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(54) **Title**: VARIANTS OF A FAMILY 44 XYLOGLUCANASE

(57) **Abstract**: The present invention relates to variants of a parent xyloglucanase.The present invention also relates to polynucleotides encoding the variant xyloglucanasesand to nucleic acid constructs, vectors, and host cells comprising the polynucleotide.

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VARIANTS OF A FAMILY 44 XYLOGLUCANASE

REFERENCE TO A SEQUENCE LISTING

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

FIELD OF THE INVENTION

The present invention relates to variants of a xyloglucanase belonging to family 44 of glycosyl hydrolases, polynucleotides encoding the variants and methods of producing the variants.

BACKGROUND OF THE INVENTION

Xyloglucan is a major structural polysaccharide in the primary (growing) cell wall of plants. Structurally, xyloglucans consists of a cellulose-like beta-1,4-linked glucose backbone which is frequently substituted with various side chains. Xyloglucan is believed to function in the primary wall of plants by cross-linking cellulose micro fibrils, forming a cellulose-xyloglucan network.

Xyloglucanses are capable of catalyzing the solubilization of xyloglucan to xyloglucan oligosaccharides. Some xyloglucanases only exhibit xyloglucanase activity, whereas others exhibit both xyloglucanase and cellulase activity. Xyloglucanses may be classified in EC 3.2.1.4 or EC. 3.2.1.151. Enzymes with xyloglucanase activity are for example described in Vincken et al. (1997) *Carbohydrate Research* 298(4):299-310, wherein three different endoglucanases EndoI, EndoV and EndoVI from *Trichoderma viride* (similar to *T. reesei*) are characterized. EndoI, EndoV and EndoVI belongs to family 5, 7 and 12 of glycosyl hydrolases, respectively, see Henrissat, B. (1991) Biochem. J. 280: 309-316, and Henrissat, B. and Bairoch, A. (1993) Biochem. J. 293: 781-788. WO 94/14953 discloses a family 12 xyloglucanase (EG II) cloned from the fungus *Aspergillus aculeatus*. WO 99/02663 discloses family 12 and family 5 xyloglucanases cloned from *Bacillus licheniformis* and *Bacillus agaradhaerens*, respectively. WO 01/062903 discloses family 44 xyloglucanases.

In particular WO 99/02663 and WO 01/062903 suggest that xyloglucanases may be used in detergents.

It is an object of the present invention to provide variants of xyloglucanases belonging to family 44 of glycosyl hydrolases with improved properties compared to its parent enzyme.

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides an isolated variant of a parent xyloglucanase, the variant comprising an alteration of the parent xyloglucanase at position number 68, which position corresponds to a position in amino acid sequence SEQ ID NO:3 and wherein

a) the alteration(s) are
   i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or
   ii) deletion of the amino acid which occupies the position, and/or
   iii) a substitution of the amino acid which occupies the position with a different amino acid;
b) the parent xyloglucanase is a family 44 xyloglucanase;
c) the variant has xyloglucanase activity, and
d) wherein the parent xyloglucanase is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7 or a xyloglucanase having at least 75% identity to the amino acid sequence of SEQ ID NO:3.

According to a second aspect, the present invention provides an isolated polynucleotide sequence encoding the variant according to the first aspect.

According to a third aspect, the present invention provides a recombinant host cell comprising an expression vector comprising the polynucleotide sequence according to the second aspect.

According to a fourth aspect, the present invention provides a method for producing a variant of a parent xyloglucanase, wherein said variant has xyloglucanase activity, said method comprising:

a) cultivating the host cell of the third aspect under conditions suitable for the expression of the variant; and
b) recovering the variant from the cultivation medium.

According to a fifth aspect, the present invention provides a method for improving the detergent stability of a xyloglucanase, comprising altering of the position 68, which position corresponds to a position in amino acid sequence SEQ ID NO:3 and wherein

a) the alteration(s) are
   i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or
   ii) deletion of the amino acid which occupies the position, and/or
   iii) a substitution of the amino acid which occupies the position with a different amino acid;
b) the parent xyloglucanase is a family 44 xyloglucanase;
c) the variant has xyloglucanase activity, and
d) wherein the parent xyloglucanase is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7 or a xyloglucanase having at least 75% identity to the amino acid sequence of SEQ ID NO:3.

According to a sixth aspect, the present invention provides a formulation comprising the variant according to the first aspect.

According to a seventh aspect, the present invention provides a variant produced by the method according to the fourth aspect.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

The present invention relates to isolated variants of a parent xyloglucanase, comprising an alteration at one or more (several) positions selected from the group consisting of position number 68, 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395, 366, 1, 374, 7, 140, 8, 14, 21, 211, 37, 45, 13, 78, 87, 436,101, 104, 111, 306, 117, 119, 414, 139, 268, 142, 159, 164, 102, 168, 176, 180, 482, 183, 202, 206, 217, 4, 222, 19, 224, 228, 232, 2, 240, 244, 5, 247, 249, 328, 252, 259, 406, 267, 269, 275, 179, 166, 278, 281, 288, 298, 301, 18, 302, 165, 80, 303, 316, 169, 322, 120, 146, 342, 348, 147, 353, 380, 468, 382, 383, 38, 384, 389, 391, 10, 392, 396, 177, 397, 399, 409, 237, 413, 253, 415, 418, 40, 443, 445, 148, 449, 225, 450,

454, 3, 455, 456, 299, 461, 470, 204, 476, 488, 347, and 507,which position corresponds to a position in amino acid sequence SEQ ID NO:3 and wherein the alteration(s) are independently

i) an insertion of an amino acid downstream of the amino acid which occupies the position,

ii) deletion of the amino acid which occupies the position, or

iii) a substitution of the amino acid which occupies the position with a different amino acid; and

the parent xyloglucanase is a family 44 xyloglucanase; and the variant has xyloglucanase activity.

The present invention also relates to isolated polynucleotides encoding the variant xyloglucanases or polypeptides having xyloglucanases activity, nucleic acid constructs, vectors, and host cells comprising the polynucleotides, and methods of producing a variant of a parent xyloglucanase or a polypeptide having xyloglucanases activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to variants of parent family 44 xyloglucanases, comprising an alteration, preferably in the form of a substitution and/or an insertion and/or a deletion at one or more (several) positions, where the numbering of the positions corresponds to the numbering of the positions of SEQ ID NO:3. The variants of the present invention have xyloglucanase activity and potentially also cellulolytic activity. The variants of the present invention have improved properties compared to the parental xyloglucanase. In one aspect, the variants have improved stability in liquid detergents, especially liquid laundry detergent compositions.

Definitions

Xyloglucanase activity: The term “xyloglucanase activity” is defined herein as an enzyme catalyzed hydrolysis of xyloglucan. The reaction involves endo hydrolysis of 1,4-beta-D-glucosidic linkages in xyloglucan. For purposes of the present invention, xyloglucanase activity is determined using AZCL-xyloglucan (from Megazyme) as the reaction substrate. The assay can be performed in

several ways, e.g. as described in Example 2 of the present application or as described in WO 01/62903. One unit of xyloglucanase activity (XyloU) is defined by reference to the assay method described in WO 01/62903, page 60, lines 3 – 17.

Cellulase activity: The term "cellulase activity" is defined herein as an enzyme catalyzed hydrolysis of 1,4-beta-D-glucosidic linkages in beta-1,4-glucan (cellulose). For purposes of the present invention, cellulase activity is determined using AZCL- HE-cellulose (from Megazyme) as the reaction substrate.

Variant: The term "variant" is defined herein as a polypeptide having xyloglucanase activity comprising an alteration, such as a substitution, insertion, and/or deletion, of one or more (several) amino acid residues at one or more (several) specific positions which positions correspond to the amino acid positions in SEQ ID NO: 3. The variants of the invention may also have cellulase activity. The altered polypeptide (variant) is obtained through human intervention by modification of the polynucleotide sequence encoding the parental enzyme. The parental enzyme may be encoded by SEQ ID NO: 1, SEQ ID NO: 4 or SEQ ID NO: 6 or a sequence which is at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95% identical to one of these sequences and which encode an active polypeptide. The variant polypeptide sequence is preferably one which is not found in nature.

Wild-Type Enzyme: The term "wild-type" xyloglucanase denotes a xyloglucanase expressed by a naturally occurring microorganism, such as bacteria, yeast, or filamentous fungus found in nature. The term wild-type may be used interchangeably with the term "naturally occurring".

Parent Enzyme: The term "parent" xyloglucanase or "parental" xyloglucanase as used herein means a xyloglucanase to which a modification, *e.g.*, substitution(s), insertion(s), deletion(s), and/or truncation(s), is made to produce the enzyme variants of the present invention. This term also refers to the polypeptide with which a variant is compared and aligned. The parent may be a naturally occurring (wild-type) polypeptide such as the enzyme of SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO: 5 or SEQ ID NO: 7 or a polypeptide which is at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95% identical to one of these sequences. The parent polypeptide may also be a variant of a naturally occurring polypeptide which has been modified or altered in the amino acid sequence. A parent may also be an allelic variant, which is a polypeptide encoded by any of two or more alternative forms of a gene occupying the same chromosomal locus.

Isolated variant or polypeptide: The term "isolated variant" or "isolated polypeptide" as used herein refers to a variant or a polypeptide that is isolated from a source, e.g. the host cell from which it is expressed or the enzyme complex it is normally present in. Preferably, the polypeptide is at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, most preferably at least 90% pure, and even most preferably at least 95% pure, as determined by SDS-PAGE.

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

Mature polypeptide: The term "mature polypeptide" is defined herein as a polypeptide having xyloglucanase activity that is in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. For the polypeptide defined by SEQ ID NO: 2, the mature xyloglucanase sequence may in theory start at position 28 of SEQ ID NO: 2. The mature sequence ends at position 551 of SEQ ID NO: 2. The theoretical mature xyloglucanase sequence is show in SEQ ID NO: 3. Depending on expression system the length of the actual mature polypeptide may vary 1 to 10 amino acids in length based on the theoretical mature polypeptide. The mature polypeptide may for example start at position 33 of SEQ ID NO: 2 and ends at position 551 of SEQ ID NO: 2.**Mature polypeptide coding sequence:** The term "mature polypeptide coding sequence" is defined herein as a nucleotide sequence that encodes a mature polypeptide having xyloglucanase activity. In one aspect, the mature polypeptide coding sequence is nucleotides 82 to 1653 of SEQ ID NO: 1. The mature polypeptide coding sequence may vary 3 to 30 nucleotides in length depending on the expression system. The mature polypeptide coding sequence can for example correspond to nucleotides 97 to 1653 of SEQ ID NO: 1.

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

For purposes of the present invention, the degree of 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 in Genetics* 16: 276-277; http://emboss.org), 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 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*; http://emboss.org), 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)

Functional fragment: The term "functional fragment of a polypeptide" is used to describe a polypeptide which is derived from a longer polypeptide, *e.g.*, a mature polypeptide, and which has been truncated either in the N-terminal region or the C-terminal region or in both regions to generate a fragment of the parent polypeptide. To be a functional polypeptide the fragment must maintain at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the xyloglucanase activity of the full-length/mature polypeptide.

Allelic variant: The term "allelic variant" denotes herein 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” as used herein refers to a polynucleotide that is isolated from a source. In one aspect, the isolated polynucleotide is at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, and even most preferably at least 95% pure, as determined by agarose electrophoresis.

Substantially pure polynucleotide: The term “substantially pure polynucleotide” as used herein refers to 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%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably 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, preferably at least 92% pure, more preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, even more preferably at least 98% pure, most preferably at least 99%, and even most preferably at least 99.5% pure by weight. The polynucleotides of the present invention are preferably in a substantially pure form, *i.e.*, that the polynucleotide preparation is essentially free of other polynucleotide material with which it is natively or recombinantly associated. The polynucleotides may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

Coding sequence: When used herein 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.

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

Host cell: The term "host cell", as used herein, includes any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or a 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.

Improved chemical stability: The term "improved chemical stability" is defined herein as a variant enzyme displaying retention of enzymatic activity after a period of incubation in the presence of a chemical or chemicals, either naturally occurring or synthetic, which reduces the enzymatic activity of the parent enzyme. Improved chemical stability may also result in variants better able to catalyze a reaction in the presence of such chemicals. In a particular aspect of the invention the improved chemical stability is an improved stability in a detergent, in particular in a liquid detergent. The improved detergent stability is in particular an improved stability of the xyloglucanase activity when a xyloglucanase variant of the present invention is mixed into a liquid detergent formulation and then stored at temperatures between 15 and 50 °C.

In the present invention liquid detergents are particular useful as liquid laundry detergents.

Conventions for Designation of Variants

For purposes of the present invention, the amino acid sequence of the xyloglucanase disclosed in SEQ ID NO: 3 is used to determine the corresponding amino acid residue in another xyloglucanase. The amino acid sequence of another xyloglucanase is aligned with the amino acid sequence of the xyloglucanase disclosed in SEQ ID NO: 3, and based on the alignment the amino acid position number corresponding to any amino acid residue in the amino acid sequence of the xyloglucanase disclosed in SEQ ID NO: 3 can be determined.

An alignment of polypeptide sequences may be made, for example, using "ClustalW" (Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, *Nucleic Acids Research* 22: 4673-4680). An alignment of DNA sequences may be done using the polypeptide alignment as a template, replacing the amino acids with the corresponding codon from the DNA sequence.

In describing the various xyloglucanase variants of the present invention, the nomenclature described below is adapted for ease of reference. In all cases, the accepted IUPAC single letter or triple letter amino acid abbreviation is employed.

Substitutions. 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.*, "G205R + S411F", representing mutations at positions 205 and 411 substituting

glycine (G) with arginine (R), and serine (S) with phenylalanine (F), respectively. Where an original amino acid may be substituted by an amino acid selected from a group it is designated as "K129R,S,A,I,F,Q" representing the substitution of a lysine (K) at position 129 with an amino acid selected from the group consisting of: arginine (R), serine (S), alanine (A), isoleucine (I), phenylalanine (F) and glutamine (Q). Alternatively, "K129R,S,A,I,F,Q" could be written as K129R or K129S, or K129A, or K129I or K129F or K129Q

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

Insertions. For an amino acid insertion, the following nomenclature is used: Asterisk (*)/position/ lower case letter/inserted amino acid, where the lower case letter indicates the addition of an amino acid down stream of the position number. Accordingly, the insertion of a glutamic acid (E) down stream of position 10 is designated "*10aE". If a second amino acid, e.g. a valine (V), is to be inserted down stream of position 10 after the glutamic acid (E) it is designated "*10aE +*10bV". Additions to the N-terminal of the polypeptide are designated with a 0 (zero). The addition of a glutamic acid (E) and a valine (V) added to the N-terminal amino acid of a polypeptide is designated as *0aE+*0bV. A "downstream" insertion can also be described as the addition of one or more amino acids between the named position and the position immediately following the named position, e.g. an insertion downstream of position 195 results in the addition of one or more amino acids between position 195 and 196, thereby generating new positions *195a, *195b and so forth.

