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(54) Title: VARIANT LIPASES AND USES THEREOF

(57) Abstract: The present disclosure relates to variant lipolytic enzymes, more particularly variant lipolytic enzymes that have improved stability and/or improved hydrolytic activity on a polyester. Such variant lipolytic enzymes find use in the degradation of polyesters, such as polyethylene terephthalate. Also provided are compositions and methods related to such variant lipolytic enzymes.

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VARIANT LIPASES AND USES THEREOF

CROSS REFERENCE

[001] This application claims the benefit of U.S. Provisional Application No. 63/216,612, filed June 30, 2021, and is incorporated by reference in its entirety.

[002] The present disclosure relates to variant lipolytic enzymes, more particularly variant lipolytic enzymes that have improved stability and/or improved hydrolytic activity on a polyester. Such variant lipolytic enzymes find use in the degradation of polyesters, such as polyethylene terephthalate. Also provided are compositions and methods related to such variant lipolytic enzymes.

BACKGROUND

[003] Polyesters, such as polyethylene terephthalate (PET), are used in a large number of products and processes, such as in the manufacture of clothes, carpets, various packaging and plastics (e.g. automobile plastics), which has led to the accumulation of polyesters in landfills and may be an ecological problem.

[004] Various enzymes, such as lipolytic enzymes, are able to catalyse the hydrolysis of a variety of polymers, including polyesters. Some of these enzymes are being investigated for use in a number of industrial applications, such as detergents for laundry and dishwashing applications, as degrading enzymes for processing biomass and food, as biocatalysts in detoxification of environmental pollutants or for the treatment of polyester fabrics in the textile industry. The use of such enzymes is of particular interest for hydrolysing polyesters, such as PET.

[005] There is a continuing need for lipolytic enzymes with improved activity and/or improved stability that can be used in compositions for treating fabrics and/or textiles and for methods for degrading polyesters.

SUMMARY

[006] In one embodiment, the present disclosure provides variant lipolytic enzymes comprising an amino acid sequence having at least 70% identity to the full length amino acid

sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and wherein the variant has esterase activity. In some embodiments, the variant lipolytic enzyme comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2. In some embodiments, the variant lipolytic enzyme is derived from a parent enzyme comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

[007] In another embodiment, the present disclosure provides polynucleotides comprising a nucleic acid sequence encoding a variant lipolytic enzyme comprising an amino acid sequence having at least 70% identity to the full length amino acid sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and wherein the variant has esterase activity. In some embodiments, the variant lipolytic enzyme comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2. In some embodiments, the variant lipolytic enzyme is derived from a parent enzyme comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

[008] In another embodiment, the disclosure provides expression vectors or cassettes comprising polynucleotides encoding the variant lipolytic enzyme and recombinant host cells containing such expression vectors or cassettes.

[009] Also provided are enzyme compositions comprising a variant lipolytic enzyme comprising an amino acid sequence having at least 70% identity to the full length amino acid

sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and wherein the variant has esterase activity. In some embodiments, the variant lipolytic enzyme comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2. In some embodiments, the variant lipolytic enzyme is derived from a parent enzyme comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

[0010] Further provided in the present disclosure are methods for degrading a polyester or polyester-containing material and methods for the enzymatic depolymerization of a polyester or polyester-containing material. Such methods find use where the polyester is selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

DESCRIPTION

[0011] The present disclosure provides variant lipolytic enzymes, compositions (e.g. enzyme and detergent compositions) comprising such variant lipolytic enzymes, and methods using such variant lipolytic enzymes and compositions, for example, for washing or treating textiles and/or fabrics, and the degradation of polyesters.

[0012] Prior to describing embodiments of present compositions and methods, the following terms are defined.

[0013] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

invention pertains. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, the preferred methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the specification as a whole. Also, as used herein, the singular terms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art.

[0014] It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[0015] As used herein, the term "polymer" refers to a chemical compound or mixture of compounds whose structure is constituted of multiple repeating units linked by covalent chemical bonds. Within the context of the disclosure, the term polymer includes natural or synthetic polymers, constituting of a single type of repeat unit (i.e., homopolymers) or of a mixture of different repeat units (i.e., block copolymers and random copolymers).

[0016] The term "polyester-containing material" or "polyester-containing product", as used herein, refer to a product, such as a textile, fabric, or plastic product, comprising at least one polyester in crystalline, semi-crystalline, or substantially amorphous forms. In some embodiments, the polyester-containing material refers to any item made from at least one plastic material, such as plastic sheet, tube, rod, profile, shape, film, block, etc., which contains at least one polyester, and possibly other substances or additives, such as plasticizers, mineral or organic fillers. In some embodiments, the polyester-containing material refers to a plastic compound, or plastic formulation, in a molten or solid state, suitable for making a plastic product. In some embodiments, the polyester-containing material refers to a textile or fabric or fibers comprising at least one polyester. In some embodiments, the polyester-containing material refers to plastic waste or fiber waste comprising at least one polyester.

[0017] As used herein, the term "polyester" refers to its monomer bonded by ester linkage. As used herein, the term "polyester" includes, but is not limited to, those polyesters selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

[0018] The term "fabric" refers to, for example, woven, knit, and non-woven material, as well as staple fibers and filaments that can be converted to, for example, yarns and woven, knit, and non-woven fabrics. The term encompasses material made from natural, as well as synthetic (e.g., manufactured) fibers, and combinations thereof.

[0019] The term "textile", as used herein, refers to any textile material including yarns, yarn intermediates, fibers, non-woven materials, natural materials, synthetic materials, and any other textile material, fabrics made of these materials and products made from fabrics (e.g., garments and other articles). The textile or fabric may be in the form of knits, wovens, denims, non-wovens, felts, yarns, and towelling. The textile may include cellulose based such as natural cellulosics, including cotton, flax/linen, jute, ramie, sisal or coir or manmade cellulosics (e.g. originating from wood pulp) including viscose/rayon, cellulose acetate fibers (tricell), lyocell or blends thereof. The textile or fabric may also be non-cellulose based such as natural polyamides including wool, camel, cashmere, mohair, rabbit and silk or synthetic polymers such as nylon, aramid, polyester, acrylic, polypropylene and spandex/elastane, or blends thereof as well as blends of cellulose based and non-cellulose based fibers. Examples of blends are blends of cotton and/or rayon/viscose with one or more companion material such as wool, synthetic fiber (e.g. polyamide fiber, acrylic fiber, polyester fiber, polyvinyl chloride fiber, polyurethane fiber, polyurea fiber, aramid fiber), and/or cellulose-containing fiber (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fiber, lyocell). Fabric may be conventional washable laundry, for example stained household laundry. When the term fabric or garment is used, it is intended to include the broader term textiles as well. In the context of the present application, the term "textile" is used interchangeably with fabric and cloth. In some embodiments, textiles include those materials that include at least one polyester.

[0020] The term "laundering" includes both household laundering and industrial laundering and means the process of treating textiles with a solution containing a cleaning or detergent composition as provided herein. The laundering process can for example be carried out using e.g. a household or an industrial washing machine or can be carried out by hand.

[0021] The term "wash cycle" refers to a washing operation in which textiles are immersed in a wash liquor, mechanical action of some kind is applied to the textile to release stains or to facilitate flow of wash liquor in and out of the textile and finally the superfluous wash liquor is removed. After one or more wash cycles, the textile is generally rinsed and dried.

[0022] The term "wash liquor" is defined herein as the solution or mixture of water and detergent components optionally including variant lipolytic enzymes as provided herein.

[0023] As used herein, "homologous genes" refers to a pair of genes from different, but usually related species, which correspond to each other and which are identical or very similar to each other. The term encompasses genes that are separated by speciation (i.e., the development of new species) (*e.g.*, orthologous genes), as well as genes that have been separated by genetic duplication (*e.g.*, paralogous genes).

[0024] As used herein, the term "variant polypeptide" refers to a polypeptide comprising an amino acid sequence that differs in at least one amino acid residue from the amino acid sequence of a parent or reference polypeptide (including but not limited to wild-type polypeptides). In some embodiments, the parent polypeptide for use herein comprises an amino acid sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 2.

Variant Lipolytic enzymes

[0025] In one embodiment, variant lipolytic enzymes are provided. In some embodiments, the variant lipolytic enzymes provided herein have hydrolytic activity on at least one polyester.

[0026] As used herein, a lipolytic enzyme includes an enzyme, polypeptide, or protein exhibiting a lipid degrading capability such as a capability of degrading a triglyceride or a phospholipid. The lipolytic enzyme can be, for example, a lipase, a phospholipase, an esterase or a cutinase. Lipolytic enzymes can be enzymes having α/β hydrolase fold. These enzymes typically have a catalytic triad of serine, aspartic acid and histidine residues. The α/β hydrolases include lipases and cutinases. Cutinases show little, if any, interfacial activation, where lipases

often undergo a conformational change in the presence of a lipid-water interface (Longhi and Cambillau (1999) Biochimica et Biophysica Acta 1441:185-96). An active fragment of a lipolytic enzyme is a portion of a lipolytic enzyme that retains a lipid degrading capability. An active fragment retains the catalytic triad. As used herein, lipolytic activity can be determined according to any procedure known in the art (see, e.g., Gupta et al., Biotechnol. Appl. Biochem., 37:63-71, 2003; U.S. Pat. No. 5,990,069; and International Patent Publication No. WO 96/18729A1).

[0027] In some embodiments, lipolytic enzymes of the present disclosure are α/β hydrolases. In some embodiments, lipolytic enzymes of the present disclosure are lipases. In some embodiments, lipolytic enzymes of the present disclosure are cutinases. In some embodiments, lipolytic enzymes of the present disclosure are esterases.

[0028] In some embodiments, lipolytic enzymes of the present disclosure are alpha/beta hydrolases. In some embodiments, lipolytic enzymes of the present disclosure are lipases. In some embodiments, lipolytic enzymes of the present disclosure are cutinases. In some embodiments, lipolytic enzymes of the present disclosure are polyesterases.

[0029] As used herein, a "a carboxylic ester hydrolase" (E.C. 3.1.1) refers to an enzyme that acts on carboxylic acid esters.

[0030] As used herein, a "lipase", "lipase enzyme", "lipolytic enzymes", "lipolytic polypeptides", or "lipolytic proteins" refers to an enzyme, polypeptide, or protein exhibiting a lipid degrading capability such as a capability of degrading a triglyceride or a phospholipid. The lipolytic enzyme may be, for example, a lipase, a phospholipase, an esterase, a polyesterase, or a cutinase. As used herein, lipolytic activity may be determined according to any procedure known in the art (see, e.g., Gupta et al, Biotechnol. Appl. Biochem., 37:63-71, 2003; U.S. Pat. No. 5,990,069; and International Patent Publication No. WO 96/1 8729A1). In one embodiment, lipolytic activity can be determined on 4-nitrophenyl butyrate (pNB) as provided in Example 2.

[0031] A used herein, "cutinase" refers to lipolytic enzymes capable of hydrolyzing cutin substrates.

[0032] Cutinases include those derived from various fungi and from bacterial sources. Cutinases include those described in P. E. Kolattukudy, "Lipases", Ed. B Borgstrom and H. L. Brockman, Elsevier 1984, 471-504; S. Longhi et al., J. of Molecular Biology, 268 (4), 779-799 (1997); U.S Pat. No. 5,827,719; WO 94/14963; WO 94/14964; WO 00/05389; Appl. Environm.

Microbiol 64, 2794-2799, 1998; Proteins: Structure, Function and Genetics 26, 442-458, 1996; J. of Computational Chemistry 17, 1783-1803, 1996; Protein Engineering 6, 157-165, 1993. Cutinases may be naturally occurring or genetically modified cutinase obtained by UV irradiation, N-methyl-N'-nitrosoguanidine (NTG) treatment, ethyl methanesulfonate (EMS) treatment, nitrous acid treatment, acridine treatment or the like, recombinant strains induced by the genetic engineering procedures such as cell fusion and gene recombination and so forth.

