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(54) Title: METHOD FOR SCREENING ALPHA-AMYLASES

(57) Abstract: The present invention relates to variants of a parent alpha-amylase.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.

METHOD FOR SCREENING ALPHA-AMYLASES

Reference to a Sequence Listing

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

Background of the Invention

Field of the Invention

The present invention relates to methods for screening alpha-amylases having high performance at low temperature, in particular having high performance at low temperatures in detergents. The invention further relates to detergent compositions comprising such amylases.

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 oligo- and polysaccharides.

There is a long history of industrial use of alpha-amylases in several known applications such as detergent, baking, brewing, starch liquefaction and saccharification e.g. in preparation of high fructose syrups or as part of ethanol production from starch. These and other applications of alpha-amylases are known and utilize alpha-amylases derived from microorganisms, in particular bacterial alpha-amylases.

Among the first bacterial alpha-amylases to be used were an alpha-amylase from *B.licheniformis,* also known as Termamyl which have been extensively characterized and the crystal structure has been determined for this enzyme. Alkaline amylases, such as SP707, form a particular group of alpha-amylases that have found use in detergents. Many of these known bacterial amylases have been modified in order to improve their functionality in a particular application.

Methods of increasing the thermostability of alpha-amylases have been well studied. Suzuki et al. (1989) disclose chimeric alpha-amylases, in which specified regions of a *B. amyloliquefaciens* alpha-amylase have been substituted for the corresponding regions of a *B. licheniformis* alpha-amylase. The chimeric alpha-amylases were constructed with the purpose of identifying regions responsible for thermostability. Such regions were found to include amino acid residues 177-186 and amino acid residues 255-270 of the *B. amyloliquefaciens* alpha-amylase. Igarashi et al. 1998 show that the thermostability of AmyS-type amylases can be increased by the deletion of two amino acid residues, R179-G180, (AmyS numbering) from a loop (F 178 to A184). However, Shiau et al. (2003) showed that an AmyS enzyme with deletion in the same loop has a lower specific activity for corn starch hydrolysis at high-temperature than the parent enzyme, negating one of the principal advantages of AmyS amylases.

For environmental reasons it has been increasingly important to lower the temperature in washing, dishwashing and/or cleaning processes. However, most enzymes including amylases have a temperature optimum which is above the temperature usually used in low temperature washing. 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. Therefore, it is important to find alpha-amylase variants, which retain their wash performance, stain removal effect and/or activity when the temperature is lowered. However, despite the efficiency of current detergents enzyme compositions, there are many stains that are difficult to completely remove. These problems are compounded by the increased use of low (e.g., cold water) wash temperatures and shorter washing cycles. Thus, it is desirable to have amylolytic enzymes that can function under low temperature and at the same time preserve or increase other desirable properties such as specific activity (amylolytic activity), stability and/or wash performance.

Thus, it is an object of the present invention to provide improved methods for screening alpha-amylases and variants which have high performance, in particular high wash performance, at low temperatures.

Summary of the Invention

In a first aspect the present invention relates to a method for screening alpha-amylases having high performance, in particular high wash performance, at low temperatures, comprising the steps of

a) Determining the binding of the enzymes to solid or immobilized substrate for the alpha-amylase, such as starch,
b) Selecting alpha-amylases having a low affinity to the substrate, in particular having a low ratio between binding to the substrate and activity.

In a further aspect the invention relates to a method for selecting variants of a parent alpha-amylase comprising the steps of

a) Generating variants by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase;
b) Testing the variants for the binding to solid or immobilized substrate for the alpha-amylase, such as starch;
c) Selecting variants having a lower binding the substrate than the parent alpha-amylase.

In a further aspect the invention relates to variants that can be selected based on the claimed method and detergent compositions comprising an alpha-amylase selected using the method of the invention.

Detailed Description of the Invention

The invention is based on the observation that there exists an inverse correlation between the binding of an amylase to raw starch and low temperature performance, in particular wash performance at low temepratures. For example using data for a number of experimental detergent amylase samples a clear correlation between binding to rice starch and wash performance in AMSA testing. Thus it has surprisingly been found that alpha-amylases having a low binding to a solid substrate have a high performance at low temperature, in particular a high performance in detergents at low temperatures.

Definitions

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.

Substrate binding: The term “Substrate binding“or “binding to a solid substrate” or grammatically equivalent terms are understood as the property of an alpha-amylase to bind to a solid substrate including a substrate immobilized to a solid material. The term is a relative term and it is in general expressed as a relative binding in comparison with a reference alpha-amylase. For variants, substrate binding is in general measured relativety to the parent alpha-amylse that has served as starting point for the variants. Substrate binding may be measured by incubating a solution of the alpha-amylase with the solid substrate, removing the substrate and determining the fraction of the initial amount of alpha-amylase that remain attached to the solid substrate, Preferred methods for determining the substrate binding to a solid substrate is disclosed below in the Materials and Methods section.

Variant: 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 (several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding 1-3 amino acids adjacent to an amino acid occupying a position.

Mutant: The term “mutant” means a polynucleotide encoding a variant.

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

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 or a variant thereof. In relation to variants the parent polypeptide is the polypeptide having exactly the same sequence as the variant except for the residues specifically altered in the variant.

Isolated variant: The term “isolated variant” means a variant that is modified by the hand of man. In one aspect, the variant is at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, and at least 90% pure, as determined by SDS-PAGE.

Substantially pure variant: The term “substantially pure variant” means a preparation that contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. Preferably, the variant is at least 92% pure, *e.g.*, at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, at least 99.5% pure, and 100% pure by weight of the total polypeptide material present in the preparation. The variants of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the variant by well known recombinant methods or by classical purification methods.

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.

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

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

For purposes of the present invention, the degree of 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 3.0.0 or later. The optional 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:

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

For purposes of the present invention, the degree of 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 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

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

Fragment: The term “fragment” means a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide; wherein the fragment has alpha-amylase activity.

Subsequence: The term “subsequence” means a polynucleotide having one or more (several) nucleotides deleted from the 5'- and/or 3'-end of a mature polypeptide coding sequence; wherein the subsequence encodes a fragment having alpha-amylase activity.

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

Isolated polynucleotide: The term “isolated polynucleotide” means a polynucleotide that is modified by the hand of man. In one aspect, the isolated polynucleotide is at least 1% pure, *e.g.*, at least 5% pure, at least 10% pure, at least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, at least 90% pure, and at least 95% pure, as determined by agarose electrophoresis. The polynucleotides may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

Substantially pure polynucleotide: The term “substantially pure polynucleotide” means a polynucleotide preparation free of other extraneous or unwanted nucleotides and in a form suitable for use within genetically engineered polypeptide production systems. Thus, a substantially pure polynucleotide contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polynucleotide material with which it is natively or recombinantly associated. A substantially pure polynucleotide may, however, include naturally occurring 5’- and 3’- untranslated regions, such as promoters and terminators. It is preferred that the substantially pure polynucleotide is at least 90% pure, *e.g.*, at least 92% pure, at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99% pure, and at least 99.5% pure by weight. The polynucleotides of the present invention are preferably in a substantially pure form.

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of its polypeptide product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

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 cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

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. The term nucleic acid construct is synonymous with the term “expression cassette” when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

Control sequences: The term “control sequences” means all components necessary for the expression of a polynucleotide encoding a variant of the present invention. Each control sequence may be native or foreign 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.

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 the expression of the coding sequence.

Expression: The term “expression” includes any step involved in the production of the 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 molecule that comprises a polynucleotide encoding a variant and is operably linked to additional nucleotides that provide for its expression.

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

Starch removing process: The expression “starch removing process” relates to any kind of process whereby starch is removed (or converted) such as in washing processes where starch is removed from textile e.g. textile cleaning such as laundry. A starch removing process could also be hard surface cleaning such as dish wash or it could be cleaning processes in general such as industrial or institutional cleaning. The expression also comprises other starch removing processes or starch conversion, ethanol production, starch liquefaction, textile desizing, paper and pulp production, beer making and detergents in general.

Improved property: The term “improved property” means a characteristic associated with a variant that is improved compared to the parent. Such improved properties include, but are not limited to, thermal activity, thermostability, pH activity, pH stability, substrate/cofactor specificity, improved surface properties, product specificity, increased stability or solubility in the presence of pretreated biomass, improved stability under storage conditions, and chemical stability.

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 intensity value (Int) defined in the description of AMSA or in the beaker wash performance test in the Methods section below.

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 an alpha-amylase having the identical amino acid sequence of said variant but not having the deletion at one or more of the specified positions or relative to the activity of an alpha-amylase having the amino acid sequence shown in SEQ ID NO 4, 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.

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.

In a first aspect the present invention relates to a method for screening alpha-amylases having high performance at low temperature, in particular having high wash performance at low termperatures, comprising the steps of

a) Determining the binding of the enzymes to solid or immobilized substrate for the alpha amylase, such as starch,
b) Selecting alpha-amylases having a low binding to the substrate, in particular alpha-amylases having a low ratio of binding to the substrate and activity.

According to this aspect of the invention relates to screening of alpha-amylases having high performance at low temperatures, in particular for screening detergent alpha-amylases having high wash performance at low temperature. The method is suitable for screening new alpha-amylases in order to find enzymes that have good performance at low temperature. The method may also with advantage be automized and adapted to a high throughput screening procedure.

In one embodiment the method is suitable for finding new alpha-amylases. One convenient way is to test a number of candidate alpha-amylases and include one known detergent alpha-amylase as a standard and then selecting new candidate alpha-amylases having a lower binding to the substrate than the standard detergent alpha-amylase. The selected alpha-amylases will be the candidates having high wash performance and can be further tested for other properties important for detergent enzymes, such as stability, specific activity etc.

Several detergent amylases are known from the literature including wild type amylases such as the alpha-amylases having SEQ ID NO: 1, 2, 3, 4, 5, 6, 8, 10, 11 and 12: variants of any of these such as the variants disclosed in WO2001066712 and WO2006002643, and any of these known detergent amylases may suitably be included in the method as a standard detergent alpha-amylase.

The alpha-amylases in this embodiment are selected to have lower binding to the substrate than the selected standard detergent amylase such as having less than 95% of the binding of the standard detergent alpha-amylase, such as less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding, more preferred less than 10% of the binding and most preferred less than 5% of the binding.

