore be expressed as the extent of color 10 change of light reflected-emitted from the washed soiled textile when illuminated with white light.

Color measurements were made with a professional flatbed scanner (Kodak iQsmart, Kodak, Midtager 29, DK-2605 Brøndby, Denmark), which was used to capture an image of the washed soiled textile. To extract a value for the light intensity from the scanned images, 24-bit pixel values 15 from the image were converted into values for red, green and blue (RGB). The intensity value (Int) was calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

$$Int = \sqrt{r^2 + g^2 + b^2}$$

Color change due to lipase activity was measured as the increase in the reflection-emitting of green light (G) relative to the intensity (Int) of the total reflected-emitted light. The wash performance 20 (RP(Wash)) of a lipase relative to a reference lipase (Lipolase™) was calculated as: RP(Wash) = (G/Int(tested lipase)-G/Int(no enzyme)) / (G/Int(lipase ref.)-G/Int(no enzyme)).

TABLE 4 shows the Relative wash performance, RP(Wash) of the tested lipase

	pH	Lipolase RP(wash)	Lipr139 RP(wash)
Buffer 0% surfactant	8	1.00	1.10
	9	1.00	1.40
	10	1.00	2.19
Composition 0% surfactant	8	1.00	1.18

	9	1.00	1.89
	10	1.00	2.47
Composition 10% surfactant	8	1.00	1.80
	9	1.00	2.89
	10	1.00	2.76
Composition 20% surfactant	8	1.00	1.90
	9	1.00	2.96
	10	1.00	3.86
Composition 60% surfactant	8	1.00	1.91
	9	1.00	1.89
	10	1.00	2.38
Composition 100% surfactant	8	1.00	2.59

Furthermore, the relative wash performance (RP(Wash)) of Lipr139 was at level with the lipase Lipex™ when tested in Compositions containing 10%-100% surfactant.

5 The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also

10 intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

**Claims**

1. An isolated polypeptide having lipase activity, selected from the group consisting of:

(a) a polypeptide having at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide of SEQ ID NO: 2;

(b) a polypeptide encoded by a polynucleotide that hybridizes under low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, or (ii) the full-length complement of (i);

10 (c) a polypeptide encoded by a polynucleotide having at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;

(d) a variant of the polypeptide of (a), (b) or (c) comprising a substitution, deletion, and/or insertion at one or more positions; and

15 (e) a fragment of the polypeptide of (a), (b), (c), or (d) that has lipase activity.

2. The polypeptide of claim 1, comprising or consisting of SEQ ID NO: 2 or the mature polypeptide of SEQ ID NO: 2.

20 3. The polypeptide of claim 2, wherein the mature polypeptide is amino acids 1 to 292 of SEQ ID NO: 2.

4. A composition comprising the polypeptide of any of claims 1-3.

25 5. The composition of claim 4, wherein said composition is more effective in removing lipid stains present at a surface in comparison with an equivalent composition lacking the lipase.

6. The composition of any of claims 4-5 further comprising one or more divalent cations, preferably selected from calcium ( $Ca^{2+}$ ), magnesium ( $Mg^{2+}$ ) and iron-II ( $Fe^{2+}$ ).

7. The composition of any of claims 4-6, further comprising one or more surfactants selected from the group consisting of an anionic surfactant, a cationic surfactant, a non-ionic surfactant, zwitterionic surfactant.

5 8. The composition of any of claims 4-7, wherein the surfactant comprises one or more surfactants selected from the group consisting of sodium dodecyl benzene sulfonate, sodium hydrogenated cocoate, sodium laureth sulfate, C12-14 pareth-7, C12-15 pareth-7, sodium C12-15 pareth sulfate, and C14-15 pareth-4.

10 9. The composition of any of claims 4-8, formulated at a pH from 8.0 to 11.

10. The composition of any of claims 4-9, wherein the composition is selected from the group consisting of a laundry cleaning composition, a dishwashing cleaning composition, a hard-surface cleaning composition and a personal care cleaning composition.

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11. The composition of any of claims 4-10, wherein the form of the composition is selected from the group consisting of a liquid, a gel, a soap bar, a powder, a granulated solid, a tablet, or any combination thereof.

20 12. The composition of any of claims 4-11, wherein the composition is effective in hydrolyzing a lipid at a temperature of from about 10°C to 90°C.