Parent xyloglucanases

In the present invention, the parent xyloglucanase is either (a) a xyloglucanase belonging to family 44 of glycosyl hydrolases also termed family 44 xyloglucanases; or (b) a polypeptide selected from the group consisting of SEQ ID NO:3, SEQ ID NO: 5 and SEQ ID NO: 7; or (c) a polypeptide comprising an amino acid sequence having at least 75% identity with the mature polypeptide of SEQ ID NO: 3; or (d) a polypeptide encoded by a polynucleotide that hybridizes under at least medium stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6 or (iii) a full-length complementary strand of (i) or (ii); or (e) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 70% identity with the mature polypeptide coding sequence of SEQ ID NO: 1.

In a first aspect, the parent xyloglucanase comprise an amino acid sequence having a degree of identity to the mature polypeptide of SEQ ID NO: 3 of preferably at least at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 96%, even more preferably at least 97%, most preferably at least 98%, or even most preferably at least 99%, which have xyloglucanase activity (hereinafter "homologous polypeptides"). In one aspect, the homologous polypeptides have an amino acid sequence that differs by ten amino acids, preferably by nine, more preferably by eight, more preferably by seven, more preferably by six, more preferably by five amino acids, more preferably by four amino acids, even more preferably by three amino acids, most preferably by two amino acids, and even most preferably by one amino acid from the mature polypeptide of SEQ ID NO: 3.

Substantially homologous parent xyloglucanases may have one or more (several) amino acid alterations such as substitutions, deletions and/or insertions. These changes are preferably of a minor nature, that is conservative amino acid substitutions and other substitutions that do not significantly affect the three-dimensional folding or activity of the protein or polypeptide; small deletions, typically of one to about 9 amino acids, preferably from one to about 15 amino acids and most preferably from one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about five to ten residues, preferably from 10 to 15 residues and most preferably from 20 to 25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tag, or protein A (Nilsson *et al.*, 1985, *EMBO J.* 4: 1075; Nilsson *et al.*, 1991, *Methods Enzymol.* 198: 3. See, also, in general, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

Although the changes described above preferably are of a minor nature, such changes may also be of a substantive nature such as fusion of larger polypeptides of up to 300 amino acids or more both as amino- or carboxyl-terminal extensions.

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

Essential amino acids in the xyloglucanase polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (i.e. xyloglucanase activity) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-4708, 1996. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-312, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with polypeptides which are related to a polypeptide according to the invention. The crystal structure of an enzyme belonging to the family 44 glycosyl hydrolases has been published by Kitago et. al, J. Biol. Chem. Vol. 282:35703-35711, 2007. Based on this structure it is possible to generate a three dimensional structure of the parent xyloglucanase (SEQ ID NO: 3) in silico. Based on comparison with the published structure the following residues in SEQ ID NO: 3 have been identified as critical for the enzymatic function E187 (Catalytic - Acid/Base), E358 (Catalytic - Nucleophile), E56 (Carboxylate group coordinating Ca2+) and D154 (Carboxylate group coordinating Ca2+). These positions should, therefore, preferably not be mutated in the parent enzyme.

The parent xyloglucanase preferably comprises the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; or a fragment thereof having xyloglucanases activity. In one aspect, the parent xyloglucanase comprises the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent xyloglucanase comprises the mature polypeptide of SEQ ID NO: 2. In another aspect, the parent xyloglucanase consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; or a fragment thereof having xyloglucanase activity. In another aspect, the parent xyloglucanase comprises the amino acid sequence of SEQ ID NO: 5, or an allelic variant thereof; or a fragment thereof having xyloglucanase activity. In another aspect, the parent xyloglucanase comprises the amino acid sequence of SEQ ID NO: 7, or an allelic variant thereof; or a fragment thereof having xyloglucanase activity. In another aspect the parent xyloglucanase comprises an amino acid sequence which is at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95% identical SEQ ID NO: 2, or SEQ ID NO: 3 or SEQ ID NO: 5.A fragment of the mature polypeptide of SEQ ID NO: 3 is a polypeptide having one or more (several)

amino acids deleted from the amino- and/or carboxyl-terminus of this amino acid sequence and still maintaining xyloglucanase activity.

In a second aspect, the parent xyloglucanases are encoded by polynucleotides that hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6, (iii) a subsequence of (i) or (ii), or (iv) a full-length complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). The subsequence may encode a polypeptide fragment having xyloglucanase activity. In one aspect, the complementary strand is the full-length complementary strand of the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6.

A subsequence of the mature polypeptide coding sequence of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6, or a homolog thereof, is a nucleotide sequence where one or more (several) nucleotides have been deleted from the 5'-and/or 3'-end, where the polypeptide encoded by the subsequence possess xyloglucanase activity.

The parent enzymes may also be allelic variants of the polypeptides that have xyloglucanase activity.

The polynucleotide of SEQ ID NO: 1 or SEQ ID NO: 4 or SEQ ID NO: 6; or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 5 or SEQ ID NO: 7; or a fragment thereof; may be used to design nucleic acid probes to identify and clone DNA encoding parent xyloglucanases 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, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, *e.g.*, nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, preferably at least 900 nucleotides in length, preferably at

least 1000 nucleotides in length, preferably at least 1100 nucleotides in length, preferably at least 1200 nucleotides in length, preferably at least 1300 nucleotides in length, preferably at least 1400 nucleotides in length, preferably at least 1500 nucleotides in length or most preferably at least 1600 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 library prepared from other organisms may be screened for DNA that hybridizes with the probes described above and encodes a parent xyloglucanase. Genomic or other DNA from 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. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, or a subsequence thereof, the carrier material is used in a Southern blot. For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleotide probe corresponding to the polynucleotide shown in SEQ ID NO: 1, its complementary strand, 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 the mature polypeptide coding sequence of SEQ ID NO: 1. In another aspect, the nucleic acid probe is nucleotides 82 to 1653 of SEQ ID NO: 1, or nucleotides 97 to 1653 of SEQ ID NO: 1. In another aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 2, or a subsequence thereof. In another aspect, the nucleic acid probe is SEQ ID NO: 1.

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.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C (medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at 65°C (high stringency), and most preferably at 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, hybridization, and washing post-hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences 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.

For short probes that are about 15 nucleotides to about 70 nucleotides in length, the carrier material is 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.

In a third aspect, the parent xyloglucanase is encoded by a polynucleotide comprising or consisting of a nucleotide sequence having a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably 96%, 97%, 98%, or 99%, which encode an active polypeptide. In one aspect, the mature polypeptide coding sequence is nucleotides 82 to 1653 of SEQ ID NO: 1, or nucleotides 97 to 1653 of SEQ ID NO: 1.

The parent xyloglucanase may be obtained from microorganisms of any genus. In one aspect, the parent xyloglucanase is secreted extracellularly.

In a further aspect the parent xyloglucanase may be a bacterial xyloglucanase. For example, the xyloglucanase may be a Gram positive bacterial polypeptide such as a *Bacillus*, preferably from the Bacillus/Lactobacillus subdivision, preferably a species from the genus Paenibacillus, especially Paenibacillus polymyxa, e.g. Paenibacillus polymyxa, ATCC 832, preferably the xyloglucanase is a family 44 xyloglucanse, e.g. as described in WO 01/62903, more preferably the xyloglucanase of SEQ ID NO: 5, more preferably the xyloglucanase of SEQ ID NO: 7, and most preferably the xyloglucanase of SEQ ID NO: 2 or the mature polypeptide thereof.

Generation of Variants

Variants of a parent xyloglucanase can be prepared according to any mutagenesis procedure known in the art, such as random and/or site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, etc.

Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide molecule of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian, *et. al.,* (Tian, *et.*

al., Nature 432:1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

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

Site-directed mutagenesis is a technique in which one or several mutations are created at a defined site in a polynucleotide molecule encoding the parent xyloglucanase. The technique can be performed *in vitro* or *in vivo*.

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 xyloglucanase 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. For further description of suitable techniques reference is made to Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990), and WO 96/34946; Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* 76: 4949-4955; and Barton *et al.*, 1990, *Nucleic Acids Research* 18: 7349-4966.

After the ligase reaction the ligation mixture may be used to transform a host cell, for cloning purposes *E. coli* cells are often used as described in Ausubel, F. M. et al. The transformed *E. coli* cells can be propagated in liquid media or on solid agar plates, plasmids can be rescued from the transformed cells and used to transform *B. subtilis* cells. Suitable competent *Bacillus* cells, such as MB1510, an 168-derivative (e.g. available from BGSC with accession no. 1A1 168 trpC2), may be transformed as described in WO 03/095658. An E. coli plasmid-borne integration cassette for library construction may be used for Bacillus transformation. The method is described in detail in WO 03/095658. Alternatively, an in vitro amplified PCR-SOE-product (Melnikov and Youngman, Nucleic Acid Research 27, 1056) may be used.

Site-directed mutagenesis can be accomplished *in vivo* by methods known in the art. See, for example, U.S. Patent Application Publication 2004/0171154; Storici *et al.*, 2001, *Nature Biotechnology* 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 of a parent xyloglucanases.

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, *Biochem.* 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 as described above can be combined with high-throughput, automated screening methods to detect the activity of cloned, mutagenized polypeptides expressed by host cells, e.g. Bacillus as described above. Mutagenized DNA molecules that encode polypeptides weith xyloglucanase activity can be recovered from the host cells and rapidly sequenced using standard methods in the art.

Variants

In the present invention, the isolated variants of a parent xyloglucanase comprise an alteration at one or more (several) positions selected from the group consisting of positions number 68, 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395, 366, 1, 374, 7, 140, 8, 14, 21, 211, 37, 45, 13, 78, 87, 436,101, 104, 111, 306, 117, 119, 414, 139, 268, 142, 159, 164, 102, 168, 176, 180, 482, 183, 202, 206, 217, 4, 222, 19, 224, 228, 232, 2, 240, 244, 5, 247, 249, 328, 252, 259, 406, 267, 269, 275, 179, 166, 278, 281, 288, 298, 301, 18, 302, 165, 80, 303, 316, 169, 322, 120, 146, 342, 348, 147, 353, 380, 468, 382, 383, 38, 384, 389, 391, 10, 392, 396, 177, 397, 399, 409, 237, 413, 253, 415, 418, 40, 443, 445, 148, 449, 225, 450, 454, 3, 455, 456, 299, 461, 470, 204, 476, 488, 347, and 507, wherein the variant having xyloglucanase activity comprises an amino acid sequence having a degree of identity of at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, more preferably at least 95%, more preferably at least about 97%, most preferably at least 98% and even more preferably 99% to the amino acid sequence of the parent xyloglucanase. The numbering of the positions are relative to the amino acid sequence of SEQ ID

NO: 3. Preferably, the variants comprising alterations at one or more of the above identified positions have an increased stability in detergent, preferably in liquid detergent as compared to the parent xyloglucanase.

In a preferred embodiment the variant comprises one or more (several) of the following combinations of alterations:

V1*+V2*+H3*;
V1Q+*1aE+*1bV;
H3A;
H3A+H436A;
K8A,Q,S;
T9D;
T9D+L34F+A83E+Q149E+H193T+S332P+R340T;
I10V+D33E+M40L+A41T+Q67M+Y73F+S76D+G78A+Q82K+T92A+L102Q+Q137E+I222V+V228I+D249N+S269N+V272A+E333A+I337L+M356L+T374A+S416A+D444Y+A469E+K470T+I473G+T517A+S522*;
I10V+F17S+D33E+M40L+A41T+Q67M+N72S+S76D+G78A+Q82K+Q137E+V219A+D249N+V272A+I337L+M356L+V397A+S416A+T421I+S424N+N441D+D444Y+V450I+K470T+I473S+V477I;
I10V+F17S+D33E+M40L+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+Q137E+H164N+N168K+T172A+V219A+I222V+V228I+D249N+S269N+V272A+E333A+I337L+M356L+N415S+T421I+S424H+N441D+D444Y+S522P+P523V+V524E;
I10V+F17S+D33E+M40L+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+Q137E+I222V+V228I+D249N+V272A+I337L+M356L+T374A+V397A+S416A+T421I+S424N+N441D+D444Y+V450I+A469E+K470T+I473G+T517A+S522P+P523V+V524E;
I10V+F17S+D33E+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+Q137E+N168K+T172A+I222V+V228I+D249N+V272A+E333A+I337L+M356L+V397A+S416A+T421I+S424H+N441D+D444Y+A469E+K470T+I473S+V477I+E489A+A490V+T517A+S522*;
I10V+F17S+M40L+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+Q137E+I222V+V228I+D249N+S269N+V272A+T320A+I337L+M356L+T374A+V397A+N415S+T421I+S424H+N441D+D444Y+A469E+K470T+I473S+V477I+T517A+S522P+P523V+V524E;
I10V+F17S+Q67M+N72S+S76D+G78A+Q82K+T104A+Q137E+N153K+R156Q+V219A+I222V+V228I+D249N+S269N+V272A+E333A+I337L+M356L+V397A+N415S+D420G+T421I+S424H+N441D+D444Y+V450I+A469E+K470T+I473G+T517A+S522*;
I10V+F17S+Q67M+N72S+S76D+G78A+Q82K+T92A+T104A+Q137E+R156Q+V159A+H164N+N168K+T172A+I222V+V228I+D249N+V272A;
I10V+F17S+Y53H+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+Q137E+T172V+A177T+I222V+V228I+D249N+S269N+I337L+M356L+V397A+S416A+T421I+S424H+N441D+D444Y+A469E+K470T+I473G+T517A+S522*;
K13A+K129A;
K13A+Q68H+T92V+K118A+Q137E+R156Y+G200P;
K13A,R;
K18R;
R20A;
K21Q+K129A;
K21Q,R,T;
Q32H+M40L+R49G+D65E+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q+T104A+Q137E+H164N+K202E+I222V+V228I+D249N+M356L+T374A;
D33V+Q68H+N168H+V450I;
L34F,I,M,V;
L34I+K129A;
D37G,N+K129A+R156Y;
E38I,V;