Alternatively using SP722, the alpha-amylase having SEQ ID NO: 1, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than SP722 preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using SP707, the alpha-amylase having SEQ ID NO: 2, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than SP707, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the bindingpreferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using AA560, the alpha-amylase having SEQ ID NO: 3, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than AA560 preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using SP690, the alpha-amylase having SEQ ID NO: 4, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than SP690 preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using KSM-AP1378, the alpha-amylase having SEQ ID NO: 5, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than KSM-AP1378, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using SP7-7, the alpha-amylase having SEQ ID NO: 6, as the standard detergent alpha-amylase the method may be used for selecting alpha-amylases having improved wash performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than SP7-7, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

The method of the invention may also be used with other parent alpha-amylases such as *Bacillus licheniformis* alpha-amylase (BLA), the alpha-amylase having SEQ ID NO: 13, for selecting alpha-amylases having improved performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than BLA, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using *Bacillus amyloliquefaciens* alpha-amylase (BAN), the alpha-amylase having SEQ ID NO: 14, as parent alpha-amylase the method may be used for selecting alpha-amylases having improved performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than BAN, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

Alternatively using *Bacillus stearothermophilus* (*Geobacillus stearothermophilus*) alpha-amylase (BSG), the alpha-amylase having SEQ ID NO: 15, as parent alpha-amylase the method may be used for selecting alpha-amylases having improved performance at low temperature by selecting for alpha-amylases having lower binding to the substrate than BSG, preferably less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

In a further aspect the invention relates to a method for selecting variants of a parent alpha-amylase comprising the steps of

a) Generating variants by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase;
b) Testing the variants for the binding to solid or immobilized substrate for the alpha-amylase, such as starch;
c) Selecting variants having a lower binding the substrate than the parent alpha-amylase.

The method may in principle be used on any known alpha-amylase for which it is desired to select variants having improved performance at low temperature, however, in a preferred embodiment the method of the invention is used for generating variants having improved wash performance at low temperature.

In other embodiments the method of the invention is used for finding amylases for selecting variants of a parent alpha-amylase having improved performance at low temperature in applications with the cleaning area, in applications such as saccharification or liquefaction of starch for degrading the starch to sugars that may be fermented to provide ethanol for consumption or for fuel, or in applications in textile manufacture. All such applications are known in the art and the skilled person will appreciate how the alpha-amylases selected according to the method of the invention may be applied in these applications.

In this preferred embodiment the parent alpha-amylase may be a detergent alpha-amylase i.e. an alpha-amylase having activity under conditions usually applied in cleaning and laundry, such as activity in the presence of surfactants, chelators and other components routinely used in detergents, and activity at an alkaline pH such as in the range of 8-11, such as 9-10.

The variants may be prepared by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase.

The skilled person will know how to identify residues to be modified by identifying residues located at the surface of the parent molecule. In this connection residues located on the surface of the molecule is understood as residues where at least a part of the residue are in direct contact with the surrounding medium in a natural form of the molecule. It will be appreciated that the skilled person can extract this kind of information from 3-D structures of the parent molecule in case that a 3-D structure of the parent molecule is available or in case that a 3-D structure of the parent is not available by aligning the parent alpha-amylase with an alpha-amylase for which the 3-D structure is available and identifying residues located on the surface of the molecule as residues of the parent alpha-amylase that in the alignment corresponds to residued located on the surface in the alpha-amylase. As more than one 3-D structure of alpha-amylases are known in the art the identification of residues located on the surface of a given parent alpha-amylase should be based on alignment of the parent alpha-amylase with the 3D structure for the alpha-amylase having the highest sequence identity to the parent alpha-amylase.

The variants are generated by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase of one or more amino acids residued in positions located on the surface of the parent alpha-amylase, such as substituting, deleting or inserting at least one residues located on the surface, such as at least two residues, e.g.at least three residues, e.g. at least four residues, e.g at least five residues, e.g. at least six residues, e.g. at least seven residues, e.g. at least eight residues, e.g. at least nine residues, e.g. at least ten residues, such as up to fiftheen residue e.g. up to twenty residues or up to thirty residues located on the surface of the parent alpha-amylase.

The variants may additionally contain further substitutions, deletions or insertions in one or more amino acid residue not located on the surface of the molecule. Many such substitutions, deletions or insertions have been disclosed in the prior art and may also be used according to the present invention. As examples of such preferred additional substitution, insertion or deletion can be mentioned the deletion of residues D183 and G184 in the parent alpha-amylase, as disclosed in WO96/23873.

Preferably variants are generated having at least 90% sequence identity to their parent alpha-amylase, preferably at least 95% sequence identity, more preferred at least 97 % sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity.

The variants are selected to have less binding to the substrate than the parent alpha-amylase such are having less than 95% of the binding of the parent alpha-amylase, such as less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

In one preferred embodiment the invention relates to a method for selecting variants of a parent alpha-amylase selected among alpha-amylases having SEQ ID NO: 1-15 and alpha-amylases having at least 80% sequence identity to one of these, preferably at least 85% identity, preferably at least 90% identity, more preferred at least 95% identity, more preferred at least 97% identity, comprising the steps of

a) Generating variants by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase, where the variants have at least 90% sequence identity to their parent alpha-amylase, preferably at least 95% sequence identity, more preferred at least 97%

sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity.

b) Testing the variants for the binding to solid or immobilized substrate for the alpha-amylase, such as starch; and

c) Selecting variants having a lower binding the substrate than the parent alpha-amylase, where the variants have less than 95% of the binding of the parent alpha-amylase, such as less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

In a further embodiment the invention relates to variants of a parent alpha-amylase selected among alpha-amylases having SEQ ID NO: 1-15 and alpha-amylases having at least 80% sequence identity to one of these, preferably at least 85% sequence identity, more preferred at least 87% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, and most preferred at least 97% sequence identity, wherein:

i) the variant has at least 80% sequence identity to the parent alpha-amylase, preferably at least 85%, preferably at least 87% sequence identity, preferably at least 90%, preferably at least 95%, preferably at least 97%, and

ii) The variant has lower binding to a solid substrate compared with the parent alpha-amylase such as less than 80% of the binding preferably less than 70%, preferably less than 60% preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding

Such variants may be provided using the method of the invention.

The variants of the invention also includes variants wherein one or more residues located on the surface of the parent alpha-amylases and being part of a substrate binding site have been substituted or deleted. Such substrate bindig sites present on the surface of parent alpha-amylases have been disclosed in the literature for a few parent alpha-amylases and the skilled person will appreciate that such substrate binding sites may be identified in other parent alpha-amylases by aligning a given parent alpha-amylase with another alpha-amylase sequence for which such substrate binging sites have been identified.

As examples of residues being part of a substrate binding site can be mentioned:

a) W140, W159, W167, Q169, W189, E194, N260, F262, W284, F289, G304, G305, R320, W347, W439, W469, G476 and G477 in SEQ ID NO: 2

b) W140, W159, W167, Q169, W189, E194, F262, W284, F289, G304, G305, K320, W347, W439, W469, G476 and G477 in SEQ ID NO: 5

c) W140, W159, W167, Q169, W189, E194, F262, W284, F289, G304, G305, K320, W347, W439, W469, G476 and G477 in SEQ ID NO: 4

d) W138, W157, W165, E167, W184, E189, E255, F257, F279, F284, G299, G300, K315, W342, R437, W467 and G475 in SEQ ID NO: 13,

e) W136, W155, W163, E165, W184, E189, E255, F257, F279, F284, G299, G300, R315, W342, R437, W467 and G475 in SEQ ID NO: 14

f) W139, W158, W166, E168, W187, E192, F260, F287, G302, G303, W345, W437, W467, G474 and G475 in SEQ ID NO: 15

The variants of the invention also includes variants including one or more modifications that introduce one or more bulky amino acid residues in a position that is close to a residue being part of a substrate binding site, such as the residues mentioned in a)-h) above. In this connection a bulky amino acid residue can be selected among amino acids having a large side chain, such as Tyr, Trp, Phe, His and Ile, where Tyr and Trp are preferred, Close to a residue being part of a substrate binding site is intended to mean that the modified residue is located less than 10 Å from the residue being part of a substrate binding site, preferably less than 6Å and most preferred less than 3Å from such residues. It is within the skilled persons capabilities to identify such residues based on available structure information.

Other preferred variants include variants having at least 80% sequence identity to SEQ ID NO: 2, preferably at least 85% sequence identity, more preferred at least 87% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% identity, more preferred at least 99% sequence identity, but less than 100% sequence identity and comprising the following substitutions:

H183*+G184*+W140F;

H183*+G184*+Q169N;

H183*+G184*+Q169A;

H183*+G184*+W189Y+E190P;

H183*+G184*+N260D;

H183*+G184*+G477E;

H183*+G184*+G477Q;

H183*+G184*+G477K;

H183*+G184*+W189E+E190P;

H183*+G184*+A51I+W140Y;

H183*+G184*+W140Y+W189E;
H183*+G184*+W140Y+N260P;
H183*+G184*+W140Y+W284D;
H183*+G184*+W140Y+G476R;
H183*+G184*+W140Y+G477E;
H183*+G184*+W189E+W439R;
H183*+G184*+W284D+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+W439R+G477E;
H183*+G184*+E194D;
H183*+G184*+W439R+D467K;
H183*+G184*+R320M+W439R;
H183*+G184*+W439R+K485R;
H183*+G184*+Y160S;
H183*+G184*+W189F+E190P;
H183*+G184*+F262A;
H183*+G184*+Y363H;
H183*+G184*+G476E;
H183*+G184*+N260P+W439R;
H183*+G184*+N260P+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+K72S+W140Y;
H183*+G184*+G109A+M202L+Y203G;
H183*+G184*+E194S;
H183*+G184*+E345D+G477R;
H183*+G184*+K302N+W439R;
H183*+G184*+R320K+W439R

H183*+G184*+W159Y+W167Y+F262P+W439R+W469Y+G477Q;
H183*+G184*+W159Y+W167F+N260D+W439Y+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439R+W469Y+G476K+G477E;
H183*+G184*+W159Y+W167F+F262P+W439V+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167F+F262P+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W167Y+N260D+W439R+G476Q+G477E;

H183*+G184*+W167Y+N260P+F262P+W439R+G476E+G477R;
H183*+G184*+W159Y+W167F+N260G+W439R+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260D+F262P+W439Y;
H183*+G184*+W159Y+W167F+N260P+W439Y+W469V+G476Q+G477Q;
H183*+G184*+W167Y+N260D+W439R+W469V+G476Q+G477Q;

H183*+G184*+W159Y+W167Y+N260P+F262P+W439R+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+W469Y+G476R;
H183*+G184*+W167F+N260D+F262P+P380Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439R+W469V+G476Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439V+W469Y;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W167Y+N260D+W439R+W469Y+G476E+G477K;
H183*+G184*+W159Y+W167Y+N260D+G476E+G477Q;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+G476K+G477Q;
H183*+G184*+N260D+F262P+W469Y+G476R+G477Q;
H183*+G184*+W167Y+L230I+N260P+W469Y;
H183*+G184*+W159Y+W167Y+N260P+E439Y+G476Q+G477Q;
H183*+G184*+W167Y+F262P+W469Y+G476R+G477Q.