[0033] As used herein, the term "polyesterase" or "PETase" refers to an enzyme that has significant capability to catalyze the hydrolysis and/or surface modification of polyester. Suitable polyesterases may be isolated from animal, plant, fungal and bacterial sources. The aforementioned microorganisms may be, in addition to being isolated from wild strains, may be isolated from any of mutant strains obtained by UV irradiation, N-methyl-N'-nitrosoguanidine (NTG) treatment, ethyl methanesulfonate (EMS) treatment, nitrous acid treatment, acridine treatment or the like, recombinant strains induced by the genetic engineering procedures such as cell fusion and gene recombination and so forth. The polyesterase may catalyze the hydrolysis and/or surface modification of a polyester selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

[0034] As used herein, "% identity or percent identity" refers to sequence similarity. Percent identity may be determined using standard techniques known in the art (*See e.g.*, Smith and Waterman, Adv. Appl. Math. 2:482 [1981]; Needleman and Wunsch, J. Mol. Biol. 48:443 [1970]; Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]; software programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI); and Devereux *et al.*, Nucl. Acid Res. 12:387-395 [1984]). One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pair-wise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (*See*, Feng and

Doolittle, J. Mol. Evol. 35:351-360 [1987]). The method is similar to that described by Higgins and Sharp (*See*, Higgins and Sharp, CABIOS 5:151-153 [1989]). Useful PILEUP parameters include a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps. Other useful algorithm is the BLAST algorithms described by Altschul *et al.*, (*See*, Altschul *et al.*, J. Mol. Biol. 215:403-410 [1990]; and Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 [1993]). The BLAST program uses several search parameters, most of which are set to the default values.

[0035] As used herein, "homologous proteins", "homologs" or "homologous proteins" refers to proteins that have distinct similarity in primary, secondary, and/or tertiary structure. Protein homology can refer to the similarity in linear amino acid sequence when proteins are aligned. Homology can be determined by amino acid sequence alignment, e.g., using a program such as BLAST, MUSCLE, or CLUSTAL. Homologous search of protein sequences can be done using BLASTP and PSI-BLAST from NCBI BLAST with threshold (E-value cut-off) at 0.001. (Altschul et al., "Gapped BLAST and PSI BLAST a new generation of protein database search programs", *Nucleic Acids Res*, Set 1;25(17):3389-402(1997)). The BLAST program uses several search parameters, most of which are set to the default values. The NCBI BLAST algorithm finds the most relevant sequences in terms of biological similarity but is not recommended for query sequences of less than 20 residues (Altschul et al., *Nucleic Acids Res*, 25:3389-3402, 1997 and Schaffer et al., *Nucleic Acids Res*, 29:2994-3005, 2001). Exemplary default BLAST parameters for a nucleic acid sequence searches include: Neighboring words threshold=11; E-value cutoff=10; Scoring Matrix=NUC.3.1 (match=1, mismatch=-3); Gap Opening=5; and Gap Extension=2. Exemplary default BLAST parameters for amino acid sequence searches include: Word size = 3; E-value cutoff=10; Scoring Matrix=BLOSUM62; Gap Opening=11; and Gap extension=1. Using this information, protein sequences can be grouped and/or a phylogenetic tree built therefrom. Amino acid sequences can be entered in a program such as the Vector NTI Advance suite and a Guide Tree can be created using the Neighbor Joining (NJ) method (Saitou and Nei, *Mol Biol Evol*, 4:406-425, 1987). The tree construction can be calculated using Kimura's correction for sequence distance and ignoring positions with gaps. A program such as AlignX can display the calculated distance values in parentheses following the molecule name displayed on the phylogenetic tree.

[0036] A percent (%) amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "reference" sequence including any gaps created by the program for optimal/maximum alignment. If a sequence is 90% identical to SEQ ID NO: A, SEQ ID NO: A is the "reference" sequence. BLAST algorithms refer the "reference" sequence as "query" sequence.

[0037] The CLUSTAL W algorithm is another example of a sequence alignment algorithm (See, Thompson et al., *Nucleic Acids Res*, 22:4673-4680, 1994). Default parameters for the CLUSTAL W algorithm include: Gap opening penalty=10.0; Gap extension penalty=0.05; Protein weight matrix=BLOSUM series; DNA weight matrix=IUB; Delay divergent sequences %=40; Gap separation distance=8; DNA transitions weight=0.50; List hydrophilic residues=GPSNDQEKR; Use negative matrix=OFF; Toggle Residue specific penalties=ON; Toggle hydrophilic penalties=ON; and Toggle end gap separation penalty=OFF. In CLUSTAL algorithms, deletions occurring at either terminus are included. For example, a variant with a five amino acid deletion at either terminus (or within the polypeptide) of a polypeptide of 500 amino acids would have a percent sequence identity of 99% (495/500 identical residues × 100) relative to the "reference" polypeptide. Such a variant would be encompassed by a variant having "at least 99% sequence identity" to the polypeptide.

[0038] In some embodiments, the variant lipase includes those derived from 2FX5_A, and those derived from the lipase disclosed in WO88/09367, US Patent Nos. 5,512,203, 5,389,536, U.S. Patent Publication No. US2003199068, European Patent Publication No. EP1543117, and WO 03/076580.

[0039] In some embodiments, the variant lipolytic enzymes provided herein comprise an amino acid sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 2. In some embodiments, the variant lipolytic enzymes have an amino acid sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 2 and has esterase activity.

[0040] The disclosure provides variant lipolytic enzymes or an active fragment thereof, comprising an amino acid sequence having at least 70% identity to the full length amino acid sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at

least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and where the variant has esterase activity.

[0041] In some embodiments, the variant lipolytic enzymes provided herein comprise an amino acid sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the full length amino acid sequence of SEQ ID NO: 2 comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and where the variant has esterase activity.

[0042] In some embodiments, the variant lipolytic enzymes provided herein comprise an amino acid sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the full length amino acid sequence of SEQ ID NO: 2 and comprises a combination of mutations selected from the group consisting of R40T-T64V-T117L-G175E-T177N-F180P-Y182A-R190L-S205G-F207L-S212D-F226L-Y239I-L249P-S252I-L258F, R40T-G61D-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Q227H-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40A-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-Q161H-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-G175A-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-S244E-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-

L258F, V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, V14S-R40A-G59Y-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, R40T-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, and V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-G175A-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2.

[0043] In some embodiments, the variant lipolytic enzymes provided herein have esterase activity (e.g. ability to catalyze the hydrolysis and/or surface modification) on at least one polyester selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof. In one embodiment, the variant lipolytic enzymes provided herein have esterase activity on PET.

[0044] Described herein is one or more isolated, non-naturally occurring, or recombinant polynucleotide comprising a nucleic acid sequence that encodes one or more variant lipolytic enzyme described herein, or recombinant polypeptide or active fragment thereof. One or more nucleic acid sequence described herein is useful in recombinant production (e.g., expression) of one or more variant lipolytic enzyme described herein, typically through expression of a plasmid expression vector comprising a sequence encoding the one or more variant lipolytic enzyme described herein or fragment thereof. One embodiment provides nucleic acids encoding one or more variant lipolytic enzyme described herein, wherein the variant is a mature form having lipolytic activity. In some embodiments, one or more variant lipolytic enzyme described herein is expressed recombinantly with a homologous pro-peptide sequence. In other embodiments, one or more variant lipolytic enzyme described herein is expressed recombinantly with a heterologous pro-peptide sequence.

[0045] One or more nucleic acid sequence described herein can be generated by using any suitable synthesis, manipulation, and/or isolation techniques, or combinations thereof. For example, one or more polynucleotide described herein may be produced using standard nucleic acid synthesis techniques, such as solid-phase synthesis techniques that are well-known to those skilled in the art. In such techniques, fragments of up to 50 or more nucleotide bases are typically synthesized, then joined (e.g., by enzymatic or chemical ligation methods) to form essentially any desired continuous nucleic acid sequence. The synthesis of the one or more polynucleotide described herein can be also facilitated by any suitable method known in the art, including but not limited to chemical synthesis using the classical phosphoramidite method (*See e.g.*, Beaucage et al. Tetrahedron Letters 22:1859-69 (1981)), or the method described in Matthes et al., EMBO J. 3:801-805 (1984) as is typically practiced in automated synthetic methods. One or more polynucleotide described herein can also be produced by using an automatic DNA synthesizer. Customized nucleic acids can be ordered from a variety of commercial sources (e.g., ATUM (DNA 2.0), Newark, CA, USA; Life Tech (GeneArt), Carlsbad, CA, USA; GenScript, Ontario, Canada; Base Clear B. V., Leiden, Netherlands; Integrated DNA Technologies, Skokie, IL, USA; Ginkgo Bioworks (Gen9), Boston, MA, USA; and Twist Bioscience, San Francisco, CA, USA). Other techniques for synthesizing nucleic acids and related principles are described by, for example, Itakura et al., Ann. Rev. Biochem. 53:323 (1984) and Itakura et al., Science 198:1056 (1984).

[0046] Recombinant DNA techniques useful in modification of nucleic acids are well known in the art, such as, for example, restriction endonuclease digestion, ligation, reverse transcription and cDNA production, and polymerase chain reaction (e.g., PCR). One or more polynucleotide described herein may also be obtained by screening cDNA libraries using one or more oligonucleotide probes that can hybridize to or PCR-amplify polynucleotides which encode one or more variant lipolytic enzyme described herein, or recombinant polypeptide or active fragment thereof. Procedures for screening and isolating cDNA clones and PCR amplification procedures are well known to those of skill in the art and described in standard references known to those skilled in the art. One or more polynucleotide described herein can be obtained by altering a naturally occurring polynucleotide backbone (e.g., that encodes one or more variant lipolytic enzyme described herein or reference lipolytic enzyme) by, for example, a known mutagenesis procedure (e.g., site-directed mutagenesis, site saturation mutagenesis, and in vitro

recombination). A variety of methods are known in the art that are suitable for generating modified polynucleotides described herein that encode one or more variant lipolytic enzyme described herein, including, but not limited to, for example, site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches.

[0047] A further embodiment is directed to one or more vector comprising one or more variant lipolytic enzyme described herein (e.g., a polynucleotide encoding one or more variant lipolytic enzyme described herein); expression vectors or expression cassettes comprising one or more nucleic acid or polynucleotide sequence described herein; isolated, substantially pure, or recombinant DNA constructs comprising one or more nucleic acid or polynucleotide sequence described herein; isolated or recombinant cells comprising one or more polynucleotide sequence described herein; and compositions comprising one or more such vector, nucleic acid, expression vector, expression cassette, DNA construct, cell, cell culture, or any combination or mixtures thereof.

[0048] Some embodiments are directed to one or more recombinant cell comprising one or more vector (e.g., expression vector or DNA construct) described herein which comprises one or more nucleic acid or polynucleotide sequence described herein. Some such recombinant cells are transformed or transfected with such at least one vector, although other methods are available and known in the art. Such cells are typically referred to as host cells. Some such cells comprise bacterial cells, including, but not limited to *Bacillus sp.* cells, such as *B. subtilis* cells. Other embodiments are directed to recombinant cells (e.g., recombinant host cells) comprising one or more variant lipolytic enzyme described herein.

[0049] In some embodiments, one or more vector described herein is an expression vector or expression cassette comprising one or more polynucleotide sequence described herein operably linked to one or more additional nucleic acid segments required for efficient gene expression (e.g., a promoter operably linked to one or more polynucleotide sequence described herein). A vector may include a transcription terminator and/or a selection gene (e.g., an antibiotic resistant gene) that enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media.

[0050] An expression vector may be derived from plasmid or viral DNA, or in alternative embodiments, contains elements of both. Exemplary vectors include, but are not limited to

pC194, pJH101, pE194, pHP13 (See, Harwood and Cutting [eds.], Chapter 3, Molecular Biological Methods for Bacillus, John Wiley & Sons (1990); suitable replicating plasmids for *B. subtilis* include those listed on p. 92). (*See also*, Perego, "Integrational Vectors for Genetic Manipulations in Bacillus subtilis"; Sonenshein et al., [eds.]; "Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics", American Society for Microbiology, Washington, D.C. (1993), pp. 615-624); and p2JM103BBI).

[0051] For expression and production of a protein of interest (e.g., one or more variant lipolytic enzyme described herein) in a cell, one or more expression vector comprising one or more copy of a polynucleotide encoding one or more variant lipolytic enzyme described herein, and in some instances comprising multiple copies, is transformed into the cell under conditions suitable for expression of the variant. In some embodiments, a polynucleotide sequence encoding one or more variant lipolytic enzyme described herein (as well as other sequences included in the vector) is integrated into the genome of the host cell, while in other embodiments, a plasmid vector comprising a polynucleotide sequence encoding one or more variant lipolytic enzyme described herein remains as autonomous extra-chromosomal element within the cell. Some embodiments provide both extrachromosomal nucleic acid elements as well as incoming nucleotide sequences that are integrated into the host cell genome. The vectors described herein are useful for production of the one or more variant lipolytic enzyme described herein. In some embodiments, a polynucleotide construct encoding one or more variant lipolytic enzyme described herein is present on an integrating vector that enables the integration and optionally the amplification of the polynucleotide encoding the variant into the host chromosome. Examples of sites for integration are well known to those skilled in the art. In some embodiments, transcription of a polynucleotide encoding one or more variant lipolytic enzyme described herein is effectuated by a promoter that is the wild-type promoter for the parent enzyme. In some other embodiments, the promoter is heterologous to the one or more variant lipolytic enzyme described herein, but is functional in the host cell. Exemplary promoters for use in bacterial host cells include, but are not limited to the amyE, amyQ, amyL, pstS, sacB, pSPAC, pAprE, pVeg, pHpaII promoters; the promoter of the *B. stearothermophilus* maltogenic amylase gene; the *B. amyloliquefaciens* (BAN) amylase gene; the *B. subtilis* alkaline protease gene; the *B. clausii* alkaline protease gene; the *B. pumilis* xylosidase gene; the *B. thuringiensis* cryIIIA; and the *B. licheniformis* alpha-amylase gene. Additional promoters include, but are not limited to the A4

promoter, as well as phage Lambda PR or PL promoters and the *E. coli* lac, trp or tac promoters.