M40L+A41T+Q67M+N72S+S76D+G78A+Q82K+Q137E+N153K+H164N+D249N+V272A+I337L+
M356L+V397A+N415S+T421I+S424N+N441D+V450I+E489A+A490V+T517A+S522*;
M40V;
L45I;
Q68H,M,N;
Q68H+G200P+N331F;
Q68H+K118A+K129A+R156Y+G200P+N331F;
Q68H+K118A+R156V+G200P+N331F;
Q68H+K118A+R156Y+H193T+D366H;
Q68H+K118R+R156F,Y;
Q68H+K118R+R156Y+G200P;
Q68H+K118S+R156F+G200P+G274D+N331F;
Q68H+K129A,T+R156K+G200P+N331F;
Q68H+R156F,V,Y+G200P+N331F;
Q68H+R156Y;
Q68H+R156Y+H193T;
Q68H+R156Y+H193T+D366H;
Q68H+R156Y+H193T+G200P+M310V;
Q68H+S76W+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92A,D,I,S,V,Y+K118A+K129A+R156Y+G200P+N331F;
Q68H+T92N+D97N+K118A+K129A+R156Y+G200P+N331F;
Q68H+T92S+K118A+K129A+R156Y+G200P+G274D+N331F;
Q68H+T92V+G200P+M310V;
Q68H+T92V+G200P+M310V+N331F;
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+N331F;
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+G200P+M310V+E446K;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331H,K,Q;
Q68H+T92V+K118A+K129A+R156Y+H193T;
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H;
Q68H+T92V+K118A+K129A+R156Y+H193T+G200P+M310V;
Q68H+T92V+K118A+Q137E+N140F+R156Y+G200P+K470T;
Q68H+T92V+K118A+Q137E+R156Y+G200P+D324N;
Q68H+T92V+K118A+Q137E+R156Y+G200P+K470T;
Q68H+T92V+K118A+Q137E+R156Y+G200P+M310L;
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A,R+R156Y,F;
Q68H+T92V+K118A+S123P,T+K129A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118R+R156Y+H193T+D366H;
Q68H+T92V+R156F+G200P+M310V+S484C;
Q68H+T92V+R156F,V,Y+G200P+M310V;
Q68H+T92V+R156F,V,Y+G200P+M310V+N331F;
Q68H+T92V+R156F,Y+H193T;
Q68H+T92V+R156F,Y+H193T+D366H;
Q68H+T92V+R156F,Y+H193T+G200P+M310V;
Q68H+T92V+R156Y;
S76E,I,K,M,R,T,V,W;
S76W+G200P;
S76W+G200P+A224P;
G78A+K118A++K129A+R156Y;

G78A+K118A+K129A+R156Y;
G78A+K118A+K129A+R156Y+G200P+N331F;
G78A+K118A+K129A+R156Y+K169A;
G78A,N,S;
G78A+T92V+K118A+K129A+R156Y;
G78A+T92V+K118A+K129A+R156Y+G200P+N331F;
G78A+T92V+K118A+K129A+R156Y+K169A;
L80V;
A83D,E,H,I,L,N,R,S,T,Y;
K87Q;
K87V+K129A+K169A;
T92I,V;
T92V+K118A+K129A+Q137E+R156Y+G200P+N331F;
T92V+K118A+K129A+R156Y;
T92V+K118A+K129A+R156Y+G200P+N331F;
T92V+K118A+K129A+R156Y+H164N+G200P+N331F;
T92V+K129A+R156Y;
K101A+K129A;
K101R;
K101R+L102I;
T104A+P111Q+A117S+K129A+R156Y;
P111Q;
K118A+K129A;
K118A+K129A+F146L+R156Y+G200P+N331F;
K118A+K129A+Q137E+R156Y+G200P+N331F;
K118A+K129A+R156Y;
K118A+K129A+R156Y+A224P;
K118A+K129A+R156Y+G200P;
K118A+K129A+R156Y+G200P+M310V+N331F;
K118A+K129A+R156Y+G200P+N331F;
K118A+K129A+R156Y+G200P+N331F+N399I;
K118A+K129A+R156Y+K169A+G200P+N331F;
K118A+K129A+R156Y+K470T;
K118A,R;
K118A+R156Y;
K118A+R156Y+G200P;
D119L;
G120A;
S123P,T;
S123T+K129A+R156Y;
K129A,F,I,K,R,S,T;
K129A+K169A;
K129A+K176P;
K129A+K275Q;
K129A+K445S;
K129A+K470T;
K129A+Q137E+R156Y;
K129A+Q137E+R156Y+G200P;
K129A+Q137E+R156Y+K470T;
K129A+Q137E+V139K+N140F+Q147S+R156Y;
K129A+R156Y;
K129A+R156Y+A177T+V179I+A183S;

K129A+R156Y+A328G;
K129A+R156Y+D247G;
K129A+R156Y+D249G,N,S;
K129A+R156Y+D303I,K,S,V;
K129A+R156Y+D324N;
K129A+R156Y+D366H+T374A;
K129A+R156Y+D461N,Q,T;
K129A+R156Y+E288Q;
K129A+R156Y+G200P;
K129A+R156Y+G200P+G204T+R211K;
K129A+R156Y+H164N;
K129A+R156Y+H436Y;
K129A+R156Y+I10V+V14I+D19E;
K129A+R156Y+I222V+A224P+V228I+V232A;
K129A+R156Y+K176P,S;
K129A+R156Y+K275T;
K129A+R156Y+K322I+K454Q;
K129A+R156Y+K406N+N415G;
K129A+R156Y+K454Q;
K129A+R156Y+L380F+N383Y+D384G+N389T;
K129A+R156Y+N298F+E299N+G301T;
K129A+R156Y+N302K+D303L,S;
K129A+R156Y+N331F;
K129A+R156Y+P507A;
K129A+R156Y+R267H;
K129A+R156Y+R409L,T;
K129A+R156Y+S443D+K445S+L449I+V450I+S455N+M456Y;
K129A+R156Y+T244D;
K129A+R156Y+V159M+H164N+F165Y;
K129A+R156Y+V259I+R267K+L268K+S269A;
Q137D,E;
N140F;
K142A,Q,R;
F146C+H164C;
F146K,L;
F146L+K322I;
L148K+N168D;
Q149E;
R156A,D,E,F,I,K,L,M,N,P,Q,R,S,T,V,W,Y;
R156Y+N331F;
V159M;
H164A,N;
L166I;
N168D;
K169A,Q,R;
K176P;
A177E,T;
K180R;
H193A,D,S,T;
R197A,L;
H199A;
G200A,C,D,E,F,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

G200P+A224P;
K202N,Q,R;
S214E;
K217A;
A221K;
G225S;
V232A;
G237A,S,V;
K240A,Q,R;
K252A,Q,R;
G253A;
R267A;
L268I;
K275A,Q,R;
L278I;
F281L;
M290R;
R295A;
K306A,R;
K307Q;
M310I,L,V;
M310V+N399I;
R314A;
G316I;
K322A,R;
D324N;
N331A,C,D,E,F,G,H,I,K,L,M,P,Q,R,S,T,V,W,Y;
S332M,P;
S332P+V397I;
R340A,N,T;
K342A;
V345I;
K347A,Q,R;
D348G;
K353Q,R;
D366H;
M373Q;
T374A;
L380F;
K382A;
N383Y;
N389A,F,N,V;
W391V;
K392G,Q;
D395G;
G396P;
V397S;
N399I;
K406N;
G413A,S;
K414A;
N415S;

T417K;
F418I;
V431E;
H436A;
N441G+A442E+S443D;
S443E,K,Q;
K445A,R,S;
K445C+K470C;
H448A;
K454R;
S467R+G468S+A469T;
G468S,Y;
K470P,R,T;
I473T;
K476Q;
K482A,Q,R;
K488A,Q,R,T;
A490R;
G498A,D,S;
R500A,T,V;
H512A;
T517A+G518D; or
G518D;

In one aspect, the number of amino acid alterations in the variants of the present invention comprise preferably the total number of 55, preferably 52, more preferably 50, more preferably 40, more preferably 30, more preferably 20, more preferably 15, more preferably ten, more preferably nine, more preferably eight, even more preferably seven, even more preferably six, even more preferably five, even more preferably four, even more preferably three, and most preferably two alterations, and most preferably one alteration. In another aspect the total number of alterations is one, preferably two, more preferably three, even more preferably four, even more preferably five, even more preferably six, even more preferably seven, even more preferably eight, even more preferably nine, most preferably ten. The alteration may be in the form of i) an insertion of an amino acid downstream of the amino acid which occupies the position; ii) deletion of the amino acid which occupies the position, or iii) a substitution of the amino acid which occupies the position with a different amino acid. The alterations may be made independently of each other, for example in one position there may be an insertion while there is a substitution at a second position and a deletion at a third position as compared to the parental xyloglucanase. In a preferred embodiment the variant only comprises substitutions.

In one aspect of the invention positions to be mutated are identified based on consensus sequence analysis. The analysis is performed by aligning SEQ ID NO: 3, with SEQ ID NO: 5 and SEQ ID NO: 7 as well as with other sequences from the uniprot database which are 30% identical to the family 44 glycosyl hydrolase region of SEQ ID NO: 3. The resulting consensus sequences are shown in

Fig. 1. Consensus sequence 1 is the sequence comprising the most abundant amino acid at a given position from the alignment, consensus sequence 2 is the sequence with the 2nd most abundant amino acid at a given position and so forth. In one aspect of the invention, one or more (several) residues of SEQ ID NO: 3 are replaced by the corresponding residue from Consensus sequence 1 or Consensus sequence 2 or Consensus sequence 3 or Consensus sequence 4. In one aspect of the present invention the variants comprise an alteration at one or more (several) of the positions selected from the group of 52 positions identified by the consensus sequence analysis consisting of position number 10, 19, 68, 80, 89, 104, 111, 117, 123, 129, 137, 139, 140, 147, 156, 159, 164, 165, 177, 179, 183, 200, 204, 211, 222, 224, 225, 228, 232, 259, 267, 268, 269, 281, 328, 345, 366, 374, 380, 383, 384, 406, 415, 436, 443, 445, 449, 450, 455, 456, 488 and 507. In a preferred embodiment the alteration is a substitution, or several substitutions, selected from the group consisting of: I10V, D19E, Q68H, L80V, G89A, T104A, P111Q, A117S, S123P, K129T, Q137E, V139K, N140F, Q147S, R156Y, V159M, H164N, F165Y, A177T, V179I, A183S, G200P, G204T, R211K, I222V, A224P, G225S, V228I, V232A, V259I, R267K, L268K, S269A, F281L, A328G, V345I, D366H, T374A, L380F, N383Y, D384G, K406N, N415G, H436Y, S443D, K445S, L449I, V450I, S455N, M456Y, K488T and P507A.

In another aspect of the invention the variant is generated by changing those amino acids in the parental peptide which have a positive charges and are situated within 20 Å of the calcium ion to neutral or negative charged amino acids. Preferred variants of the present invention comprise variants in which the overall charge within 20 Å from the calcium ion has been made more negative. In such variants positively charged amino acids may have been replaced with amino acids that are neutral or negatively charged under the application conditions. In accordance herewith, preferred variants may have an amino acid residue which is partly or fully positively charged under the “chemical stability” or application conditions, i.e. a Lys, Arg or His replaced by a negative or neutral amino acid. Preferred replacement amino acids may be negatively charged amino acids as Asp and Glu or neutral amino acids as Ala, Asn, Gln, Tyr, Trp and Phe. A preferred variant of the present invention comprises an alteration at one or more of the positions selected form the group consisting of position number 49, 87, 118, 129, 134, 142, 156, 169 and 197. In a preferred embodiment the alterations are substitutions at one or more of the positions selected form the group consisting of position number 87, 118, 129, 134, 142, 156, and 169. In a preferred embodiment the substitution is selected from the group consisting of: K87A; K129A,S,F,I; K118A; K142A,Q, R156Y,F,V,I,K,W,L,M and K169Q,A.

In one aspect, a variant of a parent xyloglucanase comprises an alteration at one or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or

76 or 331. Preferably, the variant comprises substitution at position 68 and one or more substitutions at one or more additional positions, selected from the group consisting of position number 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395 and 366.

In another aspect, a variant comprises a substitution at position 156 and one or more substitutions at one or more additional positions selected from the group consisting of position number 10, 13, 14, 19, 37, 68, 78, 92, 118, 123, 129, 137, 139, 140, 147, 159, 164, 165, 169, 176, 177, 179, 183, 200, 204, 211, 222, 224, 244, 247, 249, 259, 267, 268, 269, 275, 288, 299, 301,302, 303, 310, 324, 328, 331, 366, 380, 383, 384, 389, 406, 409, 415, 436, 443, 445, 449, 450, 454, 455, 456, 461, 470 and 507.

In another aspect, a variant of a parent xyloglucanase comprises alterations at two or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331. Preferably, the variant comprises a substitution at position 68 or 123 or 156 or 118 or 200 or 129. Even more preferably the variant comprises a substitution at position 129 and position 156.

In another aspect, a variant of a parent xyloglucanase comprises alterations at three or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331.

In another aspect, a variant of a parent xyloglucanase comprises alterations at four or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331.

In another aspect, a variant of a parent xyloglucanase comprises alterations at five or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331.

In another aspect, a variant of a parent xyloglucanase comprises alterations at six or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331.

In another aspect, a variant of a parent xyloglucanase comprises alterations at seven or more (several) positions corresponding to positions 68 or 123 or 156 or 118 or 200 or 129 or 137 or 193 or 92 or 76 or 331.

In another aspect, a variant of a parent xyloglucanase comprises alterations at the positions corresponding to positions 129 and 156 and 331 and 200 and 118.

In another aspect, a variant of a parent xyloglucanase comprises alterations at the positions corresponding to positions 68 and 129 and 156 and 331 and 200 and 118.

In another aspect, a variant of a parent xyloglucanase comprises alterations at the positions corresponding to positions 68 and 92 and 129 and 156 and 331 and 200 and 118.