Other preferred variants include variants consisting of SEQ ID NO: 2 with one of the above mentioned substitutions.

Another preferred variant of the invention is a variant having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% identity to SEQ ID NO: 13 and comprising a substitution in a position corresponding to K315 and/or W467 in SEQ ID NO: 13, preferably K315ANCQEGHILMFPSTWYVM, particular preferred K315M and/or W467AF. In a preferred embodiment the substitution in position K315 is combined with further substitutions, preferably selected among G48A, T51IL, G107A, H156Y; A181T, N190F, I201F, A209V and Q264S.

In a further aspect the invention relates to a detergent composition comprising an alpha-amylase selected using the method of the invention.

In a further embodiment the invention relates to a detergent composition comprising an variant alpha-amylase characterized in:

i) having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% identity to one of SEQ ID NO: 1-12

ii) having an binding to a solid or immobilized substrate that is less than less than 95% of the binding of the parent alpha-amylase, such as less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, preferably less than 20% of the binding and most preferred less than 10% of the binding.

In a further embodiment the invention relates to a detergent composition comprising an variant alpha-amylase characterized in:

i) having at least 80% sequence identity to one of these, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% identity to one of SEQ ID NO: 1-12

ii) having an binding to a solid or immobilized substrate that is less than 95% of the binding of the alpha-amylasehaving SEQ ID NO: 8, such as less than 90% of the binding; preferably less than 80% of the binding, preferably less than 70% of the binding, preferably less than 60% of the binding preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

The invention also relates to a detergent composition comprising an alpha-amylase where the ding of the alpha-amylase to rice starch is lower than the binding af the alpha-amylase having SEQ ID NO: 8 to rice starch, preferably less than 80%, more preferred less than 70%, more preferred less than 60%, more preferred less than 50%, more preferred less than 40%, more preferred less than 30%, more preferred less than 20% and most preferred less than 10%.

Determining the binding of alpha-amylases to solid substrate

For the purpose of the present invention binding of alpha-amylases to a solid or immobilized substrate can in principle be determined using any such method for determining binding to a substrate known in the art. In general such method involves contacting an alpha-amylase to a solid or immobilized substrate and determining the fraction of alpha-amylase bound to the substrates.

The solid or immobilized substrate may be any substrate for alpha-amylases that is substratially insoluble under the conditions of the test. The solid substrate may be a starch such as wheat starch, maize starch or rice starch, where rice starch is preferred. Alternatively may the determination be made using immobilized substrate which includes any soluble or insoluble substrates for alpha-amylases immobilized on a solid matrix. According to the invention binding of an alpha-amylase to its substrate is determined as the fraction of bound amylase measured at 20°C in the presence of starch at pH 8. Binding of an alpha-amylase to its substrate can be determined by incubating an aqueous solution of the alpha-amylase with the starch and determining the fraction of bound amylase using the method disclosed below.

Conventions for Designation of Variants

For purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 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, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 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: 2 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 3.0.0 or later.

Identification of the corresponding amino acid residue in another alpha-amylase can be confirmed by an alignment of multiple polypeptide sequences using “ClustalW” (Larkin *et al.*, 2007, *Bioinformatics* 23: 2947-2948).

When the other enzyme has diverged from the mature polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, or SEQ ID NO: 6 , SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15 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 can 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 can 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, can be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can 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 implementations of these algorithms can 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 alpha-amylase 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 abbreviation is employed.

<u>Substitutions</u>. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine with alanine at position 226 is designated as “Thr226Ala” or “T226A”. Multiple mutations are separated by addition marks (“+”), *e.g.*, “Gly205Arg + Ser411Phe” or “G205R + S411F”, representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

<u>Deletions</u>. 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*”.

<u>Insertions</u>. 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 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:

Parent:	Variant:
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 tyrosine and glutamic acid for arginine and glycine at positions 170 and 195, respectively.

Different substitutions. Where different substitutions can be introduced at a position, the different substitutions are separated by a comma, *e.g.*, "Arg170Tyr,Glu" represents a substitution of arginine with tyrosine or glutamic acid at position 170. Thus, "Tyr167Gly,Ala + Arg170Gly,Ala" designates the following variants:
"Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167Ala+Arg170Ala".

Parent Alpha-amylases

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 1, SP722.

In one aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 1 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 1.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 1. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 1.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 1.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 2, SP707 disclosed in Tsukamoto et al. 1988, Biochem. Biophys.Res Comm. 151, 25-31.

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 2.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 2.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 3, AA560

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 3 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 3.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 3. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 3.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 3.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 4, SP690 disclosed in WO9526397.

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 4 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 4.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 4. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 4.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 4.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 5, KSM-AP1378 disclosed in EP 670367..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 5 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 5.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 5. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 5.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 5.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 6, sp 7-7..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 6 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 6.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 6. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 6.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 6.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 7, SP722+T183*+G184*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 7 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 7.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 7. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 7.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 7.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 8, SP 707+G182*+H183*.

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 8 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 8.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 8. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 8.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 8.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 9, AA560+T183*+G184*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 9 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 9.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 9. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 9.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 9.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 10, SP690+T183*+G184*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 10 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 10.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 10. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 10.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 10.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 11, KSM-AP1378+D183*+G184*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 11 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 11.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 11. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 11.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 11.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 12, SP-7-7+G182*+H183*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 12 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 12.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 12. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 12.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 12.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 13., derived from *Bacillus licheniformis*.

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 13 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 13.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 13. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 13.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 13.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 14, derived from *Bacillus amyloliquefaciens*..

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 14 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 14.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 14. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 14.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 14.

The parent alpha-amylase may also be a polypeptide with at least 80% sequence identity with the mature polypeptide of SEQ ID NO: 15, derived from *Bacillus stearothermophilus* (*Geobacillus stearothermophilus*).

In another aspect, the parent has a sequence identity to the mature polypeptide of SEQ ID NO: 15 of at least 80%, *e.g.*, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which have alpha-amylase activity. In one aspect, the amino acid sequence of the parent differs by no more than ten amino acids, *e.g.*, by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 15.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 15. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 15.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 15.

The amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, *e.g.*, at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, *e.g.,* at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, ^{35}S, biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material, which is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleotide probe corresponding to a polynucleotide encoding SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 or a subsequence thereof, under low to very high stringency conditions. Molecules to which the probe hybridizes can be detected using, for example, X-ray film or any other detection means known in the art.

In one aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15 or a fragment thereof.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), 50°C (low stringency), 55°C (medium stringency), 60°C (medium-high stringency), 65°C (high stringency), or 70°C (very high stringency).

For short probes that are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48: 1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m.

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

The parent may be a bacterial alpha-amylase. For example, the parent may be a gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces* alpha-amylase, or a gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, or *Ureaplasma* alpha-amylase.

In one aspect, the parent is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* alpha-amylase.

In another aspect, the parent is a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subsp. *Zooepidemicus* alpha-amylase.

In another aspect, the parent is a *Streptomyces achromogenes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, *Streptomyces griseus*, or *Streptomyces lividans* alpha-amylase.

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

In another aspect, the parent is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* alpha-amylase.

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

In another aspect, the parent is a *Bacillus* sp. alpha-amylase, *e.g.*, the alpha-amylase of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 15.

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

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

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

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

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

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

Preparation of Variants

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

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

Site-directed mutagenesis can be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can 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 and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests at the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and 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 can 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 can 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 can 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 olgionucleotides are synthesized and assembled upon photo-programable microfluidic chips.

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

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

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 constuction 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 ampflication. Polynucleotide subsequences may then be shuffled.

Variants

Essential amino acids in a parent can 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 alpha-amylase or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent.

Polynucleotides

The present invention also relates to isolated polynucleotides that encode any of the variants of the present invention.

Nucleic Acid Constructs

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 (several) control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

A polynucleotide may be manipulated in a variety of ways to provide for expression of 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 sequence, which is recognized by a host cell for expression of the polynucleotide. The promoter sequence contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any nucleic acid sequence 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 the transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* *xylA* and *xylB* genes, *E. coli lac* operon, *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 suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are the promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (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 including a gene encoding a neutral alpha-amylase in *Aspergilli* in which the untranslated leader has been replaced by an untranslated leader from a gene encoding triose phosphate isomerase in *Aspergilli*; non-limiting examples include modified promoters including the gene encoding neutral alpha-amylase in *Aspergillus niger* in which the untranslated leader has been replaced by an untranslated leader from the gene encoding triose phosphate isomerase in *Aspergillus nidulans* or *Aspergillus oryzae*); 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 suitable transcription terminator sequence, 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 filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

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

The control sequence may also be a suitable leader sequence, 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 sequence that is functional in the host cell may be used.

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

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

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the 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.

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

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

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

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

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

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 region 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 inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region 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.

Where both signal peptide and propeptide regions are present at the N-terminus of a variant, the propeptide region is positioned next to the N-terminus of the variant and the signal peptide region is positioned next to the N-terminus of the propeptide region.

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

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more (several) 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 the 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 contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more (several) 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 the *dal* genes from *Bacillus licheniformis* or *Bacillus subtilis*, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. 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 adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

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

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

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term “origin of replication” or “plasmid replicator” means a nucleotide sequence 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 pAMß1 permitting replication in *Bacillus*.