25 13. The composition of any of claims 7-12, wherein the composition is more effective in hydrolyzing lipid substrates compared to an equivalent composition comprising *Thermomyces lanuginosus* lipase (Lipolase™) in place of Lipr139 lipase.

30 14. The composition of any of claims 4-13, further comprising one or more enzymes selected from: hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, mannanases, pectate lyases, keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases,  $\beta$ -

glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, amylases, or mixtures thereof.

15. The composition of claims 4-14, wherein the stability of the lipase with at least 92% identity to SEQ ID NO: 2 is greater than the stability of the *Thermomyces lanuginosus* lipase in an equivalent composition comprising *Thermomyces lanuginosus* lipase in place of the lipase with at least 92% identity to SEQ ID NO: 2, preferably wherein stability is measured in a final composition and/or in a final wash medium.
- 10 16. A method of producing the composition according to any of claims 4-15, comprising adding a lipase with at least 92% identity to SEQ ID NO: 2, and a surfactant.
17. A method for cleaning a surface, comprising contacting a lipid stain present on the surface to be cleaned with the composition according to any of claims 4-15.
- 15 18. A method for hydrolyzing a lipid present in a soil and/or a stain on a surface, comprising contacting the soil and/or the stain with the polypeptide of any of claims 1-3 or the composition of any of claims 4-15.
- 20 19. An isolated polynucleotide encoding the polypeptide of any of claims 1-3.
20. A nucleic acid construct or expression vector comprising the polynucleotide of claim 19 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
- 25 21. A recombinant host cell comprising the polynucleotide of claim 19 operably linked to one or more control sequences that direct the production of the polypeptide.

22. A method of producing the polypeptide of any of claims 1-3, comprising:

- (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

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23. A method of producing a polypeptide having lipase activity, comprising:

- (a) cultivating the host cell of claim 21 under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.

10

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2013/064682

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
  - a. (means)  
 on paper  
 in electronic form
  - b. (time)  
 in the international application as filed  
 together with the international application in electronic form  
 subsequently to this Authority for the purpose of search
2.  In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2013/064682

**A. CLASSIFICATION OF SUBJECT MATTER**  
INV. C11D3/386 C12N9/20  
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 C12N

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, Sequence Search, WPI Data, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARNALDO GLOGAUER ET AL: "Identification and characterization of a new true lipase isolated through metagenomic approach", MICROBIAL CELL FACTORIES, vol. 10, no. 1, 54, 1 January 2011 (2011-01-01), pages 1-15, XP055038298, ISSN: 1475-2859, DOI: 10.1186/1475-2859-10-54 abstract page 2, right-hand column, paragraph 2 page 3, left-hand column, last paragraph - page 4, right-hand column, paragraph 1 page 9, right-hand column, paragraph 2; tables 1, 29 ----- -/-	1,4-23
A	page 2, right-hand column, paragraph 2 page 3, left-hand column, last paragraph - page 4, right-hand column, paragraph 1 page 9, right-hand column, paragraph 2; tables 1, 29 ----- -/-	2,3

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier 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
4 September 2013	12/09/2013
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Niebuhr-Ebel, K

## INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/064682

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRIMONT P A D ET AL: "CEDECEA-DAVIAE NEW-GENUS NEW-SPECIES AND CEDECEA-LAPAGEI NEW-SPECIES NEW ENTEROBACTERIACEAE FROM CLINICAL SPECIMENS", INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, vol. 31, no. 3, 1981, pages 317-326, XP007921054, ISSN: 0020-7713 abstract page 322, last paragraph - page 324, last line table 5 ----- EP 0 258 068 A2 (NOVO INDUSTRI AS [DK] NOVO NORDISK AS [DK]) 2 March 1988 (1988-03-02) the whole document -----	1-23
A		1-23
1		

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No

PCT/EP2013/064682

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0258068	A2 02-03-1988	AT 110768 T DE 3750450 D1 DE 3750450 T2 EP 0258068 A2 ES 2058119 T3 JP H0633417 B2 JP S6368697 A US 4810414 A		15-09-1994 06-10-1994 05-01-1995 02-03-1988 01-11-1994 02-05-1994 28-03-1988 07-03-1989