In another aspect the variant comprises one or more (several) substitutions selected from the group consisting of: Q68H,N,L; S123P,T; R156Y,F,V,I,K,W,L,M; K118A,R; G200P,E,S,D; K129T,A,S; Q137E; H193T,S,D; T92V,I,A,S; A83E; Q149E; L34F,I,V; R340T,N; S332P; T9D; S76W,V,I,K,R,T; N331F,C; M310I,V,L; D324N; G498A,D; D395G and D366H. Preferably, the substitutions are selected from the group consisting of Q68H; S123P; R156Y,F; K118A; G200P,E; K129T,A; Q137E; H193T; T92V and N331F. More preferably, the substitutions are selected from the group consisting of Q68H; S123P; R156Y,F; K118A; G200P,E; K129T,A; Q137E; T92V and N331F. More preferably, the variant contains a substitution in nine or eight, seven or six or five or four or three or two or one position(s), where the substitutions are selected from the group consisting of Q68H; S123P; R156Y,F; K118A; G200P,E; K129T,A; Q137E; T92V and N331F.

In a further aspect the variant comprises one or more (several) of the following combinations of substitutions:

Q68H;
S123P;
R156Y;
Q68H+R156Y;
K129A+R156Y;
S123T+K129A+R156Y;
K129A+R156Y+G200P;
Q68H+K118R+R156F;
Q68H+R156Y+H193T;
Q68H+R156F+G200P+N331F;
Q68H+T92V+K118A+R156Y;
K118A+K129A+R156Y+G200P+N331F;
G78A+T92V+K118A+K129A+R156Y ;
Q68H+K129T+R156K+G200P+N331F ;
K118A+K129A+R156Y+K169A+G200P+N331F;
T92V+K118A+K129A+R156Y+G200P+N331F;
G78A+K118A+K129A+R156Y+G200P+N331F;
G78A+T92V+K118A+K129A+R156Y+K169A;
Q68H+T92V+Q137E+R156Y+G200P+N331F ;
Q68H+T92V+K118A+Q137E+R156Y+N331F ;
Q68H+T92V+R156Y+G200P+M310V+N331F ;
Q68H+K118A+K129A+R156Y+G200P+N331F;
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F ;
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H;

Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F ;
Q68H+T92V+K118A+S123P,T+K129A+Q137E+R156Y+G200P+N331F; or
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+N331F;

In a preferred embodiment all the variants described in the above are variants of a parent xyloglucanase which belong to family 44 of glycosyl hydrolases, more preferred the parent xyloglucanase is selected from a xyloglucanase having at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95% identity to the amino acid sequence of SEQ ID NO: 3, more preferred the parent xyloglucanase is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7 and most preferred the parent xyloglucanases consists of SEQ ID NO: 3.

Polynucleotides

The present invention also relates to isolated polynucleotides that encode variants of a parent xyloglucanase according to the present invention. In particular polynucleotides that encode a xyloglucanase variant as described in the variant section above, is encompassed by the present invention. Polynucleotides of the invention will hybridize to a denatured double-stranded DNA probe comprising either the full variant sequence corresponding to positions 82-1653 of SEQ ID NO: 1 or position 97 to 1653 of SEQ ID NO: 1 with proper sequence alterations corresponding to actual amino acid alterations in the variant or any probe comprising a variant subsequence thereof having a length of at least about 100 base pairs under at least medium stringency conditions, but preferably at high stringency conditions. The variant polynucleotides of the present invention may also comprise silent mutations in addition to the mutations giving rise to the amino acid alterations described in the variant section above. Silent mutations are mutations in the three letter code which does not give rise to a change in the amino acid, e.g. GTT to GAT which both code for valine.

The polynucleotides encoding the xyloglucanase variants of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. DNA and RNA encoding genes of interest can be cloned in Gene Banks or DNA libraries by means of methods known in the art. Polynucleotides encoding polypeptides having xyloglucanase activity of the invention are then identified and isolated by, for example, hybridization or PCR.

Expression vectors

The present invention also relates to expression vectors, in particular recombinant expression vectors, comprising a nucleic acid construct of the invention. Nucleic acid constructs of the invention comprise an isolated polynucleotide encoding a variant xyloglucanase of the present

invention, preferably operably linked to one or more control sequences which direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences. 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 control sequences may either be provided by the vector or by the nucleic acid construct inserted into the vector.

The control sequence may be an appropriate promoter sequence, a nucleotide sequence which is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter may be any nucleotide sequence which shows transcriptional activity in the host cell of choice 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. Such promoters are well known in the art. The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention, such terminators are well known in the art. The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention, such leader sequences are well known in the art. The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which 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 polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention. The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleotide sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention. It may also be desirable

to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which 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.

An isolated polynucleotide encoding a variant xyloglucanase of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide sequence prior to insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art. Furthermore, tags which may aid purification or immobilization of the polypeptide may be added to the polypeptide. Such a tag may for example be a polyhistidine tag (His tag). Preferably, the tag located in the N-terminal or C-terminal of the polypeptide, and may be encoded by the vector. Alternatively, the tag may be located internally in the polypeptide, as long as it does not affect the functionality of the polypeptide.

The recombinant expression vector may be any vector (*e.g.*, a plasmid, phagemid, phage or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleotide sequence. 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 vectors may be linear or closed circular plasmids. 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 which, 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 which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

The vectors of the present invention preferably contain one or more selectable markers that permit easy selection of transformed 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 subtilis* or *Bacillus licheniformis*, or markers that confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol 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), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), *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 vectors of the present invention may contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

More than one copy of a nucleotide sequence of the present invention may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleotide sequence 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 nucleotide sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleotide sequence, 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*).

In one embodiment of the present invention the plasmid vector may contain the following elements:

i) a signal peptide coding region (e.g. obtained from the genes for Bacillus NCIB 11837 maltogenic amylase, Bacillus stearothermophilus alpha-amylase, Bacillus licheniformis subtilisin, Bacillus licheniformis alpha-amylase, Bacillus stearothermophilus neutral proteases (nprT, nprS, nprM), and Bacillus subtilis prsA), followed by a polynucleotide sequence encoding the mature xyloglucanase variant. This sequence may be preceded by and operably linked to:

ii) a DNA sequence comprising a mRNA stabilising segment (e.g. derived from the CryIIIa gene, as shown in WO 99/043835);

iii) a marker gene (e.g. a chloramphenicol resistance gene); and

iv) genomic DNA from Bacillus subtilis as 5' and 3' flanking segments upstream and downstream of the polynucleotide, respectively, to enable genomic integration by homologous recombination between the flanking segments and the Bacillus genome.

The vectors describe above may also be useful in the generation and screening of the variants using the previously described mutagenesis procedures

Host cells

The present invention also relates to recombinant a host cell comprising a polynucleotide encoding a variant xyloglucanase of the invention, which are advantageously used in the recombinant production of the polypeptides. A vector comprising a polynucleotide sequence of the present invention is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier.

The host cell may be a prokaryote such as bacterial cells, an archaea or an eukaryote such as fungal cells, plant cells, insect cells, or mammalian cells.

Useful prokaryotes are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus halodurans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, or *Bacillus subtilis* cell. In another preferred embodiment, the *Bacillus* cell is an alkalophilic *Bacillus*.

The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278).

In a preferred embodiment, the host cell is a fungal cell. “Fungi” as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In*, *Ainsworth and Bisby’s Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, *In*, *Ainsworth and Bisby’s Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK, page 171) and all mitosporic fungi (Hawksworth *et al.*, *In*, *Ainsworth and Bisby’s Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK). In a more preferred embodiment, the fungal host cell is a yeast cell. “Yeast as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the

purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, eds, *Soc. App. Bacteriol. Symposium Series* No. 9, 1980).

In an even more preferred embodiment, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell. In a most preferred embodiment, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

In another more preferred embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi include all filamentous forms of the subdivision Eumycota and Oomycota (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 filamentous fungi are 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. In an even more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma*. In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal host cell is a *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*, or *Fusarium venenatum* cell. In an even most preferred embodiment, the filamentous fungal parent cell is a *Fusarium venenatum* (Nirenberg sp. nov.) cell. In another most preferred embodiment, the filamentous fungal host cell is a *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *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* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences 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 and Simon, editors, Guide to Yeast Genetics and Molecular Biology, *Methods in Enzymology* 194: 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, Journal of Bacteriology 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

A particular embodiment of the present invention is a recombinant host cell transformed with a polynucleotide encoding a variant xyloglucanase of the present invention. Preferably, such a host cell does not contain an inherent xyloglucanase encoding gene, or such a gene has been disrupted. Thereby the recombinant variant xyloglucanases is the only xyloglucanase produced by the recombinant host cell of the present invention.

Methods of Production

The present invention also relates to methods of producing a xyloglucanase 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 from the cultivation medium.

In the production methods of the present invention, the host cells are cultivated in a nutrient medium suitable for production of the xyloglucanase 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 polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

One embodiment of the present invention is a method of producing a variant of a parent xyloglucanase, wherein said variant has xyloglucanase activity, said method comprising: a) culturing a cell under conditions suitable for expression of the variant, where said cell contains a polynucleotide sequence encoding a variant of a parent xyloglucanase in which said variant is altered in one or more (several) amino acid position(s) selected from the group consisting of

positions: 68, 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395, 366, 1, 374, 7, 140, 8, 14, 21, 211, 37, 45, 13, 78, 87, 436,101, 104, 111, 306, 117, 119, 414, 139, 268, 142, 159, 164, 102, 168, 176, 180, 482, 183, 202, 206, 217, 4, 222, 19, 224, 228, 232, 2, 240, 244, 5, 247, 249, 328, 252, 259, 406, 267, 269, 275, 179, 166, 278, 281, 288, 298, 301, 18, 302, 165, 80, 303, 316, 169, 322, 120, 146, 342, 348, 147, 353, 380, 468, 382, 383, 38, 384, 389, 391, 10, 392, 396, 177, 397, 399, 409, 237, 413, 253, 415, 418, 40, 443, 445, 148, 449, 225, 450, 454, 3, 455, 456, 299, 461, 470, 204, 476, 488, 347, and 507, and said polynucleotide sequence is prepared by mutagenesis of a parent polynucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 4 and SEQ ID NO: 6, or a parent polynucleotide sequence having at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95% identity to the nucleotide sequence of SEQ ID NO: 1; and b) recovering the xyloglucanase variant from the cultivation medium.

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

The xyloglucanase variant may be detected using methods known in the art that are specific for the expressed polypeptides. 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 xyloglucanase as described herein in the Examples.

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

A xyloglucanase variant of the present invention 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 xyloglucanase variants.

Compositions

The present invention also relates to compositions comprising a variant xyloglucanase or a polypeptide having xyloglucanase activity of the present invention. Preferably, the compositions are

enriched in such a variant or polypeptide. The term "enriched" indicates that the xyloglucanase activity of the composition has been increased, *e.g.*, with an enrichment factor of 1.1 or more. Preferably, the compositions are formulated to provide desirable characteristics such as low color, low odor and acceptable storage stability.

The composition may comprise a variant or polypeptide of the present invention 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 polypeptide compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry formulation. For instance, the polypeptide may be formulated in the form of a granulate or a microgranulate. The variant or polypeptide to be included in the composition may be stabilized in accordance with methods known in the art. In a preferred embodiment the variant xyloglucanase is formulated in a liquid composition.

Uses

The present invention is also directed to methods for using the xyloglucanase variants.

The variant xyloglucanases are preferably incorporated into and/or used together with detergent compositions, for example in laundry detergent compositions, for example household laundry detergent compositions, especially liquid laundry detergent compositions. The detergent composition typically comprises conventional detergent ingredients such as surfactants (anionic, cationic, nonionic, zwitterionic, amphoteric), builders, bleaches, polymers, other enzymes and other ingredients, e.g. as described in WO2007/130562 and WO2007/149806, which are hereby incorporated by reference in its entirety.

The detergent composition can be in any form, such as a solid, liquid, gel or any combination thereof, preferably the composition is in a liquid form, preferably a liquid laundry detergent composition.

An aspect of the invention is the use of a xyloglucanase variant or of a xyloglucanase variant composition of the invention together with a detergent composition in order to impart de-pilling and/or fabric-softness and/or colour clarification and/or soil removal and/or soil anti-redeposition and/or dye transfer inhibition benefits to a fabric or garment.

Furthermore, the invention relates to a process for laundering of fabrics comprising treating fabrics with a washing solution containing a detergent composition and a xyloglucanase variant or a xyloglucanase variant composition of the invention. The laundering treatment can for example be carried out in a machine washing process or in a manual washing process. The washing solution can for example be an aqueous washing solution containing the detergent composition and with a pH between 3 and 12.

During washing and use, the surface of fabrics or garment will conventionally become contaminated with broken or loosed fibre fragments which can give the fabric a faded and worn appearance. Removal of these surface fibers from the fabric will partly restore the original colours and looks of the fabric, resulting in colour clarification and enhanced appearance. A xyloglucanase variant or xyloglucanase variant composition of the invention may be used to provide colour clarification and /or enhanced appearance by use in single or in multiple (repeated) washing cycles.

Furthermore, microfibrils protruding from the surface of the textile can gather into little balls, so-called pills or fluffs that stick to the surface and disturb the appearance of the fabric. A xyloglucanase variant or xyloglucanase variant composition of the invention may be used to remove such pills, an effect that is termed de-pilling.

Colour-clarification and de-pilling can be assessed by visual inspection using a test group panel. The effects may also be measured by light reflection or by determination of cotton fluffs by means of optical measurements. These methods are generally known in the art and briefly described in *Enzymes in Detergency*, 1997, published by Marcel Dekker, page 139 to page 140.

Especially with an increasing number of wash cycles, deposits, which can include particulate soils, soluble soils, dyes and pigments and insoluble salts, build up on the textile fibre surfaces. This can leads to a visible deterioration of the perceived cleaning performance of the washing treatments for example leading to a greyish or yellowish appearance of the fabric. This may be prevented using a xyloglucanase variant or xyloglucanase variant composition of the invention in the wash cycles. This effect is termed anti-redeposition or dye transfer inhibition or soil removal and may be assessed by optical measurements.

Soil or insoluble salt particles trapped on the surface of the fabric and between the fibers can lead to stiffening of the fabric. By including a xyloglucanase variant or xyloglucanase variant composition of the invention in the wash cycles the fabric may be softened.

