



- (51) International Patent Classification:
C11D 3/386 (2006.01) *C12N 9/28* (2006.01)
- (21) International Application Number:
PCT/EP2015/063135
- (22) International Filing Date:
12 June 2015 (12.06.2015)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
62/011,564 12 June 2014 (12.06.2014) US
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))



WO 2015/189372 A1

(54) Title: ALPHA-AMYLASE VARIANTS

(57) Abstract: The present invention relates to alpha-amylase variants. The present invention also relates to polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of using the variants.

ALPHA-AMYLASE VARIANTS

Reference to a Sequence Listing

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

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to alpha-amylase variants, polynucleotides encoding the
10 variants, methods of producing the variants, and methods of using the variants.

Description of the Related Art

Alpha-amylases (alpha-1,4-glucan-4-glucanohydrolases, E.C. 3.2.1.1) constitute a group
of enzymes, which catalyses hydrolysis of starch and other linear and branched 1,4-gluosidic
15 oligo- and polysaccharides.

Alpha-amylase is a key enzyme for use in detergent compositions and its use has
become increasingly important for removal of starchy stains during laundry washing or
dishwashing.

Some detergents, in particular dishwashing detergents, contain bleaching systems,
20 bleach activators, and bleach catalysts which are all very destabilizing for the alpha-amylases
due to oxidation of the molecules. Therefore, it is important to find alpha-amylase variants,
which are stable, have high wash performance, stain removal effect and/or activity in detergents
comprising various bleaching agents.

It is known in the art to stabilize alpha-amylases towards bleaching agents and oxidation
25 by these by substituting the methionine at position 197 (using the amylase from *B. licheniformis*
for numbering) with *e.g.* leucine. This has *e.g.* been disclosed in WO199418314. However,
these prior art oxidation stable alpha-amylases have the disadvantage that the alpha-amylase
activity is reduced.

Thus, it is an object of the present invention to provide alpha-amylase variants that
30 exhibit a high level of stability in detergents, in particular in dishwashing detergents and other
detergents comprising bleaching agents or systems but at the same time have improved alpha-
amylase activity compared to the parent alpha-amylase having the prior art solution of M197L
substitution. It is a further object to provide alpha-amylase variants which have high
performance, in particular high wash performance, in particular high dishwashing performance.

35 The present invention provides alpha-amylase variants with improved stability compared
to its parent and improved activity compared to its parent having M197L (which in the parent
amylases of the present invention corresponds to M202L).

SUMMARY OF THE INVENTION

The present invention relates to an isolated alpha-amylase variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203 and A204 of the mature polypeptide of SEQ ID NO: 1, and c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1 with any other amino acid, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity.

The present invention also relates to detergent compositions comprising the variants, isolated polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of producing the variants.

The present invention also relates to the use of the variants in a cleaning process.

The present invention also relates to a method of improving the activity of an alpha-amylase by introducing into a parent alpha-amylase a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203 and A204 of the mature polypeptide of SEQ ID NO: 1, and c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1 with any other amino acid, wherein the resulting variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2.

Definitions

Alpha-amylase: The term "alpha-amylase" as used herein, refers to an enzyme capable of catalyzing the degradation of starch. Generally, alpha-amylases (E.C. 3.2.1.1, α -D-(1->4)-glucan glucanohydrolase) are endo-acting enzymes that cleave the α -D(1->4) O-glycosidic linkages within the starch molecule in a random order.

The term "starch" as used herein, refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein X can be any number. In particular, the term refers to plant-based materials, such as rice, barley, wheat, corn, rye, potato, and the like.

Alpha-amylase activity: The term "alpha-amylase activity" means the activity of alpha-1,4-glucan-4-glucanohydrolases, E.C. 3.2.1.1, which constitute a group of enzymes, which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides. For purposes of the present invention, alpha-amylase activity is determined according to the procedure described in the Methods. In one aspect, the variants of the present

invention have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the mature polypeptide of SEQ ID NO: 1 or 2.

5 **SEQ ID NO: 1:**

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HHDGTNGTIM QYFEWNPND GQHWNRLHNN AQN LKNAGIT AIWIPPAWK
TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TKAELERAIR SLKANGIQVY
GDVVMNHKGG ADFTERVQAV EVNPQNRNQE VSGTYQIEAW TGFNFPGRGN
QHSSFKWRWY HFDGTDWDQS RQLANRIYKF RGDGKAWDWE VDTENGNYDY
10 LMYADVMDH PEVINELNRW GVWYANTLNL DGFRLDAVKH IKFSFMRDWL
GHVRGQTGKN LFAVAEYWKN DLGALENYLS KTNWTMSAFD VPLHYNLYQA
SNSSGNYDMR NLLNGTLVQR HPSHAVTFVD NHDTQPGEAL ESFVQGWFKP
LAYATILTRE QGYPQVFGD YYGIPSDGVP SYRQQIDPLL KARQQYAYGR
QHDYFDHWDV IGWTREGNAS HPNSGLATIM SDGPGGSKWM YVGRQKAGEV
15 WHDMTGNRSG TVTINQDGWG HFFVNGGSVS VWVKR
    
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SEQ ID NO: 2:

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HHDGTNGTIM QYFEWNPND GQHWNRLHNN AQN LKNAGIT AIWIPPAWK
20 TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TKAELERAIR SLKANGIQVY
GDVVMNHKGG ADFTERVQAV EVNPQNRNQE VSGTYQIEAW TGFNFPGRGN
QHSSFKWRWY HFDGTDWDQS RQLANRIYKF RG KAWDWE VDTEFGNYDY
LMYADVMDH PEVINELNRW GVWYANTLNL DGFRLDAVKH IKFSFMRDWL
GHVRGQTGKN LFAVAEYWKN DLGALENYLS KTNWTMSAFD VPLHYNLYQA
25 SNSSGNYDMR NLLNGTLVQR HPSHAVTFVD NHDTQPGEAL ESFVQGWFKP
LAYATILTRE QGYPQVFGD YYGIPSDGVP SYRQQIDPLL KARQQYAYGR
QHDYFDHWDV IGWTREGNAS HPNSGLATIM SDGPGGSKWM YVGRQKAGEV
WHDMTGNRSG TVTINQDGWG HFFVNGGSVS VWVKR
    
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30 **Allelic variant:** The term “allelic variant” means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a
 35 gene.

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

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of a variant. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG,

GTG or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Corresponding to: The term "corresponding to" as used herein, refers to way of determining the specific amino acid of a sequence wherein reference is made to a specific amino acid sequence. *E.g.* for the purposes of the present invention, when references are made to specific amino acid positions, the skilled person would be able to align another amino acid sequence to said amino acid sequence that reference has been made to, in order to determine which specific amino acid may be of interest in said another amino acid sequence. Alignment of another amino acid sequence with *e.g.* the sequence as set forth in SEQ ID NO: 1, or any other sequence listed herein, has been described elsewhere herein. Alternative alignment methods may be used, and are well-known for the skilled person.

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

Detergent composition: The term "detergent composition" as used herein, refers to a composition suitable for use within the field of detergents, such as for use in laundry and dish wash. A detergent composition may be in the form of a liquid or powder form, and may be suitable for both handwash or automated wash. Thus, the term "detergent composition" includes otherwise indicated by context, granular or powder-form all-purpose or heavy-duty washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, soap bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels, foam baths; metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types. The terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (*e.g.*, "laundry detergents"). In alternative embodiments, the term refers to other

detergents, such as those used to clean dishes, cutlery, etc. (e.g., "dishwashing detergents"). It is not intended that the present invention be limited to any particular detergent formulation or composition. The term "detergent composition" is not intended to be limited to compositions that contain surfactants. It is intended that in addition to the variants herein described, the
5 detergents compositions may comprise, e.g., surfactants, builders, chelators or chelating agents, bleach system or bleach components, polymers, fabric conditioners, foam boosters, suds suppressors, dyes, perfume, tannish inhibitors, optical brighteners, bactericides, fungicides, soil suspending agents, anti corrosion agents, enzyme inhibitors or stabilizers, enzyme activators, transferase(s), hydrolytic enzymes, oxido reductases, bluing agents and
10 fluorescent dyes, antioxidants, and/or solubilizers.

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

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

Fragment: The term "fragment" means a polypeptide having one or more (e.g., several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has alpha-amylase activity. In one aspect, a fragment contains at least 480 amino
20 acid residues, at least 481 amino acid residues, or at least 482 amino acid residues.

High stringency conditions: The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally
25 washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

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

Improved property: The term "improved property" means a characteristic associated with a variant that is improved compared to the parent or compared to the mature polypeptide of SEQ ID NO: 1 or 2. Such improved properties include, but are not limited to, catalytic efficiency, catalytic rate, chemical stability, oxidation stability, pH activity, pH stability, specific activity,
35 stability under storage conditions, substrate binding, substrate cleavage, substrate specificity, substrate stability, surface properties, thermal activity, and thermo stability, and improved wash performance. Another property that may be improved in the variants is the stability in detergent

compositions, *i.e.* detergent stability. The detergent stability (or residual activity) of a given variant may be determined by incubating the variant in a detergent model solution preferably containing chelating agents such as EDTA, EGTA, DTPA, DTMPA, MGDA, EDDS. or HEDP.

5 Preferably, the variants of the present invention have improved alpha-amylase activity compared to prior art oxidation stable alpha-amylases.

Wash performance: In the present context the term “wash performance” is used as an enzyme’s ability to remove starch or starch-containing stains present on the object to be cleaned during *e.g.* laundry or hard surface cleaning, such as dish wash. The wash performance may be quantified by calculating the so-called delta remission value (ΔRem) as described in the definition herein.

Improved wash performance: The term “improved wash performance” is defined herein as a variant enzyme displaying an alteration of the wash performance of an amylase variant relative to the wash performance of the parent amylase or relative to the alpha-amylases of the prior art *e.g.* by increased stain removal. The term “wash performance” includes cleaning in general *e.g.* hard surface cleaning as in dish wash, but also wash performance on textiles such as laundry, and also industrial and institutional cleaning. Improved wash performance may be measured by comparing of the so-called delta remission value (ΔRem) as described in the definition herein, wherein the ΔRem of the variant is compared with the ΔRem of the parent alpha-amylase.

20 **Low temperature:** “Low temperature” is a temperature of 5-35°C, preferably 5-30°C, more preferably 5-25°C, more preferably 5-20°C, most preferably 5-15°C, and in particular 5-10°C. In a preferred embodiment, “Low temperature” is a temperature of 10-35°C, preferably 10-30°C, more preferably 10-25°C, most preferably 10-20°C, and in particular 10-15°C.

Delta remission value (ΔRem): The terms “Delta remission” or “Delta remission value” are defined herein as the result of a reflectance or remission measurement at 460 nm of a test material, *e.g.* a swatch CS-28 (Center For Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands) or a hard surface. The swatch is measured with at least one other swatch, washed under identical conditions, as background. The delta remission is the remission value of the test material washed with amylase subtracted the remission value of the test material washed without amylase.

Isolated: The term “isolated” means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is

naturally associated (e.g., multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

Immunological cross reactivity: The term “a polypeptide having immunological cross reactivity with an antibody” as used herein, refers to any polypeptide which is bound by an antibody raised against the polypeptide of SEQ ID NO: 1 or 2. When a polypeptide not necessarily having the sequence as set forth in SEQ ID NO: 1 or 2 is bound by an antibody, and thereby provides cross reactivity, it indicates that the polypeptide may have similar characteristics as the polypeptides of SEQ ID NO: 1 or 2. Determination of cross reactivity may be done by ELISA comprising the steps of (i) adhering the polypeptide of interest to the ELISA plate; (ii) adding the antibody raised against the polypeptide of SEQ ID NO: 1 or 2; (iii) adding a secondary labeled antibody binding the antibody raised against the polypeptide of SEQ ID NO: 1 or 2; and (iv) measuring the signal from the bound secondary antibody. Other methods of determining the immunological cross reactivity may be used and is within the knowledge of the skilled person.

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

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 485 of SEQ ID NO: 1.

It is known in the art that a host cell may produce a mixture of two or more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature polypeptide having alpha-amylase activity.

Medium stringency conditions: The term “medium stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 55°C.

Medium-high stringency conditions: The term “medium-high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at

42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

5 **Nucleic acid construct:** The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

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

15 **Parent or parent alpha-amylase:** The term "parent" or "parent alpha-amylase" means an alpha-amylase to which an alteration is made to produce the enzyme variants of the present invention. The parent may be a naturally occurring (wild-type) polypeptide such *e.g.* as the alpha-amylase of SEQ ID NO: 1 or a variant or fragment thereof such as *e.g.* the amylase of SEQ ID NO: 2.

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

20 For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the *-nobrief* option) is used as the percent identity and is calculated as follows:

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

30 For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the *-nobrief* option) is used as the percent identity and is calculated as follows:

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

Variants: The term "variant" means a polypeptide having alpha-amylase activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (*e.g.*, several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding an amino acid adjacent to and immediately following the amino acid occupying a position. The variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the alpha-amylase activity of the mature polypeptide of SEQ ID NO: 1 or 2.

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

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

Wild-type alpha-amylase: The term "wild-type" alpha-amylase means an alpha-amylase expressed by a naturally occurring microorganism, such as a bacterium, archaea, yeast, or filamentous fungus found in nature.

Fabric: The term "fabric" encompasses any textile material. Thus, it is intended that the term encompass garments, as well as fabrics, yarns, fibers, non-woven materials, natural materials, synthetic materials, and any other textile material.

Textile: The term "textile" refers to woven fabrics, as well as staple fibers and filaments suitable for conversion to or use as yarns, woven, knit, and non-woven fabrics. The term encompasses yarns made from natural, as well as synthetic (*e.g.*, manufactured) fibers. The term, "textile materials" is a general term for fibers, yarn intermediates, yarn, fabrics, and products made from fabrics (*e.g.*, garments and other articles).

Non-fabric detergent compositions: The term "non-fabric detergent compositions" include non-textile surface detergent compositions, including but not limited to compositions for hard surface cleaning, such as dishwashing detergent compositions, oral detergent compositions, denture detergent compositions, and personal cleansing compositions.

Effective amount of enzyme: The term "effective amount of enzyme" refers to the quantity of enzyme necessary to achieve the enzymatic activity required in the specific application, *e.g.*, in a defined detergent composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the

particular enzyme used, the cleaning application, the specific composition of the detergent composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like. The term "effective amount" of a variant refers to the quantity of variant described hereinbefore that achieves a desired level of enzymatic activity, e.g., in a defined detergent
5 composition. In one embodiment, the effective amount of a protease variant is the same effective amount of an alpha-amylase, such as an alpha-amylase variant. In another embodiment, the effective amount of a protease variant is different than the effective amount of an alpha-amylase, such as an alpha-amylase variant, e.g., the effective amount of a protease variant may be more or may be less than the effective amount of an alpha-amylase, such as an
10 alpha-amylase variant.

Water hardness: The term "water hardness" or "degree of hardness" or "dH" or "°dH" as used herein refers to German degrees of hardness. One degree is defined as 10 milligrams of calcium oxide per liter of water.

Relevant washing conditions: The term "relevant washing conditions" is used herein to
15 indicate the conditions, particularly washing temperature, time, washing mechanics, detergent concentration, type of detergent and water hardness, actually used in households in a detergent market segment.

Stain removing enzyme: The term "stain removing enzyme" as used herein, describes an enzyme that aids the removal of a stain or soil from a fabric or a hard surface. Stain removing enzymes act on specific substrates, e.g., protease on protein, amylase on starch,
20 lipase and cutinase on lipids (fats and oils), pectinase on pectin and hemicellulases on hemicellulose. Stains are often depositions of complex mixtures of different components which either results in a local discolouration of the material by itself or which leaves a sticky surface on the object which may attract soils dissolved in the washing liquor thereby resulting in
25 discolouration of the stained area. When an enzyme acts on its specific substrate present in a stain the enzyme degrades or partially degrades its substrate thereby aiding the removal of soils and stain components associated with the substrate during the washing process.

Reduced amount: The term "reduced amount" means in this context that the amount of the component is smaller than the amount which would be used in a reference process under
30 otherwise the same conditions. In a preferred embodiment the amount is reduced by, e.g., at least 5%, such as at least 10%, at least 15%, at least 20% or as otherwise herein described.

Low detergent concentration: The term "low detergent concentration" system includes detergents where less than about 800 ppm of detergent components is present in the wash water. Asian, e.g., Japanese detergents are typically considered low detergent concentration
35 systems.

Medium detergent composition: The term "medium detergent concentration" system includes detergents wherein between about 800 ppm and about 2000 ppm of detergent

components is present in the wash water. North American detergents are generally considered to be medium detergent concentration systems.

High detergent concentration: The term “high detergent concentration” system includes detergents wherein greater than about 2000 ppm of detergent components is present in the wash water. European detergents are generally considered to be high detergent concentration systems.

Liquid laundry detergent composition: The term “liquid laundry detergent composition” as used herein refers to a detergent composition which is in a stabilized liquid form and used in a method for laundering a fabric. Thus, the detergent composition has been formulated to be in fluid form.

Powder laundry detergent composition: The term “powder laundry detergent composition” as used herein refers to a detergent composition which is in a solid form, such as a granulate, non-dusting granulate or powder, which is used in a method for laundering a fabric.

Liquid dishwash detergent composition: The term “liquid dishwash detergent composition” as used herein refers to a detergent composition which is in a stabilized liquid form and used in dishwash. Dishwash may be any kind of dishwash, such as manual dishwash and such as automated dishwash (ADW).

Powder dishwash detergent composition: The term “powder dishwash detergent composition” as used herein refers to a detergent composition which is in a solid form, such as a granulate, powder or compact unit and used in dishwash. A powder dishwash detergent composition is typically used in automated dishwash, but the used is not limited to such ADW, and may also be intended for used in any other kind of dishwash, such as manual dishwash.

Polynucleotide encoding: The term “polynucleotide encoding” as used herein, refers to a polynucleotide that encodes a mature polypeptide having alpha-amylase activity.

Conventions for Designation of Variants

For purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO: 1 is used to determine the corresponding amino acid residue in another alpha-amylase. The amino acid sequence of another alpha-amylase is aligned with the mature polypeptide disclosed in SEQ ID NO: 1, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO: 1 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

Identification of the corresponding amino acid residue in another alpha-amylase may be determined by an alignment of multiple polypeptide sequences using several computer programs including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later; Edgar, 2004, *Nucleic Acids Research* 32: 1792-1797), MAFFT (version 6.857 or later; Katoh and Kuma, 2002, *Nucleic Acids Research* 30: 3059-3066; Katoh *et al.*, 2005, *Nucleic Acids Research* 33: 511-518; Katoh and Toh, 2007, *Bioinformatics* 23: 372-374; Katoh *et al.*, 2009, *Methods in Molecular Biology* 537: 39-64; Katoh and Toh, 2010, *Bioinformatics* 26: 1899-1900), and EMBOSS EMMA employing ClustalW (1.83 or later; Thompson *et al.*, 1994, *Nucleic Acids Research* 22: 4673-4680), using their respective default parameters.

When the other enzyme has diverged from the mature polypeptide of SEQ ID NO: 1 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* 295: 613-615), other pairwise sequence comparison algorithms may be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Atschul *et al.*, 1997, *Nucleic Acids Res.* 25: 3389-3402). Even greater sensitivity may be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such as GenTHREADER (Jones, 1999, *J. Mol. Biol.* 287: 797-815; McGuffin and Jones, 2003, *Bioinformatics* 19: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough *et al.*, 2000, *J. Mol. Biol.* 313: 903-919, may be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments may in turn be used to generate homology models for the polypeptide, and such models may be assessed for accuracy using a variety of tools developed for that purpose.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* 33: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* 11: 739-747), and implementation of these algorithms may additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs (*e.g.*, Holm and Park, 2000, *Bioinformatics* 16: 566-567).

In describing the variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviations are employed.

Substitutions: For an amino acid substitution, the following nomenclature is used:

5 Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as “Thr226Ala” or “T226A”. In situations where the amino acid at a given position may be substituted for any other amino acid it is designated T226ACDEFGHIKLMNPQRSWVY. Accordingly, this means that threonine at position 226 may be substituted with one amino acid selected from the group of A, C, D, E, F, G, H, I, K, L, M, N,
 10 P, Q, R, S, W, V or Y. Likewise, in situations where the amino acid at a given position may be substituted for one amino acid selected from a specific group of amino acids, e.g. where the threonine at position 226 may be substituted with any of tyrosine, phenylalanine or histidine it is designated T226YFH. The different alterations at a given position may also be separated by a comma (“,”), e.g., “Arg170Tyr,Glu” or “R170Y,E” represents a substitution of arginine at position
 15 170 with tyrosine or glutamic acid. Thus, “Tyr167Gly,Ala + Arg170Gly,Ala” designates the following variants: “Tyr167Gly+Arg170Gly”, “Tyr167Gly+Arg170Ala”, “Tyr167Ala+Arg170Gly”, and “Tyr167Ala+Arg170Ala”.