[0052] One or more variant lipolytic enzyme described herein can be produced in host cells of any suitable microorganism, including bacteria and fungi. In some embodiments, one or more variant lipolytic enzyme described herein can be produced in Gram-positive bacteria. In some embodiments, the host cells are *Bacillus spp., Streptomyces spp., Escherichia spp., Aspergillus spp., Trichoderma spp., Pseudomonas spp., Corynebacterium spp., Saccharomyces spp.,* or *Pichia spp.* In some embodiments, one or more variant lipolytic enzyme described herein is produced by *Bacillus sp.* host cells. Examples of *Bacillus sp.* host cells that find use in the production of the one or more variant lipolytic enzyme described herein include, but are not limited to *B. licheniformis, B. lentus, B. subtilis, B. amyloliquefaciens, B. brevis, B. stearothermophilus, B. alkalophilus, B. coagulans, B. circulans, B. pumilis, B. thuringiensis, B. clausii,* and *B. megaterium*, as well as other organisms within the genus *Bacillus*. In some embodiments, *B. subtilis* host cells are used to produce the variants described herein. USPNs 5,264,366 and 4,760,025 (RE 34,606) describe various *Bacillus* host strains that can be used to produce one or more variant lipolytic enzyme described herein, although other suitable strains can be used.

[0053] Several bacterial strains that can be used to produce one or more variant lipolytic enzyme described herein include non-recombinant (i.e., wild-type) *Bacillus sp.* strains, as well as variants of naturally-occurring strains and/or recombinant strains. In some embodiments, the host strain is a recombinant strain, wherein a polynucleotide encoding one or more variant lipolytic enzyme described herein has been introduced into the host. In some embodiments, the host strain is a *B. subtilis* host strain and particularly a recombinant *B. subtilis* host strain. Numerous *B. subtilis* strains are known, including, but not limited to for example, 1A6 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 211strain (*See e.g.,* Hoch et al., Genetics 73:215–228 (1973); *See also*, US 4,450,235; US 4,302,544; and EP 0134048). The use of *B. subtilis* as an expression host cell is well known in the art (*See e.g.,* Palva et al., Gene 19:81-87 (1982); Fahnestock and Fischer, J. Bacteriol., 165:796–804 (1986); and Wang et al., Gene 69:39–47 (1988)).

[0054] In some embodiments, the *Bacillus* host cell is a *Bacillus sp.* that includes a mutation or deletion in at least one of the following genes: degU, degS, degR and degQ. In some

embodiments, the mutation is in a degU gene, and in some embodiments the mutation is degU(Hy)32 (*See e.g.*, Msadek et al., J. Bacteriol. 172:824-834 (1990); and Olmos et al., Mol. Gen. Genet. 253:562–567 (1997)). In some embodiments, the *Bacillus* host comprises a mutation or deletion in scoC4 (*See e.g.,* Caldwell et al., J. Bacteriol. 183:7329-7340 (2001)); spoIIE (*See e.g.,* Arigoni et al., Mol. Microbiol. 31:1407-1415 (1999)); and/or oppA or other genes of the opp operon (*See e.g.*, Perego et al., Mol. Microbiol. 5:173-185 (1991)). Indeed, it is contemplated that any mutation in the opp operon that causes the same phenotype as a mutation in the oppA gene will find use in some embodiments of the altered *Bacillus* strain described herein. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an altered *Bacillus* host cell strain that can be used to produce one or more variant lipolytic enzyme described herein is a *Bacillus* host strain that already includes a mutation in one or more of the above-mentioned genes. In addition, *Bacillus sp.* host cells that comprise mutation(s) and/or deletion(s) of endogenous protease genes find use. In some embodiments, the *Bacillus* host cell comprises a deletion of the aprE and the nprE genes. In other embodiments, the *Bacillus sp.* host cell comprises a deletion of 5 protease genes, while in other embodiments the *Bacillus sp.* host cell comprises a deletion of 9 protease genes (*See e.g.*, US 2005/0202535).

[0055] Host cells are transformed with one or more nucleic acid sequence encoding one or more variant lipolytic enzyme described herein using any suitable method known in the art. Methods for introducing a nucleic acid (e.g., DNA) into *Bacillus* cells or *E. coli* cells utilizing plasmid DNA constructs or vectors and transforming such plasmid DNA constructs or vectors into such cells are well known. In some embodiments, the plasmids are subsequently isolated from *E. coli* cells and transformed into *Bacillus* cells. However, it is not essential to use intervening microorganisms such as *E. coli*, and in some embodiments, a DNA construct or vector is directly introduced into a *Bacillus* host.

[0056] Exemplary methods for introducing one or more nucleic acid sequence described herein into *Bacillus* cells are described in, for example, Ferrari et al., "Genetics," in Harwood et al. [eds.], Bacillus, Plenum Publishing Corp. (1989), pp. 57-72; Saunders et al., J. Bacteriol. 157:718-726 (1984); Hoch et al., J. Bacteriol. 93:1925-1937 (1967); Mann et al., Current Microbiol. 13:131-135 (1986); Holubova, Folia Microbiol. 30:97 (1985); Chang et al., Mol. Gen. Genet. 168:11-115 (1979); Vorobjeva et al., FEMS Microbiol. Lett. 7:261-263 (1980); Smith et

al., Appl. Env. Microbiol. 51:634 (1986); Fisher et al., Arch. Microbiol. 139:213-217 (1981); and McDonald, J. Gen. Microbiol. 130:203 (1984)). Indeed, such methods as transformation, including protoplast transformation and transfection, transduction, and protoplast fusion are well known and suited for use herein. Methods known in the art to transform *Bacillus* cells include such methods as plasmid marker rescue transformation, which involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (*See*, Contente et al., Plasmid 2:555-571 (1979); Haima et al., Mol. Gen. Genet. 223:185-191 (1990); Weinrauch et al., J. Bacteriol. 154:1077-1087 (1983); and Weinrauch et al., J. Bacteriol. 169:1205-1211 (1987)). In this method, the incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

[0057] In addition to commonly used methods, in some embodiments, host cells are directly transformed with a DNA construct or vector comprising a nucleic acid encoding one or more variant lipolytic enzyme described herein (i.e., an intermediate cell is not used to amplify, or otherwise process, the DNA construct or vector prior to introduction into the host cell). Introduction of a DNA construct or vector described herein into the host cell includes those physical and chemical methods known in the art to introduce a nucleic acid sequence (e.g., DNA sequence) into a host cell without insertion into the host genome. Such methods include, but are not limited to calcium chloride precipitation, electroporation, naked DNA, and liposomes. In additional embodiments, DNA constructs or vector are co-transformed with a plasmid, without being inserted into the plasmid. In further embodiments, a selective marker is deleted from the altered *Bacillus* strain by methods known in the art (*See*, Stahl et al., J. Bacteriol. 158:411-418 (1984); and Palmeros et al., Gene 247:255 -264 (2000)).

[0058] In some embodiments, the transformed cells are cultured in conventional nutrient media. The suitable specific culture conditions, such as temperature, pH and the like are known to those skilled in the art and are well described in the scientific literature. Some embodiments provide a culture (e.g., cell culture) comprising one or more variant lipolytic enzyme or nucleic acid sequence described herein.

[0059] In some embodiments, host cells transformed with one or more polynucleotide sequence encoding one or more variant lipolytic enzyme described herein are cultured in a suitable nutrient medium under conditions permitting the expression of the variant, after which the resulting variant is recovered from the culture. In some embodiments, the variant produced

by the cells is recovered from the culture medium by conventional procedures, including, but not limited to, for example, separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt (e.g., ammonium sulfate), and chromatographic purification (e.g., ion exchange, gel filtration, affinity, etc.).

[0060] In some embodiments, one or more variant lipolytic enzyme produced by a recombinant host cell is secreted into the culture medium. A nucleic acid sequence that encodes a purification facilitating domain may be used to facilitate purification of the variant. A vector or DNA construct comprising a polynucleotide sequence encoding one or more variant lipolytic enzyme described herein may further comprise a nucleic acid sequence encoding a purification facilitating domain to facilitate purification of the variant (*See e.g.*, Kroll et al., DNA Cell Biol. 12:441-53 (1993)). Such purification facilitating domains include, but are not limited to, for example, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (See, Porath, Protein Expr. Purif. 3:263-281 [1992]), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system. The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (e.g., sequences available from Invitrogen, San Diego, CA) between the purification domain and the heterologous protein also find use to facilitate purification.

[0061] A variety of methods can be used to determine the level of production of one or more mature variant lipolytic enzyme described herein in a host cell. Such methods include, but are not limited to, for example, methods that utilize either polyclonal or monoclonal antibodies specific for the enzyme. Exemplary methods include, but are not limited to, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), fluorescent immunoassays (FIA), and fluorescent activated cell sorting (FACS). These and other assays are well known in the art (*See e.g.*, Maddox et al., J. Exp. Med. 158:1211 (1983)). In another embodiment, the method that can be used includes the assays provided in Examples 2 and 3.

[0062] Some other embodiments provide methods for making or producing one or more mature variant lipolytic enzyme described herein. A mature variant does not include a signal peptide or a propeptide sequence. Some methods comprise making or producing one or more variant lipolytic enzyme described herein in a recombinant bacterial host cell, such as for example, a *Bacillus sp.* cell (e.g., a *B. subtilis* cell). Other embodiments provide a method of

producing one or more variant described herein, wherein the method comprises cultivating a recombinant host cell comprising a recombinant expression vector comprising a nucleic acid sequence encoding one or more variant lipolytic enzyme described herein under conditions conducive to the production of the variant. Some such methods further comprise recovering the variant from the culture.

[0063] Further embodiments provide methods of producing one or more variant lipolytic enzyme described herein, wherein the methods comprise: (a) introducing a recombinant expression vector comprising a nucleic acid encoding the variant into a population of cells (e.g., bacterial cells, such as *B. subtilis* cells); and (b) culturing the cells in a culture medium under conditions conducive to produce the variant encoded by the expression vector. Some such methods further comprise: (c) isolating the variant from the cells or from the culture medium.

Compositions

[0064] The variant lipolytic enzymes provided herein may be used in the production of various compositions, such as enzyme compositions and cleaning or detergent compositions. Thus, in one embodiment, the present disclosure provides enzyme compositions comprising the variant lipolytic enzymes of the present disclosure, as well as cleaning or detergent compositions comprising the variant lipolytic enzymes provided herein or the enzyme compositions comprising such variant lipolytic enzymes.

[0065] As used herein, the "enzyme composition" refers to any enzyme product, preparation or composition, which comprises at least one of the variant lipolytic polypeptides provided herein. Such an enzyme composition may be a spent culture medium or filtrate containing one or more variant lipolytic enzymes, or one or more variant lipolytic enzymes and one or more additional enzymes. Spent culture medium means the culture medium of the host comprising the produced enzymes. Preferably the host cells are separated from the medium after the production. The enzyme composition may be a "whole culture broth" composition, optionally after inactivating the production host(s) or microorganism(s) without any biomass separation, down-stream processing or purification of the desired variant lipolytic enzyme(s), because the variant polypeptides can be secreted into the culture medium, and they display activity in the ambient conditions of the spent culture medium.

[0066] The enzyme composition may contain the variant lipolytic enzymes in at least partially purified and isolated form. It may even essentially consist of the desired enzyme or enzymes. If desired, the enzyme compositions may be dried, spray-dried or lyophilized, granulated or the enzymatic activity may be otherwise concentrated and/or stabilized for storage. If required, a desired enzyme may be crystallized or isolated or purified in accordance with conventional methods, such as filtration, extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

[0067] Enzyme granules may be made, for example, by rotary atomization, wet granulation, dry granulation, spray drying, disc granulation, extrusion, pan coating, spheronization, drum granulation, fluid-bed agglomeration, high-shear granulation, fluid-bed spray coating, crystallization, precipitation, emulsion gelation, spinning disc atomization and other casting approaches, and prilling processes. The core of the granule may be the granule itself or the inner nucleus of a layered granule.