The fabrics subjected to the methods of the present invention may be conventional washable laundry, for example household laundry. Preferably, the major part of the laundry is garments and fabrics, including knits, wovens, denims, yarns, and towelling, made from cotton, cotton blends or

natural or manmade cellulosics (e.g. originating from wood pulp) or blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fibers, lyocell).

It is recognized that the treatment of fabrics and/or garments with a detergent solution containing the xyloglucanase variant or xyloglucanase variant composition of the invention can be particularly relevant in connection with, for example, production of new fibers and/or fabrics and/or garments, and also during laundering of used fabrics and/or garments for example during household laundering processes or in institutional laundering processes.

The dosage of the xyloglucanase variant or the xyloglucanase variant composition of the present invention and other conditions, under which the composition is used, including the composition and concentration of the detergent solution, may be determined on the basis of methods known in the art.

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

The xyloglucanases can be used in the compositions of the present invention to effect removal of soils containing derivatives of cellulose or hemicellulose, enhance anti-redeposition and improve soil release. The xyloglucanses can also be used in the compositions of the present invention to impart soil release benefits to cotton during a subsequent laundering process. The soil release benefit is observed on cotton fabric and on all types of fabric that comprise a significant amount of cotton, such as cotton-synthetic (e.g. polyester, polyamide such as NylonTM, and elastane) blends.

EXAMPLES

Example 1 – production and purification of xyloglucanase variants

The xyloglucanase variants of the present invention were prepared by standard procedures, in brief: Introducing random and/or site-directed mutations into the gene, transforming Bacillus subtilis host cells with the mutated genes, fermenting the transformed host cells, and obtaining the xyloglucanase variant from the fermentation broth. The reference xyloglucanase (SEQ ID NO: 3) was produced recombinantly in Bacillus subtilis in a similar manner.

Fermentation was carried out in shake flask cultures at 37°C for 4 days shaking of 100 ml PS-1 medium containing one CaCO3 tablet (0,5 g) in a baffled 500ml Erlenmeyer flask. The PS-1

medium composition contains 100g/L sucrose, 40g/L Soymeal Meal, 10g/L $Na_2HPO_4*12H_2O$, 0.1 ml/L Dowfax 63N10 and antibiotic in the form of 6µg/ml chloramphenicol.

After fermentation the culture broth was harvested by centrifugation (26000 x g, 20 min). A small volume of the supernatant was sterile filtered through a 0.45µm filter, and stored frozen. The samples were allowed to thaw immediately before the stability assays described below were started.

In some cases the enzyme samples were purified before they were used for the stability test.

For enzyme purification the supernatants were filtered through a NALGENE 0.2µm Filtration unit (cat. no. 569-0020) in order to remove the rest of the host cells. The pH of the 0.2µm filtrate was adjusted to pH 5.0 with 20% CH_3COOH and the filtrate was applied to an XpressLine ProA column (UpFront chromatography A/S) equilibrated in 50mM succinic acid/NaOH, 1mM $CaCl_2$, pH 5.0. After washing the XpressLine ProA column extensively with the equilibration buffer, the xyloglucanase was eluted by a step-elution with 50mM Tris/HCl, pH 9.0. Fractions were collected during elution. Fractions from the column were analysed for xyloglucanase activity (Example 2) and fractions with activity were pooled. The pH of the pool was adjusted to pH 9.0 with 3M Tris base and the pool was diluted with demineralised water to the same (or lower) conductivity as 50mM Tris/HCl, pH 9.0. The adjusted solution was applied to a SOURCE Q column (GE Healthcare) equilibrated in 50mM Tris/HCl, pH 9.0. After washing the SOURCE Q column extensively with the equilibration buffer, the enzyme was eluted with a linear NaCl gradient (0 → 0.5M) in the same buffer over five column volumes. Fractions from the column were again analysed for xyloglucanase activity and active fractions were further analysed by SDS-PAGE. Fractions, where only one band was seen on the Coomassie stained SDS-PAGE gel, were pooled as the purified preparation.

Example 2 - Xyloglucanase assay

The xyloglucanase activity of enzyme samples, e.g. from purification, were measured in an AZCL-xyloglucan assay.

AZCL-xyloglucan (Megazyme) was incubated with the xyloglucanase and the liberated blue colour was measured at 650nm. The xyloglucanase activity was calculated as the increase in blue colour during incubation after subtraction of the proper blank value.

AZCL-xyloglucan substrate : 4 mg/ml AZCL-xyloglucan (Megazyme) homogeneously suspended in 0.01% Triton X-100 by stirring.

Assay temperature : 37°C.

Assay buffer : 50mM succinic acid/NaOH, 0.01% Triton X-100, pH 5.0.

500µl AZCL-xyloglucan substrate suspension was placed on ice in an Eppendorf tube. 500µl Assay buffer was added and the mixture was allowed to become ice-cold. 20µl enzyme sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm). The incubation was stopped by transferring the tube back to the ice bath. When the tube had become ice-cold, the tube was centrifuged shortly in an ice-cold centrifuge to precipitate unreacted substrate. 200µl supernatant was transferred to a microtiter plate and A_{650} was read. A buffer blank (20µl 0.01% Triton X-100 instead of enzyme) was included in the assay and the difference in A_{650} between enzyme sample and buffer blank was a measure of the xyloglucanase activity.

Example 3 - Stability of xyloglucanase variants

The detergent stability of the xyloglucanase variants of the present invention was assessed by measuring the activity of the variants after incubation in a liquid detergent.

The stability test was performed by adding an enzyme sample into the liquid detergent and storing it at elevated temperatures, e.g. 35 °C or 40 °C. After the prescribed storage time the enzyme activity was determined and compared with the activity of an equivalent sample stored at approximately -18°C for the same time period. The result of the stability test is the activity found in the sample stored at elevated temperature expressed as % of the activity found in the cold stored sample.

The results for the xyloglucanase variants were compared to the result for the parental xyloglucanase (SEQ ID NO:3), tested under the same conditions. The ratio between these two stability results is the Stability Improvement Factor (SIF).

Variants having a SIF >1 are more stable under the test conditions than the parental xyloglucanase. Preferred variants are those that have high SIF in this test.

Detergent

The liquid detergent used for the stability tests has the following composition

alkylethoxy sulfate	20.1%
alkylbenzene sulfonate	2.7%
alkyl sulfate	6.5%
alkyl ethoxylate	0.8%
citric acid	3.8%
fatty acid	2.0%
Borax	3.0%
Na & Ca formate	0.2%
amine ethoxylate polymers	3.4%

diethylenetriaminepentaacetic acid	0.4%
Tinopal AMS-GX	0.2%
Ethanol	2.6%
Propylene glycol	4.6%
Diethylene glycol	3.0%
polyethylene glycol	0.2%
Monoethanolamine	2,7%
NaOH	to pH 8.3
Minor ingredients (protease, amylase, perfume, dye)	2,3%
Water	balance

Storage test

The enzyme samples prepared according to Example 1 were allowed to thaw immediately before starting the storage stability test.

The enzyme samples were diluted to a concentration of approximately 0.25 mg enzyme protein per ml.

The liquid detergent was dispensed into glass bottles with a volume of approximately 12 ml, providing 1.0±0.05 gram of detergent in each glass.

For each enzyme sample two duplicate bottles were prepared. 50µl diluted enzyme and a small magnetic stirrer bar was added to the bottles and they were closed tightly (to prevent evaporation during storage). The contents were mixed with help of the magnetic stirrer bar for about 5 minutes. One bottle of the pair was placed in a freezer at approximately -18 ºC. The other bottle was placed in a suitable incubator oven at the prescribed elevated temperature, e.g. 35 °C or 40 °C, to be tested. After the prescribed storage time the bottles in the incubator oven are transferred into the freezer.

Activity assay

The activity of the enzyme samples after storage in detergent was measured using the following procedure.

Materials and reagents:

1M phosphate buffer pH7:
Dissolve 138 grams of $NaH_2PO_4 \cdot H_2O$ in about 750 ml water. Add 4N NaOH to give pH 7.0. Then make the final volume to 1000ml.

Assay buffer (50 mM phosphate pH7):

Mix 950ml water, 50 ml 1M phosphate buffer pH7 and 5ml of Berol 537 (nonionic surfactant supplied by Akzo Nobel). Adjust the final pH to 7.00±0.02.

Substrate:
Cellazyme C tablets, supplied by Megazyme International Ireland Ltd, catalogue number T-CCZ. The tablets contain cross-linked dyed HE cellulose.

Procedure

About 30 minutes prior to starting the assay the bottles were transferred from the freezer into a refrigerator at approximately 4 °C. Immediately before starting the assay the bottles were taken out of the refrigerator and placed on the laboratory bench top and opened.

10 ml assay buffer (room temperature) was added to each open bottle. The bottles were then transferred into a 30 ºC water bath equipped with a submerged multipoint magnetic stirrer. The contents were stirred gently for about 5 minutes.

One Cellazyme C tablet was added to each bottle. Stirring was continued using a stirrer speed which is just adequate to keep the substrate particles in movement and avoid sedimentation. The bottles were removed from the water bath 30 minutes after addition of the tablet and were then allowed to stand at room temperature with no stirring for 15 minutes.

With a pipette approximately 1 ml of the practically clear supernatant from the top of each bottle was transferred into a semi-micro spectrophotometer cuvette. Absorbance at 590nm was then measured using a suitable spectrophotometer. All measurements were finished within 15 minutes.

Blank samples, i.e. equivalent detergent samples but containing no added xyloglucanase enzyme, were included in the assay.

Calculation

For each enzyme sample there are two Abs590 measurements:

- A590f, which is the Abs590 value of the sample stored at -18°C
- A590w, which is the Abs590 value of the sample stored at elevated temperature.

Subtract the blank value (A590b) from both A590f (giving A590f – A590b) and from A590w (giving A590w – A590b).

The stability was calculated as:

% Stability = ((A590w – A590b) / (A590f – A590b)) x 100%.

For each enzyme the results for (A590f – A590b) must be in the range 0.1 – 1.2. If the value is outside this range the result for that enzyme must be regarded as being unreliable and the test should be repeated with a different dilution of the enzyme sample.

Finally the Stability Improvement Factor (SIF) for each enzyme variant is calculated as follows:

SIF = % stability of enzyme sample / % stability of parent enzyme (SEQ ID NO: 3)

Results

Below are the stability results of xyloglucanase variants tested under different conditions.

Table 1: Sterile filtered enzyme samples stored for 18 hours at 40 ºC.

Mutations	SIF
K8Q	1,1
K8A	1,2
K13A	1,1
K18R	1,1
K87Q	1,1
K129A	1,7
K169Q	1,3
K169R	1,4
K169A	1,3
N140F	1,2
G316I	1,1
F418I	1,1
L34I	1,1
L166I	1,1
L268I	1,1
L278I	1,3
V1*+ V2*+H3*	1,2
*0aE+*0bV	1,3
F146L	1,2
Q137E	1,6
R156Y	2,2
R156Q	1,5
K8S	1,2
K21T	1,4
K176P	1,1
K445S	1,4
K470T	1,2

Table 2: Purified enzyme samples stored for 18 hours at 40 ºC.

Mutations	SIF
K87Q	1,1
K129A	1,8
K169A	1,1
A7T+G200P+A224P+G225K+R267K+L268K+S269A	1,3

Mutations	SIF
H164N+V179I+G200A+R267K	1,2
H164N+V179I+G200A+R211K+G225D+F281L	1,5
H164N+G200A+G225N+R267K	1,2

Table 3: Sterile filtered enzyme samples stored for 24 hours at 40 ºC.

Mutations	SIF
K101R+L102I	1,1
K217A	1,1
L380F	1,1
N383Y	1,2
G78A	1,2
M310V	1,2
N399I	1,1
G498S	1,1
F146L	1,1
Q137E	1,4
R156Y	2,0
V1*+ V2*+H3*+G4*+Q5*	1,1
N331F	1,2
K8S	1,1
T92V	1,3
K176P	1,2
G253A	1,1
K445S	1,3
K470T	1,2

Table 4: Purified enzyme samples stored for 24 hours at 40 ºC.

Mutations	SIF
T92V	1,2
Q137E	1,5
R156Y	1,7
R156Q	1,2

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Table 5: Sterile filtered enzyme samples stored for 30 hours at 40 ºC.

Mutations	SIF
K118R	1,1
K118A	1,7
K129A+K169A	1,6
G200P	1,5
K129A+R156Y	2,0
K129A+Q137E+R156Y	2,2
K129A+R156Y+H164N	2,1

Table 6: Purified enzyme samples stored for 30 hours at 40 ºC.

Mutations	SIF
T92V	1,3

Mutations	SIF
R156Y	1,9
K129A+R156Y	2,1

Table 7: Sterile filtered enzyme samples stored for 48 hours at 40 ºC.