Multiple alterations are separated by addition marks (“+”), e.g., “Gly205Arg + Ser411Phe” or “G205R + S411F”, representing substitutions at positions 205 and 411 of glycine
 20 (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

Deletions: For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as “Gly195*” or “G195*”. Multiple deletions are separated by addition marks (“+”), e.g., “Gly195* + Ser411*” or “G195* + S411*”.

25 Insertions: For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated “Gly195GlyLys” or “G195GK”. An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after
 30 glycine at position 195 is indicated as “Gly195GlyLysAla” or “G195GKA”.

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

<u>Parent:</u>	<u>Variant:</u>
195	195 195a 195b
G	G - K - A

Multiple alterations: Variants comprising multiple alterations are separated by addition marks (“+”), e.g., “Arg170Tyr+Gly195Glu” or “R170Y+G195E” representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively. The term “alteration”, the term “mutation”, the term “variation”, and the term “modification” may be used interchangeably and constitute the same meaning and purpose unless otherwise stated by context.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated alpha-amylase variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1 with any other amino acid, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity. In another embodiment the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 3. In another embodiment, the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 4. In another embodiment, the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 5. In another embodiment, the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 6. In another embodiment, the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 7. In another embodiment, the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 8.

The term “a substitution with any other amino acid” as used herein, refers to substituting the amino acid originally occurring at the particular position with any of the nineteen alternative naturally-occurring amino acids, *i.e.* when the originally occurring amino acid is an A, “any other amino acid” may be any one of the following; R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, or V. Any other amino acid is not limited to naturally-occurring amino acids but may also be non-naturally-occurring amino acids.

In one embodiment the alteration b) is an insertion in the loop spanning from amino acid positions 199-204. The insertion may be selected from the list comprising: D199DX, Y200YX, L201LX, M202MX, Y203YX, A204AX, where X denotes any amino acid.

In one embodiment the alteration b) is selected from one or more of Y198FLYHQ, Y200FLSYCWPHQRIMTNKVADEG, L201FLSYCWPHQRIMTNKVADEG, Y203FLSYCWPHQRIMTNKVADEG, A204IMTSRVAG and the alteration c) is any of M202FLSYCWPHQRIMTNKVADEG.

5 In preferred embodiments, the invention relates to variants comprising an amino acid sequence of positions 198-204 (using SEQ ID NO: 1 for numbering) selected from the list consisting of:

YDYLLFA, YDWLLYA, YDWLLFA, YDWLLPA, YDYLLIA, YDYLLNA, YDYLLPA, YDYQLYA,
 YDPLLYA, YDYLLTA, YDYLLWA, YDNLLYA, YDQLLLA, YDQLLYA, YDWLLWA, YDYLLVA,
 10 YDQLLPA, YDQLLWA, YDYLLHA, YDYLLLA, YDYLLSA, YDYPLYA, YDYQPAA, YDELLYA,
 YDKLLPA, YDQLLNA, YDYELYA, YDYHLYA, YDYLLDA, YDYLPRA, YDYQLLA, YDYQLPA,
 YDYQLQA, YDDLLYA, YDKLLYA, YDWLLHA, YDWLLTA, YDWLPSA, YDWQLYA, YDYGLYA,
 YDYLLEA, YDYLLQA, YDYPLPA, YDYRLYA, YDIELSA, YDQLLIA, YDQLLSA, YDWLGYA,
 YDWLLAA, YDWQLHA, YDWLWPA, YDYELLA, YDYLFDA, YDYLLKA, YDYLYSA, YDYQLFA,
 15 YDYQYYA, YDYTLYA, YDYLYA, YDIELWA, YDIELYA, YDNLLNA, YDNLLPA, YDPLLHA,
 YDQLLVA, YDQLPYA, YDWLLRA, YDWLWYA, YDWLWGA, YDYHLIA, YDYQHIA,
 YDYQLGA, YDYQLIA, YDYWLPA, YDELLWA, YDHLLNA, YDIELLA, YDIELNA, YDIELRA,
 YDILLYA, YDKLLWA, YDLPLYA, YDNLLLA, YDPLLA, YDPLPA, YDQHLPA, YDQLLEA,
 YDQLLQA, YDQLNYA, YDQLPFA, YDQLPNA, YDQLPRA, YDTLLA, YDTLLYA, YDVLLYA,
 20 YDWLLKA, YDWLLLA, YDWLLNA, YDWLLQA, YDWLLVA, YDWLPPA, YDWLPTA,
 YDWPWYA, YDWLWLA, YDYHLFA, YDYHPSA, YDYLLNT, YDYLLYT, YDYLPFA, YDYLPNA,
 YDYLVSA, YDYLYPA, YDYLYRA, YDYPLFA, YDYPLSA, YDYPLTA, YDYPQYA, YDYQLTA,
 YDYQLWA, YDYQNYA, YDYQPRA, YDYQSHA, YDYREYA, YDYRLPA, YDYRNSA,
 YDYRPA, YDYRQYA, YDYVLYA, YDYWLSA, YDDLISA, YDELLDA, YDELLPA, YDELLTA,
 25 YDEQLEA, YDEQLYA, YDGLPHA, YDIELFA, YDIELKA, YDIELPA, YDKLLNA, YDKPPSA,
 YDLLLLFA, YDLPLLA, YDNLLKA, YDPLKFA, YDPLLEA, YDPLLFA, YDPLLKA, YDPLLWA,
 YDPPLPA, YDPPLYA, YDPTLPA, YDPTQYA, YDQELPA, YDQLDHA, YDQLEYA, YDQLLDA,
 YDQLLFA, YDQLLNT, YDQLLYS, YDQLLYT, YDQLPSA, YDQLWYA, YDQQLVA, YDTLLWA,
 YDTPLFA, YDTPLYA, YDWELYA, YDWHLYA, YDWLHSA, YDWLLEA, YDWLLIA, YDWLLSA,
 30 YDWLNIA, YDWLPFA, YDWLPRA, YDWLQPA, YDWLQYA, YDWLWGA, YDWPLHA,
 YDWQLRA, YDWQLTA, YDWSLPA, YDWSLYA, YDWWLYA, YDYELEA, YDYELNA,
 YDYGLAA, YDYHEWA, YDYHLPA, YDYHLSA, YDYHQYA, YDYHTSA, YDYLFQA, YDYLHLA,
 YDYLIEA, YDYLLFT, YDYLLRA, YDYLLYG, YDYLPDA, YDYLPQA, YDYLPWA, YDYLQEA,
 YDYPGYA, YDYPHSA, YDYPLA, YDYPLNA, YDYPNYA, YDYPSRA, YDYPWYA, YDYQEYA,
 35 YDYQLAA, YDYQLKA, YDYQLPT, YDYQPTA, YDYQPYA, YDYQQYA, YDYQWYA,
 YDYRLFA, YDYRPSA, YDYRTFA, YDYRTSA, YDYRTYA, YDYSLYA, YDYSVYA, YDYTPRA,
 YDYWLFA, YDYWLGA, YDYWLWA, YDYWLYA, YDDLILA, YDDLINA, YDDLPA, YDEHLHA,
 YDELLFA, YDELLSA, YDELQIA, YDEWPYA, YDGLLSA, YDHLLYA, YDIELHA, YDIELKT,

YDIELTA, YDIEVSA, YDIPLYA, YDIRGYA, YDIRNYA, YDIRTKA, YDIWLYA, YDKLPHA,
 YDKLQYA, YDKPLSA, YDLLLVA, YDNHLPA, YDNHLYA, YDNLGYA, YDNLLIA, YDNLLVA,
 YDNLLWA, YDNLPRa, YDNQLYA, YDNRLYA, YDPHRHA, YDPLHVA, YDPLLLDA, YDPLLLA,
 YDPLQYA, YDPPQFA, YDPQLIA, YDQELYA, YDQLFSA, YDQLKYA, YDQLLAA, YDQLLHA,
 5 YDQLNNA, YDQLPAA, YDQLPPA, YDQLQNA, YDQLWGT, YDQLWPA, YDQLYPA,
 YDQNLya, YDQPLPA, YDQQLQA, YDQTLya, YDQWLHA, YDQWLTA, YDRLLPA, YDSELYA,
 YDTLIRA, YDTLLKA, YDTLLNA, YDTPLNA, YDTPLPA, YDTPQIA, YDTRLYA, YDTSLPA,
 YDTTLPA, YDTWKYA, YDVLLPA, YDVLNTA, YDWELIA, YDWHLPA, YDWHQYA, YDWHSHA,
 YDWHTQA, YDWLHHA, YDWLHYA, YDWLNWA, YDWLPAA, YDWLPGA, YDWLPIA,
 10 YDWLQVA, YDWLTPA, YDWLTQA, YDWNLSA, YDWNWYA, YDWPGYA, YDWPLIA,
 YDWPLVA, YDWPLYA, YDWPTYA, YDWQLIA, YDWQLLA, YDWQLNA, YDWWLDA,
 YDYDLYA, YDYEKYA, YDYELIA, YDYELPA, YDYELTA, YDYELWA, YDYGWPA, YDYGWYA,
 YDYHENA, YDYHHEA, YDYHIEA, YDYHLQA, YDYHPRA, YDYHTIA, YDYHTPA, YDYHTYA,
 YDYLFPA, YDYLHWA, YDYLIRA, YDYLNDA, YDYLNPA, YDYLNQA, YDYLPEA, YDYLPHA,
 15 YDYLPYA, YDYLPLA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA,
 YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA,
 YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA,
 YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA, YDYLPYA,
 20 YDYTLsa, YDYTQsa, YDYWLEA, YDYWLGT, YDYWLHA, YDYWLLA, YDYWLTA,
 YDYWPEA, YDYWPRA, YDYYLRA.

In a preferred embodiment the alteration b) and/or c) is selected from one or more of
 Y200H, Y200Q, M202L, Y203N, Y203L, A204S, Y200H+M202L, Y200Q+M202L,
 25 M202L+Y203N, M202L+Y203L, M202L+A204S, Y200H+M202L+Y203N,
 Y200H+M202L+Y203L, Y200H+M202L+A204S, Y200Q+M202L+Y203N,
 Y200Q+M202L+Y203L, Y200Q+M202L+A204S, Y200H+M202L+Y203N+A204S,
 Y200H+M202L+Y203L+A204S, Y200Q+M202L+Y203N+A204S,
 30 Y200Q+M202L+Y203L+A204S.

In another preferred embodiment, the alteration b) and/or c) comprises or consists of a
 substitution selected from the list consisting of Y200H, Y200Q, M202L, Y203N, Y203L, A204S
 or an insertion selected from M202MG or M202GG.

In a preferred embodiment, the deletion a) is selected from the list consisting of
 35 R181*+G182*, R181*+D183*, R181*+G184*, G182*+D183*, G182*+ G184* and D183*+G184*,
 preferably D183*+G184*.

In a preferred embodiment, the deletion a) and alterations b) and c) are
 selected from the list consisting of:

- R181*+G182*+Y200H+M202L, R181*+G182*+Y200Q+M202L, R181*+G182*+M202L+Y203N,
R181*+G182*+M202L+Y203L, R181*+G182*+M202L+A204S,
- R181*+G182*+Y200H+M202L+Y203N, R181*+G182*+Y200H+M202L+Y203L,
R181*+G182*+Y200H+M202L+A204S, R181*+G182*+Y200Q+M202L+Y203N,
- 5 R181*+G182*+Y200Q+M202L+Y203L, R181*+G182*+Y200Q+M202L+A204S,
R181*+G182*+Y200H+M202L+Y203N+A204S, R181*+G182*+Y200H+M202L+Y203L+A204S,
R181*+G182*+Y200Q+M202L+Y203N+A204S, R181*+G182*+Y200Q+M202L+Y203L+A204S.
- R181*+D183*+Y200H+M202L, R181*+D183*+Y200Q+M202L, R181*+D183*+M202L+Y203N,
R181*+D183*+M202L+Y203L, R181*+D183*+M202L+A204S,
- 10 R181*+D183*+Y200H+M202L+Y203N, R181*+D183*+Y200H+M202L+Y203L,
R181*+D183*+Y200H+M202L+A204S, R181*+D183*+Y200Q+M202L+Y203N,
R181*+D183*+Y200Q+M202L+Y203L, R181*+D183*+Y200Q+M202L+A204S,
R181*+D183*+Y200H+M202L+Y203N+A204S, R181*+D183*+Y200H+M202L+Y203L+A204S,
R181*+D183*+Y200Q+M202L+Y203N+A204S, R181*+D183*+Y200Q+M202L+Y203L+A204S.
- 15 G182*+G184*+Y200H+M202L, G182*+G184*+Y200Q+M202L, G182*+G184*+M202L+Y203N,
G182*+G184*+M202L+Y203L, G182*+G184*+M202L+A204S,
G182*+G184*+Y200H+M202L+Y203N, G182*+G184*+Y200H+M202L+Y203L,
G182*+G184*+Y200H+M202L+A204S, G182*+G184*+Y200Q+M202L+Y203N,
G182*+G184*+Y200Q+M202L+Y203L, G182*+G184*+Y200Q+M202L+A204S,
- 20 G182*+G184*+Y200H+M202L+Y203N+A204S, G182*+G184*+Y200H+M202L+Y203L+A204S,
G182*+G184*+Y200Q+M202L+Y203N+A204S, G182*+G184*+Y200Q+M202L+Y203L+A204S.
- D183*+G184*+Y200H+M202L, D183*+G184*+Y200Q+M202L, D183*+G184*+M202L+Y203N,
D183*+G184*+M202L+Y203L, D183*+G184*+M202L+A204S,
D183*+G184*+Y200H+M202L+Y203N, D183*+G184*+Y200H+M202L+Y203L,
- 25 D183*+G184*+Y200H+M202L+A204S, D183*+G184*+Y200Q+M202L+Y203N,
D183*+G184*+Y200Q+M202L+Y203L, D183*+G184*+Y200Q+M202L+A204S,
D183*+G184*+Y200H+M202L+Y203N+A204S, D183*+G184*+Y200H+M202L+Y203L+A204S,
D183*+G184*+Y200Q+M202L+Y203N+A204S, and
D183*+G184*+Y200Q+M202L+Y203L+A204S.

30 In another embodiment, the alteration a) and variation b) are selected from the list consisting of N195F+M202L+R181*+G182*, N195F+M202L+R181*+D183*, N195F+M202L+R181*+G184*, N195F+M202L+G182*+D183*, N195F+M202L+D183*+G184*, N195Y+M202L+R181*+G182*, N195Y+M202L+R181*+D183*, N195Y+M202L+R181*+G184*,
35 N195Y+M202L+G182*+D183* and N195Y+M202L+D183*+G184*.

In another embodiment, the alteration a) and alteration b) are selected from the list consisting of N195F+M202L+R181*+G182*, N195F+M202L+R181*+D183*,

N195F+M202L+R181*+G184*, N195F+M202L+G182*+D183*, N195F+M202L+D183*+G184*, N195Y+M202L+R181*+G182*, N195Y+M202L+R181*+D183*, N195Y+M202L+R181*+G184*, N195Y+M202L+G182*+D183* and N195Y+M202L+D183*+G184*.

In another embodiment, the alteration a) and variation b) are selected from the list
 5 consisting of R181*+G182*+N195F+Y200H+M202L+Y203N,
 R181*+G182*+N195F+Y200Q+M202L+A204S, R181*+G182*+N195F+M202L+Y203L,
 G182*+G184*+N195F+Y200H+M202L+Y203N, G182*+G184*+N195F+Y200Q+M202L+A204S,
 G182*+G184*+N195F+M202L+Y203L, G182*+D183*+N195F+Y200H+M202L+Y203N,
 G182*+D183*+N195F+Y200Q+M202L+A204S, G182*+D183*+N195F+M202L+Y203L,
 10 R181*+D184*+N195F+Y200H+M202L+Y203N, R181*+D184*+N195F+Y200Q+M202L+A204S,
 R181*+D184*+N195F+M202L+Y203L.

In another embodiment, the alteration a) and alteration b) are selected from the list
 consisting of R181*+G182*+N195F+Y200H+M202L+Y203N,
 R181*+G182*+N195F+Y200Q+M202L+A204S, R181*+G182*+N195F+M202L+Y203L,
 15 G182*+G184*+N195F+Y200H+M202L+Y203N, G182*+G184*+N195F+Y200Q+M202L+A204S,
 G182*+G184*+N195F+M202L+Y203L, G182*+D183*+N195F+Y200H+M202L+Y203N,
 G182*+D183*+N195F+Y200Q+M202L+A204S, G182*+D183*+N195F+M202L+Y203L,
 R181*+D184*+N195F+Y200H+M202L+Y203N, R181*+D184*+N195F+Y200Q+M202L+A204S,
 R181*+D184*+N195F+M202L+Y203L.

20 In another embodiment, the alteration b) is at two or more of said positions, such as
 three or more of said positions, four or more of said positions, five or more of said positions, six
 or more of said positions, seven or more of said positions, eight or more of said positions, or
 nine or more of said positions.

In another embodiment, the number of alterations is 2-20, e.g., 2-10 and 2-5, such as 2,
 25 3, 4, 5, 6, 7, 8, 9 or 10 alterations.

In another embodiment, the variant has at least 85%, at least 86%, at least 87%, at least
 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least
 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%,
 sequence identity to the amino acid sequence of SEQ ID NO: 1.

30 In another embodiment, the variant has at least 85%, at least 86%, at least 87%, at least
 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least
 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%,
 sequence identity, to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 85%, at least 86%, at least 87%, at least
 35 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least
 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%,
 sequence identity, to the amino acid sequence of SEQ ID NO: 3.

In another embodiment, the variant has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity, to the amino acid sequence of SEQ ID NO: 40.

5 In another embodiment, the variant has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less than 100%, sequence identity, to the amino acid sequence of SEQ ID NO: 41.

10 In a preferred embodiment the variant is a variant of a parent alpha-amylase selected from the group consisting of: a. a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 1; b. a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 2; c. a fragment of the mature polypeptide of SEQ ID NO: 1, which has alpha-amylase activity; d. a fragment of the mature polypeptide of SEQ ID NO: 2, which has alpha-amylase activity; e. a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 1; f. a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 2.

20 In one embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 1.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 2.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 3.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 4.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 5.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 6.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 7.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 8.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 9.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 10.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 11.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 12.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 13.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 14.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 15.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 16.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 17.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 18.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 19.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 20.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 21.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 22.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 23.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 24.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 25.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 26.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 27.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 28.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 29.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 30.

40 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 31.

45 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 32.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 33.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 34.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 35.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 36.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 37.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 38.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 39.

40 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 40.

45 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 41.

In one embodiment, the parent alpha-amylase comprises or consists of the mature polypeptide of SEQ ID NO: 1.

5 In another embodiment, the parent alpha-amylase comprises or consists of the mature polypeptide of SEQ ID NO: 2.

In a preferred embodiment, the variant has an improved property relative to the parent alpha-amylase, wherein the improved property is selected from the group consisting of catalytic efficiency, catalytic rate, chemical stability, oxidation stability, pH activity, pH stability, specific activity, stability under storage conditions, substrate binding, substrate cleavage, substrate
10 activity, stability under storage conditions, substrate binding, substrate cleavage, substrate specificity, substrate stability, surface properties, thermal activity, thermo stability, and preferably improved washing performance at low temperature.

In a particularly preferred embodiment, the variant has improved detergent stability compared to the parent alpha-amylase.

15 In another embodiment, the variant has improved oxidation stability in detergents relative to the alpha-amylase of SEQ ID NO: 1 or 2.

In one embodiment, the variant comprises the deletion R181*+G182* of the mature polypeptide of SEQ ID NO: 1.

In one embodiment, the variant comprises the deletion R181*+D183* of the mature
20 polypeptide of SEQ ID NO: 1.

In one embodiment, the variant comprises the deletion R181*+G184* of the mature polypeptide of SEQ ID NO: 1.

In one embodiment, the variant comprises the deletion G182*+D183* of the mature polypeptide of SEQ ID NO: 1.

25 In one embodiment, the variant comprises the deletion D183*+G184* of the mature polypeptide of SEQ ID NO: 1.

In one embodiment, the variant has at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 85% sequence identity to the amino acid
30 sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 91% sequence identity to the amino acid sequence of SEQ ID NO: 1.