[0068] In some embodiments, the enzyme compositions comprise a variant lipolytic enzyme as provided herein in combination with one or more additional enzymes selected from the group consisting of acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, feruloyl esterase, galactanases, glucoamylases, hemicellulases, hexosaminidases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, metalloproteases, nucleases (e.g. deoxyribonucleases and ribonucleases), oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, polyesterases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, and any combination or mixture thereof. Generally, at least one enzyme coating layer comprises at least one variant lipolytic enzyme.

[0069] The enzyme composition can be in any form suitable. For example, the enzyme composition can be in the form of a liquid composition or a solid composition such as solution, dispersion, paste, powder, granule, granulate, coated granulate, tablet, cake, crystal, crystal slurry, gel or pellet.

[0070] The enzyme composition can be used in cleaning agents or boosters that are added on top of the detergent during or before the wash and that are for example in the form of liquid, gel, powder, granules or tablets. The enzyme composition and detergent components may also be soaked in a carrier like textiles.

[0071] The disclosure further provides cleaning or detergent compositions comprising a variant lipolytic enzyme as provided herein. The cleaning or detergent compositions generally comprise a variant lipolytic enzyme as provided herein and one or more additional detergent components, such as a surfactant.

[0072] The disclosure further includes detergent or cleaning compositions. As used herein, the term "detergent composition" or "detergent formulation" is used in reference to a composition intended for use in a wash medium (e.g. a wash liquor) for the cleaning or treatment of soiled or dirty objects, including particular textile or non-textile objects or items. Such compositions of the present invention are not limited to any particular detergent composition or formulation. Indeed, in some embodiments, the detergents of the invention comprise at least one variant lipolytic enzyme as provided herein and, in addition, one or more surfactants, transferase(s), additional hydrolytic enzymes, oxido reductases, builders (*e.g.*, a builder salt), bleaching agents, bleach activators, bluing agents, fluorescent dyes, caking inhibitors, masking agents, enzyme activators, antioxidants, and/or solubilizers. In some instances, a builder salt is a mixture of a silicate salt and a phosphate salt, preferably with more silicate (*e.g.*, sodium metasilicate) than phosphate (*e.g.*, sodium tripolyphosphate). Some compositions of the invention, such as, but not limited to, cleaning compositions or detergent compositions, do not contain any phosphate (*e.g.*, phosphate salt or phosphate builder).

[0073] The compositions having a variant lipolytic enzyme, which find use in the methods provided herein, may comprise a variant lipolytic enzyme at a concentration of in use of 0.001 to 10,000 mg/L, or 0.001 to 2000 mg/L, or 0.01 to 5000 mg/L, or 0.01 to 2000 mg/L, or 0.01 to 1300 mg/L, or 0.1 to 5000 mg/L, or 0.1 to 2000 mg/L, or 0.1 to 1300 mg/L, or 1 to 5000 mg/L, or 1 to 1300 mg/L, or 1 to 500 mg/L, or 10 to 5000 mg/L, or 10 to 1300 mg/L, or 10 to 500 mg/L. In another embodiment, the composition may contain a variant lipolytic enzyme in an amount of 0.002 to 5000 mg of protein, such as 0.005 to 1300 mg of protein, or 0.01 to 5000 mg of protein, or 0.01 to 1300 mg of protein, or 0.1 to 5000 mg of protein, or 1 to 1300 mg of protein, preferably 0.1 to 1300 mg of protein, more preferably 1 to 1300 mg of protein, even

more preferably 10 to 500 mg of protein, per liter of wash liquor, or in the amount of at least 0.01 ppm active lipase.

[0074] In one embodiment, the composition comprises a variant lipolytic enzyme as provided herein and at least one additional detergent component, and optionally one or more additional enzymes.

[0075] In some embodiments, the cleaning or detergent compositions of the present invention further comprise adjunct materials including, but not limited to, surfactants, builders, bleaches, bleach activators, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, hydrotropes, photoactivators, fluorescers, fabric conditioners, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, and pH control agents (*See e.g.*, U.S. Pat. Nos. 6,610,642, 6,605,458, 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101, all of which are incorporated herein by reference).

[0076] The detergent or cleaning compositions of the present disclosure are advantageously employed for example, in laundry applications, hard surface cleaning, dishwashing applications, as well as cosmetic applications such as dentures, teeth, hair and skin. In addition, in some embodiments, the variant lipolytic enzyme of the present invention are ideally suited for laundry applications. Furthermore, the variants of the present disclosure find use in granular and liquid compositions.

[0077] Enzyme component weights are based on total active protein. All percentages and ratios are calculated by weight unless otherwise indicated. All percentages and ratios are calculated based on the total composition unless otherwise indicated. In laundry detergent compositions, the enzyme levels are expressed in ppm, which equals mg active protein/kg detergent composition.

[0078] In some embodiments, the laundry detergent compositions described herein further comprise a surfactant. In some embodiments, the surfactant is selected from a non-ionic, ampholytic, semi-polar, anionic, cationic, zwitterionic, and combinations and mixtures thereof. In yet a further embodiment, the surfactant is selected from an anionic surfactant, a cationic

surfactant, a zwitterionic surfactant, and combinations thereof. In some embodiments, the laundry detergent compositions described herein comprise from about 0.1% to about 60%, about 1% to about 50%, or about 5% to about 40% surfactant by weight of the composition.

[0079] Exemplary surfactants include, but are not limited to sodium dodecylbenzene sulfonate, C12-14 pareth-7, C12-15 pareth-7, sodium C12-15 pareth sulfate, C14-15 pareth-4, sodium laureth sulfate (e.g., Steol CS-370), sodium hydrogenated cocoate, C12 ethoxylates (Alfonic 1012-6, Hetoxol LA7, Hetoxol LA4), sodium alkyl benzene sulfonates (e.g., Nacconol 90G), and combinations and mixtures thereof. Anionic surfactants include but are not limited to linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. Nonionic surfactants include but are not limited to alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide (e.g., as described in WO92/06154), polyoxyethylene esters of fatty acids, polyoxyethylene sorbitan esters (e.g., TWEENs), polyoxyethylene alcohols, polyoxyethylene isoalcohols, polyoxyethylene ethers (e.g., TRITONs and BRIJ), polyoxyethylene esters, polyoxyethylene-p- tert-octylphenols or octylphenyl-ethylene oxide condensates (e.g., NONIDET P40), ethylene oxide condensates with fatty alcohols (e.g., LUBROL), polyoxyethylene nonylphenols, polyalkylene glycols (SYNPERONIC F108), sugar-based surfactants (e.g., glycopyranosides, thioglycopyranosides), and combinations and mixtures thereof.

[0080] In a further embodiment, the laundry detergent compositions described herein further comprise a surfactant mixture that includes, but is not limited to 5-15% anionic surfactants, < 5% nonionic surfactants, cationic surfactants, phosphonates, soap, enzymes, perfume, butylphenyl methylpropionate, geraniol, zeolite, polycarboxylates, hexyl cinnamal, limonene, cationic surfactants, citronellol, and benzisothiazolinone.

[0081] The laundry detergent compositions described herein may additionally include one or more detergent builders or builder systems, a complexing agent, a polymer, a bleaching system, a stabilizer, a foam booster, a suds suppressor, an anti-corrosion agent, a soil-suspending agent, an anti-soil redeposition agent, a dye, a bactericide, a hydrotope, an optical brightener, a fabric conditioner, and a perfume. The laundry detergent compositions described herein may also

include additional enzymes selected from proteases, amylases, cellulases, lipases, mannanases, nucleases, pectinases, xyloglucanases, or perhydrolases, as provided in more detail herein.

[0082] In some embodiments, the laundry detergent compositions described herein further comprises from about 1%, from about 3% to about 60% or even from about 5% to about 40% builder by weight of the cleaning composition. Builders may include, but are not limited to, the alkali metals, ammonium and alkanolammonium salts of polyphosphates, alkali metal silicates, alkaline earth and alkali metal carbonates, aluminosilicates, polycarboxylate compounds, ether hydroxypolycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1,3,5-trihydroxy benzene-2,4,6-trisulphonic acid, and carboxymethyloxysuccinic acid, the various alkali metals, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates such as mellitic acid, succinic acid, citric acid, oxydisuccinic acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof.

[0083] In some embodiments, the builders form water-soluble hardness ion complexes (e.g., sequestering builders), such as citrates and polyphosphates (e.g., sodium tripolyphosphate and sodium tripolyphospate hexahydrate, potassium tripolyphosphate, and mixed sodium and potassium tripolyphosphate, etc.). Any suitable builder can find use in the compositions described herein, including those known in the art.

[0084] In some embodiments, the laundry detergent compositions described herein further comprise an adjunct ingredient including, but not limited to surfactants, builders, bleaches, bleach activators, bleach catalysts, additional enzymes, an enzyme stabilizer (including, for example, an enzyme stabilizing system), chelants, optical brighteners, soil release polymers, dye transfer agents, dye transfer inhibiting agents, catalytic materials, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal agents, structure elasticizing agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, hydrotropes, photoactivators, fluorescers, fabric conditioners, hydrolyzable surfactants, solvents, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, pH control agents, and combinations thereof. (See, e.g., US6610642, US6605458, US5705464, US5710115, US5698504, US5695679, US5686014, and US5646101). In some embodiments, one or more adjunct is incorporated for example, to assist or enhance

cleaning performance, for treatment of the substrate to be cleaned, or to modify the aesthetics of the cleaning composition as is the case with perfumes, colorants, dyes or the like. Any such adjunct ingredient is in addition to variant enzyme provided herein. In some embodiments, the adjunct ingredient is selected from surfactants, enzyme stabilizers, builder compounds, polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-soap dispersants, soil suspension agents, softening agents, anti-redeposition agents, corrosion inhibitors, and combinations thereof.

[0085] In some further embodiments, the laundry detergent compositions described herein comprise one or more enzyme stabilizer. In some embodiments, the enzyme stabilizer is a water-soluble source of calcium and/or magnesium ions. In some embodiments, the enzyme stabilizers include oligosaccharides, polysaccharides, and inorganic divalent metal salts, including alkaline earth metals, such as calcium salts. In some embodiments, the enzymes employed herein are stabilized by the presence of water-soluble sources of zinc (II), calcium (II) and/or magnesium (II) ions in the finished compositions that provide such ions to the enzymes, as well as other metal ions (e.g., barium (II), scandium (II), iron (II), manganese (II), aluminum (III), tin (II), cobalt (II), copper (II), nickel (II), and oxovanadium (IV)). Chlorides and sulfates also find use in some embodiments. Exemplary oligosaccharides and polysaccharides (e.g., dextrins) are described, for example, in WO07145964. In some embodiments, the laundry detergent compositions described herein contain reversible protease inhibitors selected from a boron-containing compound (e.g., borate, 4-formyl phenyl boronic acid, and phenyl-boronic acid derivatives, such as, e.g., are described in WO9641859); a peptide aldehyde (such as, e.g., is described in WO2009118375 and WO2013004636), and combinations thereof.

[0086] The cleaning compositions herein are typically formulated such that, during use in aqueous cleaning operations, the wash water will have a pH of from about 3.0 to about 11. Liquid product formulations are typically formulated to have a neat pH from about 5.0 to about 9.0, more preferably from about 7.5 to about 9. Granular laundry products are typically formulated to have a pH from about 8.0 to about 11.0. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

[0087] Suitable high pH cleaning compositions typically have a neat pH of from about 9.0 to about 11.0, or even a neat pH of from 9.5 to 10.5. Such cleaning compositions typically comprise

a sufficient amount of a pH modifier, such as sodium hydroxide, monoethanolamine, or hydrochloric acid, to provide such cleaning composition with a neat pH of from about 9.0 to about 11.0. Such compositions typically comprise at least one base-stable enzyme. In some embodiments, the compositions are liquids, while in other embodiments, they are solids.

[0088] In one embodiment, the cleaning compositions include those having a pH of from 7.4 to pH 11.5, or pH 7.4 to pH 11.0, or pH 7.5 to pH 11.5, or pH 7.5 to pH 11.0, or pH 7.5 to pH 10.5, or pH 7.5 to pH 10.0, or pH 7.5 to pH 9.5, or pH 7.5 to pH 9.0, or pH 7.5 to pH 8.5, or pH 7.5 to pH 8.0, or pH 7.6 to pH 11.5, or pH 7.6 to pH 11.0, or pH 7.6 to pH 10.5, or pH 8.7 to pH 10.0, or pH 8.0 to pH 11.5, or pH 8.0 to pH 11.0, or pH 8.0 to pH 10.5, or pH 8.0 to pH 10.0.