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 can be accomplished according to the methods disclosed in WO 00/24883.

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

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

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide of the present invention operably linked to one or more (several) control sequences that direct the production of a variant of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the 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*, *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 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, *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, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), by using competent cells (see, *e.g.*, Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may, for instance, 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, for instance, be effected by protoplast transformation and electroporation (see, *e.g.*, Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), by conjugation (see, *e.g.*, Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or by 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, for instance, be effected by electroporation (see, *e.g.*, Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or by conjugation (see, *e.g.*, Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may, for instance, be effected by natural competence (see, *e.g.*, Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), by protoplast transformation (see, *e.g.*, Catt and Jollick, 1991, *Microbios* 68: 189-2070, by electroporation (see, *e.g.*, Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804) or by 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.

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).

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, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series* No. 9, 1980).

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

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

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.

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 sarcochroum*, *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 and Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474. 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 the 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 polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be 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 can be recovered directly from the medium. If the variant is not secreted, it can 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 may include 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 by 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*, J.-C. Janson and Lars 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 a variant is used as a source of the variant.

Compositions

The present invention also relates to compositions comprising a variant of the present invention. 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 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, 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*, *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.*, *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 methods known in the art.

Detergent compositions

In one embodiment, the invention is directed to detergent compositions comprising an enzyme of the present invention in combination with 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 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 artisan.

Enzyme of the present invention

In one embodiment of the present invention, the a polypeptide of the present invention may be added to a detergent composition in an amount corresponding to 0.001-100 mg of protein, such as 0.01-100 mg of protein, preferably 0.005-50 mg of protein, more preferably 0.01-25 mg of protein, even more preferably 0.05-10 mg of protein, most preferably 0.05-5 mg of protein, and even most preferably 0.01-1 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 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 detergent composition may comprise one or more surfactants, which may be anionic and/or cationic and/or non-ionic and/or semi-polar and/or zwitterionic, or a mixture thereof. In a particular embodiment, the detergent composition includes a mixture of one or more nonionic surfactants and one or more anionic surfactants. The surfactant(s) is typically present at a level of from about 0.1% to 60% by weight, such as about 1% to about 40%, or about 3% to about 20%, or about 3% to about 10%. The surfactant(s) is chosen based on the desired cleaning application, and includes any conventional surfactant(s) known in the art. Any surfactant known in the art for use in laundry detergents may be utilized.

When included therein the detergent will usually contain from about 1% to about 40% by weight, such as from about 5% to about 30%, including from about 5% to about 15%, or from about 20% to about 25% of an anionic surfactant. Non-limiting examples of anionic surfactants include sulfates and sulfonates, in particular, linear alkylbenzenesulfonates (LAS), isomers of LAS, branched alkylbenzenesulfonates (BABS), phenylalkanesulfonates, alpha-olefinsulfonates (AOS), olefin sulfonates, alkene sulfonates, alkane-2,3-diylbis(sulfates), hydroxyalkanesulfonates and disulfonates, alkyl sulfates (AS) such as sodium dodecyl sulfate (SDS), fatty alcohol sulfates (FAS), primary alcohol sulfates (PAS), alcohol ethersulfates (AES or AEOS or FES, also known as alcohol ethoxysulfates or fatty alcohol ether sulfates), secondary alkanesulfonates (SAS), paraffin sulfonates (PS), ester sulfonates, sulfonated fatty acid glycerol esters, alpha-sulfo fatty acid methyl esters (alpha-SFMe or SES) including methyl ester sulfonate (MES), alkyl- or alkenylsuccinic acid, dodecenyl/tetradecenyl succinic acid (DTSA), fatty acid derivatives of amino acids, diesters and monoesters of sulfo-succinic acid or soap, and combinations thereof.

When included therein the detergent will usually contain from about 0 % to about 40% by weight of a cationic surfactant. Non-limiting examples of cationic surfactants include alklydimethylehanolamine quat (ADMEAQ), cetyltrimethylammonium bromide (CTAB), dimethyldistearylammonium chloride (DSDMAC), and alkylbenzyldimethylammonium, and combinations thereof, Alkyl quaternary ammonium compounds, Alkoxylated quaternary ammonium (AQA),

When included therein the detergent will usually contain from about 0.2% to about 40% by weight of a non-ionic surfactant, for example from about 0.5% to about 30%, in particular from about 1% to about 20%, from about 3% to about 10%, such as from about 3% to about 5%, or from about 8% to about 12%. Non-limiting examples of non-ionic surfactants include alcohol ethoxylates (AE or AEO), alcohol propoxylates, propoxylated fatty alcohols (PFA), alkoxylated fatty acid alkyl esters, such as ethoxylated and/or propoxylated fatty acid alkyl esters, alkylphenol ethoxylates (APE), nonylphenol ethoxylates (NPE), alkylpolyglycosides (APG), alkoxylated amines, fatty acid monoethanolamides (FAM), fatty acid diethanolamides (FADA), ethoxylated fatty acid monoethanolamides (EFAM), propoxylated fatty acid monoethanolamide (PFAM), polyhydroxy alkyl fatty acid amides, or N-acyl N-alkyl derivatives of glucosamine (glucamides, GA, or fatty acid glucamide, FAGA), as well as products available under the trade names SPAN and TWEEN, and combinations thereof.

When included therein the detergent will usually contain from about 0_% to about 40% by weight of a semipolar surfactant. Non-limiting examples of semipolar surfactants include amine oxides (AO) such as alkyldimethylamineoxide, *N*-(coco alkyl)-*N,N*-dimethylamine oxide and *N*-(tallow-alkyl)-*N,N*-bis(2-hydroxyethyl)amine oxide, fatty acid alkanolamides and ethoxylated fatty acid alkanolamides, and combinations thereof.

When included therein the detergent will usually contain from about 0_% to about 40% by weight of a zwitterionic surfactant. Non-limiting examples of zwitterionic surfactants include betaine, alkyldimethylbetaine, and sulfobetaine, and combinations thereof.

Hydrotropes

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 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 miceller, 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 containing 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 as phase separation or high viscosity.

The detergent may contain 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 laundry detergents may be utilized. Non-limiting examples of hydrotropes include sodium benzene sulfonate, sodium p-toluene sulfonates (STS), sodium xylene sulfonates (SXS), sodium cumene sulfonates (SCS), sodium cymene sulfonate, amine oxides, alcohols and polyglycolethers, sodium hydroxynaphthoate, sodium hydroxynaphthalene sulfonate, sodium ethylhexyl sulfate, and combinations thereof.

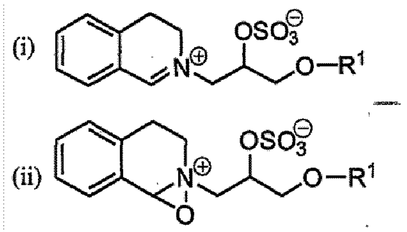
Builders and Co-Builders

The detergent composition may contain about 0-65% by weight, such as about 10% to about 40% of a detergent builder or co-builder, or a mixture thereof. In a dish wash deteregent, 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 laundry or dish washing detergents or detergents used for industrial or institutional (?) cleaning may be utilized. Non-limiting examples of builders include zeolites, diphosphates (pyrophosphates), triphosphates such as sodium triphosphate (STP or STPP), carbonates such as sodium carbonate, soluble silicates such as sodium metasilicate, layered silicates (*e.g.*, SKS-6 from Hoechst), ethanolamines such as 2-aminoethan-1-ol (MEA), iminodiethanol (DEA) and 2,2',2"-nitrilotriethanol (TEA), and carboxymethylinulin (CMI), and combinations thereof.

The detergent composition may also contain 0-50% by weight, such as about 10% to about 40%, of a detergent co-builder, or a mixture thereof. The detergent composition may include 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), etheylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), iminodisuccinic acid (IDS), ethylenediamine-N,N'-disuccinic acid (EDDS), methylglycinediacetic acid (MGDA), glutamic acid-N,N-diacetic acid (GLDA), 1-hydroxyethane-1,1-diylbis(phosphonic acid) (HEDP), ethylenediaminetetrakis(methylene)tetrakis(phosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylene)pentakis(phosphonic acid) (DTPMPA), N-(2-hydroxyethyl)iminodiacetic acid (EDG), aspartic acid-N-monoacetic acid (ASMA), aspartic acid-N,N-diacetic acid (ASDA), aspartic acid-N- monopropionic acid (ASMP) , iminodisuccinic acid (IDA), N- (2-sulfomethyl) aspartic acid (SMAS), N- (2-sulfoethyl) aspartic acid (SEAS), N- (2-sulfomethyl) glutamic acid (SMGL), N- (2- sulfoethyl) glutamic acid (SEGL), N- methyliminodiacetic acid (MIDA), 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-(hydroxyethyl)-ethylidenediaminetriacetate (HEDTA), diethanolglycine (DEG), Diethylenetriamine Penta (Methylene Phosphonic acid) (DTPMP), aminotris(methylenephosphonic acid) (ATMP), and combinations and salts thereof. Further exemplary builders and/or co-builders are described in, e.g., WO 09/102854, US 5977053 Incrustation inhibitors such as phosphonates.