35 In another embodiment, the variant has at least 92% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 93% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 94% sequence identity to the amino acid sequence of SEQ ID NO: 1.

5 In another embodiment, the variant has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 96% sequence identity to the amino acid sequence of SEQ ID NO: 1.

10 In another embodiment, the variant has at least 97% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 98% sequence identity to the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the variant has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 1.

15 In one embodiment, the variant has at least 80% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 85% sequence identity to the amino acid sequence of SEQ ID NO: 2.

20 In another embodiment, the variant has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 91% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 92% sequence identity to the amino acid sequence of SEQ ID NO: 2.

25 In another embodiment, the variant has at least 93% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 94% sequence identity to the amino acid sequence of SEQ ID NO: 2.

30 In another embodiment, the variant has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 96% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 97% sequence identity to the amino acid sequence of SEQ ID NO: 2.

35 In another embodiment, the variant has at least 98% sequence identity to the amino acid sequence of SEQ ID NO: 2.

In another embodiment, the variant has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 2.

The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

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

Essential amino acids in a polypeptide may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for alpha-amylase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction may also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids may also be inferred from an alignment with a related polypeptide.

In an embodiment, the variant has improved catalytic efficiency compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved catalytic rate compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved chemical stability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved oxidation stability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

5 In an embodiment, the variant has improved detergent stability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved pH activity compared to the alpha-amylase of SEQ ID NO: 1 or 2.

10 In an embodiment, the variant has improved pH stability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved specific activity compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved stability under storage conditions compared to the alpha-amylase of SEQ ID NO: 1 or 2.

15 In an embodiment, the variant has decreased substrate binding compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved substrate specificity compared to the alpha-amylase of SEQ ID NO: 1 or 2.

20 In an embodiment, the variant has improved substrate stability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved surface properties compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In an embodiment, the variant has improved thermal activity compared to the alpha-amylase of SEQ ID NO: 1 or 2.

25 In an embodiment, the variant has improved thermostability compared to the alpha-amylase of SEQ ID NO: 1 or 2.

In another embodiment, the variant has improved wash performance, in particular improved wash performance at low temperature compared to the alpha-amylase of SEQ ID NO: 1 or 2.

30

Parent alpha-amylases

The parent alpha-amylase may be (a) a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 1; (b) a fragment of the mature polypeptide of SEQ ID NO: 1, which has alpha-amylase activity; or (c) a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 1.

35

In another aspect, the parent alpha-amylase may be (a) a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 2; (b) a fragment of the mature

polypeptide of SEQ ID NO: 2, which has alpha-amylase activity; or (c) a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 2.

5 In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 1 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 1.

10 In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 2.

15 In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 3. In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 4. In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 5. In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 6 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 6. In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 7 of at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 7. In an aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 8 of at

least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%. In one aspect, the amino acid sequence of the parent differs by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO:8.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 9.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 10.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 11.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 12.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 13.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 14.

30 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 15.

35 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 16.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 35.

5 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 36.

10 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 37.

15 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 38.

20 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 39.

In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 40.

25 In another embodiment, the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% or 100% sequence identity to SEQ ID NO: 41.

30 In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 1. In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 3. In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 4. In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 5.
35 In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 6. In another aspect, the parent alpha-amylase comprises or consists of the

amino acid sequence of SEQ ID NO: 7. In another aspect, the parent alpha-amylase comprises or consists of the amino acid sequence of SEQ ID NO: 8.

In yet another embodiment, the parent alpha-amylase is an allelic variant of the mature polypeptide of SEQ ID NO: 1 or 2.

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

A fusion polypeptide may further comprise a cleavage site between the two
15 polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381;
20 Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

The parent alpha-amylase may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a
25 given source shall mean that the parent encoded by a polynucleotide is produced by the source or by a strain in which the polynucleotide from the source has been inserted. In one aspect, the parent alpha-amylase is secreted extracellularly.

The parent alpha-amylase may be a bacterial alpha-amylase. For example, the parent alpha-amylase may be a Gram-positive bacterial polypeptide such as a *Bacillus* alpha-amylase.
30 In one aspect, the parent alpha-amylase is a *Bacillus sp. AAI-10* alpha-amylase *e.g.*, the alpha-amylase of SEQ ID NO: 1.

The alpha-amylases of SEQ ID NOs 1 and 2 as well as the variants hereof may be artificially manufactured by methods known in the art.

35 **Preparation of Variants**

The present invention also relates to a method of improving the activity of an alpha-amylase by introducing into a parent alpha-amylase a) a deletion at two or more positions

corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203 and A204 of the mature polypeptide of SEQ ID NO: 1, and c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1 with any other amino acid, wherein the resulting variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2; and recovering the variant.

The variants may be prepared using any mutagenesis procedure known in the art, such as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, etc.

Site-directed mutagenesis is a technique in which one or more (e.g., several) mutations are introduced at one or more defined sites in a polynucleotide encoding the parent.

Site-directed mutagenesis may be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis may also be performed *in vitro* by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent alpha-amylase and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and the insert to ligate to one another. See, e.g., Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* 76: 4949-4955; and Barton *et al.*, 1990, *Nucleic Acids Res.* 18: 7349-4966.

Site-directed mutagenesis may also be accomplished *in vivo* by methods known in the art. See, e.g., U.S. Patent Application Publication No. 2004/0171154; Storici *et al.*, 2001, *Nature Biotechnol.* 19: 773-776; Kren *et al.*, 1998, *Nat. Med.* 4: 285-290; and Calissano and Macino, 1996, *Fungal Genet. Newslett.* 43: 15-16.

Any site-directed mutagenesis procedure may be used in the present invention. There are many commercial kits available that can be used to prepare variants.

Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis may be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian *et al.* (2004, *Nature* 432: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

Single or multiple amino acid substitutions, deletions, and/or insertions may be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR,

phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204) and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

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

10 Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized *de novo*, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR
15 amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

20 The present invention also relates to isolated polynucleotides encoding a variant of the present invention. Accordingly, the present invention relates to isolated polynucleotides encoding a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position
25 corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity.

Nucleic Acid Constructs

30 The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences. Accordingly, the present invention relates to nucleic acid constructs comprising a polynucleotide encoding a variant comprising a) a deletion at two or
35 more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c)

substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity, wherein the polynucleotide is operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

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

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

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic alpha-amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis cryIIIA* gene (Agaïsse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli lac* operon, *E. coli trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*. Examples of tandem promoters are disclosed in WO 99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-alpha-amylase, *Aspergillus niger* acid stable alpha-alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoalpha-amylase (*glaA*), *Aspergillus oryzae* TAKA alpha-amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900),

Rhizomucor miehei lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

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

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

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

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

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

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

Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis cryIIIA* gene (WO 94/25612) and a *Bacillus subtilis* SP82 gene (Hue *et al.*, 1995, *Journal of Bacteriology* 177: 3465-3471).

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

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

10 Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

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

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

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

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

35 Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus*

stearothermophilus alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

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

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

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a variant. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally
15 inactive and may be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

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

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

35

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant of the present invention, a promotor, and transcriptional and translational stop signals. Accordingly, the present invention relates to a recombinant expression vector comprising a polynucleotide encoding a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity, and wherein the vector further comprises a promotor, and transcriptional and translational stop signals.

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

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

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

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

Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis* *dal* genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3.

5 Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or
10 *Aspergillus oryzae* *amdS* and *pyrG* genes and a *Streptomyces hygrosopicus* *bar* gene.

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

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

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

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*.

35 Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene may be accomplished according to the methods disclosed in WO 00/24883.

5 More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a variant. An increase in the copy number of the polynucleotide may be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells comprising amplified copies of the selectable marker gene, and
10 thereby additional copies of the polynucleotide, may be selected for by cultivating the cells in the presence of the appropriate selectable agent.

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

15 The alpha-amylase variants of the present invention may be expressed as described in WO2010/115021.

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide
20 encoding a variant of the present invention operably linked to one or more control sequences that direct the production of a variant of the present invention. Accordingly, the present invention relates to recombinant host cells, comprising a polynucleotide encoding a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions
25 corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO:
30 1 or 2, and wherein the variant has alpha-amylase activity, wherein the polynucleotide is operably linked to one or more control sequences that direct the production of the variant.

A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.
35 The choice of a host cell will to a large extent depend upon the gene encoding the variant and its source.

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

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

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

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

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

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation
20 (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987,
25 *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation
30 (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397), or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by
35 natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or

conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

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

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

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

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

20 The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

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

35 For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium*

pannicola, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a variant, comprising: (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant; and (b) recovering the variant. Accordingly, the present invention relates to methods of producing a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity, wherein the method comprises (a) cultivating a host cell expressing the variant under conditions suitable for expression of the variant; and (b) recovering the variant.

The host cells are cultivated in a nutrient medium suitable for production of the variant using methods known in the art. For example, the cell may be cultivated by shake flask

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

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

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

The variant may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure variants.

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

Compositions

The present invention also relates to compositions comprising a variant of the present invention. Accordingly, the present invention relates to compositions comprising a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity.

Preferably, the compositions are enriched in such a variant. The term "enriched" means that the alpha-amylase activity of the composition has been increased, e.g., with an enrichment factor of 1.1.

The composition may comprise a variant as the major enzymatic component, e.g., a
5 mono-component composition. Alternatively, the composition may comprise multiple enzymatic activities, such as an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, laccase, lipase, mannosidase, oxidase, pectinolytic enzyme,
10 peptidoglutaminase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The additional enzyme(s) may be produced, for example, by a microorganism belonging to the genus *Aspergillus*, e.g., *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, or *Aspergillus oryzae*; *Fusarium*, e.g., *Fusarium bactridioides*,
15 *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sulphureum*, *Fusarium toruloseum*, *Fusarium trichothecioides*, or *Fusarium venenatum*; *Humicola*, e.g., *Humicola insolens* or *Humicola lanuginosa*; or *Trichoderma*, e.g.,
20 *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride*.

The compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the composition may be in the form of a granulate or a microgranulate. The variant may be stabilized in accordance with
25 methods known in the art.

Detergent compositions

In one embodiment, the invention is directed to detergent compositions comprising an alpha-amylase variant of the present invention in combination with one or more additional cleaning
30 composition components. Accordingly, the present invention relates to detergent compositions comprising a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to
35 position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity, and wherein the detergent

compositions further comprises one or more additional cleaning composition components.

The choice of additional components is within the skill of the artisan and includes conventional ingredients, including the exemplary non-limiting components set forth below. The choice of components may include, for fabric care, the consideration of the type of fabric to be
5 cleaned, the type and/or degree of soiling, the temperature at which cleaning is to take place, and the formulation of the detergent product. Although components mentioned below are categorized by general header according to a particular functionality, this is not to be construed as a limitation, as a component may comprise additional functionalities as will be appreciated by the skilled
10 artisan.

Concentration of the enzyme of the present invention

In one embodiment of the present invention, the polypeptide of the present invention may be used in the dishwashing composition in an amount corresponding to 0.001-200 mg of protein, such as 0.005-100 mg of protein, preferably 0.01-50 mg of protein, more preferably
15 0.05-20 mg of protein, even more preferably 0.1-10 mg of protein per liter of wash liquor.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, *e.g.* a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, *e.g.* an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be
20 formulated as described in, for example, WO92/19709 and WO92/19708.

A polypeptide of the present invention may also be incorporated in the detergent formulations disclosed in WO97/07202, which is hereby incorporated by reference.

Surfactants

The dish washing composition may comprise at least one non-ionic surfactant. Suitable nonionic surfactants include, but are not limited to low-foaming nonionic (LFNI) surfactants. An LFNI surfactant is most typically used in an automatic dishwashing composition because of the improved water- sheeting action (especially from glassware) which they confer to the automatic
25 dishwashing composition. They also may encompass non-silicone, phosphate or nonphosphate polymeric materials which are known to defoam food soils encountered in automatic dishwashing. The LFNI surfactant may have a relatively low cloud point and a high hydrophilic-lipophilic balance (HLB). Cloud points of 1% solutions in water are typically below about 32°C alternatively lower, *e.g.*, 0°C, for optimum control of sudsing throughout a full range of water
30 temperatures. If desired, a biodegradable LFNI surfactant having the above properties may be used.

A LFNI surfactant may include, but is not limited to: alkoxyated surfactants, especially ethoxylates derived from primary alcohols, and blends thereof with more sophisticated

surfactants, such as the polyoxypropylene/polyoxyethylene/polyoxypropylene reverse block polymers. Suitable block polyoxyethylene-polyoxypropylene polymeric compounds that meet the requirements may include those based on ethylene glycol, propylene glycol, glycerol, trimethylolpropane and ethylenediamine, and mixtures thereof. Polymeric compounds made from a sequential ethoxylation and propoxylation of initiator compounds with a single reactive hydrogen atom, such as C 12- is aliphatic alcohols, do not generally provide satisfactory suds control in Automatic dishwashing compositions. However, certain of the block polymer surfactant compounds designated as PLURONIC(R) and TETRONIC(R) by the BASF-Wyandotte Corp., Wyandotte, Mich., are suitable in Automatic dishwashing compositions.

The LFNI surfactant may optionally include a propylene oxide in an amount up to about 15% by weight. Other LFNI surfactants may be prepared by the processes described in U.S. Pat. No. 4,223,163. The LFNI surfactant may also be derived from a straight chain fatty alcohol containing from about 16 to about 20 carbon atoms (C16-C20 alcohol), alternatively a Ci8 alcohol, condensed with an average of from about 6 to about 15 moles, or from about 7 to about 12 moles, and alternatively, from about 7 to about 9 moles of ethylene oxide per mole of alcohol. The ethoxylated nonionic surfactant so derived may have a narrow ethoxylate distribution relative to the average.

In certain embodiments, an LFNI surfactant having a cloud point below 30°C may be present in an amount from about 0.01% to about 60%, or from about 0.5% to about 10% by weight, and alternatively, from about 1% to about 5% by weight of the composition.

In preferred embodiments, the surfactant is a non-ionic surfactant or a non-ionic surfactant system having a phase inversion temperature, as measured at a concentration of 1% in distilled water, between 40 and 70°C, preferably between 45 and 65°C. By a "non-ionic surfactant system" is meant herein a mixture of two or more non-ionic surfactants. Preferred for use herein are non-ionic surfactant systems. They seem to have improved cleaning and finishing properties and stability in product than single non-ionic surfactants. Suitable nonionic surfactants include: i) ethoxylated non-ionic surfactants prepared by the reaction of a monohydroxy alkanol or alkylphenol with 6 to 20 carbon atoms with preferably at least 12 moles particularly preferred at least 16 moles, and still more preferred at least 20 moles of ethylene oxide per mole of alcohol or alkylphenol; ii) alcohol alkoxyated surfactants having from 6 to 20 carbon atoms and at least one ethoxy and propoxy group. Preferred for use herein are mixtures of surfactants i) and ii).

Another suitable non-ionic surfactants are epoxy-capped poly(oxyalkylated) alcohols represented by the formula:

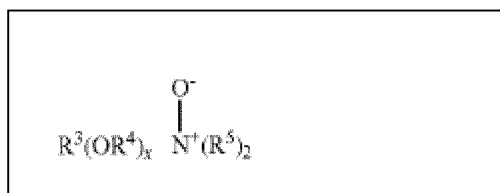


wherein R₁ is a linear or branched, aliphatic hydrocarbon radical having from 4 to 18 carbon atoms; R₂ is a linear or branched aliphatic hydrocarbon radical having from 2 to 26

carbon atoms; x is an integer having an average value of from 0.5 to 1.5, more preferably about 1; and y is an integer having a value of at least 15, more preferably at least 20. Preferably, the surfactant of formula I has at least about 10 carbon atoms in the terminal epoxide unit $[\text{CH}_2\text{CH}(\text{OH})\text{R}_2]$. Suitable surfactants of formula I are Olin Corporation's POLY-
 5 TERGENT(R) SLF- 18B nonionic surfactants, as described, for example, in WO 94/22800, published October 13, 1994 by Olin Corporation.

Preferably, non-ionic surfactants and/or system herein have a Draves wetting time of less than 360 seconds, preferably less than 200 seconds, more preferably less than 100 seconds and especially less than 60 seconds as measured by the Draves wetting method
 10 (standard method ISO 8022 using the following conditions; 3-g hook, 5-g cotton skein, 0.1% by weight aqueous solution at a temperature of 25 °C). Amine oxides surfactants are also useful in the present invention as anti-redeposition surfactants include linear and branched compounds having the formula:

15



wherein R³ is selected from an alkyl, hydroxyalkyl, acylamidopropyl and alkyl phenyl
 20 group, or mixtures thereof, containing from 8 to 26 carbon atoms, preferably 8 to 18 carbon atoms; R⁴ is an alkylene or hydroxyalkylene group containing from 2 to 3 carbon atoms, preferably 2 carbon atoms, or mixtures thereof; x is from 0 to 5, preferably from 0 to 3; and each R⁵ is an alkyl or hydroxyalkyl group containing from 1 to 3, preferably from 1 to 2 carbon atoms, or a polyethylene oxide group containing from 1 to 3, preferable 1, ethylene oxide groups. The
 25 R⁵ groups may be attached to each other, e.g., through an oxygen or nitrogen atom, to form a ring structure.

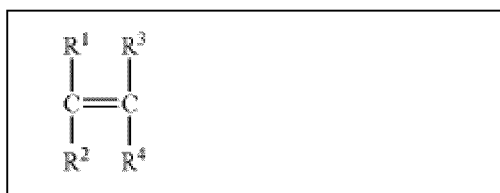
These amine oxide surfactants in particular include C₁₀-C₁₈ alkyl dimethyl amine oxides and C₈-C₁₈ alkoxy ethyl dihydroxyethyl amine oxides. Examples of such materials include dimethyloctylamine oxide, diethyldecylamine oxide, bis-(2-hydroxyethyl)dodecylamine oxide,
 30 dimethyldodecylamine oxide, dipropyltetradecylamine oxide, methylethylhexadecylamine oxide, dodecylamidopropyl dimethylamine oxide, cetyl dimethylamine oxide, stearyl dimethylamine oxide, tallow dimethylamine oxide and dimethyl-2-hydroxyoctadecylamine oxide. Preferred are C₁₀-C₁₈ alkyl dimethylamine oxide, and C₁₀-C₁₈ acylamido alkyl dimethylamine oxide. Surfactants and especially non-ionic surfactants may be present in amounts from 0 to 10% by
 35 weight, preferably from 0.1% to 10%, and most preferably from 0.25% to 6%.

Sulfonated polymer

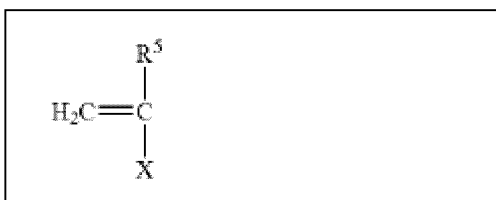
The polymer, if used, is used in any suitable amount from about 0.1% to about 20%, preferably from 1% to about 15%, more preferably from 2% to 10% by weight of the composition. Sulfonated/carboxylated polymers are particularly suitable for the compositions
5 contained in a pouch.

Suitable sulfonated/carboxylated polymers described herein may have a weight average molecular weight of less than or equal to about 100,000 Da, or less than or equal to about 75,000 Da, or less than or equal to about 50,000 Da, or from about 3,000 Da to about 50,000, preferably from about 5,000 Da to about 45,000 Da.

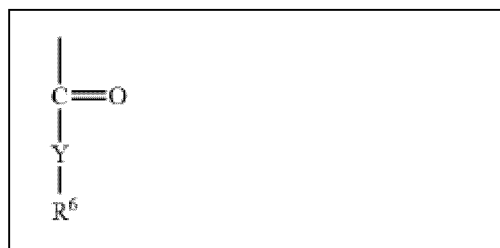
As noted herein, the sulfonated/carboxylated polymers may comprise (a) at least one structural unit derived from at least one carboxylic acid monomer having the general formula (I):
10



wherein R^1 to R^4 are independently hydrogen, methyl, carboxylic acid group or CH_2COOH and wherein the carboxylic acid groups may be neutralized; (b) optionally, one or more structural units derived from at least one nonionic monomer having the general formula
20 (II):

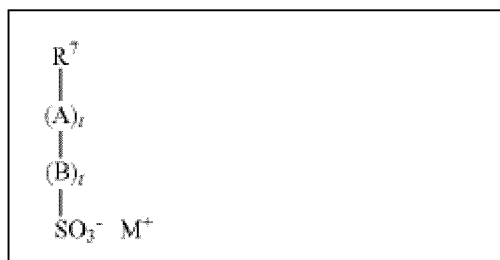


wherein R^5 is hydrogen, C_1 to C_6 alkyl, or C_1 to C_6 hydroxyalkyl, and X is either aromatic (with R^5 being hydrogen or methyl when X is aromatic) or X is of the general formula (III):
30



wherein R^6 is (independently of R^5) hydrogen, C_1 to C_6 alkyl, or C_1 to C_6 hydroxyalkyl, and Y is O or N; and at least one structural unit derived from at least one sulfonic acid monomer having the general formula (IV):
35

5



10

wherein R^7 is a group comprising at least one sp^2 bond, A is O, N, P, S or an amido or ester linkage, B is a mono- or polycyclic aromatic group or an aliphatic group, each t is independently 0 or 1, and M^+ is a cation. In one aspect, R^7 is a C_2 to C_6 alkene. In another aspect, R^7 is ethene, butene or propene.