[0089] Concentrations of detergent compositions in typical wash solutions throughout the world vary from less than about 800 ppm of detergent composition ("low detergent concentration geographies"), for example about 667 ppm in Japan, to between about 800 ppm to about 2000 ppm ("medium detergent concentration geographies"), for example about 975 ppm in U.S. and about 1500 ppm in Brazil, to greater than about 2000 ppm ("high detergent concentration geographies"), for example about 4500 ppm to about 5000 ppm in Europe and about 6000 ppm in high suds phosphate builder geographies.

[0090] In some embodiments, the detergent compositions described herein may be utilized at a temperature of from about 10°C to about 60°C, or from about 20°C to about 60°C, or from about 30°C to about 60°C, from about 40°C to about 60°C, from about 40°C to about 55°C, or all ranges within 10°C to 60°C. In some embodiments, the detergent compositions described herein are used in "cold water washing" at temperatures of from about 10°C to about 40°C, or from about 20°C to about 30°C, from about 15°C to about 25°C, from about 15°C to about 35°C, or all ranges within 10°C to 40°C.

[0091] As a further example, different geographies typically have different water hardness. Water hardness is usually described in terms of the grains per gallon mixed Ca^{2+}/Mg^{2+}. Hardness is a measure of the amount of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the water. Most water in the United States is hard, but the degree of hardness varies. Moderately hard (60-120 ppm) to hard (121-181 ppm) water has 60 to 181 parts per million (parts per million converted to grains per U.S. gallon is ppm # divided by 17.1 equals grains per gallon) of hardness minerals.

Table I. Water Hardness Levels

Water	Grains per gallon	Parts per million
Soft	less than 1.0	less than 17
Slightly hard	1.0 to 3.5	17 to 60
Moderately hard	3.5 to 7.0	60 to 120
Hard	7.0 to 10.5	120 to 180
Very hard	greater than 10.5	greater than 180

[0092] European water hardness is typically greater than about 10.5 (for example about 10.5 to about 20.0) grains per gallon mixed Ca^{2+}/Mg^{2+} (e.g., about 15 grains per gallon mixed Ca^{2+}/Mg^{2+}). North American water hardness is typically greater than Japanese water hardness, but less than European water hardness. For example, North American water hardness can be between about 3 to about 10 grains, about 3 to about 8 grains or about 6 grains. Japanese water hardness is typically lower than North American water hardness, usually less than about 4, for example about 3 grains per gallon mixed Ca^{2+}/Mg^{2+}.

[0093] In other embodiments, the composition described herein comprises one or more additional enzyme. The one or more additional enzyme is selected from acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, DNases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hexoaminidases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, metalloproteases, nucleases (e.g. deoxyribonucleases and ribonucleases), oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, polyesterases, additional proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, and any combination or mixture thereof. Some embodiments are directed to a combination of enzymes (i.e., a "cocktail") comprising enzymes like amylase, protease, lipase, mannanase, and/or nuclease in conjunction with one or more variant lipolytic enzyme in the compositions provided herein.

[0094] In some embodiments, the compositions provided herein comprise a variant lipolytic enzyme in combination with a protease. The protease for use in combination with the variant lipolytic enzyme in the compositions of the instant disclosure include any polypeptide having

protease activity. In one embodiment, the additional protease is a serine protease. In another embodiment, the additional protease is a metalloprotease, a fungal subtilisin, or an alkaline microbial protease or a trypsin-like protease. Suitable proteases include those of animal, vegetable or microbial origin. In some embodiments, the protease is a microbial protease. In other embodiments, the protease is a chemically or genetically modified mutant. In another embodiment, the protease is subtilisin like protease or a trypsin-like protease. In other embodiments, the additional protease does not contain cross-reactive epitopes with the variant as measured by antibody binding or other assays available in the art. Exemplary subtilisin proteases include those derived from for example, *Bacillus* (e.g., e.g., BPN', Carlsberg, subtilisin 309, subtilisin 147, and subtilisin 168), or fungal origin, such as, for example, those described in US Patent No. 8,362,222. Exemplary additional proteases include but are not limited to those described in WO92/21760, WO95/23221, WO2008/010925, WO09/149200, WO09/149144, WO09/149145, WO 10/056640, WO10/056653, WO2010/0566356, WO11/072099, WO2011/13022, WO11/140364, WO 12/151534, WO2015/038792, WO2015/089447, WO2015/089441, WO 2017/215925, US Publ. No. 2008/0090747, US 5,801,039, US 5,340,735, US 5,500,364, US 5,855,625, RE 34,606, US 5,955,340, US 5,700,676 US 6,312,936, US 6,482,628, US 8,530,219, US Provisional Appl Nos. 62/180673 and 62/161077, and PCT Appl Nos. PCT/US2015/021813, PCT/US2015/055900, PCT/US2015/057497, PCT/US2015/057492, PCT/US2015/057512, PCT/US2015/057526, PCT/US2015/057520, PCT/US2015/057502, PCT/US2016/022282, and PCT/US16/32514, International publications WO2016001449, WO2016087617, WO2016096714, WO2016203064, WO2017089093, and WO2019180111, as well as metalloproteases described in WO1999014341, WO1999033960, WO1999014342, WO1999034003, WO2007044993, WO2009058303, WO 2009058661, WO2014071410, WO2014194032, WO2014194034, WO 2014194054, and WO 2014/194117. Exemplary additional proteases include, but are not limited to trypsin (e.g., of porcine or bovine origin) and the *Fusarium* protease described in WO89/06270. Exemplary commercial proteases include, but are not limited to MAXATASE®, MAXACAL™, MAXAPEM™, OPTICLEAN®, OPTIMASE®, PROPERASE®, PURAFECT®, PURAFECT® OXP, PURAMAX™, EXCELLASE™, PREFERENZ™ proteases (e.g. P100, P110, P280), EFFECTENZ™ proteases (e.g. P1000, P1050, P2000), EXCELLENZ™ proteases (e.g. P1000), ULTIMASE®, and PURAFAST™ (DuPont); ALCALASE®, BLAZE®, BLAZE® variants, BLAZE® EVITY®, BLAZE® EVITY® 16L,

CORONASE®, SAVINASE®, SAVINASE® ULTRA, SAVINASE® EVITY®, SAVINASE® EVERIS®, PRIMASE®, DURAZYM™, POLARZYME®, OVOZYME®, KANNASE®, LIQUANASE®, LIQUANASE EVERIS®, NEUTRASE®, PROGRESS UNO®, RELASE®, and ESPERASE® (Novozymes); BLAP™ and BLAP™ variants (Henkel); LAVERGY™ PRO 104 L, LAVERGY™ PRO 106 LS , LAVERGY™ PRO 114 LS (BASF), KAP (*B. alkalophilus* subtilisin (Kao)) and BIOTOUCH® (AB Enzymes).

[0095] In some embodiments, the compositions provided herein comprise a variant lipolytic enzyme in combination with one or more amylases. In one embodiment, the composition comprises from about 0.00001% to about 10%, about 0.0001% to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% amylase by weight composition. Any amylase (e.g., alpha and/or beta) suitable for use in alkaline solutions may be useful to include in such composition. An exemplary amylase can be a chemically or genetically modified mutant. Exemplary amylases include, but are not limited to those of bacterial or fungal origin, such as, for example, amylases described in GB 1,296,839, WO9100353, WO9402597, WO94183314, WO9510603, WO9526397, WO9535382, WO9605295, WO9623873, WO9623874, WO 9630481, WO9710342, WO9741213, WO9743424, WO9813481, WO 9826078, WO9902702, WO 9909183, WO9919467, WO9923211, WO9929876, WO9942567, WO 9943793, WO9943794, WO 9946399, WO0029560, WO0060058, WO0060059, WO0060060, WO 0114532, WO0134784, WO 0164852, WO0166712, WO0188107, WO0196537, WO02092797, WO 0210355, WO0231124, WO 2004055178, WO2004113551, WO2005001064, WO2005003311, WO 2005018336, WO2005019443, WO2005066338, WO2006002643, WO2006012899, WO2006012902, WO2006031554, WO 2006063594, WO2006066594, WO2006066596, WO2006136161, WO 2008000825, WO2008088493, WO2008092919, WO2008101894, WO2008/112459, WO2009061380, WO2009061381, WO 2009100102, WO2009140504, WO2009149419, WO 2010/059413, WO 2010088447, WO2010091221, WO2010104675, WO2010115021, WO10115028, WO2010117511, WO 2011076123, WO2011076897, WO2011080352, WO2011080353, WO 2011080354, WO2011082425, WO2011082429, WO 2011087836, WO2011098531, WO2013063460, WO2013184577, WO 2014099523, WO2014164777, and WO2015077126. Exemplary commercial amylases include, but are not limited to AMPLIFY®, DURAMYL®, TERMAMYL®, FUNGAMYL®, STAINZYME®, STAINZYME PLUS®, STAINZYME PLUS®,

STAINZYME ULTRA® EVITY®, and BAN™ (Novozymes); EFFECTENZ™ S 1000, POWERASE™, PREFERENZ™ S 100, PREFERENZ™ S 110, EXCELLENZ™ S 2000, RAPIDASE® and MAXAMYL® P (DuPont).

[0096] In some embodiments, the compositions provided herein comprise a variant lipolytic enzyme in combination with one or more additional lipases. In some embodiments, the composition comprises from about 0.00001% to about 10%, about 0.0001% to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% lipase by weight composition. An exemplary lipase can be a chemically or genetically modified mutant. Exemplary lipases include, but are not limited to, e.g., those of bacterial or fungal origin, such as, e.g., *H. lanuginosa* lipase (*see, e.g.*, EP 258068 and EP 305216), *T. lanuginosa* lipase (*see, e.g.*, WO 2014/059360 and WO2015/010009), *Rhizomucor miehei* lipase (*see, e.g.*, EP 238023), *Candida* lipase, such as *C. antarctica* lipase (e.g., *C. antarctica* lipase A or B) (*see, e.g.*, EP 214761), *Pseudomonas* lipases such as *P. alcaligenes* and *P. pseudoalcaligenes* lipase (*see, e.g.*, EP 218272), *P. cepacia* lipase (*see, e.g.*, EP 331376), *P. stutzeri* lipase (*see, e.g.*, GB 1,372,034), *P. fluorescens* lipase, *Bacillus* lipase (e.g., *B. subtilis* lipase (Dartois et al., Biochem. Biophys. Acta 1131:253-260 (1993)), *B. stearothermophilus* lipase (*see, e.g.*, JP 64/744992), and *B. pumilus* lipase (*see, e.g.*, WO 91/16422)). Exemplary cloned lipases include, but are not limited to *Penicillium camembertii* lipase (*See*, Yamaguchi et al., Gene 103:61-67 (1991)), *Geotrichum candidum* lipase (*See*, Schimada et al., J. Biochem., 106:383-388 (1989)), and various *Rhizopus* lipases, such as, *R. delemar* lipase (*See*, Hass et al., Gene 109:117-113 (1991)), *R. niveus* lipase (Kugimiya et al., Biosci. Biotech. Biochem. 56:716-719 (1992)) and *R. oryzae* lipase. Other lipolytic enzymes, such as cutinases, may also find use in one or more composition described herein, including, but not limited to, e.g., cutinase derived from *Pseudomonas mendocina* (*see*, WO 88/09367) and/or *Fusarium solani pisi* (*see*, WO90/09446). Exemplary commercial lipases include, but are not limited to M1 LIPASE™, LUMA FAST™, and LIPOMAX™ (DuPont); LIPEX®, LIPOCLEAN®, LIPOLASE® and LIPOLASE® ULTRA (Novozymes); and LIPASE P™ (Amano Pharmaceutical Co. Ltd).

[0097] In some embodiments, the compositions provided herein comprise a variant lipolytic enzyme in combination with one or more mannanases. In one embodiment, the composition comprises from about 0.00001% to about 10%, about 0.0001% to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% mannanase by weight

composition. An exemplary mannanase can be a chemically or genetically modified mutant. Exemplary mannanases include, but are not limited to, those of bacterial or fungal origin, such as, for example, those described in WO 2016/007929; USPNs 6,566,114; 6,602,842; and 6,440,991: and US Provisional Appl. Nos. 62/251516, 62/278383, and 62/278387. Exemplary commercial mannanases include, but are not limited to MANNAWAY® (Novozymes) and EFFECTENZ™ M 1000, EFFECTENZ™ M 2000, PREFERENZ® M 100, MANNASTAR®, and PURABRITE™ (DuPont).

[0098] In some embodiments, the compositions and methods provided herein comprise variant lipolytic enzyme in combination with a nuclease, such as a DNase or RNase. Exemplary nucleases include, but are not limited to, those described in WO2015181287, WO2015155350, WO2016162556, WO2017162836, WO2017060475 (e.g. SEQ ID NO: 21), WO2018184816, WO2018177936, WO2018177938, WO2018/185269, WO2018185285, WO2018177203, WO2018184817, WO2019084349, WO2019084350, WO2019081721, WO2018076800, WO2018185267, WO2018185280, and WO2018206553. Other nucleases which can be used in combination with the variant lipolytic enzymes in the compositions and methods provided herein include those described in Nijland R, Hall MJ, Burgess JG (2010) Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase. PLoS ONE 5(12) and Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., Mattick, J.S. (2002) Extracellular DNA required for bacterial biofilm formation. Science 295: 1487.