Bleaching Systems

The detergent may contain 0-20% by weight, such as about 0 % to about 10%, of a bleaching system. Any bleaching system known in the art for use in laundry + dish wash + I&I detergents may be utilized. Suitable bleaching system components include bleaching catalysts, photobleaches, bleach activators, sources of hydrogen peroxide such as sodium percarbonate and sodium perborates, preformed peracids and mixtures thereof. Suitable preformed peracids include, but are not limited to, peroxycarboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts, for example, Oxone (R), and mixtures thereof. Non-limiting examples of bleaching systems include peroxide-based bleaching systems, which may comprise, for example, an inorganic salt, including alkali metal salts such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, persulfate, perphosphate, persilicate salts, in combination with a peracid-forming bleach activator. By Bleach activator is meant herin a compound which reacts with peroxygen bleach like hydrogen peroxide to form a Peracid. The peracid thus formed constitutes the activated bleach. Suitable bleach activators to be used herin include those belonging to the class of esters amides, imides or anhydrides, Suitable examples are tetracetyl athylene diamine (TAED), sodium 3,5,5 trimethyl hexanoyloxybenzene sulphonat, diperoxy dodecanoic acid, 4-(dodecanoyloxy)benzenesulfonate (LOBS), 4-(decanoyloxy)benzenesulfonate, 4-(decanoyloxy)benzoate (DOBS), 4-(3,5,5-trimethylhexanoyloxy)benzenesulfonate (ISONOBS), tetraacetylethylenediamine (TAED) and 4-(nonanoyloxy)benzenesulfonate (NOBS), and/or those disclosed in WO98/17767. A particular family of bleach activators of interest was disclosed in EP624154 and particulary preferred in that family is acetyl triethyl citrate (ATC). ATC or a short chain triglyceride like Triacin has the advantage that it is environmental friendly as it eventually degrades into citric acid and alcohol. Furthermore acethyl triethyl citrate and triacetin has a good hydrolytical stability in the product upon storage and it is an efficient bleach activator. Finally ATC provides a good building capacity to the laundry additive. Alternatively, the bleaching system may comprise peroxyacids of, for example, the amide, imide, or sulfone type. The bleaching system may also comprise peracids such as 6-(phthaloylamino)percapronic acid (PAP). The bleaching system may also include a bleach catalyst. In some embodiments the bleach component may be an organic catalyst selected from the group consisting of organic catalysts having the following formulae:



(iii) and mixtures thereof; wherein each R^1 is independently a branched alkyl group containing from 9 to 24 carbons or linear alkyl group containing from 11 to 24 carbons, preferably each R^1 is independently a branched alkyl group containing from 9 to 18 carbons or linear alkyl group containing from 11 to 18 carbons, more preferably each R^1 is independently selected from the group consisting of 2-propylheptyl, 2-butyloctyl, 2-pentylnonyl, 2-hexyldecyl, n- dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, iso-nonyl, iso-decyl, iso- tridecyl and iso-pentadecyl. Other exemplary bleaching systems are described, e.g., in WO2007/087258, WO2007/087244, WO2007/087259, WO2007/087242. Suitable photobleaches may for example be sulfonated zinc phthalocyanine

Polymers

The detergent may contain 0-10% by weight, such as 0.5-5%, 2-5%, 0.5-2% or 0.2-1% of a polymer. Any polymer known in the art for use in laundry, dish wash and I&I detergents may be utilized. The polymer may function as a co-builder as mentioned above, or may provide antiredeposition, fiber protection, soil release, dye transfer inhibition and/or grease cleaning properties. Exemplary antiredeposition polymers include (carboxymethyl)cellulose (CMC), poly(vinyl alcohol) (PVA), poly(vinylpyrrolidone) (PVP), poly(ethyleneglycol) or poly(ethylene oxide) (PEG), ethoxylated poly(ethyleneimine), and polycarboxylates such as PAA, PAA/PMA, and lauryl methacrylate/acrylic acid copolymers. Exemplary fiber protection polymers include hydrophobically modified CMC (HM-CMC) and silicones. Exemplary soil release polymers include copolymers of terephthalic acid and oligomeric glycols. Exemplary dye transfer inhibition polymers include PVP, poly(vinylimidazole) (PVI) and poly(vinylpyridin-N-oxide) (PVPO or PVPNO). Other exemplary polymers are disclosed in, e.g., WO 2006/130575.

Fabric hueing agents

The detergent compositions of the present invention may also include fabric hueing agents such as dyes or pigments which when formulated in detergent compositions can deposit onto a fabric when said fabric is contacted with a wash liquor comprising said detergent compositions thus altering the tint of said fabric through absorption/reflection of visible light. Fluorescent whitening agents emit at least some visible light. In contrast, fabric hueing agents alter the tint of a surface as they absorb at least a portion of the visible light spectrum. Suitable fabric hueing agents include dyes and dye-clay conjugates, and may also include pigments. Suitable dyes include small molecule dyes and polymeric dyes. Suitable small molecule dyes include small molecule dyes selected from the group consisting of dyes falling into the Colour Index (C.I.) classifications of Direct Blue, Direct Red, Direct Violet, Acid Blue, Acid Red, Acid Violet, Basic Blue, Basic Violet and Basic Red, or mixtures thereof, for example as described in WO2005/03274, WO2005/03275, WO2005/03276 and EP1876226 (hereby incorporated by reference). The detergent composition preferably comprises from about 0.00003 wt% to about 0.2 wt%, from about 0.00008 wt% to about 0.05 wt%, or even from about 0.0001 wt% to about 0.04 wt% fabric hueing agent. The composition may comprise from 0.0001 wt% to 0.2 wt% fabric hueing agent, this may be especially preferred when the composition is in the form of a unit dose pouch. Suitable hueing agents are also disclosed in, e.g., WO 2007/087257, WO2007/087243.

(Additional) 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 PCT/DK98/00299.

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

Proteases: Suitable proteases include those of animal, vegetable or microbial origin.

Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metalloprotease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, *e.g.*, subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (*e.g.*, of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235, and 274.

Preferred commercially available protease enzymes include Alcalase™, Savinase™, Primase™, Duralase™, Esperase™, and Kannase™ (Novozymes A/S), Maxatase™, Maxacal™, Maxapem™, Properase™, Purafect™, Purafect OxP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), *e.g.*, from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, *e.g.*, from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, *e.g.*, from *B. subtilis* (Dartois *et al.*, 1993, *Biochemica et Biophysica Acta*, 1131: 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase™, Lipolase Ultra™, and Lipex™ (Novozymes A/S).

Amylases: Suitable amylases (α and/or β) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, α-amylases obtained from *Bacillus*, *e.g.*, a special strain of *Bacillus licheniformis*, described in more detail in GB 1,296,839.

Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are Stainzyme™, Stainzyme™ Plus, Natalase™, Duramyl™, Termamyl™, Fungamyl™ and BAN™ (Novozymes A/S), Rapidase™ , Powerase™ and Purastar™ (from Genencor International Inc.).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, *e.g.*, from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme™ (Novozymes A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing 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, can be formulated, for example, as a granulate, liquid, slurry, etc. Preferred detergent 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 1000 to 20000; 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 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

Any detergent components known in the art for use in laundry, dish wash or I&I 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 in laundry, dish wash or I&I 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 can also contain 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, polyvinyloxazolidones 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 contain 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-sulphonic acid derivatives, diarylpyrazoline derivatives and bisphenyl-distyryl derivatives. Examples of the diaminostilbene-sulphonic 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'-disulphonate; 4,4'-bis-(2,4-dianilino-s-triazin-6-ylamino) stilbene-2.2'-disulphonate; 4,4'-bis-(2-anilino-4(N-methyl-N-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate, 4,4'-bis-(4-phenyl-2,1,3-triazol-2-yl)stilbene-2,2'-disulphonate; 4,4'-bis-(2-anilino-4(1-methyl-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate and 2-(stilbyl-4"-naptho-1.,2':4,5)-1,2,3-trizole-2"-sulphonate. 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 disulphonate. Tinopal CBS is the disodium salt of 2,2'-bis-(phenyl-styryl) disulphonate. 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 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 include one or more soil release polymers which aid the removal of soils from fabrics such as cotton and polyester based fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil release polymers may for example be nonionic or anionic terephthalte 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 alkoxylated grease cleaning polymers comprising a core structure and a plurality of alkoxylate 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 2006/113314 (hereby incorporated by reference). Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose deriviatives 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 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.

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.

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, structurants for liquid detergents and/or structure elasticizing agents.

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 regular or compact powder, a granule, a paste, a gel, or a regular, compact or concentrated liquid.

Detergent formulation forms: Layers (same or different phases), Pouches, versus forms for Machine dosing unit.

Pouches can be configured as single or multicompartments. It can 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 can be devided into compartments of the pouch. Preferred films are polymeric materials preferably polymers which are formed into a film or sheet. Preferred polymers, copolymers or derivates therof 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, hydroxyprpyl methyl cellulose (HPMC). Preferably the level of polymer in the film for example PVA is at least about 60%. Preferred average molecular weight will typically be about 20,000 to about 150,000. Films can also be of blend compositions comprising hydrolytically degradable and water soluble polymer blends such as polyactide and polyvinyl alcohol (known under the Trade reference M8630 as sold by Chris Craft In. Prod. Of Gary, Ind., US) plus plasticisers like glycerol, ethylene glycerol, Propylene glycol, sorbitol and mixtures thereof. The pouches can 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 can be different in composition than compartments containing solids. Ref: (US2009/0011970 A1)

Detergent ingredients can 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 can also give rise to delayed dissolution of selected components in the wash solution.

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,

Uses

The present invention is also directed to methods for using the compositions thereof.

Laundry/textile/fabric (House hold laundry washing, Industrial laundry washing)

Hard surface cleaning (ADW, car wash, Industrial surface)

Use in detergents. The polypeptides of the present invention may be added to and thus become a component of a 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. In another aspect, the present invention provides a detergent suited to cleaning at temperatures at or below 35 °C.

Methods

Wash performance of alpha-amylase using AMSA

In order to assess the wash performance of the alpha-amylase variants in a detergent base composition, washing experiments may be performed. The enzymes are tested using the 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 embodiments" at page 23-24.