15

Preferred carboxylic acid monomers include one or more of the following: acrylic acid, maleic acid, itaconic acid, methacrylic acid, or ethoxylate esters of acrylic acids, acrylic and methacrylic acids being more preferred. Preferred sulfonated monomers include one or more of the following: sodium (meth) allyl sulfonate, vinyl sulfonate, sodium phenyl (meth) allyl ether sulfonate, or 2-acrylamido-methyl propane sulfonic acid. Preferred non-ionic monomers include one or more of the following: methyl (meth) acrylate, ethyl (meth) acrylate, t-butyl (meth) acrylate, methyl (meth) acrylamide, ethyl (meth) acrylamide, t-butyl (meth) acrylamide, styrene, or [alpha]-methyl styrene.

20

Preferably, the polymer comprises the following levels of monomers: from about 40 to about 90%, preferably from about 60 to about 90% by weight of the polymer of one or more carboxylic acid monomer; from about 5 to about 50%, preferably from about 10 to about 40% by weight of the polymer of one or more sulfonic acid monomer; and optionally from about 1 % to about 30%, preferably from about 2 to about 20% by weight of the polymer of one or more non-ionic monomer. An especially preferred polymer comprises about 70% to about 80% by weight of the polymer of at least one carboxylic acid monomer and from about 20% to about 30% by weight of the polymer of at least one sulfonic acid monomer.

25

30

The carboxylic acid is preferably (meth)acrylic acid. The sulfonic acid monomer is preferably one of the following: 2-acrylamido methyl- 1-propanesulfonic acid, 2-methacrylamido-2-methyl- 1-propanesulfonic acid, 3-methacrylamido-2-hydroxypropanesulfonic acid, allylsulfonic acid, methallylsulfonic acid, allyloxybenzenesulfonic acid, methallyloxybenzenesulfonic acid, 2-hydroxy-3-(2-propenyloxy)propanesulfonic acid, 2-methyl-2-propene-1-sulfonic acid, styrene sulfonic acid, vinylsulfonic acid, 3-sulfopropyl acrylate, 3-sulfopropyl methacrylate, sulfomethylacrylamid, sulfomethylmethacrylamide, and water soluble salts thereof. The unsaturated sulfonic acid monomer is most preferably 2-acrylamido-2-propanesulfonic acid (AMPS).

35

Preferred commercial available polymers include: Alcosperse 240, Aquatreat AR 540 and Aquatreat MPS supplied by Alco Chemical; Acumer 3100, Acumer 2000, Acusol 587G and

Acusol 588G supplied by Rohm & Haas; Goodrich K-798, K-775 and K-797 supplied by BF Goodrich; and ACP 1042 supplied by ISP technologies Inc. Particularly preferred polymers are Acusol 587G and Acusol 588G supplied by Rohm & Haas.

5 In the polymers, all or some of the carboxylic or sulfonic acid groups may be present in neutralized form, *i.e.* the acidic hydrogen atom of the carboxylic and/or sulfonic acid group in some or all acid groups may be replaced with metal ions, preferably alkali metal ions and in particular with sodium ions.

Hydrotropes

10 A hydrotrope is a compound that solubilises hydrophobic compounds in aqueous solutions (or oppositely, polar substances in a non-polar environment). Typically, hydrotropes have both hydrophilic and a hydrophobic character (so-called amphiphilic properties as known from surfactants); however the molecular structure of hydrotropes generally do not favor spontaneous self-aggregation, see *e.g.* review by Hodgdon and Kaler (2007), Current Opinion in
15 Colloid & Interface Science 12: 121-128. Hydrotropes do not display a critical concentration above which self-aggregation occurs as found for surfactants and lipids forming micellar, lamellar or other well defined meso-phases. Instead, many hydrotropes show a continuous-type aggregation process where the sizes of aggregates grow as concentration increases. However, many hydrotropes alter the phase behavior, stability, and colloidal properties of systems
20 comprising substances of polar and non-polar character, including mixtures of water, oil, surfactants, and polymers. Hydrotropes are classically used across industries from pharma, personal care, food, to technical applications. Use of hydrotropes in detergent compositions allow for example more concentrated formulations of surfactants (as in the process of compacting liquid detergents by removing water) without inducing undesired phenomena such
25 as phase separation or high viscosity.

The detergent may comprise 0-10% by weight, for example 0-5% by weight, such as about 0.5 to about 5%, or about 3% to about 5%, of a hydrotrope. Any hydrotrope known in the art for use in detergents may be utilized. Non-limiting examples of hydrotropes include sodium benzenesulfonate, sodium *p*-toluene sulfonate (STS), sodium xylene sulfonate (SXS), sodium
30 cumene sulfonate (SCS), sodium cymene sulfonate, amine oxides, alcohols and polyglycoethers, sodium hydroxynaphthoate, sodium hydroxynaphthalene sulfonate, sodium ethylhexyl sulfate, and combinations thereof.

Builders and Co-Builders

35 The detergent composition may comprise about 0-65% by weight, such as about 5% to about 50% of a detergent builder or co-builder, or a mixture thereof. In a dish wash detergent, the level of builder is typically 40-65%, particularly 50-65%. The builder and/or co-builder may

particularly be a chelating agent that forms water-soluble complexes with Ca and Mg. Any builder and/or co-builder known in the art for use in ADW detergents may be utilized. Non-limiting examples of builders include zeolites, diphosphates (pyrophosphates), triphosphates such as sodium triphosphate (STP or STPP), carbonates such as sodium carbonate, soluble silicates such as sodium metasilicate, layered silicates (e.g., SKS-6 from Hoechst), ethanolamines such as 2-aminoethan-1-ol (MEA), diethanolamine (DEA, also known as 2,2'-iminodiethan-1-ol), triethanolamine (TEA, also known as 2,2',2''-nitrilotriethan-1-ol), and (carboxymethyl)inulin (CMI), and combinations thereof.

The detergent composition may also comprise 0-50% by weight, such as about 5% to about 30%, of a detergent co-builder. The detergent composition may include a co-builder alone, or in combination with a builder, for example a zeolite builder. Non-limiting examples of co-builders include homopolymers of polyacrylates or copolymers thereof, such as poly(acrylic acid) (PAA) or copoly(acrylic acid/maleic acid) (PAA/PMA). Further non-limiting examples include citrate, chelators such as aminocarboxylates, aminopolycarboxylates and phosphonates, and alkyl- or alkenylsuccinic acid. Additional specific examples include 2,2',2''-nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), iminodisuccinic acid (IDS), ethylenediamine-*N,N*-disuccinic acid (EDDS), methylglycinediacetic acid (MGDA), glutamic acid-*N,N*-diacetic acid (GLDA), 1-hydroxyethane-1,1-diphosphonic acid (HEDP), ethylenediaminetetra(methylenephosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylenephosphonic acid) (DTMPA or DTPMPA), *N*-(2-hydroxyethyl)iminodiacetic acid (EDG), aspartic acid-*N*-monoacetic acid (ASMA), aspartic acid-*N,N*-diacetic acid (ASDA), aspartic acid-*N*-monopropionic acid (ASMP), iminodisuccinic acid (IDA), *N*-(2-sulfomethyl)-aspartic acid (SMAS), *N*-(2-sulfoethyl)-aspartic acid (SEAS), *N*-(2-sulfomethyl)-glutamic acid (SMGL), *N*-(2-sulfoethyl)-glutamic acid (SEGL), *N*-methyliminodiacetic acid (MIDA), α -alanine-*N,N*-diacetic acid (α -ALDA), serine-*N,N*-diacetic acid (SEDA), isoserine-*N,N*-diacetic acid (ISDA), phenylalanine-*N,N*-diacetic acid (PHDA), anthranilic acid-*N,N*-diacetic acid (ANDA), sulfanilic acid-*N,N*-diacetic acid (SLDA), taurine-*N,N*-diacetic acid (TUDA) and sulfomethyl-*N,N*-diacetic acid (SMDA), *N*-(2-hydroxyethyl)ethylenediamine-*N,N',N''*-triacetic acid (HEDTA), diethanolglycine (DEG), diethylenetriamine penta(methylenephosphonic acid) (DTPMP), aminotris(methylenephosphonic acid) (ATMP), and combinations and salts thereof. Further exemplary builders and/or co-builders are described in, e.g., WO 09/102854, US 5977053

Bleaching Systems

Inorganic and organic bleaches are suitable cleaning actives for use herein. Inorganic bleaches include perhydrate salts such as perborate, percarbonate, perphosphate, persulfate and persilicate salts. The inorganic perhydrate salts are normally the alkali metal salts. The

inorganic perhydrate salt may be included as the crystalline solid without additional protection. Alternatively, the salt may be coated.

Alkali metal percarbonates, particularly sodium percarbonate are preferred perhydrates for use herein. The percarbonate is most preferably incorporated into the products in a coated form which provides in-product stability. A suitable coating material providing in product stability comprises mixed salt of a water-soluble alkali metal sulphate and carbonate. Such coatings together with coating processes have previously been described in GB 1,466,799. The weight ratio of the mixed salt coating material to percarbonate lies in the range from 1:200 to 1:4, more preferably from 1:99 to 1:9, and most preferably from 1:49 to 1:19. Preferably, the mixed salt is of sodium sulphate and sodium carbonate which has the general formula $\text{Na}_2\text{SO}_4 \cdot n \cdot \text{Na}_2\text{CO}_3$ wherein n is from 0.1 to 3, preferably n is from 0.3 to 1.0 and most preferably n is from 0.2 to 0.5.

Another suitable coating material providing in product stability, comprises sodium silicate of SiO_2 : Na_2O ratio from 1.8:1 to 3.0:1, preferably 1.8:1 to 2.4:1, and/or sodium metasilicate, preferably applied at a level of from 2% to 10%, (normally from 3% to 5%) of SiO_2 by weight of the inorganic perhydrate salt. Magnesium silicate may also be included in the coating. Coatings that comprise silicate and borate salts or boric acids or other inorganics are also suitable.

Other coatings which contain waxes, oils, fatty soaps can also be used advantageously within the present invention.

Potassium peroxymonopersulfate is another inorganic perhydrate salt of utility herein. Typical organic bleaches are organic peroxyacids including diacyl and tetraacylperoxides, especially diperoxydodecanedioic acid, diperoxytetradecanedioic acid, and diperoxyhexadecanedioic acid. Dibenzoyl peroxide is a preferred organic peroxyacid herein. Mono- and diperazelaic acid, mono- and diperbrassylic acid, and Nphthaloylaminoperoxicaproic acid are also suitable herein. The diacyl peroxide, especially dibenzoyl peroxide, should preferably be present in the form of particles having a weight average diameter of from about 0.1 to about 100 microns, preferably from about 0.5 to about 30 microns, more preferably from about 1 to about 10 microns. Preferably, at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, most preferably at least about 90%, of the particles are smaller than 10 microns, preferably smaller than 6 microns. Diacyl peroxides within the above particle size range have also been found to provide better stain removal especially from plastic dishware, while minimizing undesirable deposition and filming during use in automatic dishwashing machines, than larger diacyl peroxide particles. The preferred diacyl peroxide particle size thus allows the formulator to obtain good stain removal with a low level of diacyl peroxide, which reduces deposition and filming. Conversely, as diacyl peroxide particle size increases, more diacyl peroxide is needed for good stain removal, which increases deposition on surfaces encountered during the dishwashing process.

Further typical organic bleaches include the peroxy acids, particular examples being the alkylperoxy acids and the arylperoxy acids. Preferred representatives are (a) peroxybenzoic acid and its ring-substituted derivatives, such as alkylperoxybenzoic acids, but also peroxy-[alpha]-naphthoic acid and magnesium monoperoxophthalate, (b) the aliphatic or substituted aliphatic peroxy acids, such as peroxy lauric acid, peroxy stearic acid, [epsilon]-phthalimidoperoxycaproic acid [phthalimidoperoxycaproic acid (PAP)], o-carboxybenzamidoperoxycaproic acid, N-nonenylamidoperadipic acid and N-nonenylamidopersuccinates, and (c) aliphatic and araliphatic peroxydicarboxylic acids, such as 1,12-diperoxydicarboxylic acid, 1,9-diperoxyazelaic acid, diperoxysebacic acid, diperoxybrassylic acid, the diperoxyphthalic acids, 2-decyldiperoxybutane-1,4-dioic acid, N,N-terephthaloyldi(6-aminopercaproic acid).

Bleach activators

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 peroxydicarboxylic acids having preferably from 1 to 10 carbon atoms, in particular from 2 to 4 carbon atoms, and/or optionally substituted perbenzoic acid. Suitable substances bear O-acyl and/or N-acyl groups of the number of carbon atoms specified and/or optionally substituted benzoyl groups. Preference is given to polyacylated alkylenediamines, in particular tetraacetylenediamine (TAED), acylated triazine derivatives, in particular 1,5-diacetyl-2,4-dioxohexahydro-1,3,5-triazine (DADHT), acylated glycolurils, in particular tetraacetylglycoluril (TAGU), N-acylimides, in particular N-nonanoylsuccinimide (NOSI), acylated phenolsulfonates, in particular n-nonanoyl- or isononanoyloxybenzenesulfonate (n- or iso- NOBS), carboxylic anhydrides, in particular phthalic anhydride, acylated polyhydric alcohols, in particular triacetin, ethylene glycol diacetate and 2,5-diacetoxy-2,5-dihydrofuran and also triethylacetyl citrate (TEAC). Bleach activators if included in the compositions of the invention are in a level of from about 0.1 to about 10%, preferably from about 0.5 to about 2% by weight of the composition.

Bleach catalyst

Bleach catalysts preferred for use herein include the manganese triazacyclononane and related complexes (US-A-4246612, US-A-5227084); Co, Cu, Mn and Fe bispyridylamine and related complexes (US-A-5114611); and pentamine acetate cobalt(III) and related complexes (US-A-4810410). A complete description of bleach catalysts suitable for use herein can be found in WO 99/06521, pages 34, line 26 to page 40, line 16. Bleach catalyst if included in the compositions of the invention are in a level of from about 0.1 to about 10%, preferably from about 0.5 to about 2% by weight of the composition.

Oxidoreductases, for example oxidases, oxygenases, catalases, peroxidases such as halo-, chloro-, bromo-, lignin, glucose, or manganese peroxidases, dioxygenases, or laccases (phenoloxidases, polyphenoloxidases), may also be used according to the present invention to intensify the bleaching effect. Advantageously, preferably organic, particularly preferably aromatic compounds that interact with the enzymes are additionally added in order to enhance the activity of the relevant oxidoreductases (enhancers) or, if there is a large difference in redox potentials between the oxidizing enzymes and the stains, to ensure electron flow (mediators).

Silicates

Preferred silicates are sodium silicates such as sodium disilicate, sodium metasilicate and crystalline phyllosilicates. Silicates if present are at a level of from about 1 to about 20%, preferably from about 5 to about 15% by weight of composition.

Metal care agents

Metal care agents may prevent or reduce the tarnishing, corrosion or oxidation of metals, including aluminium, stainless steel and non-ferrous metals, such as silver and copper. Suitable examples include one or more of the following:

(a) benzotriazoles, including benzotriazole or bis-benzotriazole and substituted derivatives thereof. Benzotriazole derivatives are those compounds in which the available substitution sites on the aromatic ring are partially or completely substituted. Suitable substituents include linear or branch-chain C₁-C₂₀- alkyl groups and hydroxyl, thio, phenyl or halogen such as fluorine, chlorine, bromine and iodine.

(b) metal salts and complexes chosen from the group consisting of zinc, manganese, titanium, zirconium, hafnium, vanadium, cobalt, gallium and cerium salts and/or complexes, the metals being in one of the oxidation states II, III, IV, V or VI. In one aspect, suitable metal salts and/or metal complexes may be chosen from the group consisting of Mn(II) sulphate, Mn(II) citrate, Mn(II) stearate, Mn(II) acetylacetonate, K⁺TiF₆, K⁺ZrF₆, CoSO₄, Co(NO₂)₂ and Ce(NO₂)₃, zinc salts, for example zinc sulphate, hydrozincite or zinc acetate;

(c) silicates, including sodium or potassium silicate, sodium disilicate, sodium metasilicate, crystalline phyllosilicate and mixtures thereof.

Further suitable organic and inorganic redox-active substances that act as silver/copper corrosion inhibitors are disclosed in WO 94/26860 and WO 94/26859. Preferably the composition of the invention comprises from 0.1 to 5% by weight of the composition of a metal care agent, preferably the metal care agent is a zinc salt.

Enzymes

The detergent additive as well as the detergent composition may comprise one or more additional enzymes such as a protease, lipase, cutinase, an amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, oxidase, e.g., a laccase, and/or peroxidase.

In general, the properties of the selected enzyme(s) should be compatible with the selected detergent, (i.e., pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Cellulases

Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*, *Acremonium*, e.g., the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having color care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and WO99/001544.

Other cellulases are endo-beta-1,4-glucanase enzyme having a sequence of at least 97% identity to the amino acid sequence of position 1 to position 773 of SEQ ID NO:2 of WO 2002/099091 or a family 44 xyloglucanase, which a xyloglucanase enzyme having a sequence of at least 60% identity to positions 40-559 of SEQ ID NO: 2 of WO 2001/062903.

Commercially available cellulases include Celluzyme™, and Carezyme™ (Novozymes A/S) Carezyme Premium™ (Novozymes A/S), Celluclean™ (Novozymes A/S), Celluclean Classic™ (Novozymes A/S), Cellusoft™ (Novozymes A/S), Whitezyme™ (Novozymes A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Proteases

Suitable proteases include those of bacterial, fungal, plant, viral or animal origin e.g. vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered variants are included. It may be an alkaline protease, such as a serine protease or a metalloprotease. A serine protease may for example be of the S1 family, such as trypsin, or the S8 family such as subtilisin. A metalloprotease protease may for example be a thermolysin from e.g. family M4 or other metalloprotease such as those from M5, M7 or M8 families.

The term "subtilases" refers to a sub-group of serine protease according to Siezen et al., Protein Engng. 4 (1991) 719-737 and Siezen et al. Protein Science 6 (1997) 501-523. Serine proteases are a subgroup of proteases characterized by having a serine in the active site, which forms a covalent adduct with the substrate. The subtilases may be divided into 6 sub-divisions, *i.e.* the Subtilisin family, the Thermitase family, the Proteinase K family, the Lantibiotic peptidase family, the Kexin family and the Pyrolysin family.

Examples of subtilases are those derived from *Bacillus* such as *Bacillus lentus*, *B. alkalophilus*, *B. subtilis*, *B. amyloliquefaciens*, *Bacillus pumilus* and *Bacillus gibsonii* described in; US7262042 and WO09/021867, and *subtilisin lentus*, *subtilisin Novo*, *subtilisin Carlsberg*, *Bacillus licheniformis*, *subtilisin BPN'*, *subtilisin 309*, *subtilisin 147* and *subtilisin 168* described in WO89/06279 and protease PD138 described in (WO93/18140). Other useful proteases may be those described in WO92/175177, WO01/016285, WO02/026024 and WO02/016547. Examples of trypsin-like proteases are trypsin (*e.g.* of porcine or bovine origin) and the *Fusarium* protease described in WO89/06270, WO94/25583 and WO05/040372, and the chymotrypsin proteases derived from *Cellulomonas* described in WO05/052161 and WO05/052146.

A further preferred protease is the alkaline protease from *Bacillus lentus* DSM 5483, as described for example in WO95/23221, and variants thereof which are described in WO92/21760, WO95/23221, EP1921147 and EP1921148.

Examples of metalloproteases are the neutral metalloprotease as described in WO07/044993 (Genencor Int.) such as those derived from *Bacillus amyloliquefaciens*.