[0099] Yet a still further embodiment is directed to a composition comprising one or more variant lipolytic enzymes described herein and one or more cellulase. In one embodiment, the composition comprises from about 0.00001% to about 10%, 0.0001% to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% cellulase by weight of composition. Any suitable cellulase may find use in a composition described herein. An exemplary cellulase can be a chemically or genetically modified mutant. Exemplary cellulases include but are not limited, to those of bacterial or fungal origin, such as, for example, those described in WO2005054475, WO2005056787, US 7,449,318, US 7,833,773, US 4,435,307; EP 0495257; and US Provisional Appl. No. 62/296,678. Exemplary commercial cellulases include, but are not limited to, CELLUCLEAN®, CELLUZYME®, CAREZYME®, ENDOLASE®, RENOZYME®, and CAREZYME® PREMIUM (Novozymes); REVITALENZ™ 100, REVITALENZ™ 200/220, and REVITALENZ® 2000 (DuPont); and KAC-500(B)™ (Kao

Corporation). In some embodiments, cellulases are incorporated as portions or fragments of mature wild-type or variant cellulases, wherein a portion of the N-terminus is deleted (*see, e.g.,* US 5,874,276).

[00100] In some embodiments, the laundry detergent compositions described herein comprise at least one chelating agent. Suitable chelating agents may include, but are not limited to copper, iron, and/or manganese chelating agents, and mixtures thereof. In some embodiments, the laundry detergent compositions described herein comprises from about 0.1% to about 15% or even from about 3.0% to about 10% chelating agent by weight of composition.

[00101] In some still further embodiments, the laundry detergent compositions described herein comprise at least one deposition aid. Suitable deposition aids include, but are not limited to, polyethylene glycol, polypropylene glycol, polycarboxylate, soil release polymers such as polyterephthalic acid, clays such as kaolinite, montmorillonite, attapulgite, illite, bentonite, halloysite, and mixtures thereof.

[00102] In some embodiments, the laundry detergent compositions described herein comprise at least one anti-redeposition agent.

[00103] In some embodiments, the laundry detergent compositions described herein comprise one or more dye transfer inhibiting agent. 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. In some embodiments, the laundry detergent compositions described herein comprise from about 0.0001% to about 10%, from about 0.01% to about 5%, or even from about 0.1% to about 3% dye transfer inhibiting agent by weight of composition.

[00104] In some embodiments, the laundry detergent compositions described herein comprise one or more silicates. In some such embodiments, sodium silicates (*e.g.,* sodium disilicate, sodium metasilicate, and crystalline phyllosilicates) find use. In some embodiments, the laundry detergent compositions described herein comprise from about 1% to about 20% or from about 5% to about 15% silicate by weight of the composition.

[00105] In yet further embodiments, the laundry detergent compositions described herein comprise one or more dispersant. Suitable water-soluble organic materials include, but are not limited to 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.

[00106] In some embodiments, the laundry detergent compositions described herein comprise one or more bleach, bleach activator, and/or bleach catalyst. In some embodiments, the laundry detergent compositions described herein comprise inorganic and/or organic bleaching compound(s). Inorganic bleaches may include, but are not limited to perhydrate salts (*e.g.*, perborate, percarbonate, perphosphate, persulfate, and persilicate salts). In some embodiments, inorganic perhydrate salts are alkali metal salts. In some embodiments, inorganic perhydrate salts are included as the crystalline solid, without additional protection, although in some other embodiments, the salt is coated. Suitable salts include, for example, those described in EP2100949. Bleach activators are typically organic peracid precursors that enhance the bleaching action in the course of cleaning at temperatures of 60°C and below. Bleach activators suitable for use herein include compounds which, under perhydrolysis conditions, give aliphatic peroxycarboxylic acids having preferably from about 1 to about 10 carbon atoms, in particular from about 2 to about 4 carbon atoms, and/or optionally substituted perbenzoic acid. Bleach catalysts typically include, for example, manganese triazacyclononane and related complexes, and cobalt, copper, manganese, and iron complexes, as well as those described in US4246612, US5227084, US4810410, WO9906521, and EP2100949.

[00107] In some embodiments, the laundry detergent compositions described herein comprise one or more catalytic metal complex. In some embodiments, a metal-containing bleach catalyst finds use. In other embodiments, the metal bleach catalyst comprises a catalyst system comprising a transition metal cation of defined bleach catalytic activity (*e.g.*, copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations), an auxiliary metal cation having little or no bleach catalytic activity (*e.g.*, 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 are used (*See, e.g.*, US4430243). In some embodiments, the laundry detergent compositions described herein are catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art (*See, e.g.*, US5576282). In additional embodiments, cobalt bleach catalysts find use in the laundry detergent compositions described herein. Various cobalt bleach catalysts are known in the art (*See, e.g.*, US5597936 and

US 5595967) and are readily prepared by known procedures.

[00108] Polyesters as used herein include polymers that contain at least one ester repeating unit in their main chain polymers. In their simplest form, polyesters are produced by polycondensation reaction of a glycol (diol) with a dicarboxylic acid (diacid) or its diester. Polyesters include naturally occurring chemicals, such as in the cutin of plant cuticles, as well as synthetics through step-growth polymerization such as polybutyrate.

[00109] Polyesters that can be contacted with the variant lipases provided herein (e.g. in the methods provided herein), or a composition including such variant lipase include any ester bond-containing polymer. Such polyesters include aliphatic and aromatic polyesters. The aliphatic polyesters include: polyhydroxyalkanoates (PHA), which can be divided into polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), and their copolymers; polylactide (PLA); poly(ε-caprolactone) (PCL); polybutylenesuccinate (PBS) and its derivative poly(butylenesuccinate adipate) (PBSA). The aromatic polyesters include: modified poly(ethylene terephthalate) (PET) such as poly(butylene adipate/terephthalate) (PBAT) and poly(tetramethylene adipate-coterephthalate) (PTMAT); and aliphatic-aromatic copolyesters (AAC). In some embodiments, polyesters may be partially or substantially biodegradable. In other embodiments, the polyesters may be partially or substantially resistant to microbial and enzymatic attack.

[00110] In some embodiments, a polyester may be an aliphatic polyester. In some embodiments, a polyester may be an aromatic polyester. In some embodiments, an aromatic polyester maybe a polyethylene terephthalate (PET). In some embodiments, an aromatic polyester maybe a polytrimethylene terephthalate (PTT).

[00111] Accordingly, in one embodiment, the polyesters that find use in the methods provided herein include those selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

[00112] In another embodiment, the fabrics or textiles that find use in the methods provided herein include fabrics and textiles that contain at least one polyester selected from the group

consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

[00113] In some embodiments, the disclosure provides methods for treating a fabric or a textile comprising contacting a fabric or a textile with a variant lipolytic enzyme as provided herein, or a composition comprising such variant lipolytic enzyme and optionally rinsing the fabric or textile.

[00114] In some embodiments, the contacting steps of the methods provided herein comprise a variant lipolytic enzyme in an amount selected from the group consisting of 0.002 to 10,000 mg of protein, 0.005 to 5000 mg of protein, 0.01 to 5000 mg of protein, 0.05 to 5000 mg of protein, 0.05 to 1300 mg of protein, 0.1 to 1300 mg of protein, 0.1 to 500 mg of protein, 0.1 to 100 mg of protein, per liter of wash liquor.

Esterase for surface modification

[00115] In some embodiments, a polyester (e.g. PET)-containing textile, fabric, or film may have a hydrolyzable polymer end or a loop on their surface. The variant lipolytic enzymes provided herein find use in methods for surface modification of polyester (e.g. PET) fibers, which may improve factors such as finishing fastness, dyeability, wettability, de-pilling and preventing pilling. In some embodiments, polymer chains that protrude or form a loop on the surface of a polyester (e.g. PET)-containing textile, fiber or film may be hydrolyzed by the variant lipases herein to carboxylic acid and hydroxyl residues, thus increasing surface hydrophilicity. Pilling is the formation of small, fuzzy balls on the surface of polyester (e.g. PET) fabrics resulting in an unsightly worn appearance of the textile. Generally, these nodules are produced by loose fibers in the fabric or those which have been released from the tissue.

[00116] Thus, in some embodiments, the variant lipolytic enzymes of the present disclosure can be used in methods for finishing fastness, dyeability, wettability, de-pilling, and preventing pilling of polyester (e.g. PET) textiles, fabrics, and films. In other embodiments, the variant lipolytic enzymes of the present disclosure may be used in a detergent composition in order to

reduce pilling during textile cleaning. In some embodiments, the variant lipolytic enzymes of the present disclosure have PETase activity.

[00117] In one embodiment, methods for degrading a polyester or a polyester-containing material are provided, where the methods comprise contacting a polyester-containing material with a variant lipolytic enzyme or composition comprising a variant lipolytic enzyme as provided herein. In some embodiments, the polyester-containing material is a polyester textile or fabric.

[00118] In another embodiment, the disclosure provides a method for the enzymatic depolymerization of a polyester-containing material, where the method comprises contacting a polyester-containing material with a variant lipolytic enzyme or composition comprising a variant lipolytic enzyme as provided herein, and recovering monomers and/or oligomers of the polyester. In some embodiments, the polyester-containing material is a polyester textile or fabric.

[00119] In other embodiments, the variant lipolytic enzymes of the present disclosure can be used in methods for cleaning or conditioning a textile or fabric, improving the thermophysiological properties (e.g. heat or moisture management, or wear comfort) of a textile or fabric comprising a polyester, and increasing the hydrophilicity of a textile or fabric comprising a polyester. In other embodiments, the variant lipolytic enzymes of the present disclosure may be used in a detergent composition in order to clean or condition a textile or fabric, improve the thermophysiological properties (e.g. heat or moisture management, or wear comfort) of a textile or fabric comprising a polyester, and increase the hydrophilicity of a textile or fabric comprising a polyester. In some embodiments, the variant lipolytic enzymes of the present disclosure have PETase activity.

[00120] In other embodiments, the variant lipolytic enzymes of the present disclosure can be used in methods for reducing the pilling effects and/or increasing the anti-graying effect of a cleaning composition on a textile or fabric comprising a polyester. In other embodiments, the variant lipolytic enzymes of the present disclosure may be used in a detergent composition in order to reduce the pilling effects and/or increasing the anti-graying effect of a detergent composition on a textile or fabric comprising a polyester. In some embodiments, the variant lipolytic enzymes of the present disclosure have PETase activity. In some embodiments, the variant lipolytic enzyme of the present disclosure is combined with a second enzyme, for

example a cellulase.

[00121] The textile or fabric can be contacted with the variant lipolytic enzyme or a composition comprising the variant lipolytic enzyme in a washing machine or in a manual wash tub (e.g. for handwashing). In one embodiment, the textile or fabric is contacted with the variant lipolytic enzyme or a composition comprising the variant lipolytic enzyme in a wash liquor. In another embodiment, a solution containing the variant lipolytic enzyme is incubated with or flowed over the polyester-containing material, such as by pumping the solution through tubing or pipes or by filling a reservoir with the solution.

[00122] In some embodiments, the textiles or articles are contacted with the variant lipolytic enzyme or a composition comprising the variant lipolytic enzyme under conditions having a temperature that allows for activity of the variant lipolytic enzyme. In some embodiments, the temperature in the methods disclosed herein include those between 10° to 60° C, between 10° to about 45° C, between 15° to about 55° C, between 15° to about 50° C, between 15° to about 45° C, between 20° to about 60° C, between 20° to about 50° C and between 20° to about 45° C.

[00123] The polypeptides, compositions, and methods provided herein have utility in a wide array of applications in which degrading polyester (e.g. PET) is desired, such as household cleaning, including in washing machines, dishwashers, and on household surfaces.

[00124] Other aspects and embodiments of the present compositions and methods will be apparent from the foregoing description and following examples. Various alternative embodiments beyond those described herein can be employed in practicing the invention without departing from the spirit and scope of the invention. Accordingly, the claims, and not the specific embodiments described herein, define the scope of the invention and as such methods and structures within the scope of the claims and their equivalents are covered thereby.

EMBODIMENTS

[00125] Embodiment 1. A variant lipolytic enzyme comprising an amino acid sequence having at least 70% identity to the full length amino acid sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, where the positions

are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and where the variant has esterase activity.