General wash performance description

A test solution comprising water (15°dH), 0.8 g/L detergent, e.g. model detergent A as described below, or 50 mM HCO^{3-}, and the enzyme of the invention, e.g. at concentration of 0,1 0.2, 0.3, 0.4, 0.8 and/or 1.2 mg enzyme protein/L, is prepared. Fabrics stained with starch (e.g. CS-28 from Center For Testmaterials BV, P.O. Box 120, 3133 KT, Vlaardingen, The Netherlands) is added and washed for 30 minutes at 20°C, or alternatively 20 minutes at 15 °, or alternatively 45 minutes at 15 °C or alternatively 20 minutes at 15°C or 40°C as specified in the examples. After thorough rinse under running tap water and drying in the dark, the light intensity or reflectance values of the stained fabrics are subsequently measured as a measure for wash performance. The test with 0 mg enzyme protein/L is used as a blank to obtain a delta remission value (ΔREM). Alternatively, the wash performance is compared to that of the parent alpha-amylase, with the performance result of the parent alpha-amylase is assigned the value of 100 and the results of the variants are compared to this value. 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:

Table A: Experimental condition

Detergent	Model detergent A (see below)
Detergent dosage	0.8 g/L
Test solution volume	160 micro L
pH	As is
Wash time	20, 30 or 45 minutes

Temperature	15 °C, 20°C or 40°C
Water hardness	15°dH
Enzyme concentration in test solution	0.1 to 1.2 mg/L
Test material	CS-28 (Rice starch cotton)

Table B: Model detergent A

Compound	**Amount g/100g**	**% active ingredient**
	Surfactants	
Na-LAS (92%) (Nacconol 90G) (anionic)	21.74	20
STEOL CS-370E (70%) (anionic)	14.28	10
Bio-soft N25-7 (99.5%) (non-ionic)	10	10
Oleic acid (fatty acid)	4	4
	Solvents	
H_2O	25	~33
Ethanol	0.5	0.5
STS (sodium p-toluene sulfonate (40%)	3.75	1.5
Mono propylene glycol	7	7
	Builder	
Tri-sodium-citrate	8	8
Triethanolamine (TEA)	0.5	0.5
	Inhibitor	
Boric acid	1.5	1.5
	Minors	
10N NaOH (for adjustment to pH 8.5)	2	

Water hardness was adjusted to 15°dH by addition of $CaCl_2$, $MgCl_2$, and $NaHCO_3$ (Ca^{2+}:Mg^{2+}:HCO_3^- = 4:1:7.5) to the test system. After washing the textiles were flushed in tap water and dried.

Table C: Experimental condition

Detergent	Model detergent X (see below)
Detergent dosage	1,75 g/L
Test solution volume	160 micro L
pH	9.9

Wash time	20 minutes
Temperature	15 °C or 40 °C
Water hardness	12°dH
Enzyme concentration in test solution	0.1; 0.3; 0.6; 1.2 mg/L
Test material	CS-28 (Rice starch cotton)

Table D: Model detergent X

Compound	wt%
Surfactant system	
LAS	15
AEO	2*
soap	0
Builder system	
sodium carbonate	20
sodium disilicate	12
zeolite A	15
PCA	1
sodium sulfate	37

*Model X is mixed without AEO. AEO is added to the wash separately.

Approximate wash pH in 12 °dH water (Ca: Mg: HCO_3 =2:1:4.5)

Water hardness was adjusted to 12 °dH by addition of $CaCl_2$, $MgCl_2$, and $NaHCO_3$ (Ca: Mg: HCO_3 =2:1:4.5) to the test system. After washing the textiles were flushed in tap water and dried.

The wash performance is measured as the brightness of the colour of the textile washed. 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.

Colour measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak), which is 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:

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

Textiles:

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

Wash performance test using beakers

This assay is a small scale model of a top loaded washing machine and used to evaluate the washing performance of amylases.

The beaker wash performance test, using 250 mL beakers and a paddle stirrer providing oscillating rotational motion, 180° in each direction, with a frequency of 80 per minute, comprises the following steps: providing 100 mL wash solution (6 °C, 15 °dH, , pH 8.0) containing 50 mM $NaHCO_3$ and 0.4 mg/L enzyme; adding two swatches of CS-28 (5x5 cm) and two swatches of EMPA 162 (5x5 cm) to the wash solution to start the wash; setting the agitation speed to 80 rpm; stopping the agitation after 60 minutes, rinsing the swatches under cold running tap water; drying the rinsed swatches in the dark over night; and evaluating the wash performance by measuring the remission of incident light at 460 nm using Color Eye as described below.

Equipment and material

Water bath (5 °C) with circulation; glass beakers (250 mL); one rotating arm per beaker with capacity of 100 mL of washing solution; test swatches: CS-28 (rice starch on cotton) from Center for Testmaterials BV, Vlaardingen, The Netherlands and EMPA 162 (rice starch on cotton/polyester) from EMPA Testmaterials AG, St. Gallen, Switzerland, the swatches are cut into 5 x 5 cm.

Wash solution: 50 mM $NaHCO_3$ buffer, pH 8.0, water hardness: 15 °dH, Calcium:Magnesium ratio 4:1.

Amylase stock solution: 1 mg enzyme protein per mL. - A solution of 0.1 % (w/v) Triton X-100 and 0.1 mM $CaCl_2$ in ultrapure water (MilliQ water) is used for dilution of amylase (amylase dilution buffer).

Color Eye measurement

Wash performance is expressed as a delta remission value (ΔRem). Light reflectance evaluations of the swatches were done using a Macbeth Color Eye 7000 reflectance spectrophotometer with very small oval aperture, i.e. 0.7 cm^2 (~0.7 x 1.0 cm) . The measurements were made without UV in the incident light and remission at 460 nm was extracted. The swatch to be measured was placed on top of another swatch of the same type before being measured to reduce reflection from the piston pushing the swatch up against the measuring opening. Delta remission values for individual swatches were calculated by subtracting the remission value of the swatch washed without added amylase (control) from the remission value of the swatch washed with amylase.

Wash performance alpha-amylases using Mini-wash robot:

Mini-wash robot is a small scale model of washing machine and used to evaluate the washing performance of amylases.

To 100 mL beakers are added 60 mL wash solution, which is heated to 15°C or 40°C. Then enzyme (Concentrations: 0.00; 0.015; 0.05; 0.25; 0.50; 1.00 mg enzyme protein/L) is added. Textile (CS-28; rice starch on cotton) on rack is submerged into the wash solution containing a certain enzyme concentration and washed for 20 minutes. After wash the textile on rack is dried in drying cupboards without heat. The remission of the textile is measured at 460 nm by use of ZEISS MCS 521 VIS Spectrophotometer. Delta remission values for individual textile were calculated by subtracting the remission value of the textile washed without added amylase (control) from the remission value of the textile washed with amylase.

pNP-G7 assay for determination of alpha-amylase activity

The alpha-amylase activity may be determined by a method employing the G7-pNP substrate. G7-pNP which is an abbreviation for 4,6-ethylidene(G_7)-*p*-nitrophenyl(G_1)-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 p-nitrophenol (pNP) molecule which has a yellow color and thus can be measured by visible spectophometry at λ = 405 nm (400-420 nm). Kits containing G7-pNP substrate and alpha-Glucosidase is 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 contains 52.4 mM HEPES, 87 mM NaCl, 12.6 mM $MgCl_2$, 0.075 mM $CaCl_2$, $\geq$ 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 MOPS, 0.05% (w/v) Triton X100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether ($C_{14}H_{22}O(C_2H_4O)_n$ (n = 9-10))), 1 mM $CaCl_2$, pH 8.0.

PROCEDURE:

The amylase sample to be analyzed was diluted in dilution buffer to ensure the pH in the diluted sample is 7. The assay was performed by transferring 20 µl diluted enzyme samples to 96 well microtiter plate and adding 80µl 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.

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 binding to starch

Assay principle:

Amylase variants are incubated in the presence or absence of insoluble raw rice starch at a selected pH value, in the range of pH 4.0 to 11.0 depending on the intended pH value for the application of the alpha-amylases to be selected,; e.g. for detergent applications the pH is suitably selected in the alkaline area such as pH 8.0 or pH10.0; at a selected time, in general between 5 minutes and one hour, preferably in the range of 10 to 30 min such as 10 min or 30 minutes; and at a sselected temperature, in general in the range of 0°C to 30°C, preferably at 4°C. After centrifugation, amylase activity is determined in the supernatants. Difference in activity in the samples incubated in the presence and absence of rice starch is a measure of binding of amylase to insoluble starch.

Materials and methods:

Rice starch (Sigma Inc, Cat No. S7260), HEPES, Calcium chloride, Triton X-100, Glycine, EnzChek Ultra Amylase Assay Kit (Life Technologies, Cat No. E33651), 96 microwell plates for incubation and dilution (Nunc, Cat No. 269620) and 96 well half-area black plates for fluorescence measurements (Corning, Cat No. 3694).

Assay buffer contained 50 mM HEPES (pH 8.0), 0.1 mM $CaCl_2$ and 0.01% Triton X-100. Enzyme protein solutions were diluted to 0.15 mg/ml with the assay buffer. High pH binding buffer contained 50 mM Glycine-NaOH (pH 10.0) and 0.01% Triton X-100. Rice starch solution

(2.5%) was prepared in the assay buffer for the variants of SEQ ID NO: 14 and in high pH binding buffer for the variants OF SEQ ID NO: 2. EnzCheck Ultra Amylase substrate solution was prepared according to the manufacturer's instructions and diluted to 50 µg/ml in the assay buffer.

a) Buffer with or without rice starch (2.5%), 100 µl was added to 96 microwell plate and preincubated at 4°C for 30 min
b) Enzyme solutions, 20 µl were added to the above wells and the plate was placed on a mixer and mixed at 900 rpm for 30 min at 4°C.
c) The plate was centrifuged at 2000 rpm for 5 min at 4°C for the starch to settle down and the supernatant was carefully removed and diluted to about 1250 times in the assay buffer so that the enzyme protein concentration is about 20 ng/ml.
d) Diluted enzyme samples, 25 µl were added to 96 well half-area black plates containing 25 µl of EnzCheck Ultra Amylase substrate solution and the plate was immediately placed in a plate reader for fluorescence measurements.
e) Change in fluorescence intensity (Δ F.I.) was measured at 25°C for 30 min at an excitation wavelength of 485 nm and emission wavelength of 512 nm. Fluorescence readings between the time intervals of 0.5 to 5 min were taken for the calculation of activity (i.e.) change in fluorescence intensity per min (Δ F.I./min).

$$Binding\ (\%) = \frac{Activity\ for\ without\ starch - Activity\ for\ with\ starch}{Activity\ for\ without\ starch} \times 100$$

$$Binding\ relative\ to\ parent = \frac{Binding\ for\ variants}{Binding\ for\ parent}$$

EXAMPLES

Example 1: Determination of binding to amylose and wash performance

Using the method of measuring the binding to starch and the AMSA wash performance test described in the methods section, a number of amylases and variants thereof were analyzed for binding to amylose and wash performance.