Mutations	SIF
K118A	3,0
K252Q	1,1
K252R	1,2
K252A	1,1
K275Q	1,1
K275R	1,2
K275A	1,1
K306R	1,1
K306A	1,1
K347Q	1,1
K347R	1,1
K347A	1,1
K382A	1,1
K414A	1,2
K445R	1,3
K454R	1,1
K476Q	1,1
K482Q	1,1
K482A	1,1
K488Q	1,1
K488R	1,1
K488A	1,1
M40V	1,4
R156Y	2,9
G200P	1,8
K129A+R156Y	3,5
K129A+Q137E+R156Y+K470T	3,7
K406N	1,1
K445S	1,2
K488T	1,2
T92V+K129A+R156Y	3,7
K118A+K129A+R156Y	3,8
T92V+K118A+K129A+R156Y	3,9
K129A+R156Y+P507A	3,2
K129A+R156Y+S443D+K445S+L449I+V450I+S455N+M456Y	3,8
K129A+R156Y+H436Y	3,9
K129A+R156Y+K406N+N415G	3,5
K129A+R156Y+L380F+N383Y+D384G+N389T	3,5
K129A+R156Y+D366H+T374A	3,4
K129A+R156Y+A328G	3,5
K129A+R156Y+V259I+R267K+L268K+S269A	3,5
K129A+R156Y+T244D	3,4
K129A+R156Y+I222V+A224P+V228I+V232A	2,0
K129A+R156Y+G200P+G204T+R211K	3,6

Mutations	SIF
K129A+R156Y+A177T+V179I+A183S	2,9
K129A+R156Y+V159M+H164N+F165Y	2,8
K129A+R156Y+I10V+V14I+D19E	4,0
T104A+P111Q+A117S+K129A+R156Y	2,1
S123T+K129A+R156Y	3,8
K129A+Q137E+V139K+N140F+Q147S+R156Y	2,9
K129A+R156Y+D324N	3,4
K129A+R156Y+K176P	3,2
K129A+R156Y+D249N	3,2
K129A+R156Y+D249G	3,3
K129A+R156Y+D249S	3,1
K129A+R156Y+D461N	3,6
K129A+R156Y+D461T	3,9
K129A+R156Y+D461Q	4,0
K129A+R156Y+R409T	3,8
K129A+R156Y+R409L	3,6
K129A+R156Y+D247G	1,4
K129A+R156Y+E288Q	2,7
D37G+K129A+R156Y	3,9
D37N+K129A+R156Y	3,6
K129A+R156Y+R267H	3,8
K129A+R156Y+D303I	4,1
K129A+R156Y+D303K	3,7
K129A+R156Y+K275T	3,5
K129A+R156Y+G200P	3,9
K129A+R156Y+N331F	3,8
R156Y+N331F	3,2
K118A+K129A+R156Y+K470T	4,4
K470R	1,1
K470P	1,2
G413A	1,1
K118A+K129A+R156Y+A224P	3,9
D119L	1,3
K87V+K129A+K169A	1,9
K129A+K445S	1,8
K118A+K129A+R156Y+G200P	3,8
K118A+K129A+R156Y+G200P+N331F	4,2
G78A+K118A+K129A+R156Y	3,8
G78A+T92V+K118A+K129A+R156Y	3,8
T92V+K118A+K129A+R156Y	3,7
M310V+N399I	1,7
L34I+K129A	1,9
K101A+K129A	1,8
K13A+K129A	2,0
K129A+K470T	1,8
K129A+K176P	1,9
G78A+T92V+K118A+K129A+R156Y+K169A	4,8
K118A+K129A+R156Y+K169A+G200P+N331F	4,7
K118A+K129A+R156Y+G200P+M310V+N331F	4,7
K129A+R156Y+K454Q	3,8

Mutations	SIF
G78A+K118A+K129A+R156Y+G200P+N331F	4,2
T92V+K118A+K129A+R156Y+G200P+N331F	4,3
K129A+R156Y+N302K+D303S	2,9
K129A+R156Y+N302K+D303L	2,7
S332P+V397I	1,1
K129A+R156Y+K322I+K454Q	2,3
Q68H+K118A+K129A+R156Y+G200P+N331F	4,1
Q68H+T92S+K118A+K129A+R156Y+G200P+N331F	5,2
Q68H+T92A+K118A+K129A+R156Y+G200P+N331F	4,7
Q68H+K118A+K129A+R156Y+G200P+N331F	5,0
Q68H+K118A+K129A+R156Y+G200P+N331F	5,7
Q68H+T92D+K118A+K129A+R156Y+G200P+N331F	3,3
Q68H+T92I+K118A+K129A+R156Y+G200P+N331F	4,4
Q68H+K118A+K129A+R156Y+G200P+N331F	4,4
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	4,2
K129S	1,1
K129A	1,5
R156M	1,3
R156F	2,3
R156W	1,6
R156L	1,4
R156V	2,2
G396P	1,3
G413S	1,1
A177T	1,1
E38I	1,1
E38V	1,2
G36V+D37A+E38*+N39*	1,2
T104A	1,2
L102A+T104V+*104P	1,3
Q68L	1,3
Q68H	3,6
N389A	1,1
G468Y	1,1
G237V	1,1

Table 8: Purified enzyme samples stored for 48 hours at 40 ºC.

Mutations	SIF
K118A	2,3
R156Y	2,5
K129A+K169A	1,7
G200P	1,5
K129A+R156Y	1,7
K129A+Q137E+R156Y	3,7
K129A+R156Y+H164N	3,5
K129A+Q137E+R156Y+K470T	4,2
T92V+K129A+R156Y	4,5
K118A+K129A+R156Y	3,8
K129A+R156Y+G200P	4,8

Mutations	SIF
K129A+R156Y+N331F	4,1
R156Y+N331F	3,5
K118A+K129A+R156Y+G200P,	4,2
K118A+K129A+R156Y+G200P+N331F	4,5
G78A+K118A,+K129A+R156Y	4,0
G78A+T92V+K118A+K129A+R156Y	4,3
Q68H	3,7

Table 9: Sterile filtered enzyme samples stored for 72 hours at 40 ºC.

Mutations	SIF
K13R	1,3
K206Q	1,1
K129A+R156Y	5,1
K129A+Q137E+R156Y+K470T	6,4
T92V+K129A+R156Y	6,6
K118A+K129A+R156Y	7,2
K129A+R156Y+G200P	7,7
K129A+R156Y+N331F	5,9
R156Y+N331F	5,3

Table 10: Sterile filtered enzyme samples stored for one week at 35 ºC.

Mutations	SIF
K8Q	1,4
K8A	1,1
K13Q	1,1
K18Q	1,1
K18A	1,4
K21Q	1,4
K21R	1,4
K21A	1,4
K87Q	1,3
K101R	1,3
K101A	1,6
K118R	1,4
K118A	2,3
K101R+L102I	1,1
K129A	2,1
K169Q	1,4
K169R	1,5
K169A	1,5
K220Q	1,3
K220A	1,2
K252Q	1,1
K252R	1,1
K275Q	1,1
K275R	1,1
K275A	1,1
K306R	1,1

Mutations	SIF
K306A	1,1
K307Q	1,2
K307R	1,1
K454Q	1,6
K454R	1,2
K476Q	1,3
K476R	1,3
K476A	1,2
K482Q	1,2
K482A	1,2
K488Q	1,2
K488R	1,2
K488A	1,1
N140F	1,7
G78A	1,2
M310V	1,3
G316I	1,1
W391V	1,1
N399I	1,4
L34I	1,3
L268I	1,1
L278I	1,2
G498S	1,2
*0aE+*0bV	1,4
F146L	2,3
Q137E	2,0
R156Y	3,2
R156Q	1,7
N331F	1,5
K8S	1,3
K21T	1,5
K176P	1,2
G253A	1,1
K445S	1,5
K470T	1,6
F146C	1,3
K129A+K169A	1,8
G200P	1,7
A224P	1,1
K129A+R156Y	2,6
K129A+Q137E+R156Y	2,6
K129A+R156Y+H164N	2,6
K406N	1,3
K445S	1,2
K488T	1,2
K129R	1,1
R156F	2,0

Table 11: Purified enzyme samples stored for one week at 35 °C.

Mutations	SIF
K101R	1,1
K101A	1,1
K118A	2,3
K129A	1,8
K169R	1,2
K169A	1,1
T92V	2,0
F418I	1,1
V1*+ V2*+H3*+G4*+Q5*;	1,2
Q137E	1,6
R156Y	2,5
R156Q	1,2
K21T	1,1
G200P	1,7
K129A+R156Y	2,7
K129A+Q137E+R156Y	3,0
K129A+R156Y+H164N	3,1
A7T+G200P+A224P+G225K+R267K+L268K+S269A	1,3
H164N+V179I+G200A+R267K	1,3
H164N+V179I+G200A+R211K+G225D+F281L	1,8
H164N+G200A+G225N+R267K	1,6

Table 12: Purified enzyme samples stored for 16 hours at 44 ºC.

Mutation	SIF
Q68H	5,8
S123P	4,4
R156Y	4,0
K118A	2,9
G200P	2,6
K129A	2,4
Q137E	2,4
H193T	2,1
T92V	2,0
S76W	1,7

Example 4 - Stability of xyloglucanase variants

The detergent stability of the xyloglucanase variants of the present example was assessed by measuring the activity of the variants after incubation in a liquid detergent.

The stability test was performed by adding an enzyme sample into the liquid detergent and storing it at elevated temperatures, e.g. 35 °C or 46 °C. After the prescribed storage time the enzyme activity was determined and compared with the activity of an identical sample that had been stored cold at approximately +5°C for the same time period. The result of the stability test is the activity

found in the sample stored at elevated temperature (the stressed sample) expressed as % of the activity found in the equivalent cold-stored sample (the unstressed sample).

The results for the xyloglucanase variants were compared to the result for the parental xyloglucanase (SEQ ID NO:3), tested under the same conditions.

Detergent

The liquid detergent used for the stability tests has the following composition

alkylethoxy sulfate	20.1%
alkylbenzene sulfonate	2.7%
alkyl sulfate	6.5%
alkyl ethoxylate	0.8%
citric acid	3.8%
fatty acid	2.0%
Borax	3.0%
Na & Ca formate	0.2%
amine ethoxylate polymers	3.4%
diethylenetriaminepentaacetic acid	0.4%
Tinopal AMS-GX	0.2%
Ethanol	2.6%
Propylene glycol	4.6%
Diethylene glycol	3.0%
polyethylene glycol	0.2%
Monoethanolamine	2,7%
NaOH	to pH 8.3
Minor ingredients (protease, amylase, perfume, dye)	2,3%
Water	balance

Storage test

The enzyme samples prepared according to Example 1 were allowed to thaw immediately before starting the storage stability test.

The enzyme samples were used without further dilution.

The liquid detergent was dispensed into a round-bottom polystyrene 96-well microtiter plate (Plate 1) providing 190 µl of detergent per well.

Ten µl enzyme sample and a small magnetic stirrer bar was added to each well and the plate was closed tightly (to prevent evaporation) using adhesive aluminium foil lids (Beckman Coulter). The contents were mixed with the magnetic stirrer bars for about 30 minutes.

From each well of Plate 1, 20 µl detergent-enzyme mixture was then transferred into a new empty identical plate (Plate 2). Both plates were then sealed.

The original plate (Plate 1) was placed in an incubator oven at the prescribed elevated temperature, e.g. 35 °C or 46 °C, to be tested. The other plate (Plate 2) was placed in a refrigerator at approximately 5°C.

Following incubation for the prescribed period, the plates were removed from the refrigerator and the incubator oven. The plates were placed on the laboratory bench for at least half an hour to allow all wells to reach room-temperature.

Then 20 µl from each well of Plate 1 was transferred into a new empty round bottom 96-well plate (Plate 1a).

Plate 1a now contains 20 µl stressed samples and Plate 2 contains 20 µl unstressed samples.

Activity assay

The activity of the enzyme samples after storage in detergent was measured using the following procedure at room temperature.

Assay principle:

Para-nitrophenol-beta-D-cellotetraoside (pNP-beta-D-cellotetraoside) is a synthetic substrate that is hydrolysed by the catalytic action of certain xyloglucanase enzymes.

The substrate itself is colourless; however upon hydrolysis of the terminal reducing end glycoside bond, para-nitrophenol is released which is yellow in a pH8 buffer due to a strong absorbance at 405 nm.

pNP-beta-D-cellotetraoside itself is very stable under the given assay conditions. Thus increasing absorbance at 405 nm is an attribute of enzymatic activity.

We found that the parental xyloglucanase (SEQ ID NO:3) accepted pNP-beta-D-cellotetraoside as substrate, as evidenced by the strong absorbance increase at 405 nm.

Materials and reagents:

Assay Buffer: 100 mM EPPS; 0.01% Tween 20; pH 8.0.

pNP-beta-D-cellotetraoside (CAS-#: 129411-62-7; Toronto Research Chemicals; Canada)

Substrate solution: 1 mM pNP-beta-D-cellotetraoside in assay buffer.

Procedure:

Plate 1a contains 20 µl stressed samples and Plate 2 contains 20 µl unstressed samples.

The samples were diluted by adding 50 µl assay buffer to all wells in Plate 1a and Plate 2, and mixed for one hour using a microtiter plate shaker. Then an additional 50 µl assay buffer was added to all wells and the shaking was continued for an additional 10 minutes.

20 µl of the factor 6 diluted samples were transferred to a transparent 384 well polystyrene microtiter plate, and 20 µl substrate solution was added to all wells. The samples were mixed by shaking the microtiter plate briefly. The kinetic measurement of enzymatic activity was initiated immediately by observing the rate of increasing absorbance at 405 nm using a 384-well spectrophotometric reader.

The initial velocity (Abs/min) of the reaction was determined. The initial velocity of the reaction was a measure of the enzymatic activity in the sample as verified by a linear standard curve within relevant enzyme concentrations.

Calculation:

% residual activity was calculated as enzymatic activity in the stressed sample divided by enzymatic activity in the identical unstressed sample.

% residual activity = “Abs/min (stressed sample)” / “Abs/min (not stressed sample)” * 100%.

Results

Below are the stability results of xyloglucanase variants tested under different conditions.

Table 13. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	**% Residual Activity**
SEQ ID NO: 3	7
K118A	24
R156Y	36
K129A+K169A	19
G200P	26
K129A+R156Y	51
K129A+Q137E+R156Y	72
K129A+R156Y+H164N	63

Table 14. Sterile filtered enzyme samples stored for 16 hours at +47 °C.