[00126] Embodiment 2. The variant lipolytic enzyme of Embodiment 1, where the variant comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

[00127] Embodiment 3. The variant lipolytic enzyme of Embodiments 1 or 2, where the variant is derived from a parent enzyme comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

[00128] Embodiment 4. The variant lipolytic enzyme of any of the preceding Embodiments, where the variant comprises a combination of substitutions selected from the group consisting of R40T-T64V-T117L-G175E-T177N-F180P-Y182A-R190L-S205G-F207L-S212D-F226L-Y239I-L249P-S252I-L258F, R40T-G61D-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Q227H-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40A-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-Q161H-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-G175A-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-S244E-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, V14S-R40A-G59Y-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, R40T-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, and

V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-G175A-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, where the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2.

[00129] Embodiment 5. The variant lipolytic enzyme of any of the preceding Embodiments, where the variant has one or more improved properties when compared to a parent or reference lipolytic enzyme, where the improved property is selected from improved stability, improved hydrolytic activity on a polyester, or combinations thereof.

[00130] Embodiment 6. The variant lipolytic enzyme of any of the preceding Embodiments, where the improved property is:

(i) improved stability, where said variant has a residual activity at least 5% when measured in accordance with the stability assay of Example 3 and/or

(ii) improved hydrolytic activity on a polyester, wherein said variant has a PI $\geq$ 1.2 compared to the lipolytic enzyme having the amino acid sequence of SEQ ID NO: 2 having the substitutions R40T-T64V-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Y239I-L249P-S252I-L258F when measured in accordance with the PET assay of Example 2.

[00131] Embodiment 7. The lipolytic enzyme of any of Embodiments 1-6, where the variant has hydrolytic activity on a polyester selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

[00132] Embodiment 8. A polynucleotide comprising a nucleic acid sequence encoding a variant lipolytic enzyme of any one of Embodiments 1-7.

[00133] Embodiment 9. The polynucleotide of Embodiment 8, where the nucleic acid sequence is operably linked to a promoter.

[00134] Embodiment 10. An expression vector or cassette comprising the polynucleotide of

Embodiment 8 or 9.

[00135] Embodiment 11. A recombinant host cell comprising the expression vector or cassette of Embodiment 10.

[00136] Embodiment 12. An enzyme composition comprising a variant lipolytic enzyme of any one of Embodiments 1-7.

[00137] Embodiment 13. The enzyme composition of Embodiment 12, where the composition further comprises at least at least one additional enzyme selected from the group consisting of: acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, feruloyl esterase, galactanases, glucoamylases, hemicellulases, hexosaminidases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, metalloproteases, nucleases (e.g. deoxyribonucleases and ribonucleases), oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, polyesterases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, and any combination or mixture thereof.

[00138] 14. The enzyme composition of Embodiment 13, wherein the at least one additional enzyme is selected from the group consisting of a protease, an alpha-amylase, a cellulase, and a mannanase.

[00139] Embodiment 15. A method for degrading a polyester or a polyester containing material comprising

i) contacting the polyester containing material with a variant lipolytic enzyme according to any one of Embodiments 1-7 or a composition comprising a variant lipolytic enzyme according to any one of Embodiments 1-7, and, optionally,

ii) rinsing said polyester containing material.

[00140] Embodiment 16. A method for the enzymatic depolymerization of a polyester or a polyester containing material comprising,

i) contacting the polyester or polyester containing material with a variant lipolytic

enzyme according to any one of Embodiments 1-7 or a composition comprising a variant lipolytic enzyme according to any one of Embodiments 1-7, and, optionally,

ii) recovering monomers and/or oligomers of the polyester.

[00141] Embodiment 17. The method of Embodiment 15 or 16, where the polyester is selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

EXAMPLES

EXAMPLE 1

Recombinant expression and generation of *P. mendocina* lipase variants

[00142] A synthetic, codon-optimized gene (SEQ ID NO:1) encoding the wild-type *Pseudomonas mendocina* lipase (SEQ ID NO:2) was made and served as template for the construction of plasmids expressing variant polypeptides thereof. Lipase genes were produced by either GeneArt AG (Regensburg, Germany) or Twist Bioscience (San Francisco, U.S.A.) and cloned into the pSB expression vector (Babé, L.M., *et al.* (1998) *Biotechnol Appl Biochem.* 27:117-24) using standard molecular biology techniques resulting in expression plasmids suitable for expression in *Bacillus subtilis*. Elements of the constructs included: DNA fragments comprising the aprE promoter sequence (SEQ ID NO:3), the nucleotide sequence encoding either the aprE signal peptide sequence (SEQ ID NO:4) or a hybrid aprE-*P. mendocina* lipase signal peptide sequence (SEQ ID NO:5), the sequence corresponding to the gene encoding a mature lipase, the BPN' terminator (SEQ ID: NO:6), and additional elements from pUB110 (McKenzie *et al.* (1986) *Plasmid* 15: 93-103) including a replicase gene (reppUB), a neomycin/kanamycin resistance gene (neo), and a bleomycin resistance marker (bleo).

[00143] A suitable *B. subtilis* host strain was transformed with the pSB expression plasmid using a method known in the art (WO 02/14490). The transformation mixtures were plated onto

LA plates containing 10ppm neomycin sulfate and incubated overnight at 37°C. Single colonies were picked and grown in Luria broth at 37°C under antibiotic selection.

[00144] To generate the enzyme samples for screening, the transformed *B. subtilis* cells were grown in 96 well microtiter plates (MTPs, manufacture) at 37°C for 68 hours in cultivation medium (enriched semi-defined media based on MOPS buffer, with urea as the major nitrogen source, glucose as the main carbon source, and supplemented with 1% soytone for robust cell growth) in each well. Cultures were harvested by centrifugation at 3600 rpm for 15 min and filtered through Multiscreen® filter plates (EMD Millipore, Billerica, MA, USA) using a Millipore vacuum system. The filtered culture supernatants were used for the assays described below. Typically, the culture broth was diluted in 100mM Tris pH8 in 96 well plate (NUNC, 267245). Enzyme concentration was determined by separation of protein components using a Zorbax 300 SB-C3 column (Agilent) and running a linear gradient of 0.1% Trifluoroacetic acid in water (Buffer A) and 0.1% Trifluoroacetic acid in Acetonitrile (Buffer B) with detection at 220nm column on UHPLC. The enzyme concentration of the samples was calculated using a standard curve of the purified reference enzyme PEV132.

EXAMPLE 2

Enzyme activity of *P. mendocina* lipase variants

[00145] The enzymatic activity of *P. mendocina* lipase variants (listed on Table 1) was tested on PET (Polyethylene terephthalate) substrate by measuring the hydrolysis of PET Pellet substrate in solution. PET pellets were purchased from Scientific Polymer Products (Cat#138). One PET pellet (20-30mg) was added to each well of a microtiter plate (Nunc, 267245) and detergent solutions were added. Specifically, the detergent solution consists of two hundred microliters of Formula A HDL (3.0g/L) (composition in Table 2) prepared in 10 mM Tris-HCl buffer with 6 gpg water hardness Ca:Mg = 3:1, pH 8.

Table 1: List of variants constructed with sample ID and amino acid substitutions with respect to *P. mendocina* wild type enzyme	
sample ID	**Amino acid substitution with respect to *P. mendocina* wild-type lipase**
PEV132	R40T-T64V-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Y239I-L249P-S252I-L258F
PEV132v1	R040A-T064V-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Y239I-L249P-S252I-L258F
PEV132v2	R040T-T064V-T117L-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Y239I-L249P-S252I-L258F
PEV176	R40T-T64V-T117L-G175E-T177N-F180P-Y182A-R190L-S205G-F207L-S212D-F226L-Y239I-L249P-S252I-L258F
PEV192	R40T-G61D-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Q227H-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV194	R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV304	R40A-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV314	R40T-T64V-S70E-T117L-Q161H-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV315	R40T-T64V-S70E-T117L-G175A-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV318	R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F
PEV319	R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-S244E-L249P-S252I-E254Q-L258F
PEV320	R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F
PEV325	V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F
PEV328	V14S-R40A-G59Y-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F
PEV329	R40T-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F
PEV334	V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-G175A-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F

Table 2: Composition of Custom-made Formula A HDL Detergent		
Chemical name	WT % of active substance in the raw material	WT % of active substance in the formulation
Water demin.	100	Ad 100
Alkyl benzene sulfonic acid (LAS)	96	3-7
Anionic surfactant (FAEOS)	70	2-6
Coconut fatty acid	30	0.3-1
Non-ionic surfactant (FAEO)	100	3-7
Citric acid	100	0.1-2
NaOH	50	0.3-1
Glycerol	99,5	0.3-1
Preserving agent	100	0.05-0.1
Boric acid	100	0.3-1
Optical Brightener	90	0.01-0.08
Thickener	25	1-3
Enzymes (except lipase)	100	0.5-2
Dequest	40	0.1-2
Dye, Perfume, Antifoam	t.q.	minors
pH = 8.2 to 8.6 Dosage = 50 mL		

[00146] A set of plates without PET in the well were also set up to serve as controls for enzyme background. Twenty microliters of each enzyme sample were added per well of the assay plate to initiate the reaction. The reaction was carried out at 40 °C for 24 hours with shaking (180 rpm) in incubation shaker (Infors HT, Multitron). After incubation, 100ul of the reaction supernatant was transferred into a new UV-transparent plate (Corning 3635) and measured at 240 nm on Microplate reader (Molecular devices, SpectraMax plus 384). The resulting absorbance after subtracting absorbance from enzyme background plate was taken as a measure of PET hydrolysis activity. The absorbance values were plotted against enzyme concentration. Each variant was assayed in triplicates. PET activity is reported as Performance Index (PI) values, which were calculated by dividing the PET activity of each variant by that of the parent, tested at the same protein concentration. Table 3 shows the polyesterase activity on PET substrate (performance index) of variants from Table 1. Theoretical values for the PET activity of the parent enzyme at the relevant protein concentrations were calculated using the parameters extracted from a Langmuir fit of measured values from a standard curve of the parent enzyme activity.

Table 3: PET Activity of *P. mendocina* lipase variants in Formula A HDL. Relative activity on PET substrate is reported as Performance Index values (PI) calculated relative to *P. mendocina* lipase PEV132	
sample ID	**Polyesterase Activity (PI)**
PEV132	1.0
PEV132v1	1.1
PEV132v2	1.0
PEV318	1.4
PEV319	1.5
PEV314	1.7
PEV194	1.8
PEV329	1.8
PEV192	1.9
PEV328	1.9
PEV315	1.9
PEV334	1.9
PEV325	2.1
PEV320	2.2
PEV304	2.2

EXAMPLE 3

Thermal stability of *P. mendocina* variants

[00147] The stability of *P. mendocina* lipase variants (shown on Table 1) was tested under stress condition in a 50% (v/v) aqueous solution of Formula A HDL detergent by measuring the residual activity of samples after incubation at 56°C for 16 hours. A 67% (v/v) aqueous solution of the detergent was prepared and enzyme samples from filtered culture supernatants were mixed with the appropriate volume of this detergent solution to achieve 50% (v/v) final detergent concentration. To measure the initial (unstressed) activity, aliquots of this mixture were immediately diluted in 100mM Tris-HCl, 0.1% Triton X-100, pH8 and assayed for activity on pNB substrate. pNB substrate (4-nitropheyl butyrate, Sigma) solution (1 mM) was prepared by adding 0.2 mL of pNB stock solution (100 mM in DMSO) to 20mL of buffer (100mM Tris-HCl, 0.1% Triton X-100, pH8). Ten microliters of diluted enzyme solution were mixed into 190ul of 1mM pNB in assay buffer in 96 well plate (Costar, #9017, ThermoFisher) to start the reaction.

The plate was mixed thoroughly, and absorbance was monitored at OD 405 nm every 12 seconds for 3 minutes in a Microplate reader (Molecular devices, SpectraMax plus 384). The Vmax in mOD/min value of a sample not containing enzyme (blank) was subtracted from Vmax values of the enzyme - containing samples. The resulting Vmax in mOD/min was recorded as enzyme activity on pNB substrate. Once stressed and unstressed activity values were measured by hydrolysis of pNB substrate as described above, the percent (%) residual activities were calculated by taking a ratio of the stressed to unstressed activity and multiplying by 100. Table 4 shows the % residual activity of the *P. mendocina* lipase variants tested.

Table 4: Stability of *P. mendocina* lipase variants in Formula A liquid detergent. Stability is reported as % residual activity remaining after incubation at 56°C for 16 hours.

sample ID	% Residual activity
PEV132	20
PEV176	90
PEV192	20
PEV194	40
PEV304	40
PEV314	50
PEV315	50
PEV318	60
PEV319	80
PEV320	100
PEV325	90
PEV328	100
PEV329	80
PEV334	90

[00148] Although the disclosure has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

[00149] All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if

each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present disclosure. To the extent that section headings are used, they should not be construed as necessarily limiting.