Examples of useful proteases are the variants described in: WO92/19729, WO96/034946, WO98/20115, WO98/20116, WO99/011768, WO01/44452, WO03/006602, WO04/03186, WO04/041979, WO07/006305, WO11/036263, WO11/036264, especially the variants with substitutions in one or more of the following positions: 3, 4, 9, 15, 27, 36, 57, 68, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 118, 120, 123, 128, 129, 130, 160, 167, 170, 194, 195, 199, 205, 206, 217, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274 using the BPN' numbering. More preferred the subtilase variants may comprise the mutations: S3T, V4I, S9R, A15T, K27R, *36D, V68A, N76D, N87S,R, *97E, A98S, S99G,D,A, S99AD, S101G,M,R S103A, V104I,Y,N, S106A, G118V,R, H120D,N, N123S, S128L, P129Q, S130A, G160D, Y167A, R170S, A194P, G195E, V199M, V205I, L217D, N218D, M222S, A232V, K235L, Q236H, Q245R, N252K, T274A (using BPN' numbering).

Suitable commercially available protease enzymes include those sold under the trade names Alcalase®, Duralase™, Durazym™, Relase®, Relase® Ultra, Savinase®, Savinase® Ultra, Primase®, Polarzyme®, Kannase®, Liquanase®, Liquanase® Ultra, Ovozyme®, Coronase®, Coronase® Ultra, Neutrase®, Everlase® and Esperase® (Novozymes A/S), those sold under the tradename Maxatase®, Maxacal®, Maxapem®, Purafect®, Purafect Prime®,

Preferenz™, Purafect MA®, Purafect Ox®, Purafect OxP®, Puramax®, Properase®, Effectenz™, FN2®, FN3®, FN4®, Excellase®, Opticlean® and Optimase® (Danisco/DuPont), Axapem™ (Gist-Brocades N.V.), BLAP (sequence shown in Figure 29 of US5352604) and variants hereof (Henkel AG) and KAP (*Bacillus alkalophilus subtilisin*) from Kao.

- 5 Savinase® is marketed by NOVOZYMES A/S. It is subtilisin 309 from *B. Lentus* and differs from BAALKP only in one position (N87S).

Lipases and Cutinases

Suitable lipases and cutinases include those of bacterial or fungal origin. Chemically
10 modified or protein engineered variant enzymes are included. Examples include lipase from
Thermomyces, e.g. from *T. lanuginosus* (previously named *Humicola lanuginosa*) as described
in EP258068 and EP305216, cutinase from *Humicola*, e.g. *H. insolens* (WO96/13580), lipase
from strains of *Pseudomonas* (some of these now renamed to *Burkholderia*), e.g. *P. alcaligenes*
or *P. pseudoalcaligenes* (EP218272), *P. cepacia* (EP331376), *P. sp.* strain SD705
15 (WO95/06720 & WO96/27002), *P. wisconsinensis* (WO96/12012), GDSL-type *Streptomyces*
lipases (WO10/065455), cutinase from *Magnaporthe grisea* (WO10/107560), cutinase from
Pseudomonas mendocina (US5,389,536), lipase from *Thermobifida fusca* (WO11/084412),
Geobacillus stearothermophilus lipase (WO11/084417), lipase from *Bacillus subtilis*
(WO11/084599), and lipase from *Streptomyces griseus* (WO11/150157) and *S. pristinaespiralis*
20 (WO12/137147).

Other examples are lipase variants such as those described in EP407225,
WO92/05249, WO94/01541, WO94/25578, WO95/14783, WO95/30744, WO95/35381,
WO95/22615, WO96/00292, WO97/04079, WO97/07202, WO00/34450, WO00/60063,
WO01/92502, WO07/87508 and WO09/109500.

- 25 Preferred commercial lipase products include include Lipolase™, Lipex™; Lipolex™
and Lipoclean™ (Novozymes A/S), Lumafast (originally from Genencor) and Lipomax (originally
from Gist-Brocades).

Still other examples are lipases sometimes referred to as acyltransferases or
perhydrolases, e.g. acyltransferases with homology to *Candida antarctica* lipase A
30 (WO10/111143), acyltransferase from *Mycobacterium smegmatis* (WO05/56782),
perhydrolases from the CE 7 family (WO09/67279), and variants of the *M. smegmatis*
perhydrolase in particular the S54V variant used in the commercial product Gentle Power
Bleach from Huntsman Textile Effects Pte Ltd (WO10/100028).

35 Additional Amylases

Suitable amylases which may be used together with the enzyme preparation of the
invention may be an alpha-amylase, a pullulanase or a glucoamylase and may be of bacterial or

fungal origin. Chemically modified or protein engineered variants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus*, e.g., a special strain of *Bacillus licheniformis*, described in more detail in GB 1,296,839.

5 Suitable amylases include amylases having SEQ ID NO: 2 in WO 95/10603 or variants having 90% sequence identity to SEQ ID NO: 3 thereof. Preferred variants are described in WO 94/02597, WO 94/18314, WO 97/43424 and SEQ ID NO: 4 of WO 99/019467, such as variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 178, 179, 181, 188, 190, 197, 201, 202, 207, 208, 209, 211, 243, 264, 304, 305, 391, 408, and 444.

10 Different suitable amylases include amylases having SEQ ID NO: 6 in WO 02/010355 or variants thereof having 90% sequence identity to SEQ ID NO: 6. Preferred variants of SEQ ID NO: 6 are those having a deletion in positions 181 and 182 and a substitution in position 193.

Other amylases which are suitable are hybrid alpha-amylase comprising residues 1-33 of the alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 6 of WO 15 2006/066594 and residues 36-483 of the *B. licheniformis* alpha-amylase shown in SEQ ID NO: 4 of WO 2006/066594 or variants having 90% sequence identity thereof. Preferred variants of this hybrid alpha-amylase are those having a substitution, a deletion or an insertion in one of more of the following positions: G48, T49, G107, H156, A181, N190, M197, I201, A209 and Q264. Most preferred variants of the hybrid alpha-amylase comprising residues 1-33 of the 20 alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 6 of WO 2006/066594 and residues 36-483 of SEQ ID NO: 4 are those having the substitutions: M197T; H156Y+A181T+N190F+A209V+Q264S; or G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S.

Further amylases which are suitable are amylases having SEQ ID NO: 6 in WO 25 99/019467 or variants thereof having 90% sequence identity to SEQ ID NO: 6. Preferred variants of SEQ ID NO: 6 are those having a substitution, a deletion or an insertion in one or more of the following positions: R181, G182, H183, G184, N195, I206, E212, E216 and K269. Particularly preferred amylases are those having deletion in positions R181 and G182, or positions H183 and G184.

30 Additional amylases which may be used are those having SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 2 or SEQ ID NO: 7 of WO 96/023873 or variants thereof having 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7. Preferred variants of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7 are those having a substitution, a deletion or an insertion in one or more of the following positions: 140, 181, 182, 183, 184, 195, 35 206, 212, 243, 260, 269, 304 and 476, using SEQ ID 2 of WO 96/023873 for numbering. More preferred variants are those having a deletion in two positions selected from 181, 182, 183 and 184, such as 181 and 182, 182 and 183, or positions 183 and 184. Most preferred amylase

variants of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 7 are those having a deletion in positions 183 and 184 and a substitution in one or more of positions 140, 195, 206, 243, 260, 304 and 476.

Other amylases which may be used are amylases having SEQ ID NO: 2 of WO 08/153815, SEQ ID NO: 10 in WO 01/66712 or variants thereof having 90% sequence identity to SEQ ID NO: 2 of WO 08/153815 or 90% sequence identity to SEQ ID NO: 10 in WO 01/66712. Preferred variants of SEQ ID NO: 10 in WO 01/66712 are those having a substitution, a deletion or an insertion in one or more of the following positions: 176, 177, 178, 179, 190, 201, 207, 211 and 264.

Further suitable amylases are amylases having SEQ ID NO: 2 of WO 09/061380 or variants having 90% sequence identity to SEQ ID NO: 2 thereof. Preferred variants of SEQ ID NO: 2 are those having a truncation of the C-terminus and/or a substitution, a deletion or an insertion in one or more of the following positions: Q87, Q98, S125, N128, T131, T165, K178, R180, S181, T182, G183, M201, F202, N225, S243, N272, N282, Y305, R309, D319, Q320, Q359, K444 and G475. More preferred variants of SEQ ID NO: 2 are those having the substitution in one or more of the following positions: Q87E,R, Q98R, S125A, N128C, T131I, T165I, K178L, T182G, M201L, F202Y, N225E,R, N272E,R, S243Q,A,E,D, Y305R, R309A, Q320R, Q359E, K444E and G475K and/or deletion in position R180 and/or S181 or of T182 and/or G183. Most preferred amylase variants of SEQ ID NO: 2 are those having the substitutions:

N128C+K178L+T182G+Y305R+G475K;
N128C+K178L+T182G+F202Y+Y305R+D319T+G475K;

S125A+N128C+K178L+T182G+Y305R+G475K; or

S125A+N128C+T131I+T165I+K178L+T182G+Y305R+G475K wherein the variants are C-terminally truncated and optionally further comprises a substitution at position 243 and/or a deletion at position 180 and/or position 181.

Other suitable amylases are the alpha-amylase having SEQ ID NO: 12 in WO01/66712 or a variant having at least 90% sequence identity to SEQ ID NO: 12. Preferred amylase variants are those having a substitution, a deletion or an insertion in one or more of the following positions of SEQ ID NO: 12 in WO01/66712: R28, R118, N174; R181, G182, D183, G184, G186, W189, N195, M202, Y298, N299, K302, S303, N306, R310, N314; R320, H324, E345, Y396, R400, W439, R444, N445, K446, Q449, R458, N471, N484. Particular preferred amylases include variants having a deletion of D183 and G184 and having the substitutions R118K, N195F, R320K and R458K, and a variant additionally having substitutions in one or more position selected from the group: M9, G149, G182, G186, M202, T257, Y295, N299, M323, E345 and A339, most preferred a variant that additionally has substitutions in all these positions.

Other examples are amylase variants such as those described in WO2011/098531, WO2013/001078 and WO2013/001087.

Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™, Stainzyme™, Stainzyme Plus™, Natalase™, Liquozyme X and BAN™ (from Novozymes A/S), and
5 Rapidase™, Purastar™/Effectenz™, Powerase, Preferenz S100, Preferenz S110 and Preferenz S1000 (from Genencor International Inc./DuPont).

Peroxidases/Oxidases

A peroxidase is an enzyme comprised by the enzyme classification EC 1.11.1.7, as set
10 out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), or any fragment derived therefrom, exhibiting peroxidase activity.

Suitable peroxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinopsis*, e.g., from *C. cinerea* (EP 179,486), and variants thereof as those
15 described in WO 93/24618, WO 95/10602, and WO 98/15257.

A peroxidase also includes a haloperoxidase enzyme, such as chloroperoxidase, bromoperoxidase and compounds exhibiting chloroperoxidase or bromoperoxidase activity. Haloperoxidases are classified according to their specificity for halide ions. Chloroperoxidases (E.C. 1.11.1.10) catalyze formation of hypochlorite from chloride ions.

In an embodiment, the haloperoxidase is a chloroperoxidase. Preferably, the
20 haloperoxidase is a vanadium haloperoxidase, i.e., a vanadate-containing haloperoxidase, optionally wherein the vanadate-containing haloperoxidase is combined with a source of chloride ion.

Haloperoxidases have been isolated from many different fungi, in particular from the
25 fungus group dematiaceous hyphomycetes, such as *Caldariomyces*, e.g., *C. fumago*, *Alternaria*, *Curvularia*, e.g., *C. verruculosa* and *C. inaequalis*, *Drechslera*, *Ulocladium* and *Botrytis*.

Haloperoxidases have also been isolated from bacteria such as *Pseudomonas*, e.g., *P. pyrocinia* and *Streptomyces*, e.g., *S. aureofaciens*.

In an preferred embodiment, the haloperoxidase is derivable from *Curvularia* sp., in
30 particular *Curvularia verruculosa* or *Curvularia inaequalis*, such as *C. inaequalis* CBS 102.42 as described in WO 95/27046; or *C. verruculosa* CBS 147.63 or *C. verruculosa* CBS 444.70 as described in WO 97/04102; or from *Drechslera hartlebii* as described in WO 01/79459, *Dendryphiella salina* as described in WO 01/79458, *Phaeotrichoconis crotalarie* as described in WO 01/79461, or *Geniculosporium* sp. as described in WO 01/79460.

An oxidase according to the invention include, in particular, any laccase enzyme
35 comprised by the enzyme classification EC 1.10.3.2, or any fragment derived therefrom exhibiting laccase activity, or a compound exhibiting a similar activity, such as a catechol

oxidase (EC 1.10.3.1), an o-aminophenol oxidase (EC 1.10.3.4), or a bilirubin oxidase (EC 1.3.3.5).

Preferred laccase enzymes are enzymes of microbial origin. The enzymes may be derived from plants, bacteria or fungi (including filamentous fungi and yeasts).

5 Suitable examples from fungi include a laccase derivable from a strain of *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g., *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinopsis*, e.g., *C. cinerea*, *C. comatus*, *C. friesii*, and *C. plicatilis*, *Psathyrella*, e.g., *P. condelleana*, *Panaeolus*, e.g., *P. papilionaceus*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, e.g., *S. thermophilum*, *Polyporus*, e.g., *P. pinsitus*, *Phlebia*, e.g., *P. radiata* (WO 92/01046), or *Coriolus*,
10 e.g., *C. hirsutus* (JP 2238885).

Suitable examples from bacteria include a laccase derivable from a strain of *Bacillus*.

A laccase derived from *Coprinopsis* or *Myceliophthora* is preferred; in particular a laccase derived from *Coprinopsis cinerea*, as disclosed in WO 97/08325; or from
15 *Myceliophthora thermophila*, as disclosed in WO 95/33836.

The detergent enzyme(s) may be included in a detergent composition by adding separate additives comprising one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e., a separate additive or a combined additive, may be formulated, for example, as a granulate, liquid, slurry, etc. Preferred detergent
20 additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g. as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of
25 1,000 to 20,000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a
30 polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

Adjunct materials

35 Any detergent component known in the art for use in ADW detergents may also be utilized. Other optional detergent components include anti-corrosion agents, anti-shrink agents, anti-soil redeposition agents, anti-wrinkling agents, bactericides, binders, corrosion inhibitors,

disintegrants/disintegration agents, dyes, enzyme stabilizers (including boric acid, borates, CMC, and/or polyols such as propylene glycol), fabric conditioners including clays, fillers/processing aids, fluorescent whitening agents/optical brighteners, foam boosters, foam (suds) regulators, perfumes, soil-suspending agents, softeners, suds suppressors, tarnish inhibitors, and wicking agents, either alone or in combination. Any ingredient known in the art for use ADW detergents may be utilized. The choice of such ingredients is well within the skill of the artisan.

Dispersants

The detergent compositions of the present invention may also comprise dispersants. In particular, powdered detergents may comprise dispersants. Suitable water-soluble organic materials include the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated from each other by not more than two carbon atoms. Suitable dispersants are for example described in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc.

Dye Transfer Inhibiting Agents

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

Fluorescent whitening agent

The detergent compositions of the present invention will preferably also comprise additional components that may tint articles being cleaned, such as fluorescent whitening agent or optical brighteners. Where present the brightener is preferably at a level of about 0.01% to about 0.5%. Any fluorescent whitening agent suitable for use in a laundry detergent composition may be used in the composition of the present invention. The most commonly used fluorescent whitening agents are those belonging to the classes of diaminostilbene-sulfonic acid derivatives, diarylpyrazoline derivatives and bisphenyl-distyryl derivatives. Examples of the diaminostilbene-sulfonic acid derivative type of fluorescent whitening agents include the sodium salts of: 4,4'-bis-(2-diethanolamino-4-anilino-*s*-triazin-6-ylamino) stilbene-2,2'-disulfonate, 4,4'-bis-(2,4-dianilino-*s*-triazin-6-ylamino) stilbene-2,2'-disulfonate, 4,4'-bis-(2-anilino-4-(*N*-methyl-*N*-2-hydroxyethylamino)-*s*-triazin-6-ylamino) stilbene-2,2'-disulfonate, 4,4'-bis-(4-phenyl-1,2,3-triazol-2-

yl)stilbene-2,2'-disulfonate and sodium 5-(2*H*-naphtho[1,2-*d*][1,2,3]triazol-2-yl)-2-[(*E*)-2-phenylvinyl]benzenesulfonate. Preferred fluorescent whitening agents are Tinopal DMS and Tinopal CBS available from Ciba-Geigy AG, Basel, Switzerland. Tinopal DMS is the disodium salt of 4,4'-bis-(2-morpholino-4-anilino-*s*-triazin-6-ylamino) stilbene-2,2'-disulfonate. Tinopal
5 CBS is the disodium salt of 2,2'-bis-(phenyl-styryl)-disulfonate. Also preferred are fluorescent whitening agents is the commercially available Parawhite KX, supplied by Paramount Minerals and Chemicals, Mumbai, India. Other fluorescers suitable for use in the invention include the 1-3-diaryl pyrazolines and the 7-alkylaminocoumarins.

Suitable fluorescent brightener levels include lower levels of from about 0.01, from
10 0.05, from about 0.1 or even from about 0.2 wt % to upper levels of 0.5 or even 0.75 wt%.

Soil release polymers

The detergent compositions of the present invention may also comprise one or more soil release polymers which aid the removal of soils from fabrics such as cotton and polyester
15 based fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil release polymers may for example be nonionic or anionic terephthalate based polymers, polyvinyl caprolactam and related copolymers, vinyl graft copolymers, polyester polyamides see for example Chapter 7 in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc. Another type of soil release polymers are amphiphilic alkoxyated grease cleaning
20 polymers comprising a core structure and a plurality of alkoxyate groups attached to that core structure. The core structure may comprise a polyalkylenimine structure or a polyalkanolamine structure as described in detail in WO 2009/087523 (hereby incorporated by reference). Furthermore random graft co-polymers are suitable soil release polymers. Suitable graft co-polymers are described in more detail in WO 2007/138054, WO 2006/108856 and WO
25 2006/113314 (hereby incorporated by reference). Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose derivatives such as those described in EP 1867808 or WO 2003/040279 (both are hereby incorporated by reference). Suitable cellulosic polymers include cellulose, cellulose ethers, cellulose esters, cellulose amides and mixtures thereof. Suitable cellulosic polymers include
30 anionically modified cellulose, nonionically modified cellulose, cationically modified cellulose, zwitterionically modified cellulose, and mixtures thereof. Suitable cellulosic polymers include methyl cellulose, carboxy methyl cellulose, ethyl cellulose, hydroxyl ethyl cellulose, hydroxyl propyl methyl cellulose, ester carboxy methyl cellulose, and mixtures thereof.

35 Anti-redeposition agents

The detergent compositions of the present invention may also include one or more anti-redeposition agents such as carboxymethylcellulose (CMC), polyvinyl alcohol (PVA),

polyvinylpyrrolidone (PVP), polyoxyethylene and/or polyethyleneglycol (PEG), homopolymers of acrylic acid, copolymers of acrylic acid and maleic acid, and ethoxylated polyethyleneimines. The cellulose based polymers described under soil release polymers above may also function as anti-redeposition agents.

5

Rheology Modifiers

The detergent compositions of the present invention may also comprise one or more rheology modifiers, structurants or thickeners, as distinct from viscosity reducing agents. The rheology modifiers are selected from the group consisting of non-polymeric crystalline, hydroxy-
10 functional materials, polymeric rheology modifiers which impart shear thinning characteristics to the aqueous liquid matrix of a liquid detergent composition. The rheology and viscosity of the detergent may be modified and adjusted by methods known in the art, for example as shown in EP 2169040.

15 **Other suitable adjunct materials** include, but are not limited to, anti-shrink agents, anti-wrinkling agents, bactericides, binders, carriers, dyes, enzyme stabilizers, fabric softeners, fillers, foam regulators, hydrotropes, perfumes, pigments, sod suppressors, solvents, and structurants for liquid detergents and/or structure elasticizing agents.

20 Formulation of detergent products

The detergent composition of the invention may be in any convenient form, e.g., a bar, a homogenous tablet, a tablet having two or more layers, a pouch having one or more compartments, a regular or compact powder, a granule, a paste, a gel, or a regular, compact or concentrated liquid.