Mutations	**% Residual Activity**
SEQ ID NO: 3	< 5
Q68H+T92S+K118A+K129A+R156Y+G200P+N331F	77
Q68H+T92A+K118A+K129A+R156Y+G200P+N331F	83
Q68H+K118A+K129A+R156Y+G200P+N331F	91
Q68H+T92D+K118A+K129A+R156Y+G200P+N331F	49
Q68H+T92Y+K118A+K129A+R156Y+G200P+N331F	78
Q68H+T92I+K118A+K129A+R156Y+G200P+N331F	89

Mutations	% Residual Activity
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	95
Q68H+T92S+K118A+K129A+R156Y+G200P+G274D+N331F	67
Q68H+T92N+D97N+K118A+K129A+R156Y+G200P+N331F	81
Q68H	52
K118A+K129A+R156Y	52
T92V+K118A+K129A+R156Y	88
K129A+R156Y+G200P+G204T+R211K	68
S123T+K129A+R156Y	65
K129A+R156Y+G200P	73
K118A+K129A+R156Y+G200P+N331F	90
G78A+K118A+K129A+R156Y+G200P+N331F	98
T92V+K118A+K129A+R156Y+G200P+N331F	95

Table 15. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	22
R156Y	59
K13R	34
K307Q	31
K414A	34
G253A	33
G498S	31
M310V	38
N399I	30
V1*+ V2*+H3*+G4*+Q5*	31
F146L	34
K445S	30
K470T	30

Table 16. Sterile filtered enzyme samples stored for 16 hours at +45 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	6
R156Y	34
K129A+R156Y	55
K101R+L102I	12
K118A+K129A+R156Y	72
K129A+R156Y+P507A	57
K129A+R156Y+D366H+T374A	44
K129A+R156Y+V259I+R267K+L268K+S269A	40
K129A+R156Y+G200P+G204T+R211K	49
K129A+R156Y+V159M+H164N+F165Y	30
T104A+P111Q+A117S+K129A+R156Y	39
S123T+K129A+R156Y	70
K129A+R156Y+D324N	60
K129A+R156Y+D461N	59
K129A+R156Y+D461T	61
K129A+R156Y+D461Q	59
D37G+K129A+R156Y	60

Mutations	% Residual Activity
D37N+K129A+R156Y	64
K129A+R156Y+R267H	64
K129A+R156Y+D303I	62
K129A+R156Y+D303K	65
K129A+R156Y+K275T	68
K129A+R156Y+G200P	92
K118A+K129A+R156Y+K470T	80
H164N	< 5
K129A+R156Y+N302K+D303S	66
K129A+R156Y+N302K+D303L	64

Table 17. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	26
R156Y	58
K118A+R156Y+G200P	84
K118A+K129A+Q137E+R156Y+G200P+N331F	92
K445C+K470C	32
F281L	32
D366H	35
K392G	26
D395G	35
S76W	47
G498D	32
G498A	36
D324N	39
S123T	36
Q68Y	6
Q68C	13
K129A+R156Y	89
K118A+K129A+R156Y+G200P+N331F	100

Table 18. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	34
R156Y	66
R156M	39
R156F	63
R156W	44
R156L	34
R156P	< 5
R156V	50
R156T	35
R156S	27
R156A	36
R156D	34
R156K	52
R156N	29

Mutations	% Residual Activity
R156I	50
T92I	39
R156Q	34

Table 19. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	25
R156Y	70
R156E	66
R156F	65
T92V	43
R156P	< 5
R156V	53
R156K	38
R156I	31

Table 20. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	31
R156Y	65
N415S	34
S443E	33
S443K	32
S443Q	35
K129T	46
K129A	50
G468Y	32
G237A	34
G237S	34
G237V	25
G468S	32

Table 21. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	21
R156Y	45
S332P	41
K129A+R156Y+K176S	73
K129A+R156Y+D303V	77
K129A+R156Y+D303S	81
R197L	20
R340N	41
R340T	43
H193S	51
H193D	49
H193T	66
L34F	43

Mutations	% Residual Activity
Q137D	24
Q149E	48
T9D	40
A83E	49
S214E	25
K129A+R156Y	98
T92V	49
T92I	36

Table 22. Sterile filtered enzyme samples stored for 16 hours at +47 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	29
Q68H+R156V+G200P+N331F	93
Q68H+R156F+G200P+N331F	Approx. 100
Q68H+G200P+N331F	Approx. 100
Q68H+T92V+R156V+G200P+M310V	86
Q68H+T92V+R156Y+G200P+M310V	86
Q68H+T92V+R156F+G200P+M310V	91
Q68H+T92V+R156F+G200P+M310V+S484C	82
Q68H+T92V+G200P+M310V	82
Q68H+T92V+R156V+G200P+M310V+N331F	Approx. 100
Q68H+T92V+R156Y+G200P+M310V+N331F	Approx. 100
Q68H+T92V+R156F+G200P+M310V+N331F	86
Q68H+T92V+G200P+M310V+N331F	80
D366H	< 5
K118A+K129A+R156Y+G200P+N331F	81
Q68H+K118A+K129A+R156Y+G200P+N331F	87
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	80
M40L+A41T+Q67M+N72S+S76D+G78A+Q82K+Q137E+N153K+ H164N+D249N+V272A+I337L+M356L+V397A+N415S+T421I+ S424N+N441D+V450I+E489A+A490V+T517A+S522*	41
I10V+F17S+D33E+M40L+Q67M+N72S+S76D+G78A+Q82K+ T92A+L102Q+Q137E+I222V+V228I+D249N+V272A+I337L+M356L +T374A+V397A+S416A+T421I+S424N+N441D+D444Y+V450I+ A469E+K470T+I473G+T517A+S522P+P523V+V524E	52
Q32H+M40L+R49G+D65E+Q67M+N72S+S76D+G78A+Q82K+92A +L102Q+T104A+Q137E+H164N+K202E+I222V+V228I+D249N+ M356L+T374A	41
I10V+F17S+Y53H+Q67M+N72S+S76D+G78A+Q82K+T92A+L102Q +Q137E+T172V+A177T+I222V+V228I+D249N+S269N+I337L+ M356LV397A+S416A+T421I+S424H+N441D+D444Y+A469E+ K470T+I473G+T517A+S522*	26

Table 23. Sterile filtered enzyme samples stored for 64 hours at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5

Mutations	% Residual Activity
Q68H+R156V+G200P+N331F	80
Q68H+R156F+G200P+N331F	84
Q68H+G200P+N331F	63
Q68H+T92V+R156V+G200P+M310V	52
Q68H+T92V+R156Y+G200P+M310V	67
Q68H+T92V+R156F+G200P+M310V	63
Q68H+T92V+R156F+G200P+M310V+S484C	68
Q68H+T92V+G200P+M310V	48
Q68H+T92V+R156V+G200P+M310V+N331F	93
Q68H+T92V+R156Y+G200P+M310V+N331F	100
Q68H+T92V+R156F+G200P+M310V+N331F	91
Q68H+T92V+G200P+M310V+N331F	80
K118A+K129A+R156Y+G200P+N331F	56
Q68H+K118A+K129A+R156Y+G200P+N331F	86
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	88

Table 24. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	16
R156Y	52
T374A	27
F146L+K322I	24
K129A+Q137E+R156Y+G200P	87
Q68S	14
Q68T	< 5
K129A+R156Y	71
F146L	26
K129A+R156Y+G200P	82
Q68H	77

Table 25. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	19
R156Y	53
K101A+K129A	47
K129A+K470T	46
S332P	29
G413A	30
K118A+K129A+R156Y+A224P	81
K129A+K176P	50
K118A+K129A+R156Y+K169A+G200P+N331F	89
K118A+K129A+R156Y+G200P+M310V+N331F	86
K129A+R156Y+K454Q	86
K13A+K129A	49
G78A+T92V+K118A+K129A+R156Y+K169A	93
K129A+R156Y+K322I+K454Q	76
K129A	47
K129A+R156Y	74

Mutations	% Residual Activity
K118A+K129A+R156Y	77
K118A+K129A+R156Y+G200P+N331F	Approx. 100
G78A+T92V+K118A+K129A+R156Y	93

Table 26. Sterile filtered enzyme samples stored for 6 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
Q68H+R156V+G200P+N331F	50
Q68H+R156Y+G200P+N331F	60
Q68H+R156F+G200P+N331F	64
Q68H+G200P+N331F	40
Q68H+T92V+R156V+G200P+M310V	32
Q68H+T92V+R156Y+G200P+M310V	42
Q68H+T92V+R156F+G200P+M310V	43
Q68H+T92V+R156F+G200P+M310V+S484C	34
Q68H+T92V+G200P+M310V	27
Q68H+T92V+R156F+G200P+M310V+N331F	93
Q68H+T92V+G200P+M310V+N331F	58
K118A+K129A+R156Y+G200P+N331F	27
Q68H+K118A+K129A+R156Y+G200P+N331F	75
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	70

Table 27. Sterile filtered enzyme samples stored for 64 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	9
K101A+K129A	6
K129A+K470T	4
S332P	< 5
G413A	< 5
K118A+K129A+R156Y+A224P	51
K129A+K176P	6
K118A+K129A+R156Y+K169A+G200P+N331F	67
K118A+K129A+R156Y+G200P+M310V+N331F	63
K129A+R156Y+K454Q	52
K13A+K129A	5
G78A+T92V+K118A+K129A+R156Y+K169A	72
K129A	5
K129A+R156Y	32
K118A+K129A+R156Y	30
K118A+K129A+R156Y+G200P+N331F	63
G78A+T92V+K118A+K129A+R156Y	72

Table 28. Sterile filtered enzyme samples stored for 64 hours at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5

Mutations	% Residual Activity
R156Y	4
G78A+T92V+K118A+K129A+R156Y+G200P+N331F	71
K118A+K129A+R156Y+G200P+N331F+N399I	59
K118A+K129A+F146L+R156Y+G200P+N331F	62
T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	74
T92V+K118A+K129A+R156Y+H164N+G200P+N331F	70
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	87
Q68H+T92V+K118A+S123T+K129A+Q137E+R156Y+G200P+ N331F	90
T92V+K118A+K129A+R156Y+G200P+N331F	66
K118A+K129A+R156Y+G200P+N331F	68
Q68H T92V K118A K129A R156Y G200P N331F	83

Table 29. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	19
R156Y	51
S123P	69
V159M	21
V345I	34
G225S	30
V232A	<10

Table 30. Sterile filtered enzyme samples stored for 10 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
G78A+T92V+K118A+K129A+R156Y+G200P+N331F	32
K118A+K129A+R156Y+G200P+N331F+N399I	16
K118A+K129A+F146L+R156Y+G200P+N331F	23
T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	34
T92V+K118A+K129A+R156Y+H164N+G200P+N331F	31
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	67
Q68H+T92V+K118A+S123T+K129A+Q137E+R156Y+G200P+N331F	81
T92V+K118A+K129A+R156Y+G200P+N331F	23
K118A+K129A+R156Y+G200P+N331F	25
Q68H T92V K118A K129A R156Y G200P N331F	61

5

Table 31. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	15
R156Y	51
Q68F	< 5
Q68N	69
Q68Y	< 5
Q68D	< 10
Q68C	< 10
Q68G	< 10

Mutations	% Residual Activity
Q68S	< 10
Q68E	< 5
Q68A	< 5
Q68M	27
Q68W	< 10
Q68H	82

Table 32. Sterile filtered enzyme samples stored for 7 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+ N331F	81
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F	74
Q68H+T92V+Q137E+R156Y+G200P+N331F	80
Q68H+T92V+K118A+Q137E+G200P+N331F	65
Q68H+T92V+K118A+Q137E+R156Y+N331F	80
Q68H+T92V+K118A+Q137E+R156Y+G200P	67
G78A+K118A+K129A+R156Y+K169A	14
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	73
K129A+R156Y	< 5
G78A+K118A+K129A+R156Y	7

Table 33. Sterile filtered enzyme samples stored for 48 hours at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	9
K118A+K129A+R156Y+G200P+N331F	67
Q68H+K118A+K129A+R156Y+G200P+N331F	79
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	85
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H	73
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H	72
Q68H+T92V+R156Y+H193T+D366H	78
Q68H+T92V+R156F+H193T+D366H	78
Q68H+R156Y+H193T+D366H	68
Q68H+T92V+K118A+K129A+R156Y+H193T	67
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T	80
Q68H+T92V+R156Y+H193T	84
Q68H+T92V+R156F+H193T	66
Q68H+R156Y+H193T	66
Q68H+R156Y+H193T+G200P+M310V	93
Q68H+T92V+R156F+H193T+G200P+M310V	82
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+G200P +M310V+E446K	76
Q68H+T92V+R156Y+H193T+G200P+M310V	73
Q68H+T92V+K118A+K129A+R156Y+H193T+G200P+M310V	89
Q68H+K129T+R156K+G200P+N331F	95
Q68H+K129A+R156K+G200P+N331F	86

Mutations	% Residual Activity
Q68H+K118A+R156V+G200P+N331F	81
Q68H+K118S+R156F+G200P+G274D+N331F	68

Table 34. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	22
R156Y	61
S123T+K129A+R156Y	83
H193T	44
G78A+T92V+K118A+K129A+R156Y	91
S123T	55
S123P	73
V232A	< 10
K129A+R156Y	64
K118A+K129A+R156Y	68

Table 35. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	17
R156Y	60
N140F	25
H164A	7
H193A	23
R500T	30
R500A	33
R500V	29
H199A	< 10
H3A	26
H436A	26
H448A	< 10
H512A	25
H96A	14
H3A+H436A	27

5

Table 36. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	27
R156Y	66
N399I	33
L34F	35
Q149E	35
S332P	36
K129A	50
K21Q+K129A	54
K129A+K275Q	56
Q68F	6
T9D+L34F+A83E+Q149E+H193T+S332P+R340T	53

Table 37. Sterile filtered enzyme samples stored for 12 days at +37 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	8
K118A+K129A+R156Y+G200P+N331F	52
Q68H+K118A+K129A+R156Y+G200P+N331F	47
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	67
Q68H+R156Y+G200P+N331F	47
Q68H+R156F+G200P+N331F	66
Q68H+T92V+R156Y+G200P+M310V	41
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H	54
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H	44
Q68H+T92V+R156Y+H193T+D366H	44
Q68H+T92V+R156F+H193T+D366H	37
Q68H+R156Y+H193T+D366H	36
Q68H+T92V+K118A+K129A+R156Y+H193T	50
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T	56
Q68H+T92V+R156Y+H193T	37
Q68H+T92V+R156F+H193T	37
Q68H+R156Y+H193T	44
Q68H+R156Y+H193T+G200P+M310V	34
Q68H+T92V+R156F+H193T+G200P+M310V	28
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+G200P +M310V+E446K	47
Q68H+T92V+R156Y+H193T+G200P+M310V	47
Q68H+T92V+K118A+K129A+R156Y+H193T+G200P+M310V	56

Table 38. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	19
R156Y	49
G200S	28
G200D	25
G200Y	12
G200L	< 5
G200P	37
G200W	< 5
G200I	< 5
G200N	9
G200F	< 5
G200V	9
G200H	12
G200Q	19
G200C	17
G200A	24
G200M	6
G200K	11
G200E	48

Mutations	% Residual Activity
G200R	< 5
G200T	5

Table 39. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	13
R156Y	45
K21Q+K129A	34
K129A+K275Q	39
T9D+L34F+A83E+Q149E+H193T+S332P+R340T	43
N399I	24
L34F	22
Q149E	23
S332P	24
K129A	58
G518D	19
K118A+K129A	73
K118A	48
K129A+K169A	40

Table 40. Purified enzyme samples stored for 5 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	<5
R156Y	<5
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H	73
Q68H+R156Y+H193T	63
Q68H	13
Q68H+T92V+K118A+Q137E+R156Y+N331F	70
G78A+T92V+K118A+K129A+R156Y	44
K118A+K129A+R156Y+G200P+N331F	46
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	83
Q68H+K129T+R156K+G200P+N331F	77
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H	85

5

Table 41. Sterile filtered enzyme samples stored for 5 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331K	70
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331H	42
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331Q	24
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T	33
Q68H+K118A+Q137E+R156Y+G200P+N331F	74
Q68H+S76W+T92V+K118A+Q137E+R156Y+G200P+N331F	87
K13A+Q68H+T92V+K118A+Q137E+R156Y+G200P	54
Q68H+T92V+K118A+Q137E+R156Y+G200P+D324N	53
Q68H+T92V+K118A+Q137E+R156Y+G200P+K470T	69

Mutations	% Residual Activity
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F	75
Q68H+T92V+K118A+Q137E+R156Y+G200P	52

Table 42. Sterile filtered enzyme samples stored for 16 hours at +44°C.