SEQUENCE LISTING

SEQ ID NO:1 (codon-optimized gene sequence for wild-type lipase from *P. mendocina*)

GCTCCTCTTCCTGATACACCGGGAGCGCCATTTCCTGCTGTCGCAAACTTCGACCGC
AGCGGCCCTTACACTGTTTCTAGCCAGTCAGAAGGGCCGAGCTGTCGCATCTATAGA
CCTCGCGACCTGGGTCAGGGAGGCGTACGCCATCCGGTTATTCTTTGGGGCAACGGC
ACTGGTGCTGGACCGTCTACATATGCAGGCTTGCTTTCACACTGGGCAAGCCACGGT
TTCGTTGTAGCGGCTGCGGAAACATCTAACGCTGGTACCGGACGCGAAATGCTCGCC
TGCCTGGACTATCTGGTACGTGAGAACGACACCCCCTACGGCACCTATTCCGGCAAG
CTCAATACCGGGCGAGTCGGCACTTCTGGGCATTCTCAAGGTGGAGGCGGGTCAATC
ATGGCTGGCCAGGATACGAGAGTACGTACAACGGCGCCGATCCAGCCTTACACTCT
TGGCCTGGGACACGACAGCGCTTCTCAACGCCGCCAACAGGGACCGATGTTCCTTAT
GTCTGGTGGCGGAGACACAATCGCTTTCCCTTACCTCAACGCTCAGCCGGTCTACCG
CCGTGCAAACGTACCTGTATTCTGGGGCGAAAGACGTTACGTTTCACACTTCGAACC
GGTAGGTAGCGGTGGGGCTTATCGCGGCCCGTCTACAGCATGGTTCCGCTTCCAACT
TATGGATGACCAAGACGCTCGCGCTACATTCTACGGCGCGCAGTGCAGCCTTTGCAC
TTCTTTACTTTGGTCAGTCGAACGCCGCGGGCTTTAA

SEQ ID NO:2 (amino acid sequence of wild-type lipase from *P. mendocina*)

APLPDTPGAPFPAVANFDRSGPYTVSSQSEGPSCRIYRPRDLGQGGVRHPVILWGNGTG
AGPSTYAGLLSHWASHGFVVAAAETSNAGTGREMLACLDYLVRENDTPYGTYSGKLN
TGRVGTSGHSQGGGGSIMAGQDTRVRTTAPIQPYTLGLGHDSASQRRQQGPMFLMSGG
GDTIAFPYLNAQPVYRRANVPVFWGERRYVSHFEPVGSGGAYRGPSTAWFRFQLMDDQ
DARATFYGAQCSLCTSLLWSVERRGL

SEQ ID NO:3 (aprE promoter DNA sequence)

GAATTCTCCATTTTCTTCTGCTATCAAAATAACAGACTCGTGATTTTCCAAACGAGCT
TTCAAAAAAGCCTCTGCCCCTTGCAAATCGGATGCCTGTCTATAAAATTCCCGATAT
TGGTTAAACAGCGGCGCAATGGCGGCCGCATCTGATGTCTTTGCTTGGCGAATGTTC
ATCTTATTTCTTCCTCCCTCTCAATAATTTTTTCATTCTATCCCTTTTCTGTAAAGTTT

ATTTTTCAGAATACTTTTATCATCATGCTTTGAAAAAATATCACGATAATATCCATTG
TTCTCACGGAAGCACACGCAGGTCATTTGAACGAATTTTTTCGACAGGAATTTGCCG
GGACTCAGGAGCATTTAACCTAAAAAAGCATGACATTTCAGCATAATGAACATTTAC
TCATGTCTATTTTCGTTCTTTTCTGTATGAAAATAGTTATTTCGAGTCTCTACGGAAA
TAGCGAGAGATGATATACCTAAATAGAGATAAAATCATCTCAAAAAAAATGGGTCTA
CTAAAATATTATTCCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAGTCT
ACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGA

SEQ ID NO:4 (aprE signal peptide DNA sequence)

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGTCTGCGCAGGCT

SEQ ID NO:5 (hybrid aprE-*P. mendocino* lipase signal peptide DNA sequence)

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAGCGGCTTCT
TGCCTGAGCGTCTGTGCAACTGTAGCTGCA

SEQ ID NO:6 (BPN' terminator DNA sequence)

ACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCCTCCGCATGT
TCAATCCGCTCCATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGC
GGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCGCT
TCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGG
GAGACGGCATTCGTAATC

Claims

What is claimed is:

1. A variant lipolytic enzyme comprising an amino acid sequence having at least 70% identity to the full length amino acid sequence of SEQ ID NO: 2, comprising the substitutions T064V-T117L-T177N/R-I178L-F180P-Y182A-R190L-S205G-S212D-F226L-Y239I-L249P-S252I-L258F, and further comprising at least one additional substitution selected from the group consisting of V014S, R040A/T, G059Y, G061D, A066D, S070E, Q161H, G175A/E, F207TL/T, V210I, Q227H, A236P, S244E, E254Q, and R256K, wherein the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2, and wherein the variant has esterase activity.

2. The variant lipolytic enzyme of claim 1, wherein the variant comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

3. The variant lipolytic enzyme of claims 1 or 2, wherein the variant is derived from a parent enzyme comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, or 99% identity to the full length amino acid sequence of SEQ ID NO: 2.

4. The variant lipolytic enzyme of any of the preceding claims, wherein the variant comprises a combination of substitutions selected from the group consisting of R40T-T64V-T117L-G175E-T177N-F180P-Y182A-R190L-S205G-F207L-S212D-F226L-Y239I-L249P-S252I-L258F, R40T-G61D-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Q227H-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40A-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-Q161H-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-G175A-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F,

R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-S244E-L249P-S252I-E254Q-L258F, R40T-T64V-S70E-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, V14S-R40A-G59Y-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, R40T-G61D-T64V-S70E-T117L-Q161H-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, and V14S-R40A-G59Y-G61D-T64V-A66D-S70E-T117L-Q161H-G175A-T177R-I178L-F180P-Y182A-R190L-S205G-F207T-V210I-S212D-F226L-A236P-Y239I-L249P-S252I-E254Q-R256K-L258F, wherein the positions are numbered by reference to the amino acid sequence of SEQ ID NO: 2.

5. The variant lipolytic enzyme of any of the preceding claims, wherein the variant has one or more improved properties when compared to a parent or reference lipolytic enzyme, wherein the improved property is selected from improved stability, improved hydrolytic activity on a polyester, or combinations thereof.

6. The variant lipolytic enzyme of any of the preceding claims, wherein the improved property is:

(i) improved stability, wherein said variant has a residual activity at least 5% when measured in accordance with the stability assay of Example 3 and/or

(ii) improved hydrolytic activity on a polyester, wherein said variant has a PI $\geq$ 1.2 compared to the lipolytic enzyme having the amino acid sequence of SEQ ID NO: 2 having the substitutions R40T-T64V-T117L-T177N-I178L-F180P-Y182A-R190L-S205G-F207T-S212D-F226L-Y239I-L249P-S252I-L258F when measured in accordance with the PET assay of Example 2.

7. The lipolytic enzyme of any of claims 1-6, wherein the variant has hydrolytic activity on

a polyester selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

8. A polynucleotide comprising a nucleic acid sequence encoding a variant lipolytic enzyme of any one of claims 1-7.

9. The polynucleotide of claim 8, wherein the nucleic acid sequence is operably linked to a promoter.

10. An expression vector or cassette comprising the polynucleotide of claim 8 or 9.

11. A recombinant host cell comprising the expression vector or cassette of claim 10.

12. An enzyme composition comprising a variant lipolytic enzyme of any one of claims 1-7.

13. The enzyme composition of claim 12, wherein the composition further comprises at least at least one additional enzyme selected from the group consisting of: acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, feruloyl esterase, galactanases, glucoamylases, hemicellulases, hexosaminidases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, metalloproteases, nucleases (e.g. deoxyribonucleases and ribonucleases), oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, perhydrolases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, polyesterases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, xylosidases, and any combination or mixture thereof.

14. The enzyme composition of claim 13, wherein the at lease one additional enzyme is

selected from the group consisting of a protease, an alpha-amylase, a cellulase, and a mannanase.

15. A method for degrading a polyester or a polyester containing material comprising

i) contacting the polyester containing material with a variant lipolytic enzyme according to any one of claims 1-7 or a composition comprising a variant lipolytic enzyme according to any one of claims 1-7, and, optionally,

ii) rinsing said polyester containing material.

16. A method for the enzymatic depolymerization of a polyester or a polyester containing material comprising,

i) contacting the polyester or polyester containing material with a variant lipolytic enzyme according to any one of claims 1-7 or a composition comprising a variant lipolytic enzyme according to any one of claims 1-7, and, optionally,

ii) recovering monomers and/or oligomers of the polyester.

17. The method of claim 15 or 16, wherein the polyester is selected from the group consisting of polyethylene terephthalate (PET), polytrimethylene terephthalate (PTT), polybutylene terephthalate (PBT), polyethylene isosorbide terephthalate (PEIT), polylactic acid (PLA), polyhydroxy alkanoate (PHA), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA), polybutylene adipate terephthalate (PBAT), polyethylene furanoate (PEF), polycaprolactone (PCL), polyethylene naphthalate (PEN), polyester polyurethane, poly(ethylene adipate) (PEA), and combinations thereof.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/035080

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/18 C08J11/10 C11D3/386 C12N15/52
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)
C12N C09J C11D C08J

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, EMBASE, 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.
Y	BOSTON M ET AL: "STRUCTURE AND FUNCTION OF ENGINEERED PSEUDOMONAS MENDOCINA LIPASE", METHODS IN ENZYMOLOGY; [METHODS IN ENZYMOLOGY, ISSN 0076-6879], ELSEVIER, ACADEMIC PRESS, NL, vol. 284, 1 January 1997 (1997-01-01), pages 298-317, XP008044067, DOI: 10.1016/S0076-6879(97)84020-7 ISBN: 978-0-12-805382-9 page 304, 3rd paragraph table 1 page 303 ----- -/--	1-17

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

[x] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 2022	06/12/2022

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/035080

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SARAVANAN PARAMESWARAN ET AL: "Emulating structural stability ofPseudomonas mendocinalipase:in silicomutagenesis and molecular dynamics studies", JOURNAL OF MOLECULAR MODELING, SPRINGER BERLIN HEIDELBERG, BERLIN/HEIDELBERG, vol. 20, no. 11, 1 November 2014 (2014-11-01), pages 1-12, XP035382370, ISSN: 1610-2940, DOI: 10.1007/S00894-014-2501-4 [retrieved on 2014-11-01] abstract ----- SIBILLE NATHALIE ET AL: "Comparative NMR study on the impact of point mutations on protein stability of Pseudomonas mendocina lipase", PROTEIN SCIENCE, vol. 15, no. 8, 1 August 2006 (2006-08-01) , pages 1915-1927, XP055981600, US ISSN: 0961-8368, DOI: 10.1110/ps.062213706 abstract ----- WO 03/076580 A2 (GENENCOR INT [US]; BOTT RICHARD R [US] ET AL.) 18 September 2003 (2003-09-18) example 1; tables 1,3; sequence 2 -& DATABASE Geneseq [Online] 29 January 2004 (2004-01-29), Bott RR et al.: "Pseudomonas mendocina cutinase protein SEQ ID NO:2.", XP055930363, retrieved from EBI accession no. GSP:ADE36705 Database accession no. ADE36705 abstract ----- WO 01/34899 A1 (GENENCOR INT [US]; DYSON WADE [US] ET AL.) 17 May 2001 (2001-05-17) example 1; tables 1-3 -----	1-17 1-17 1-17 1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/035080

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03076580 A2	18-09-2003	AT 444355 T	15-10-2009
		AU 2003216540 A1	22-09-2003
		EP 1543117 A2	22-06-2005
		ES 2333789 T3	01-03-2010
		PT 1543117 E	07-01-2010
		WO 03076580 A2	18-09-2003
WO 0134899 A1	17-05-2001	AT 510957 T	15-06-2011
		AU 1076101 A	06-06-2001
		CA 2389913 A1	17-05-2001
		DK 1242671 T3	01-08-2011
		EP 1242671 A1	25-09-2002
		ES 2365207 T3	26-09-2011
		JP 4996027 B2	08-08-2012
		JP 2003514138 A	15-04-2003
		KR 20020059691 A	13-07-2002
		PT 1242671 E	03-08-2011
		US 6933140 B1	23-08-2005
		US 2003082755 A1	01-05-2003
		WO 0134899 A1	17-05-2001