25 Pouches may be configured as single or multicompartments. It may be of any form, shape and material which is suitable for hold the composition, e.g. without allowing the release of the composition to release of the composition from the pouch prior to water contact. The pouch is made from water soluble film which encloses an inner volume. Said inner volume may be divided into compartments of the pouch. Preferred films are polymeric materials preferably polymers which
30 are formed into a film or sheet. Preferred polymers, copolymers or derivates thereof are selected polyacrylates, and water soluble acrylate copolymers, methyl cellulose, carboxy methyl cellulose, sodium dextrin, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, malto dextrin, poly methacrylates, most preferably polyvinyl alcohol copolymers and, hydroxypropyl methyl cellulose (HPMC). Preferably the level of polymer in the film for example PVA is at least
35 about 60%. Preferred average molecular weight will typically be about 20,000 to about 150,000. Films may also be of blended compositions comprising hydrolytically degradable and water soluble polymer blends such as polylactide and polyvinyl alcohol (known under the Trade reference M8630

as sold by MonoSol LLC, Indiana, USA) plus plasticisers like glycerol, ethylene glycerol, propylene glycol, sorbitol and mixtures thereof. The pouches may comprise a solid laundry cleaning composition or part components and/or a liquid cleaning composition or part components separated by the water soluble film. The compartment for liquid components may be different in composition than compartments containing solids: US2009/0011970 A1.

Detergent ingredients may be separated physically from each other by compartments in water dissolvable pouches or in different layers of tablets. Thereby negative storage interaction between components can be avoided. Different dissolution profiles of each of the compartments may also give rise to delayed dissolution of selected components in the wash solution.

A liquid or gel detergent, which is not unit dosed, may be aqueous, typically comprising at least 20% by weight and up to 95% water, such as up to about 70% water, up to about 65% water, up to about 55% water, up to about 45% water, up to about 35% water. Other types of liquids, including without limitation, alkanols, amines, diols, ethers and polyols may be included in an aqueous liquid or gel. An aqueous liquid or gel detergent may contain from 0-30% organic solvent.

Granular detergent formulations

A granular detergent may be formulated as described in WO09/092699, EP1705241, EP1382668, WO07/001262, US6472364, WO04/074419 or WO09/102854. Other useful detergent formulations are described in WO09/124162, WO09/124163, WO09/117340, WO09/117341, WO09/117342, WO09/072069, WO09/063355, WO09/132870, WO09/121757, WO09/112296, WO09/112298, WO09/103822, WO09/087033, WO09/050026, WO09/047125, WO09/047126, WO09/047127, WO09/047128, WO09/021784, WO09/010375, WO09/000605, WO09/122125, WO09/095645, WO09/040544, WO09/040545, WO09/024780, WO09/004295, WO09/004294, WO09/121725, WO09/115391, WO09/115392, WO09/074398, WO09/074403, WO09/068501, WO09/065770, WO09/021813, WO09/030632, and WO09/015951.

WO2011025615, WO2011016958, WO2011005803, WO2011005623, WO2011005730, WO2011005844, WO2011005904, WO2011005630, WO2011005830, WO2011005912, WO2011005905, WO2011005910, WO2011005813, WO2010135238, WO2010120863, WO2010108002, WO2010111365, WO2010108000, WO2010107635, WO2010090915, WO2010033976, WO2010033746, WO2010033747, WO2010033897, WO2010033979, WO2010030540, WO2010030541, WO2010030539, WO2010024467, WO2010024469, WO2010024470, WO2010025161, WO2010014395, WO2010044905,

WO2010145887, WO2010142503, WO2010122051, WO2010102861, WO2010099997, WO2010084039, WO2010076292, WO2010069742, WO2010069718, WO2010069957, WO2010057784, WO2010054986, WO2010018043, WO2010003783, WO2010003792,

WO2011023716, WO2010142539, WO2010118959, WO2010115813, WO2010105942, WO2010105961, WO2010105962, WO2010094356, WO2010084203,

WO2010078979, WO2010072456, WO2010069905, WO2010076165, WO2010072603,
WO2010066486, WO2010066631, WO2010066632, WO2010063689, WO2010060821,
WO2010049187, WO2010031607, WO2010000636.

5 Uses

The present invention is also directed to methods for using the alpha-amylase variants. Accordingly, the present invention relates to methods for using a variant comprising a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203, and A204 of the mature polypeptide of SEQ ID NO: 1, and c) substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1, wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity.

The alpha-amylase variants of the invention are useful in detergent compositions, laundry washing, dishwashing and/or cleaning processes at low temperature as well as hard surface cleaning (ADW, car wash, Industrial surface). The alpha-amylase variants of the invention are particularly useful in dish wash detergent compositions because they are stable towards oxidation by the bleaching agents and have improved activity.

Use in detergents. The polypeptides of the present invention may be added to and thus become a component of a detergent composition, in particular a dish wash detergent composition.

The detergent composition of the present invention may be formulated, for example, as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

The detergent composition may further be formulated in unit dosage form or in form a soap bar or a laundry bar.

In a specific aspect, the present invention provides a detergent additive comprising a polypeptide of the present invention as described herein.

METHODS

Amylase expression: the alpha-amylase variants of the present invention may be expressed as disclosed in WO2010115021 or US 6,623,948.

Strain: e.g. *B.subtilis*, *B.licheniformis*, carrying the amylase in an expression cassette either on a plasmid or integrated on the bacillus chromosome, e.g. in the *Pel* or *Amy* locus.

Media: e.g., **LB, TY, Media-16**

Media 16

- Glycerol – 5% w/v
- Tryptone – 0.5% w/v
- 10 • Beef Extract – 0.5% w/v
- Sodium Nitrate – 1% w/v
- Na₂HPO₄ – 1.7% w/v
- KH₂PO₄ – 0.4% w/v
- NH₄Cl – 0.1% w/v
- 15 • NaCl – 0.05% w/v
- Adjust to pH 7 and autoclave.

Autoclaved separately and added just before inoculation

- 1.47% CaCl₂ – 0.4ml for 100ml media
- 2.465% MgSO₄.7H₂O – 0.4ml for 100ml media
- 20 • 1.39% FeSO₄ – 0.04ml for 100ml media
- 0.2% Na₂MoO₄.2H₂O – 0.04ml for 100ml media
- Vitamin Mix (containing 0.25% Thiamine and 0.25% Ascorbic Acid) – 0.4ml of Vitamin Mix for 100ml media
- Trace Elements (containing 0.5% MnCl₂.4H₂O, 0.2% ZnCl₂ and 0.1% CuSO₄.5H₂O) –
- 25 0.04ml of Trace sol for 100ml media

Construction of variants of SEQ ID NO: 2

The wild type amylase was isolated as described in US 6,623,948 and cloned into the *Pel* locus of *B. subtilis*, so that the up-stream fragment including the upper *Pel* locus and a down-stream fragment including the lower *Pel* logi, so that the amylase upon transformation in *B. subtilis* will integrate in the *Pel* locus by double cross-over replacement.

The upper fragment further comprises a triple promoter system (as described in WO 99/43835) consisting of the promoters from *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), and the *Bacillus thuringiensis* cryIIIA promoter including stabilizing sequence controlling the amylase expression, and the signal sequence of the *B. licheniformis* amylase signal to direct export out of the cells. The down-

stream fragment further comprises the *cat* gene for selection on Chloramphenicol containing media.

The double deletion R181*+G182*, and the N195F mutations found in the sequence ID no 2, were introduced by megaprimer mutagenesis method using a mutagenesis oligo coding for the desired amino acid change, and cloning into the expression cassette as for the reference amylase described above. The sequence was confirmed by DNA sequencing of the amylase gene.

Production and Purification of amylases

The amylase expressing clones can be fermented in media-16 at 37°C with 180rpm for 72 hours and the broths centrifuged at 13131g for 25 minutes to remove the cell mass, and then filtered using a 0.7 micro meter Glass filter GF-F, Whatman using tarsons filtration assembly.

Reference and variant amylases can be purified from the supernatant by 24 well plate protein purification method: 3 ml of a 50% slurry of butyl toyopearl resin in milli Q water is to be added into each well of 24 well filter plate and the plate subjected to vacuum to pack the column plate. The resin should be equilibrated by adding 8 mL of equilibration buffer (50 mM HEPES, pH 8.0 + 1 M ammonium sulphate +1mM CaCl₂) and 8 ml of the amylase samples can be then added into the wells of filter plate and incubate on plate mixer at 350 rpm for 8 min. The unbound fraction can be removed by vacuum and the resin washed by 4 cycles of adding 8 mL of equilibration buffer (50 mM HEPES, pH 8.0 + 1 M ammonium sulphate +1mM CaCl₂) followed by mixing and incubation and finally removing the wash buffers by vacuum.

The amylase can be eluted by adding elution buffer (50 mM HEPES, pH 8.0 + 1mM CaCl₂), mixed and incubated prior to collecting the amylase solution in a collection tray by vacuum.

Assays for measurement of amylolytic activity (alpha-amylase activity)

EnzChek Assay

The amylase activity or residual amylase activity can be determined by the following EnzCheck assay. The substrate is a corn starch derivative, DQTM starch (corn starch BODIPY FL conjugate), which is corn starch labeled with BODIPY® FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) dye to such a degree that the fluorescence is quenched. One vial containing approx. 1 mg lyophilized substrate is dissolved in 100 µL 50 mM sodium acetate pH 4.0. The vial is vortexed for 20 seconds and left at room temperature, in the dark, with occasional mixing until dissolved. Then 950 µL 10 mM sodium acetate, 0.01 % (w/v) Triton X100 ((polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (C₁₄H₂₂O(C₂H₄O)_n (n = 9-10)), pH 5.0 is added, vortexed thoroughly and stored at room temperature, in the dark until ready to use.

From 1 mL of this solution, the substrate working solution was prepared by mixing with 5 mL 50 mM HEPES, 0.01 % (w/V) Triton X100, 1 mM CaCl₂, pH 7.0.

The enzyme comprising detergent is diluted to a concentration of 15 ng enzyme protein/ml (6826.7 times dilution) in 50 mM HEPES, 0.01 % Triton X100, 1 mM CaCl₂, pH 7.0.

5 For the assay, 25 μ L of the substrate working solution is mixed for 10 second with 25 μ L of the diluted enzyme in a black 384 well microtiter plate. The fluorescence intensity is measured (excitation: 485 nm, emission: 555 nm) once every second minute for 30 minutes in each well at 25 °C and the V_{max} is calculated as the slope of the plot of fluorescence intensity against time. The plot should be linear and the residual activity assay has to been adjusted so that the diluted reference
10 enzyme solution is within the linear range of the activity assay.

In a few instances there is a significant interference from the detergent without amylase on the assay. In such cases alternative amylase assays can be used. Interference from a detergent on an amylase assay can be tested by adding a known amount of amylase to the detergent at two levels and then measure the activity of the two samples. If the difference in the measured activities
15 corresponds to the differences in the levels between the added amylases, the assay can be used to determine the residual activity of the amylase after storage.

PNP-G7 assay

The alpha-amylase activity may be determined by a method employing the PNP-G7 substrate. PNP-G7 which is an abbreviation for 4,6-ethylidene(G7)-p-nitrophenyl(G1)- α ,D-maltoheptaoside, a blocked oligosaccharide which can be cleaved by an endo-amylase, such as an alpha-amylase. Following the cleavage, the alpha-Glucosidase included in the kit digest the hydrolysed substrate further to liberate a free PNP molecule which has a yellow color and thus can be measured by visible spectrophometry at λ =405nm (400-420 nm.). Kits containing PNP-G7
25 substrate and alpha-Glucosidase are manufactured by Roche/Hitachi (cat. No.11876473).

REAGENTS:

The G7-PNP substrate from this kit contains 22 mM 4,6-ethylidene-G7-PNP and 52.4 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), pH 7.0) .

The alpha-Glucosidase reagent comprises 52.4 mM HEPES, 87 mM NaCl, 12.6 mM
30 MgCl₂, 0.075 mM CaCl₂, > 4 kU/L alpha-glucosidase).

The substrate working solution is made by mixing 1 mL of the alpha-Glucosidase reagent with 0.2 mL of the G7-PNP substrate. This substrate working solution is made immediately before use.

Dilution buffer: 50 mM EPPS, 0.01% (w/v) Triton X100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (C₁₄H₂₂O(C₂H₄O)_n (n = 9-10))), 1mM CaCl₂, pH7.0.
35

PROCEDURE:

The amylase sample to be analysed was diluted in dilution buffer to ensure the pH in the

diluted sample is 7. The assay was performed by transferring 20 μ l diluted enzyme samples to 96 well microtiter plate and adding 80 μ l substrate working solution. The solution was mixed and pre-incubated 1 minute at room temperature and absorption is measured every 20 sec. over 5 minutes at OD 405 nm.

5 The slope (absorbance per minute) of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question under the given set of conditions. The amylase sample should be diluted to a level where the slope is below 0.4 absorbance units per minute.

Determination of percentage point (pp)

10 The percentage point (pp) improvement in residual activity (stability) of the variant relative to the parent is calculated as the difference between the residual activity of the variant and the residual activity of the parent, *i.e.* the residual activity of the variant minus the residual activity of the parent.

15 **Amylazyme activity assay:**

The alpha-amylase activity may also be determined by a method using the Amylazyme substrate (from Megazyme, Ireland). An Amylazyme tablet includes interlinked amylose polymers that are in the form of globular microspheres that are insoluble in water. A blue dye is covalently bound to these microspheres. The interlinked amylose polymers in the microsphere are degraded at a speed that is proportional to the alpha-amylase activity. When the alpha-amylase degrades the amylose polymers, the released blue dye is water soluble and concentration of dye can be determined by measuring absorbance at 650nm. The concentration of blue is proportional to the alpha-amylase activity in the sample.

20 The amylase sample to be analysed is diluted in activity buffer with the desired pH. One substrate tablet is suspended in 5mL activity buffer and mixed on magnetic stirrer. During mixing of substrate transfer 150 μ l to microtiter plate (MTP). Add 30 μ l diluted amylase sample to 150 μ l substrate and mix. Incubate for 15 minutes at 37°C. The reaction is stopped by adding 30 μ l 1M NaOH and mix. Centrifuge MTP for 5 minutes at 4000xg. Transfer 100 μ l to new MTP and measure absorbance at 620nm.

30 The amylase sample should be diluted so that the absorbance at 650nm is between 0 and 2.2, and is within the linear range of the activity assay.

Phadebas activity assay

35 The alpha-amylase activity may also be determined by a method using the Phadebas substrate (from for example Magle Life Sciences, Lund, Sweden). A Phadebas tablet includes interlinked starch polymers that are in the form of globular microspheres which are insoluble in water. A blue dye is covalently bound to these microspheres. The interlinked starch polymers in the

microsphere are degraded at a speed that is proportional to the alpha-amylase activity. When the alpha-amylase degrades the starch polymers, the released blue dye is water soluble and concentration of dye can be determined by measuring absorbance at 650nm. The concentration of blue is proportional to the alpha-amylase activity in the sample.

5 The amylase sample to be analysed is diluted in activity buffer with the desired pH. One substrate tablet is suspended in 5mL activity buffer and mixed on magnetic stirrer. During mixing of substrate transfer 150µl to microtiter plate (MTP). Add 30µl diluted amylase sample to 150µl substrate and mix. Incubate for 15 minutes at 37°C. The reaction is stopped by adding 30µl 1M NaOH and mix. Centrifuge MTP for 5 minutes at 4000xg. Transfer 100µl to new MTP and measure
10 absorbance at 620nm.

 The amylase sample should be diluted so that the absorbance at 650nm is between 0 and 2.2, and is within the linear range of the activity assay.

Reducing sugar activity assay

15 The alpha-amylase activity may also be determined by reducing sugar assay with for example corn starch substrate. The number of reducing ends formed by the alpha-amylase hydrolysing the alpha-1,4-glycosidic linkages in starch is determined by reaction with p-Hydroxybenzoic acid hydrazide (PHBAH). After reaction with PHBAH the number of reducing ends can be measured by absorbance at 405nm and the concentration of reducing ends is proportional
20 to the alpha-amylase activity in the sample.

 The corns starch substrate (3mg/ml) is solubilised by cooking for 5 minutes in milliQ water and cooled down before assay. For the stop solution prepare a Ka-Na-tartrate/NaOH solution (K-Na-tartrate (Merck 8087) 50g/l, NaOH 20g/l) and prepare freshly the stop solution by adding p-Hydroxybenzoic acid hydrazide (PHBAH, Sigma H9882) to Ka-Na-tartrate/NaOH solution to
25 15mg/ml.

 In PCR-MTP 50µl activity buffer is mixed with 50µl substrate. Add 50µl diluted enzyme and mix. Incubate at the desired temperature in PCR machine for 5 minutes. Reaction is stopped by adding 75µl stop solution (Ka-Na-tartrate/NaOH/PHBAH). Incubate in PCR machine for 10 minutes at 95°C. Transfer 150µl to new MTP and measure absorbance at 405nm.

30 The amylase sample should be diluted so that the absorbance at 405nm is between 0 and 2.2, and is within the linear range of the activity assay.

Wash performance of alpha-amylases using Automatic Mechanical Stress Assay

 In order to assess the wash performance of the alpha-amylases in a detergent base
35 composition, washing experiments may be performed using Automatic Mechanical Stress Assay (AMSA). With the AMSA test the wash performance of a large quantity of small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions

and a lid firmly squeezing the textile swatch to be washed against all the slot openings. During the washing time, the plate, test solutions, textile and lid are vigorously shaken to bring the test solution in contact with the textile and apply mechanical stress in a regular, periodic oscillating manner. For further description see WO 02/42740, especially the paragraph "Special method
5 embodiments" at page 23-24.

General wash performance description

A test solution comprising water (6°dH), 0.79 g/L detergent, e.g. model detergent J as described below, and the enzyme of the invention at concentration of 0 or 0.2 mg enzyme
10 protein/L, is prepared. Fabrics stained with starch (CS-28 from Center For Test materials BV, P.O. Box 120, 3133 KT, Vlaardinggen, The Netherlands) is added and washed for 20 minutes at 15°C and 30°C, or alternatively 20 minutes at 15°C and 40°C as specified in the examples. After thorough rinse under running tap water and drying in the dark, the light intensity values of the stained fabrics are subsequently measured as a measure for wash performance. The test with 0
15 mg enzyme protein/L is used as a blank and corresponds to the contribution from the detergent. Preferably mechanical action is applied during the wash step, e.g. in the form of shaking, rotating or stirring the wash solution with the fabrics. The AMSA wash performance experiments were conducted under the experimental conditions specified below:

20 Table A: Experimental condition

Detergent	Liquid Model detergent J (see Table B)
Detergent dosage	0.79 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C or 30°C
Water hardness	6°dH
Enzyme concentration in test	0.2 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

Table B: Model detergent J

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	5.15	5.00
AS	5.00	4.50

AEOS	14.18	10.00
Coco fatty acid	1.00	1.00
AEO	5.00	5.00
MEA	0.30	0.30
MPG	3.00	3.00
Ethanol	1.50	1.35
DTPA (as Na5 salt)	0.25	0.10
Sodium citrate	4.00	4.00
Sodium formate	1.00	1.00
Sodium hydroxide	0.66	0.66
H ₂ O, ion exchanged	58.95	58.95

Water hardness was adjusted to 6°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the textiles were flushed in tap water and dried.

5 Table C: Experimental condition

Detergent	Liquid Model detergent A (see Table D)
Detergent dosage	3.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C or 40°C
Water hardness	15°dH
Enzyme concentration in test	0.2 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

Table D: Model detergent A

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	12.00	11.60
AEOS, SLES	17.63	4.90
Soy fatty acid	2.75	2.48
Coco fatty acid	2.75	2.80
AEO	11.00	11.00
Sodium hydroxide	1.75	1.80

Ethanol / Propan-2-ol	3.00	2.70/0.30
MPG	6.00	6.00
Glycerol	1.71	1.70
TEA	3.33	3.30
Sodium formate	1.00	1.00
Sodium citrate	2.00	2.00
DTMPA	0.48	0.20
PCA	0.46	0.18
Phenoxy ethanol	0.50	0.50
H ₂ O, ion exchanged	33.64	33.64

Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 4:1:7.5) to the test system. After washing the textiles were flushed in tap water and dried.

5

Table E: Experimental condition

Detergent	Powder Model detergent X (see Table F)
Detergent dosage	1.75 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	15°C or 30°C
Water hardness	12°dH
Enzyme concentration in test	0.2 mg enzyme protein/L
Test material	CS-28 (Rice starch cotton)

10 Table F: Model detergent X

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	16.50	15.00
AEO*	2.00	2.00
Sodium carbonate	20.00	20.00
Sodium (di)silicate	12.00	9.90
Zeolite A	15.00	12.00

Sodium sulfate	33.50	33.50
PCA	1.00	1.00

*Model detergent X is mixed without AEO. AEO is added separately before wash.

Water hardness was adjusted to 12°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:HCO₃⁻ = 2:1:4.5) to the test system. After washing the textiles were flushed in tap water and dried.