The binding analysis was done at a pH of 8.0, binding period of 10 minutes and at a temperature of 4°C. The binding was tested using a 5 % (w/v) amylose (Sigma A0512), and the wash performance test was carried out as described for the AMSA wash performance test using Model detergent A and an enzyme dosage of 0.4 mg enzyme protein/L wash solution and a washing temperature of 15 °C. Wash time was 45 minutes.

The results are presented in table 1 below.

Alpha-amylase	Fraction bound	Wash performance (Delta-REM)	Reduced binding compared with parent
SP707	89	4.8	
SP707 G182* H183*	66	13.9	74%

Alpha-amylase	Fraction bound	Wash performance (Delta-REM)	Reduced binding compared with parent
SP690	92	8.6	
SP690 T183* G184*	85	11.3	92%

From these results it is clearly seen that there is an inverse correlation between the fraction bound to the starch and the wash performance.

Example 2: Determination of binding to raw rice starch and wash performance

Principle:

Same principle as described in Example 1, only a suspension of 5 % (w/v) rice starch (Sigma S7260) was used instead of amylose. The binding analysis was done at a pH of 8.0, binding period of 10 minutes and at a temperature of 4°C.

Alpha-amylase	Fraction bound	Wash performance (Delta-REM)	Reduced binding compared with parent
SP707	49	4.8	
SP707 G182* H183*	13	13.9	27%

Example 3: Determination of binding to amylopectin

Same principle as described in Example 1, only a suspension of 5 % (w/v) amylopectin (from Fluka) from corn was used instead of amylose.

Alpha-amylase	Fraction bound	Wash performance (Delta-REM)	Reduced binding compared with parent
SP707	51	4.8	
SP707 G182* H183*	19	13.9	37%

Example 4: Variants of *Bacillus licheniformis* alpha-amylase (SEQ ID NO: 13).

Variants of B. licheníformis alpha-amylase having SEQ ID NO: 13, were made. The variants tested had lower binding to starch and better wash performance in comparison with the parent alpha-amylase having SEQ ID NO: 13.

The binding analysis was done at a pH of 8 with 5% (w/v) insoluble rice starch, binding period of 10 minutes and at a temperature of 5°C.

The wash performance of the variants were tested by use of mini-wash using the conditions described under "**Wash performance alpha-amylases using Mini-wash robot**" and showed that the variants have improved wash performance using 0.5 mg/L at 40°C compared with the parent alpha-amylase from *Bacillus licheniformis.* Washing time was 20 minutes.

Wash performance = Value(Variant – Blank) / Value(Termamyl-Blank) * 100

Alpha-amylase having SEQ ID NO: 13 and following substitutions	Fraction bound	Wash performance in model detergent X
Ref.	32	100
W467A	24	370
K315M	23	353
W467F	27	340

Example 5: Variants of *Bacillus sp* 707 alpha-amylase (SEQ ID NO: 2)

Additional variants of the alpha-amylase having SEQ ID NO: 2 was generated and the variants tested for substrate binding and AMSA wash performance tested using 0.3 mg enzyme protein/L at 15°C for 20 minutes in Model detergent X.

The binding analysis was done at a pH of 10.0 with 2.5% (w/v) insoluble rice starch, binding period of 10 minutes and at a temperature of 4°C.

The wash performance is indicated relative to the wash performance of the parent alpha-amylase having SEQ ID NO: 2 with the modifications H183*+G184*

Alpha-amylase having SEQ ID NO: 2 and following substitutions	Fraction bound	Wash performance
H183*+G184* (REF)	1.0	100
H183*+G184*+W140F	0.4	303
H183*+G184*+Q169N	0.8	163
H183*+G184*+Q169A	0.7	266
H183*+G184*+W189Y+E190P	0.7	209
H183*+G184*+N260D	0.7	198
H183*+G184*+G477E	0.5	273
H183*+G184*+G477Q	0.6	194
H183*+G184*+G477K	0.5	176
H183*+G184*+A51I+W140Y	0.5	184
H183*+G184*+W140Y+W189E	0.4	116
H183*+G184*+W140Y+N260P	0.5	140
H183*+G184*+W140Y+W284D	0.1	107
H183*+G184*+W140Y+G476R	0.6	222
H183*+G184*+W140Y+G477E	0.3	147
H183*+G184*+W189E+W439R	0.6	130
H183*+G184*+W284D+G477E	0.2	236
H183*+G184*+W439R+G477E	0.2	287
H183*+G184*+E194D	0.9	125
H183*+G184*+W439R+D467K	0.4	230
H183*+G184*+R320M+W439R	0.3	315
H183*+G184*+W439R+K485R	0.5	232
H183*+G184*+F262A	0,3	129
H183*+G184*+Y363H	0,5	143
H183*+G184*+G476E	0,7	129

H183*+G184*+W439R+G476R	0,2	157
H183*+G184*+K72S+W140Y	0,4	129
H183*+G184*+R320K+W439R	0,4	129

Example 6: Variants of *Bacillus sp* 707 alpha-amylase (SEQ ID NO: 2)

Further variants of the alpha-amylase having SEQ ID NO: 2 was generated and the variants tested for substrate binding and wash performance at low temperature, and variants selected having lower substrate binding than the parent alpha-amylase having SEQ ID NO: 2 with the modifications H183*+G184*. AMSA wash tests were performed using 0.3 mg enzyme protein/L at 15°C for 20 minutes in Model detergent X.

The wash performance is indicated relative to the wash performance of the parent alpha-amylase having SEQ ID NO: 2 with the modifications H183*+G184*.

Alpha-amylase having SEQ ID NO: 2 and following substitutions	Relative wash performance
H183*+G184* (Ref.)	100
H183*+G184*+W159Y+W167Y+N260P+F262P+W439R+G476E+G477K	215
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+W469Y+G476R	199
H183*+G184*+W167F+N260D+F262P+P380Q+G477K	182
H183*+G184*+W167Y+N260D+F262P+W439R+W469V+G476Q+G477K	182
H183*+G184*+W167Y+N260D+F262P+W439V+W469Y	192
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K	177
H183*+G184*+W167Y+N260D+W439R+W469Y+G476E+G477K	180
H183*+G184*+W159Y+W167Y+N260D+G476E+G477Q	153
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+G476K+G477Q	217
H183*+G184*+N260D+F262P+W469Y+G476R+G477Q	198
H183*+G184*+W167Y+L230I+N260P+W469Y	133
H183*+G184*+W159Y+W167Y+N260P+E439Y+W469Y+G476Q+G477Q	198
H183*+G184*+W167Y+F262P+W469Y+G476R+G477Q	190

Example 7: Variants of *Bacillus sp* 707 alpha-amylase (SEQ ID NO: 2)

Further variants of the alpha-amylase having SEQ ID NO: 2 was generated and the variants tested for substrate binding and wash performance at low temperature, and variants selected having lower substrate binding than the parent alpha-amylase having SEQ ID NO: 2 with the modifications H183*+G184*. AMSA wash tests were performed using 0.3 mg enzyme protein/L at 15°C for 20 minutes in Model detergent X.

The binding analysis was done at a pH of 8.0, binding period of 10 minutes and at a temperature of 4°C.

The wash performance is indicated relative to the wash performance of the parent alpha-amylase having SEQ ID NO: 2 with the modifications H183*+G184*.

Alpha-amylase having SEQ ID NO: 2 and following substitutions	Wash performance test
H183*+G184*+W159Y+W167Y+N260P+F262P+W439R+G476E+G477K	215
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+W469Y+G476R	199
H183*+G184*+W167F+N260D+F262P+P380Q+G477K	182
H183*+G184*+W167Y+N260D+F262P+W439R+W469V+G476Q+G477K	182
H183*+G184*+W167Y+N260D+F262P+W439V+W469Y	192
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;	177
H183*+G184*+W167Y+N260D+W439R+W469Y+G476E+G477K	180
H183*+G184*+W159Y+W167Y+N260D+G476E+G477Q	153
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+G476K+G477Q	217
H183*+G184*+N260D+F262P+W469Y+G476R+G477Q	198
H183*+G184*+W167Y+L230I+N260P+W469Y	133
H183*+G184*+W159Y+W167Y+N260P+E439Y+W469Y+G476Q+G477Q	198
H183*+G184*+W167Y+F262P+W469Y+G476R+G477Q	190

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 conflict, the present disclosure including definitions will control.

CLAIMS

1. A method for screening alpha-amylases having high performance at low temperature, comprising the steps of

a) determining the binding of the alpha-amylases to solid or immobilized substrate for the alpha amylase, such as starch;
b) selecting alpha-amylases having a low binding to the substrate.

2. The method of claim 1, wherein the alpha-amylases has high wash performance at low temperature.

3. The method of claim 1 or 2, wherein the alpha-amylases have less than 90% of the binding to starch than the alpha-amylase having SEQ ID NO: 8, preferably less than 80%, preferably less than 70%, preferably less than 65%, preferably less than 60%, preferably less than 50% of the binding, preferably less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

4. A method for selecting variants of a parent alpha-amylase comprising the steps of
a) generating variants by substituting, deleting or inserting one or more amino acid residue in one or more amino acids located at the surface of the parent alpha-amylase;
b) testing the variants for the binding to solid or immobilized substrate for the alpha-amylase, such as starch;
c) selecting variants having a lower binding the substrate than the parent alpha-amylase.

5. The method of claim 3, wherein the parent alpha-amylase is selected among amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% identity to one of SEQ ID NO: 1-15.

6. The method of claim 4 or 5, wherein the variants have less than 90% of the binding to starch than the parent alpha-amylase, preferably less than 80%, preferably less than 70%, preferably less than 65%, preferably less than 60%, preferably less than 50% of the binding, preferably

less than 40% of the binding, preferably less than 30% of the binding, more preferred less than 20% of the binding and most preferred less than 10% of the binding.

7 A variant polypeptide having alpha-amylase activity and having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97 % sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to one of SEQ ID NO: 1-15, the variant is derived from a parent alpha-amylase by one or more (e.g. several) modifications selected from substitutions, insertions and deletions, wherein the variant alpha-amylase has lower binding to solid starch and higher wash performance at low temperature e.g. 15 °C, compared with the patent alpha-amylase.

8. The variant alpha-amylase of claim 7, wherein an amino acid residue in one or more substrate binding sites at the surface of the molecule has been modified.