Model detergents 1 and 2

	Model 1	Model 2
Component	% w/w	% w/w
LAS	12	12
AEOS	4,9	4,9
Soap (cocoa)	2,75	2,75
Soap (soya)	2,75	2,75
AEO N25-7	11	11
NaOH	1,75	1,75
Ethanol	3	3
MPG	6	6
Glycerol	1,7	1,7
TEA	3,3	3,3
Sodium formate	1	1
Sodium citrate	2	2
HEDP	0	0,5
PCA (Sokalan CP-5)	0,18	0,18
Ion exchanged water	34,2	34,2
DTMPA	0,2	0

10 The wash performance is measured as the brightness expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower, than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance.

15 Color measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak) used to capture an image of the washed textile.

To extract a value for the light intensity from the scanned images, 24-bit pixel values

from the image are converted into values for red, green and blue (rgb). The intensity value (Int) is calculated by adding the rgb values together as vectors and then taking the length of the resulting vector:

5
$$Int = \sqrt{r^2 + g^2 + b^2}$$

Textile:

Textile sample CS-28 (rice starch on cotton) is obtained from Center For Test materials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands.

10

Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash

Washing experiments are performed in order to assess the wash performance of selected alpha-amylase variants in dishwash detergent compositions. The alpha-amylase variants of the present application may be tested using the Automatic Mechanical Stress Assay (AMSA). With the AMSA, the wash performance of many small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid that firmly squeezes the melamine tile to be washed against the slot openings. During the wash, the plate, test solutions, melamine tile and lid are vigorously shaken to bring the test solution in contact with the soiled melamine tile and apply mechanical stress in a regular, periodic oscillating manner. For further description see WO 02/42740 especially the paragraph "Special method embodiments" at page 23-24.

20

The experiment may be conducted under the experimental conditions as specified in the table(s) below:

ADW model detergent with MGDA	MGDA (40 %) 30 % Sodium carbonate 20 % Sodium percarbonate 10 % Sodium disilicate 5 % TAED 5% Sokalan CP5 (39.5 %) 10 % Surfac 23-6.5 (100 %) 5 % Sodium Sulfate 15 %
Detergent dosage	3.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes

Temperature	50 °C
Water hardness	17 °dH
Enzyme concentration in test solution	0.925, 1.85, 5.55, 11 mg enzyme protein/liter
Test material	melamine tiles with starch such as DM-77 and DM-78

ADW model detergent with STPP	STPP 50 % Sodium carbonate 20% Sodium percarbonate 10% Sodium disilicate 5% TAED 2% Sokalan CP5 (39.5%) 5% Surfac 23-6.5 (100%) 2% Phosphonate 6%
Detergent dosage	3.33 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20 minutes
Temperature	50°C
Water hardness	17°dH
Enzyme concentration in test solution	0.925, 1.85, 5.55, 11 mg enzyme protein/liter
Test material	melamine tiles with starch such as DM-77 and DM-78

Water hardness is adjusted to 17°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺ = 4:1:10) to the test system. After washing the melamine tiles were flushed in tap water and dried.

The performance of the enzyme variant is measured as the brightness of the color of the melamine tile washed with that specific alpha-amylase. Brightness can also be expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower, than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance of a protease.

Color measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak, Midtager 29, DK-2605 Brøndby, Denmark), which is used to capture an image of the washed melamine tiles.

To extract a value for the light intensity from the scanned images, a special designed software application is used (*Novozymes Colour Vector Analyzer*). The program retrieves the 24 bit pixel values from the image and converts them into values for red, green and blue (RGB). The intensity value (Int) is calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

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

Textiles:

Standard melamine tiles with starch such as DM-77 and DM-78 may be obtained from Center For Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands.

Terg-O-tometer (TOM) wash assay

The Tergo-To-Meter (TOM) is a medium scale model wash system that can be applied to test 12 different wash conditions simultaneously. A TOM is basically a large temperature controlled water bath with up to 12 open metal beakers submerged into it. Each beaker constitutes one small top loader style washing machine and during an experiment, each of them will comprise a solution of a specific detergent/enzyme system and the soiled and unsoiled fabrics its performance is tested on. Mechanical stress is achieved by a rotating stirring arm, which stirs the liquid within each beaker. Because the TOM beakers have no lid, it is possible to withdraw samples during a TOM experiment and assay for information on-line during wash.

The TOM model wash system is mainly used in medium scale testing of detergents and enzymes at US or LA/AP wash conditions. In a TOM experiment, factors such as the ballast to soil ratio and the fabric to wash liquor ratio can be varied. Therefore, the TOM provides the link between small scale experiments, such as AMSA and mini-wash, and the more time consuming full scale experiments in top loader washing machines.

Equipment: The water bath with 12 steel beakers and 1 rotating arm per beaker with capacity of 500 or 1200 mL of detergent solution. Temperature ranges from 5 to 80°C. The water bath has to be filled up with deionised water. Rotational speed can be set up to 70 to 120 rpm/min.

TOM wash performance

Water hardness is adjusted to the strength described below by addition of CaCl_2 , MgCl_2 and NaHCO_3 . Wash solutions are prepared with desired amount of detergent, temperature and water hardness in a bucket as described below. Detergent is dissolved during magnet stirring for 10 min.

Temperature and rotation (rpm) in the water bath in the Terg-O-Tometer is set according to the settings below. When temperature is adjusted according to settings (tolerance is +/- 0.5°C) wash solution is added to TOM beaker according to the amount described below.

5 Agitation in the beaker is at 120 rpm. 2 rice starch swatches (CS-28) and soil ballast are added to each of the beakers and wash carried out according to time stated below. Swatches are rinsed in cold tap water for 5 min. The swatches are left to dry in the dark overnight.

Textile: Textile sample CS-28 (rice starch on cotton) is obtained from Center for Test materials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands.

10 Soil ballast: Soil ballast Rice starch on cotton/polyester (EMPA 162) is obtained from Center for Test materials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands. Bistro gravy (063KC), Frij Chocolate milkshake, Heinz spaghetti (113KC), Herseys double chocolate is obtained from Warwick Equest Ltd, Unit 55, Consett Business Park, Consett, County Durham, DH8 6BN UK

15 Experimental conditions TOM

	European (EU) conditions	Northern America (US) conditions
Detergent dosage	5.77 g/L (liquid detergent)	0.78 g/L (liquid detergent)
Water hardness	15°dH (Ca ²⁺ :Mg ²⁺ :HCO ₃ ⁻ = 4:1:7.5)	6°dH (Ca ²⁺ :Mg ²⁺ :HCO ₃ ⁻ = 2:1:4.5)
Enzyme concentration in wash solution	0.25 mg enzyme protein/L	0.08 mg enzyme protein/L
Test solution volume	500 ml	800 ml
Wash time	30 minutes	18 minutes
Rotation	120 rpm	
pH	as is	
Temperature	15°C	

Detergents and test materials may be as follows:

Laundry liquid detergent	May be as described below
Test material	CS-28 (Rice starch on cotton)
Soil ballast	Rice starch on polyester/cotton (EMPA 162), Bistro gravy (063KC), Frij Chocolate milkshake, Heinz spaghetti (113KC), Herseys double chocolate (2 swatches of each)

The wash performance is measured as the brightness of the color of the textile washed, expressed in remission values. Remission measurements are made using a Macbeth 7000 Color Eye spectrophotometer. Each of the dry swatches is to be measured. As there is a risk of interference from the back-ground, the swatches are placed on top of 4 layers of fabric during the measurement of the remission. The remission is measured at 460 nm. The UV filter is not included. An average result for remission for the swatches is calculated.

EXAMPLES

10 Example 1: Identification of variants having alpha-amylase activity

A library of multiple substitutions in the loop spanning from amino acids 198-204 when using SEQ ID NO: 1 for numbering was designed, the amylase DNAs generated by Slonomics Technology using SEQ ID NO: 2 as starting point, and the amylase expression cassette was transformed into *B.subtilis* to provide a library. Clones expressing significant amount of amylase activity using the Enzcheck substrate were isolated and the combination of substitutions identified by sequencing. Table G shows the amino acid sequence of amino acids 198-204 for variants of SEQ. ID NO 2 (using SEQ ID NO: 1 for numbering).

Table G: amino acid sequence of amino acids 198-204 using SEQ ID NO: 1 for numbering

YDYLLFA	YDWLLYA	YDWLLFA	YDWLLPA	YDYLLIA
YDYLLNA	YDYLLPA	YDYQLYA	YDPLLYA	YDYLLTA
YDYLLWA	YDNLLYA	YDQLLLA	YDQLLYA	YDWLLWA
YDYLLVA	YDQLLPA	YDQLLWA	YDYLLHA	YDYLLLA
YDYLLSA	YDYPLYA	YDYQPAA	YDELLYA	YDKLLPA
YDQLLNA	YDYELYA	YDYHLYA	YDYLLDA	YDYLPRA
YDYQLLA	YDYQLPA	YDYQLQA	YDDLLYA	YDKLLYA
YDWLLHA	YDWLLTA	YDWLPSA	YDWQLYA	YDYGLYA
YDYLLFA	YDYLLQA	YDYPLPA	YDYRLYA	YDIELSA
YDQLLIA	YDQLLSA	YDWLGYA	YDWLLAA	YDWQLHA
YDWLWPA	YDYELLA	YDYLFTA	YDYLLKA	YDYLYSA
YDYQLFA	YDYQYYA	YDYTLYA	YDYLYYA	YDIELWA
YDIELYA	YDNLLNA	YDNLLPA	YDPLLHA	YDQLLVA
YDQLPYA	YDWLLRA	YDWLWYA	YDWLWGA	YDYHLIA
YDYQHYA	YDYQLGA	YDYQLIA	YDYWLPA	YDELLWA
YDHLLNA	YDIELLA	YDIELNA	YDIELRA	YDILLYA
YDKLLWA	YDLPLYA	YDNLLLA	YDPLLAA	YDPLLPA
YDQHLPA	YDQLLEA	YDQLLQA	YDQLNYA	YDQLPFA
YDQLPNA	YDQLPRA	YDTLLLA	YDTLLYA	YDVLLYA
YDWLLKA	YDWLLLA	YDWLLNA	YDWLLQA	YDWLLVA
YDWLPPA	YDWLPTA	YDWPWYA	YDWLWLA	YDYHLFA
YDYHPSA	YDYLLNT	YDYLLYT	YDYLPFA	YDYLPFA
YDYLVSA	YDYLYPA	YDYLYRA	YDYPLFA	YDYPLSA
YDYPLTA	YDYQYYA	YDYQLTA	YDYQLWA	YDYQNYA
YDYQPRA	YDYQSHA	YDYREYA	YDYRLPA	YDYRNSA
YDYRPRA	YDYTQYA	YDYVLYA	YDYWLSA	YDDLLSA

YDELLDA	YDELLPA	YDELLTA	YDEQLEA	YDEQLYA
YDGLPHA	YDIELFA	YDIELKA	YDIELPA	YDKLLNA
YDKPPSA	YDLLLFA	YDLPLLA	YDNLLKA	YDPLKFA
YDPLLEA	YDPLLFA	YDPLLKA	YDPLLWA	YDPPLPA
YDPPLYA	YDPTLPA	YDPTQYA	YDQELPA	YDQLDHA
YDQLEYA	YDQLLDA	YDQLLFA	YDQLLNT	YDQLLYS
YDQLLYT	YDQLPSA	YDQLWYA	YDQQLVA	YDTLLWA
YDTPLFA	YDTPLYA	YDWELYA	YDWHLYA	YDWLHSA
YDWLLEA	YDWLLIA	YDWLLSA	YDWLNIA	YDWLPFA
YDWLPRA	YDWLQPA	YDWLQYA	YDWLWGA	YDWPLHA
YDWQLRA	YDWQLTA	YDWSLPA	YDWSLYA	YDWWLYA
YDYELEA	YDYELNA	YDYGLAA	YDYHEWA	YDYHLPA
YDYHLSA	YDYHQYA	YDYHTSA	YDYLFQA	YDYHLHA
YDYLIEA	YDYLLFT	YDYLLRA	YDYLLYG	YDYLPDA
YDYLPQA	YDYLPWA	YDYLQEA	YDYPGYA	YDYPHSA
YDYPLLA	YDYPLNA	YDYPNYA	YDYPSRA	YDYPWYA
YDYQEYA	YDYQLAA	YDYQLKA	YDYQLPT	YDYQPTA
YDYQPYA	YDYQQYA	YDYQWYA	YDYRLFA	YDYRPSA
YDYRTFA	YDYRTSA	YDYRTYA	YDYSLYA	YDYSVYA
YDYTPRA	YDYWLFA	YDYWLGA	YDYWLWA	YDYWLYA
YDDLILA	YDDLINA	YDDLPIA	YDEHLHA	YDELLFA
YDELLSA	YDELQIA	YDEWPIA	YDGLLSA	YDHLLYA
YDIELHA	YDIELKT	YDIELTA	YDIEVSA	YDIPLYA
YDIRGYA	YDIRNYA	YDIRTKA	YDIWLYA	YDKLPHA
YDKLQYA	YDKPLSA	YDLLLVA	YDNHLPA	YDNHLYA
YDNLGYA	YDNLLIA	YDNLLVA	YDNLLWA	YDNLPRP
YDNQLYA	YDNRLYA	YDPRRHA	YDPLHVA	YDPLLDA
YDPLLLA	YDPLQYA	YDPPQFA	YDPLIA	YDQELYA
YDQLFSA	YDQLKYA	YDQLLAA	YDQLLHA	YDQLNNA
YDQLPAA	YDQLPPA	YDQLQNA	YDQLWGT	YDQLWPA
YDQLYPA	YDQNLIA	YDQPLPA	YDQQLQA	YDQTLYA
YDQWLHA	YDQWLTA	YDRLLPA	YDSELYA	YDTLIRA
YDTLLKA	YDTLLNA	YDTPLNA	YDTPLPA	YDTPQIA
YDTRLYA	YDTSLPA	YDTTLP	YDTWKYA	YDVLLPA
YDVLNTA	YDWELIA	YDWHLPA	YDWHQYA	YDWHSHA
YDWHNTA	YDWLHHA	YDWLHIA	YDWNWA	YDWLPAA
YDWLPGA	YDWLPIA	YDWLQVA	YDWLTPA	YDWLTQA
YDWNLSA	YDWNWYA	YDWPQYA	YDWPLIA	YDWPLVA
YDWPLYA	YDWPTYA	YDWQLIA	YDWQLLA	YDWQLNA
YDWWLDA	YDYDLIA	YDYEKYA	YDYELIA	YDYELPA
YDYELTA	YDYELWA	YDYGWPA	YDYGWYA	YDYHENA
YDYHHEA	YDYHIEA	YDYHLQA	YDYHPRA	YDYHTIA
YDYHTPA	YDYHTYA	YDYLFPA	YDYLHWA	YDYLIRA
YDYLNDA	YDYLNPA	YDYLNQA	YDYLPEA	YDYLPHA
YDYLPPIA	YDYLPPLA	YDYLPPTA	YDYLQNA	YDYLQRA
YDYLRQA	YDYLWGA	YDYLWLA	YDYLWPA	YDYPEPA
YDYPIIDA	YDYPIRA	YDYPLAA	YDYPLQA	YDYPPRA
YDYPQHA	YDYPSYA	YDYPTAA	YDYPTDA	YDYQHRA
YDYQHSA	YDYQIYA	YDYQLEA	YDYQLLT	YDYQNPA
YDYQQNA	YDYQQSA	YDYQTVA	YDYQWPA	YDYQYRA
YDYRHTA	YDYRLNA	YDYRQYA	YDYRRSA	YDYRSDA
YDYSGYA	YDYSNYA	YDYSTFA	YDYTEYA	YDYTLISA
YDYTQSA	YDYWLEA	YDYWLGT	YDYWLHA	YDYWLLA

YDYWLTA	YDYWPEA	YDYWPRA	YDYYLRA	
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Example 2: Test of amylase activity

5 The generated variants were tested in the above-described pNP-G7 assay in order to determine the amylase activity after binding to an antibody.

10 The antibody was diluted in Phosphate buffered saline (PBS) (0.010 M Phosphate buffer pH7.4, 0.0027M KCl, 0.14M NaCl) buffer to a concentration of 10µg/ml. A maxisorp microtiter plate was coated with antibody by adding 100µl diluted antibody (10µg/ml) to each well and incubated for 1h at room temperature (RT) followed by mixing at 800 rpm. After incubation the microtiter plate was washed (using Bio-Tek ELx405 ELISA washer) with 3x 200µl Phosphate buffered saline with 0.05% Tween (PBST) (0.010 M Phosphate buffer pH7.4, 0.0027M KCl, 0.14M NaCl, 0.05% Tween 20) buffer.

15 Microtiter plates with amylase variants culture broths were spun down and supernatants transferred to new microtiter plates and diluted 4x in PBST buffer. 100µl diluted supernatant was transferred to the antibody coated maxisorp microtiter plate and incubated for 1h at RT and mixing at 800rpm. After incubation microtiter plates were washed in PBST buffer (3x 200µl, ELISA washer).

20 The amylase activity of the amylase variants bound to the antibody was measured by addition of 100µl pNP-G7 substrate to all wells and mixed for 1 minute before absorbance at 405nm was measured. The slope (absorbance per minute) was determined and only the linear range of curve was used.

Results were compared to a reference sample and samples with higher activity than the reference sample indicates an improved specific activity on the pNP-G7 substrate under the tested conditions.

25

Table H; Results of specific activity assay

Variants of SEQ ID NO: 2 (SEQ ID NO: 1 numbering)	Average Activity	Improvement factor (IF)
SEQ ID NO: 2+ M202L (reference)	18,22	1
SEQ ID NO: 2 + Y200Q+M202L+A204S	18,91	1,06
SEQ ID NO: 2 + M202L+Y203L	22,80	1,22
SEQ ID NO: 2 + Y200H+M202L+Y203N	26,08	1,38

30 The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of

the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of
5 conflict, the present disclosure including definitions will control.

CLAIMS

1. An alpha-amylase variant comprising:
- 5 a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and
- b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203 and A204 of the mature polypeptide of SEQ ID NO: 1, and
- c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1,
- 10 wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity.
2. The variant of claim 1, wherein the substitution b) is selected from one or more of
- 15 Y198FLHQ, Y200FLSCWPHQRIMTNKVADEG, L201FSYCWPHQRIMTNKVADEG, Y203FLSCWPHQRIMTNKVADEG, A204IMTSRVG.
3. The variant of claims 1 or 2, wherein the substitution c) is any of
- 20 M202FLSYCWPHQRITNKVADEG.
4. The variant of any of the preceding claims, wherein the substitutions b) and/or c) is selected from one or more of: Y200H, Y200Q, M202L, Y203N, Y203L, A204S, Y200H+M202L, Y200Q+M202L, M202L+Y203N, M202L+Y203L, M202L+A204S, Y200H+M202L+Y203N, Y200H+M202L+Y203L, Y200H+M202L+A204S, Y200Q+M202L+Y203N,
- 25 Y200Q+M202L+Y203L, Y200Q+M202L+A204S, Y200H+M202L+Y203N+A204S, Y200H+M202L+Y203L+A204S, Y200Q+M202L+Y203N+A204S, Y200Q+M202L+Y203L+A204S.
5. The variant of any of the preceding claims, the variant comprising the alterations
- 30 selected from the list consisting of:
- R181*+G182*+Y200H+M202L, R181*+G182*+Y200Q+M202L, R181*+G182*+M202L+Y203N, R181*+G182*+M202L+Y203L, R181*+G182*+M202L+A204S,
- R181*+G182*+Y200H+M202L+Y203N, R181*+G182*+Y200H+M202L+Y203L,
- R181*+G182*+Y200H+M202L+A204S, R181*+G182*+Y200Q+M202L+Y203N,
- 35 R181*+G182*+Y200Q+M202L+Y203L, R181*+G182*+Y200Q+M202L+A204S,
- R181*+G182*+Y200H+M202L+Y203N+A204S, R181*+G182*+Y200H+M202L+Y203L+A204S,
- R181*+G182*+Y200Q+M202L+Y203N+A204S, R181*+G182*+Y200Q+M202L+Y203L+A204S.

- R181*+D183*+Y200H+M202L, R181*+D183*+Y200Q+M202L, R181*+D183*+M202L+Y203N,
R181*+D183*+M202L+Y203L, R181*+D183*+M202L+A204S,
- R181*+D183*+Y200H+M202L+Y203N, R181*+D183*+Y200H+M202L+Y203L,
R181*+D183*+Y200H+M202L+A204S, R181*+D183*+Y200Q+M202L+Y203N,
- 5 R181*+D183*+Y200Q+M202L+Y203L, R181*+D183*+Y200Q+M202L+A204S,
R181*+D183*+Y200H+M202L+Y203N+A204S, R181*+D183*+Y200H+M202L+Y203L+A204S,
R181*+D183*+Y200Q+M202L+Y203N+A204S, R181*+D183*+Y200Q+M202L+Y203L+A204S.
- G182*+G184*+Y200H+M202L, G182*+G184*+Y200Q+M202L, G182*+G184*+M202L+Y203N,
G182*+G184*+M202L+Y203L, G182*+G184*+M202L+A204S,
- 10 G182*+G184*+Y200H+M202L+Y203N, G182*+G184*+Y200H+M202L+Y203L,
G182*+G184*+Y200H+M202L+A204S, G182*+G184*+Y200Q+M202L+Y203N,
G182*+G184*+Y200Q+M202L+Y203L, G182*+G184*+Y200Q+M202L+A204S,
G182*+G184*+Y200H+M202L+Y203N+A204S, G182*+G184*+Y200H+M202L+Y203L+A204S,
G182*+G184*+Y200Q+M202L+Y203N+A204S, G182*+G184*+Y200Q+M202L+Y203L+A204S.
- 15 D183*+G184*+Y200H+M202L, D183*+G184*+Y200Q+M202L, D183*+G184*+M202L+Y203N,
D183*+G184*+M202L+Y203L, D183*+G184*+M202L+A204S,
D183*+G184*+Y200H+M202L+Y203N, D183*+G184*+Y200H+M202L+Y203L,
D183*+G184*+Y200H+M202L+A204S, D183*+G184*+Y200Q+M202L+Y203N,
D183*+G184*+Y200Q+M202L+Y203L, D183*+G184*+Y200Q+M202L+A204S,
- 20 D183*+G184*+Y200H+M202L+Y203N+A204S, D183*+G184*+Y200H+M202L+Y203L+A204S,
D183*+G184*+Y200Q+M202L+Y203N+A204S, D183*+G184*+Y200Q+M202L+Y203L+A204S
N195F+M202L+R181*+G182*, N195F+M202L+R181*+D183*, N195F+M202L+R181*+G184*,
N195F+M202L+G182*+D183*, N195F+M202L+D183*+G184*, N195Y+M202L+R181*+G182*,
N195Y+M202L+R181*+D183*, N195Y+M202L+R181*+G184*, N195Y+M202L+G182*+D183*,
25 N195Y+M202L+D183*+G184* R181*+G182*+N195F+Y200H+M202L+Y203N,
R181*+G182*+N195F+Y200Q+M202L+A204S, R181*+G182*+N195F+M202L+Y203L,
G182*+G184*+N195F+Y200H+M202L+Y203N, G182*+G184*+N195F+Y200Q+M202L+A204S,
G182*+G184*+N195F+M202L+Y203L, G182*+D183*+N195F+Y200H+M202L+Y203N,
G182*+D183*+N195F+Y200Q+M202L+A204S, G182*+D183*+N195F+M202L+Y203L,
- 30 R181*+D184*+N195F+Y200H+M202L+Y203N, R181*+D184*+N195F+Y200Q+M202L+A204S,
and R181*+D184*+N195F+M202L+Y203L.

6. The variant of any of the preceding claims, where the alterations consists of the alterations selected from the list consisting of:

- 35 R181*+G182*+Y200H+M202L, R181*+G182*+Y200Q+M202L, R181*+G182*+M202L+Y203N,
R181*+G182*+M202L+Y203L, R181*+G182*+M202L+A204S,
R181*+G182*+Y200H+M202L+Y203N, R181*+G182*+Y200H+M202L+Y203L,

- R181*+G182*+Y200H+M202L+A204S, R181*+G182*+Y200Q+M202L+Y203N,
- R181*+G182*+Y200Q+M202L+Y203L, R181*+G182*+Y200Q+M202L+A204S,
- R181*+G182*+Y200H+M202L+Y203N+A204S, R181*+G182*+Y200H+M202L+Y203L+A204S,
- R181*+G182*+Y200Q+M202L+Y203N+A204S, R181*+G182*+Y200Q+M202L+Y203L+A204S.
- 5 R181*+D183*+Y200H+M202L, R181*+D183*+Y200Q+M202L, R181*+D183*+M202L+Y203N,
- R181*+D183*+M202L+Y203L, R181*+D183*+M202L+A204S,
- R181*+D183*+Y200H+M202L+Y203N, R181*+D183*+Y200H+M202L+Y203L,
- R181*+D183*+Y200H+M202L+A204S, R181*+D183*+Y200Q+M202L+Y203N,
- R181*+D183*+Y200Q+M202L+Y203L, R181*+D183*+Y200Q+M202L+A204S,
- 10 R181*+D183*+Y200H+M202L+Y203N+A204S, R181*+D183*+Y200H+M202L+Y203L+A204S,
- R181*+D183*+Y200Q+M202L+Y203N+A204S, R181*+D183*+Y200Q+M202L+Y203L+A204S.
- G182*+G184*+Y200H+M202L, G182*+G184*+Y200Q+M202L, G182*+G184*+M202L+Y203N,
- G182*+G184*+M202L+Y203L, G182*+G184*+M202L+A204S,
- G182*+G184*+Y200H+M202L+Y203N, G182*+G184*+Y200H+M202L+Y203L,
- 15 G182*+G184*+Y200H+M202L+A204S, G182*+G184*+Y200Q+M202L+Y203N,
- G182*+G184*+Y200Q+M202L+Y203L, G182*+G184*+Y200Q+M202L+A204S,
- G182*+G184*+Y200H+M202L+Y203N+A204S, G182*+G184*+Y200H+M202L+Y203L+A204S,
- G182*+G184*+Y200Q+M202L+Y203N+A204S, G182*+G184*+Y200Q+M202L+Y203L+A204S.
- D183*+G184*+Y200H+M202L, D183*+G184*+Y200Q+M202L, D183*+G184*+M202L+Y203N,
- 20 D183*+G184*+M202L+Y203L, D183*+G184*+M202L+A204S,
- D183*+G184*+Y200H+M202L+Y203N, D183*+G184*+Y200H+M202L+Y203L,
- D183*+G184*+Y200H+M202L+A204S, D183*+G184*+Y200Q+M202L+Y203N,
- D183*+G184*+Y200Q+M202L+Y203L, D183*+G184*+Y200Q+M202L+A204S,
- D183*+G184*+Y200H+M202L+Y203N+A204S, D183*+G184*+Y200H+M202L+Y203L+A204S,
- 25 D183*+G184*+Y200Q+M202L+Y203N+A204S, D183*+G184*+Y200Q+M202L+Y203L+A204S
- N195F+M202L+R181*+G182*, N195F+M202L+R181*+D183*, N195F+M202L+R181*+G184*,
- N195F+M202L+G182*+D183*, N195F+M202L+D183*+G184*, N195Y+M202L+R181*+G182*,
- N195Y+M202L+R181*+D183*, N195Y+M202L+R181*+G184*, N195Y+M202L+G182*+D183*,
- N195Y+M202L+D183*+G184* R181*+G182*+N195F+Y200H+M202L+Y203N,
- 30 R181*+G182*+N195F+Y200Q+M202L+A204S, R181*+G182*+N195F+M202L+Y203L,
- G182*+G184*+N195F+Y200H+M202L+Y203N, G182*+G184*+N195F+Y200Q+M202L+A204S,
- G182*+G184*+N195F+M202L+Y203L, G182*+D183*+N195F+Y200H+M202L+Y203N,
- G182*+D183*+N195F+Y200Q+M202L+A204S, G182*+D183*+N195F+M202L+Y203L,
- R181*+D184*+N195F+Y200H+M202L+Y203N, R181*+D184*+N195F+Y200Q+M202L+A204S,
- 35 and R181*+D184*+N195F+M202L+Y203L.

7. The variant of any of the preceding claims, the variant comprising an amino acid sequence of the positions corresponding to positions 198-204 of SEQ ID NO: 1 selected from the list consisting of: YDYLLFA, YDWLLYA, YDWLLFA, YDWLLPA, YDYLLIA, YDYLLNA, YDYLLPA, YDYQLYA, YDPLLYA, YDYLLTA, YDYLLWA, YDNLLYA, YDQLLA, YDQLLYA, 5 YDWLLWA, YDYLLVA, YDQLLPA, YDQLLWA, YDYLLHA, YDYLLLA, YDYLLSA, YDYPLYA, YDYQPAA, YDELLYA, YDKLLPA, YDQLLNA, YDYELYA, YDYHLYA, YDYLLDA, YDYLPRA, YDYQLLA, YDYQLPA, YDYQLQA, YDDLLYA, YDKLLYA, YDWLLHA, YDWLLTA, YDWLPSA, YDWQLYA, YDYGLYA, YDYLLA, YDYLLQA, YDYPLPA, YDYRLYA, YDIELSA, YDQLLIA, YDQLLSA, YDWLGYA, YDWLLAA, YDWQLHA, YDWWLPA, YDYELLA, YDYLFPA, YDYLLKA, 10 YDYLYSA, YDYQLFA, YDYQYYA, YDYTYA, YDYLYA, YDIELWA, YDIELYA, YDNLLNA, YDNLLPA, YDPLLHA, YDQLLVA, YDQLPYA, YDWLLRA, YDWLWYA, YDWWLGA, YDYHLIA, YDYQHYA, YDYQLGA, YDYQLIA, YDYWLPA, YDELLWA, YDHLLNA, YDIELLA, YDIELNA, YDIELRA, YDILLYA, YDKLLWA, YDLPLYA, YDNLLLA, YDPLLA, YDPLPA, YDQHLPA, YDQLLEA, YDQLLQA, YDQLNYA, YDQLPFA, YDQLPNA, YDQLPRA, YDTLLA, YDTLLYA, 15 YDVLLYA, YDWLLKA, YDWLLA, YDWLLNA, YDWLLQA, YDWLLVA, YDWLPPA, YDWLPTA, YDWPWYA, YDWWLWA, YDYHLFA, YDYHPSA, YDYLLNT, YDYLLYT, YDYLPFA, YDYLPWA, YDYLVSA, YDYLYPA, YDYLYRA, YDYPLFA, YDYPLSA, YDYPLTA, YDYQYA, YDYQLTA, YDYQLWA, YDYQNYA, YDYQPRA, YDYQSHA, YDYREYA, YDYRLPA, YDYRNSA, YDYRPA, YDYTQYA, YDYVLYA, YDYWLSA, YDDLLSA, YDELLDA, YDELLPA, YDELLTA, 20 YDEQLEA, YDEQLYA, YDGLPHA, YDIELFA, YDIELKA, YDIELPA, YDKLLNA, YDKPPSA, YDLLLFA, YDLPLLA, YDNLLKA, YDPLKFA, YDPLLEA, YDPLLFA, YDPLLKA, YDPLLWA, YDPPLPA, YDPPLYA, YDPTLPA, YDPTQYA, YDQELPA, YDQLDHA, YDQLEYA, YDQLLDA, YDQLLFA, YDQLLNT, YDQLLYS, YDQLLYT, YDQLPSA, YDQLWYA, YDQQLVA, YDTLLWA, YDTPLFA, YDTPLYA, YDWELYA, YDWHLYA, YDWLHSA, YDWLLEA, YDWLLIA, YDWLLSA, 25 YDWLNIA, YDWLPFA, YDWLPRA, YDWLQPA, YDWLQYA, YDWLWGA, YDWPLHA, YDWQLRA, YDWQLTA, YDWSLPA, YDWSLYA, YDWWLYA, YDYELEA, YDYELNA, YDYGLAA, YDYHEWA, YDYHLPA, YDYHLSA, YDYHQYA, YDYHTSA, YDYLFQA, YDYLHLA, YDYLIEA, YDYLLFT, YDYLLRA, YDYLLYG, YDYLPDA, YDYLPQA, YDYLPWA, YDYLQEA, YDYPGYA, YDYPHSA, YDYPLA, YDYPLNA, YDYPNYA, YDYPSRA, YDYPWYA, YDYQEYA, 30 YDYQLAA, YDYQLKA, YDYQLPT, YDYQPTA, YDYQPYA, YDYQQYA, YDYQWYA, YDYRLFA, YDYRPSA, YDYRTFA, YDYRTSA, YDYRTYA, YDYSLYA, YDYSVYA, YDYTPRA, YDYWLFA, YDYWLGA, YDYWLWA, YDYWLYA, YDLLLLA, YDPLLNA, YDDLPA, YDEHLHA, YDELLFA, YDELLSA, YDELQIA, YDEWPYA, YDGLLSA, YDHLLYA, YDIELHA, YDIELKT, YDIELTA, YDIEVSA, YDIPLYA, YDIRGYA, YDIRNYA, YDIRTKA, YDIWLYA, YDKLPHA, 35 YDKLQYA, YDKPLSA, YDLLLVA, YDNHLPA, YDNHLYA, YDNLGYA, YDNLLIA, YDNLLVA, YDNLLWA, YDNLPA, YDNLQYA, YDNLRYA, YDPLRHA, YDPLHVA, YDPLLDA, YDPLLLA, YDPLQYA, YDPPQFA, YDPQLIA, YDQELYA, YDQLFSA, YDQLKYA, YDQLLAA, YDQLLHA,

YDQLNNA, YDQLPAA, YDQLPPA, YDQLQNA, YDQLWGT, YDQLWPA, YDQLYPA,
 YDQNLQA, YDQPLPA, YDQQLQA, YDQTLQA, YDQWLHA, YDQWLTA, YDRLLPA, YDSELYA,
 YDTLIRA, YDTLLKA, YDTLLNA, YDTPLNA, YDTPLPA, YDTPQIA, YDTRLYA, YDTSLPA,
 YDTTLPA, YDTWKYA, YDVLLPA, YDVLNTA, YDWELIA, YDWHLPA, YDWHQYA, YDWHSHA,
 5 YDWHTQA, YDWLHHA, YDWLHHA, YDWLHHA, YDWLNWA, YDWLPAA, YDWLPGA, YDWLPIA,
 YDWLQVA, YDWLTPA, YDWLTQA, YDWNLSA, YDWNWYA, YDWPGYA, YDWPLIA,
 YDWPLVA, YDWPLYA, YDWPTYA, YDWQLIA, YDWQLLA, YDWQLNA, YDWWLDA,
 YDYDLQA, YDYEKYA, YDYELIA, YDYELPA, YDYELTA, YDYELWA, YDYGWPA, YDYGWYA,
 YDYHENA, YDYHHEA, YDYHIEA, YDYHLQA, YDYHPRA, YDYHTIA, YDYHTPA, YDYHTYA,
 10 YDYLFPA, YDYLHWA, YDYLIRA, YDYLNDA, YDYLNPA, YDYLNQA, YDYLPEA, YDYLPHA,
 YDYLPPIA, YDYLPPLA, YDYLPPTA, YDYLQNA, YDYLQRA, YDYLRQA, YDYLWGA, YDYLWLA,
 YDYLWPA, YDYPEPA, YDYPIPA, YDYPIRA, YDYPLAA, YDYPLQA, YDYPPRA, YDYPPHA,
 YDYPSYA, YDYPTAA, YDYPTDA, YDYQHRA, YDYQHSA, YDYQIYA, YDYQLEA, YDYQLLA,
 YDYQNPA, YDYQQNA, YDYQQSA, YDYQTVQA, YDYQWPA, YDYQYRA, YDYRHTA,
 15 YDYRLNA, YDYRQYA, YDYRRA, YDYRSDA, YDYSGYA, YDYSNYA, YDYSTFA, YDYTEYA,
 YDYTLQA, YDYTQSA, YDYWLEA, YDYWLGT, YDYWLHA, YDYWLLA, YDYWLTA,
 YDYWPEA, YDYWPRA, YDYYLRA.

8. The variant of any of the preceding claims, wherein the deletion a) is selected from the
 20 list consisting of R181*+G182*, R181*+D183*, R181*+G184*, G182*+D183*, G182*+G184*,
 and D183*+G184*, preferably R181*+G182*.

9. The variant of any of the preceding claims, wherein the alteration b) is at two or more of
 said positions, such as three or more of said positions, four or more of said positions, five or
 25 more of said positions, six or more of said positions..

10. The variant of any of the preceding claims, wherein the number of alterations is 2-20,
 e.g., 2-10 and 2-5, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 alterations.

30 11. The variant of any of the preceding claims, which has at least 85%, at least 86%, at least
 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least
 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less
 than 100%, sequence identity to the amino acid sequence of SEQ ID NO: 1.

35 12. The variant of any of the preceding claims, which has at least 85%, at least 86%, at least
 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least
 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99%, but less
 than 100%, sequence identity, to the amino acid sequence of SEQ ID NO: 2.

13. The variant of any of the preceding claims, which is a variant of a parent alpha-amylase selected from the group consisting of:
- a. a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 1;
 - 5 b. a polypeptide having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 2;
 - c. a fragment of the mature polypeptide of SEQ ID NO: 1, which has alpha-amylase activity;
 - d. a fragment of the mature polypeptide of SEQ ID NO: 2, which has alpha-amylase
10 activity;
 - e. a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 1;
 - f. a polypeptide having immunological cross reactivity with an antibody raised against the mature polypeptide of SEQ ID NO: 2.
- 15
14. The variant of any of the preceding claims wherein the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 1.
- 20
15. The variant of any of the preceding claims, wherein the parent alpha-amylase has at least 85%, such as at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95% identity, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 2.
- 25
16. The variant of any of the preceding claims, wherein the parent alpha-amylase comprises or consists of the mature polypeptide of SEQ ID NO: 1.
17. The variant of any of the preceding claims wherein the parent alpha-amylase comprises
30 or consists of the mature polypeptide of SEQ ID NO: 2.
18. The variant of any of the preceding claims, which has an improved property relative to the parent, wherein the improved property is selected from the group consisting of catalytic efficiency, catalytic rate, chemical stability, oxidation stability, pH activity, pH stability, specific
35 activity, stability under storage conditions, substrate binding, substrate cleavage, substrate specificity, substrate stability, surface properties, thermal activity, thermo stability, and preferably improved washing performance at low temperature.

19. The variant of any of the preceding claims, wherein the variant has improved alpha-amylase activity compared to the alpha-amylase of SEQ ID NO: 1 or 2.
- 5 20. A detergent composition comprising a variant alpha-amylase of any of the claims 1-19.
21. A dish wash composition comprising a variant alpha-amylase of any of the claims 1-19.
22. Use of a variant alpha-amylase according to any of the claims 1-19 in a cleaning process
10 such as laundry or hard surface cleaning including automated dish wash.
23. An isolated polynucleotide encoding the variant of any of claims 1-19.
24. A nucleic acid construct comprising the polynucleotide of claim 23.
15
25. An expression vector comprising the polynucleotide of claim 23.
26. A host cell comprising the polynucleotide of claim 23.
- 20 27. A method of producing an alpha-amylase variant, comprising:
a) cultivating the host cell of claim 26 under conditions suitable for expression of the variant; and
b) recovering the variant.
- 25 28. A method for obtaining an alpha-amylase variant, comprising introducing into a parent alpha-amylase having at least 80% sequence identity to SEQ ID NO: 1:
a) a deletion at two or more positions corresponding to positions R181, G182, D183 and G184 of the mature polypeptide of SEQ ID NO: 1, and
b) a substitution at one or more positions corresponding to positions Y198, Y200, L201, Y203 and A204 of the mature polypeptide of SEQ ID NO:
30 1, and
c) a substitution of the methionine at the position corresponding to position M202 of the mature polypeptide of SEQ ID NO: 1,
wherein the variant has at least 80%, such as at least 90%, but less than 100% sequence
35 identity with the mature polypeptide of SEQ ID NO: 1 or 2, and wherein the variant has alpha-amylase activity; and recovering the variant.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063135

A. CLASSIFICATION OF SUBJECT MATTER
INV. C11D3/386 C12N9/28
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C11D C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/001087 A2 (NOVOZYMES AS [DK]; KAASGAARD SVEND [DK]; LARSEN SIGNE ESKILDSEN [DK];) 3 January 2013 (2013-01-03) cited in the application claims 10,11; sequences 1,4	1-28
A	WO 00/60058 A2 (NOVO NORDISK AS [DK]) 12 October 2000 (2000-10-12) claims 1-26; sequence 4	1-28
A	WO 96/23873 A1 (NOVO NORDISK AS [DK]; BISGAARD FRANTZEN HENRIK [DK]; SVENDSEN ALLAN [D]) 8 August 1996 (1996-08-08) cited in the application claims 1-9	1-28
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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"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

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Date of the actual completion of the international search 8 October 2015	Date of mailing of the international search report 20/10/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer van Klompenburg, Wim

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063135

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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