9. The variant alpha-amylase of claim 7, selected among:

a) variant alpha-amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 2, and comprising a deletion of positions 181+182, or 182+183, or 183+184 and further one or two or more modifications in any of positions corresponding to W140, W159, W167, Q169, W189, E194, N260, F262, W284, F289, G304, G305, R320, W347, W439, W469, G476 and G477 in SEQ ID NO: 2;

b) variant alpha-amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 5, and comprising a deletion of positions 181+182, or 182+183, or 183+184 and further comprising one or two or more modifications in any of positions corresponding to W140, W159, W167, Q169, W189, E194, F262, W284, F289, G304, G305, K320, W347, W439, W469, G476 and G477 in SEQ ID NO: 5;

c) variant alpha-amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97%

sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 4, and comprising a deletion of positions 181+182, or 182+183, or 183+184 and further comprising one or two or more modifications in any of positions corresponding to W140, W159, W167, Q169, W189, E194, F262, W284, F289, G304, G305, K320, W347, W439, W469, G476 and G477 in SEQ ID NO: 4;

d) variant alpha-amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 13, and comprising a modification in any of positions corresponding to W138, W157, W165, E167, W184, E189, E255, F257, F279, F284, G299, G300, K315, W342, R437, W467 and G475 in SEQ ID NO: 13;

e) variant alpha-amylases having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 14, and comprising a deletion of positions 176+177 or 177+178, or 178+179 and further comprising one or two or more modifications in any of positions corresponding to W136, W155, W163, E165, W184, E189, E255, F257, F279, F284, G299, G300, R315, W342, R437, W467 and G475 in SEQ ID NO: 14; and

f) variant alpha-amylases having having at least 80% sequence identity, preferably at least 85% sequence identity, more preferred at least 90% sequence identity, more preferred at least 95% sequence identity, more preferred at least 97% sequence identity, more preferred at least 98% sequence identity and most preferred at least 99% sequence identity to SEQ ID NO: 15, and comprising a deletion of positions 179+180 or 180+181, or 181+182 and further comprising one or two or more modifications in any of positions corresponding to W139, W158, W166, E168, W187, E192, F260, F287, G302, G303, W345, W437, W467, G474 and G475 in SEQ ID NO: 15,

wherein the modifications in a-f preferably are substitutions.

10. The variant alpha-amylase of any one of claims 7 to 9, having at least 90% sequence identity to SEQ ID NO: 2 and comprising modifications in positions corresponding to the following positions in SEQ ID NO: 2:

H183*+G184*+W140F;
H183*+G184*+Q169N;
H183*+G184*+Q169A;
H183*+G184*+W189Y+E190P;
H183*+G184*+N260D;
H183*+G184*+G477E;
H183*+G184*+G477Q;
H183*+G184*+G477K;
H183*+G184*+W189E+E190P;
H183*+G184*+A51I+W140Y;
H183*+G184*+W140Y+W189E;
H183*+G184*+W140Y+N260P;
H183*+G184*+W140Y+W284D;
H183*+G184*+W140Y+G476R;
H183*+G184*+W140Y+G477E;
H183*+G184*+W189E+W439R;
H183*+G184*+W284D+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+W439R+G477E;
H183*+G184*+E194D;
H183*+G184*+W439R+D467K;
H183*+G184*+R320M+W439R;
H183*+G184*+W439R+K485R;
H183*+G184*+Y160S;
H183*+G184*+W189F+E190P;
H183*+G184*+F262A;
H183*+G184*+Y363H;
H183*+G184*+G476E;
H183*+G184*+N260P+W439R;
H183*+G184*+N260P+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+K72S+W140Y;
H183*+G184*+G109A+M202L+Y203G;
H183*+G184*+E194S;
H183*+G184*+E345D+G477R;
H183*+G184*+K302N+W439R;
H183*+G184*+R320K+W439R

H183*+G184*+W159Y+W167Y+F262P+W439R+W469Y+G477Q;
H183*+G184*+W159Y+W167F+N260D+W439Y+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439R+W469Y+G476K+G477E;
H183*+G184*+W159Y+W167F+F262P+W439V+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167F+F262P+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W167Y+N260D+W439R+G476Q+G477E;
H183*+G184*+W167Y+N260P+F262P+W439R+G476E+G477R;
H183*+G184*+W159Y+W167F+N260G+W439R+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260D+F262P+W439Y;
H183*+G184*+W159Y+W167F+N260P+W439Y+W469V+G476Q+G477Q;
H183*+G184*+W167Y+N260D+W439R+W469V+G476Q+G477Q;

H183*+G184*+W159Y+W167Y+N260P+F262P+W439R+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+W469Y+G476R;
H183*+G184*+W167F+N260D+F262P+P380Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439R+W469V+G476Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439V+W469Y;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W167Y+N260D+W439R+W469Y+G476E+G477K;
H183*+G184*+W159Y+W167Y+N260D+G476E+G477Q;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+G476K+G477Q;
H183*+G184*+N260D+F262P+W469Y+G476R+G477Q;
H183*+G184*+W167Y+L230I+N260P+W469Y;
H183*+G184*+W159Y+W167Y+N260P+E439Y+G476Q+G477Q; and
H183*+G184*+W167Y+F262P+W469Y+G476R+G477Q.

11. The variant alpha-amylase of claim 10, which consists of SEQ ID NO: 2 with a modification selected among:

H183*+G184*+W140F;
H183*+G184*+Q169N;
H183*+G184*+Q169A;
H183*+G184*+W189Y+E190P;
H183*+G184*+N260D;

H183*+G184*+G477E;
H183*+G184*+G477Q;
H183*+G184*+G477K;
H183*+G184*+W189E+E190P;
H183*+G184*+A51I+W140Y;
H183*+G184*+W140Y+W189E;
H183*+G184*+W140Y+N260P;
H183*+G184*+W140Y+W284D;
H183*+G184*+W140Y+G476R;
H183*+G184*+W140Y+G477E;
H183*+G184*+W189E+W439R;
H183*+G184*+W284D+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+W439R+G477E;
H183*+G184*+E194D;
H183*+G184*+W439R+D467K;
H183*+G184*+R320M+W439R;
H183*+G184*+W439R+K485R;
H183*+G184*+Y160S;
H183*+G184*+W189F+E190P;
H183*+G184*+F262A;
H183*+G184*+Y363H;
H183*+G184*+G476E;
H183*+G184*+N260P+W439R;
H183*+G184*+N260P+G477E;
H183*+G184*+W439R+G476R;
H183*+G184*+K72S+W140Y;
H183*+G184*+G109A+M202L+Y203G;
H183*+G184*+E194S;
H183*+G184*+E345D+G477R;
H183*+G184*+K302N+W439R;
H183*+G184*+R320K+W439R

H183*+G184*+W159Y+W167Y+F262P+W439R+W469Y+G477Q;
H183*+G184*+W159Y+W167F+N260D+W439Y+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439R+W469Y+G476K+G477E;

H183*+G184*+W159Y+W167F+F262P+W439V+W469Y+G476K+G477Q;
H183*+G184*+W159Y+W167F+F262P+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W159Y+W167Y+N260G+W439R+W469Y;
H183*+G184*+W167Y+N260D+W439R+G476Q+G477E;
H183*+G184*+W167Y+N260P+F262P+W439R+G476E+G477R;
H183*+G184*+W159Y+W167F+N260G+W439R+W469Y+G476R;
H183*+G184*+W159Y+W167Y+N260D+F262P+W439Y;
H183*+G184*+W159Y+W167F+N260P+W439Y+W469V+G476Q+G477Q;
H183*+G184*+W167Y+N260D+W439R+W469V+G476Q+G477Q;

H183*+G184*+W159Y+W167Y+N260P+F262P+W439R+G476E+G477K;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+W469Y+G476R;
H183*+G184*+W167F+N260D+F262P+P380Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439R+W469V+G476Q+G477K;
H183*+G184*+W167Y+N260D+F262P+W439V+W469Y;
H183*+G184*+W159Y+W167Y+N260D+W439R+W469V+G476E+G477K;
H183*+G184*+W167Y+N260D+W439R+W469Y+G476E+G477K;
H183*+G184*+W159Y+W167Y+N260D+G476E+G477Q;
H183*+G184*+W159Y+W167F+N260P+F262P+W439Y+G476K+G477Q;
H183*+G184*+N260D+F262P+W469Y+G476R+G477Q;
H183*+G184*+W167Y+L230I+N260P+W469Y;
H183*+G184*+W159Y+W167Y+N260P+E439Y+G476Q+G477Q; and
H183*+G184*+W167Y+F262P+W469Y+G476R+G477Q.

12. The variant alpha-amylase of any one of claims 7 to 9, having at least 90% sequence identity to SEQ ID NO: 14 and comprising modifications in positions corresponding to the following positions in SEQ ID NO: 14:

E178*+G179*;
E178*+G179*+T36N;
E178*+G179*+W136Y;
E178*+G179*+W155Y;
E178*+G179*+W155F;
E178*+G179*+W163Y;
E178*+G179*+G299E;
E178*+G179*+G299K;
E178*+G179*+G299R;

E178*+G179*+R315M;
E178*+G179*+R315A;
E178*+G179*+R315Q; and
E178*+G179*+W342Y.

13. The variant of claim 12 consisting of SEQ ID NO: 14 with a modification selected among:
E178*+G179*;
E178*+G179*+T36N;
E178*+G179*+W136Y;
E178*+G179*+W155Y;
E178*+G179*+W155F;
E178*+G179*+W163Y;
E178*+G179*+G299E;
E178*+G179*+G299K;
E178*+G179*+G299R;
E178*+G179*+R315M;
E178*+G179*+R315A;
E178*+G179*+R315Q; and
E178*+G179*+W342Y.

14. The variant alpha-amylase of claim 7, having at least 90% sequence identity to SEQ ID NO: 13 and comprising modifications in positions corresponding to position K315 and/or W467 in SEQ ID NO:13.

15. The variant of claim 14, consisting of SEQ ID NO: 13 with a substitution K315M.

16. A detergent composition comprising a variant alpha-amylase of any of the claims 7-15.

17. Use of a variant alpha-amylase according to any of the claims 7-15 in a cleaning process such as laundry or hard surface cleaning including automated dish wash.