Mutations	% Residual Activity
SEQ ID NO: 3	13
R156Y	43
S76M	21
S76I	36
S76E	19
S76R	26
S76K	27
S76V	39
S76R	24

Table 43. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	20
R156Y	51
K118A+R156Y	62
R197A	< 5
R20A	26
R267A	26
R295A	23
R314A	< 10
R340A	23
A221K	25
M290R	23
M373Q	25
V397S	25
T417K	27
N441G+A442E+S443D	30
S467R+G468S+A469T	29
I473T	24
A490R	32
T517A+G518D	31
V431E	29
S76W+G200P+A224P	58
S76W+G200P	59
G200P+A224P	56
S76T	42
M310V	31
G200P	47
G200E	59
M310V+N399I	< 10
Q68W	< 5

Table 44. Sterile filtered enzyme samples stored for 16 hours at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	8
R156Y	40
Q68H+T92V+K118A+Q137E+N140F+R156Y+G200P+K470T	89
Q68H+T92V+K118A+S123P+K129A+Q137E+R156Y+G200P+ N331F	88
T92V+K118A+Q137E+R156Y+G200P+N331F	88
S76W+G200P+A224P	44
S76W+G200P	45
G200P+A224P	48
S76T	26
Q68H+T92V+K118A+Q137E+R156Y+G200P+M310L	91
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F	95
G200P	39

Table 45. Sterile filtered enzyme samples stored for 9 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331K	46
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331H	19
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331Q	9
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T	17
Q68H+K118A+Q137E+R156Y+G200P+N331F	48
Q68H+S76W+T92V+K118A+Q137E+R156Y+G200P+N331F	65
K13A+Q68H+T92V+K118A+Q137E+R156Y+G200P	31
Q68H+T92V+K118A+Q137E+R156Y+G200P+D324N	30
Q68H+T92V+K118A+Q137E+R156Y+G200P+K470T	41
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F	50
Q68H+T92V+K118A+Q137E+R156Y+G200P	30

Table 46. Purified enzyme samples stored for 9 days at +46 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	< 5
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+ D366H	52
Q68H+R156Y+H193T	34
Q68H+T92V+K118A+Q137E+R156Y+N331F	45
G78A+T92V+K118A+K129A+R156Y	14
K118A+K129A+R156Y+G200P+N331F	18
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	56
Q68H+K129T+R156K+G200P+N331F	47
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H	52
Q68H+R156Y+H193T	31

5

Table 47. Sterile filtered enzyme samples stored for 30 days at +37 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5

Mutations	% Residual Activity
R156Y	< 5
K118A+K129A+R156Y+G200P+N331F	33
Q68H+K118A+K129A+R156Y+G200P+N331F	42
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	52
Q68H+R156Y+G200P+N331F	41
Q68H+R156F+G200P+N331F	58
Q68H+T92V+R156Y+G200P+M310V	41
Q68H+T92V+R156F+G200P+M310V	42
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H	50
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H	32
Q68H+T92V+R156Y+H193T+D366H	33
Q68H+T92V+R156F+H193T+D366H	28
Q68H+R156Y+H193T+D366H	25
Q68H+T92V+K118A+K129A+R156Y+H193T	41
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T	43
Q68H+T92V+R156Y+H193T	27
Q68H+T92V+R156F+H193T	23
Q68H+R156Y+H193T	33
Q68H+R156Y+H193T+G200P+M310V	28
Q68H+T92V+R156F+H193T+G200P+M310V	21
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+G200P+ M310V+E446K	35
Q68H+T92V+R156Y+H193T+G200P+M310V	35
Q68H+T92V+K118A+K129A+R156Y+H193T+G200P+M310V	46

Table 48. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	15
R156Y	49
A83S	15
A83N	9
A83Y	10
A83H	14
A83I	8
A83L	10
A83R	16
A83D	17
A83T	12
A83E	31
L34V	22
L34M	19
L34I	24
M310I	21
M310V	20
M310L	18

Table 49. Sterile filtered enzyme samples stored for 3 days at +35 °C.

Mutations	% Residual Activity

Mutations	% Residual Activity
SEQ ID NO: 3	61
R156Y	89
N331K	57
N331R	54
N331L	39
N331H	62
N331G	59
N331M	70
N331W	55
N331S	58
N331V	57
N331T	46
N331Y	55
N331I	47
N331A	87
N331Q	82
N331C	70
N331E	58
N331D	63
N331P	26
N331F	51

Table 50. Sterile filtered enzyme samples stored for 16 hours at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	20
R156Y	58
I10V+F17S+Q67M+N72S+S76D+G78A+Q82K+T104A+Q137E+ N153K+R156Q+V219A+I222V+V228I+D249N+S269N+V272A+ E333A+I337L+M356L+V397A+N415S+D420G+T421I+S424H+ N441D+D444Y+V450I+A469E+K470T+I473G+T517A+S522*	72
I10V+D33E+M40L+A41T+Q67M+Y73F+S76D+G78A+Q82K+ T92A+L102Q+Q137E+I222V+V228I+D249N+S269N+V272A+ E333A+I337L+M356L+T374A+S416A+D444Y+A469E+K470T+ I473G+T517A+S522*	71
I10V+F17S+D33E+M40L+Q67M+N72S+S76D+G78A+Q82K+ T92A+L102Q+Q137E+H164N+N168K+T172A+V219A+I222V+ V228I+D249N+S269N+V272A+E333A+I337L+M356L+N415S+ T421I+S424H+N441D+D444Y+S522P+P523V+V524E	78
I10V+F17S+D33E+Q67M+N72S+S76D+G78A+Q82K+T92A+ L102Q+Q137E+N168K+T172A+I222V+V228I+D249N+V272A+ E333A+I337L+M356L+V397A+S416A+T421I+S424H+N441D+ D444Y+A469E+K470T+I473S+V477I+E489A+A490V+T517A+ S522*	74
I10V+F17S+M40L+Q67M+N72S+S76D+G78A+Q82K+T92A+ L102Q+Q137E+I222V+V228I+D249N+S269N+V272A+T320A+ I337L+M356L+T374A+V397A+N415S+T421I+S424H+N441D+ D444Y+A469E+K470T+I473S+V477I+T517A+S522P+P523V+ V524E	73
I10V+F17S+D33E+M40L+A41T+Q67M+N72S+S76D+G78A+	64

Mutations	% Residual Activity
Q82K+Q137E+V219A+D249N+V272A+I337L+M356L+V397A+ S416A+T421I+S424N+N441D+D444Y+V450I+K470T+I473S+ V477I	
I10V+F17S+Q67M+N72S+S76D+G78A+Q82K+T92A+T104A+ Q137E+R156Q+V159A+H164N+N168K+T172A+I222V+V228I+ D249N+V272A	66
K118A+K129A+R156Y+G200P+N331F	98
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	Approx 100

Table 51. Sterile filtered enzyme samples stored for 2 days at +44 °C.

Mutations	% Residual Activity
SEQ ID NO: 3	< 5
R156Y	20
Q68H+R156Y	61
Q68H+T92V+K118A+R156Y	66
Q68H+T92V+R156Y	68
Q68H+K118A+R156Y+H193T+D366H	74
Q68H+T92V+K118R+R156Y+H193T+D366H	65
Q68H+T92V+K118R+R156F	63
Q68H+K118R+R156Y	68
Q68H+T92V+R156Y+H193T+D366H	69
Q68H+K118R+R156Y+G200P	74
Q68H+K118R+R156F	66
K118A+K129A+R156Y+G200P+N331F	79
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F	91
Q68H	55
D33V+Q68H+N168H+V450I	70
S123T	10
K129A	10

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. An isolated variant of a parent xyloglucanase, the variant comprising an alteration of the parent xyloglucanase at position number 68, which position corresponds to a position in amino acid sequence SEQ ID NO:3 and wherein

a) the alteration(s) are

i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or

ii) deletion of the amino acid which occupies the position, and/or

iii) a substitution of the amino acid which occupies the position with a different amino acid;

b) the parent xyloglucanase is a family 44 xyloglucanase;

c) the variant has xyloglucanase activity, and

d) wherein the parent xyloglucanase is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7 or a xyloglucanase having at least 75% identity to the amino acid sequence of SEQ ID NO:3.

2. The variant according to claim 1, wherein the parent xyloglucanase is further altered in one or more positions selected from the group consisting of position number 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395, 366, 1, 374, 7, 140, 8, 14, 21, 211, 37, 45, 13, 78, 87, 436,101, 104, 111, 306, 117, 119, 414, 139, 268, 142, 159, 164, 102, 168, 176, 180, 482, 183, 202, 206, 217, 4, 222, 19, 224, 228, 232, 2, 240, 244, 5, 247, 249, 328, 252, 259, 406, 267, 269, 275, 179, 166, 278, 281, 288, 298, 301, 18, 302, 165, 80, 303, 316, 169, 322, 120, 146, 342, 348, 147, 353, 380, 468, 382, 383, 38, 384, 389, 391, 10, 392, 396, 177, 397, 399, 409, 237, 413, 253, 415, 418, 40, 443, 445, 148, 449, 225, 450, 454, 3, 455, 456, 299, 461, 470, 204, 476, 488, 347, and 507, which positions corresponds to positions in amino acid sequence SEQ ID NO:3 and wherein the alteration(s) are

i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or

ii) deletion of the amino acid which occupies the position, and/or

iii) a substitution of the amino acid which occupies the position with a different amino acid.

3. The variant of claim 1 or claim 2, wherein the variant comprises one or more of the

following combinations of alterations:

K13A+Q68H+T92V+K118A+Q137E+R156Y+G200P;

D33V+Q68H+N168H+V450I;

Q68H,M,N;

Q68H+G200P+N331F;

Q68H+K118A+K129A+R156Y+G200P+N331F;

Q68H+K118A+R156V+G200P+N331F;

Q68H+K118A+R156Y+H193T+D366H;

Q68H+K118R+R156F,Y;

Q68H+K118R+R156Y+G200P;

Q68H+K118S+R156F+G200P+G274D+N331F;

Q68H+K129A,T+R156K+G200P+N331F;

Q68H+R156F,V,Y+G200P+N331F;

Q68H+R156Y;

Q68H+R156Y+H193T;

Q68H+R156Y+H193T+D366H;

Q68H+R156Y+H193T+G200P+M310V;

Q68H+S76W+T92V+K118A+Q137E+R156Y+G200P+N331F;

Q68H+T92A,D,I,S,V,Y+K118A+K129A+R156Y+G200P+N331F;

Q68H+T92N+D97N+K118A+K129A+R156Y+G200P+N331F;

Q68H+T92S+K118A+K129A+R156Y+G200P+G274D+N331F;

Q68H+T92V+G200P+M310V;

Q68H+T92V+G200P+M310V+N331F;

Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+N331F;

Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F;

Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T;

Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H;

Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+G200P+M310V+E446K;

Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+N331H,K,Q;

Q68H+T92V+K118A+K129A+R156Y+H193T;

Q68H+T92V+K118A+K129A+R156Y+H193T+D366H;

Q68H+T92V+K118A+K129A+R156Y+H193T+G200P+M310V;

Q68H+T92V+K118A+Q137E+N140F+R156Y+G200P+K470T;

Q68H+T92V+K118A+Q137E+R156Y+G200P+D324N;

Q68H+T92V+K118A+Q137E+R156Y+G200P+K470T;

Q68H+T92V+K118A+Q137E+R156Y+G200P+M310L;
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A,R+R156Y,F;
Q68H+T92V+K118A+S123P,T+K129A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118R+R156Y+H193T+D366H;
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Q68H+T92V+R156F,V,Y+G200P+M310V+N331F;
Q68H+T92V+R156F,Y+H193T;
Q68H+T92V+R156F,Y+H193T+D366H;
Q68H+T92V+R156F,Y+H193T+G200P+M310V;
Q68H+T92V+R156Y.

4. The variant according to claim 0 or claim 2 comprising an alteration at one or more of the positions corresponding to position 123, or position156, or position 118, or position 200, or position 129, or position 137, or position 193, or position 92, or position 76, or position 331 which position corresponds to a position in amino acid sequence SEQ ID NO: 3.

5. The variant according to claim 1 or claim 2, wherein the variant comprises one or more substitutions at one or more additional positions, selected from the group consisting of position number 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395 and 366.

6. The variant according to claim 1 or claim 2, wherein the variant comprises substitution at position 156 and one or more substitutions at one or more additional positions, selected from the group consisting of position number 10, 13, 14, 19, 37, 68, 78, 92, 118, 123, 129,137, 139, 140, 147, 159, 164, 165, 169, 176, 177, 179, 183, 200, 204, 211, 222, 224, 244, 247, 249, 259, 267, 268, 269, 275, 288, 299, 301,302, 303, 310, 324, 328, 331, 366, 380, 383, 384, 389, 406, 409, 415, 436, 443, 445, 449, 450, 454, 455, 456, 461, 470 and 507.

7. The variant to claim 6, where the variant comprises substitutions at position 129 and position 156.

8. The variant according to any one of claims 1 to 7, wherein the variant comprises one or more substitutions selected from the group consisting of: Q68H,N,L; S123P,T; R156Y,F,V,I,K,W,L,M; K118A,R; G200P,E,S,D; K129T,A,S; Q137E; H193T,S,D; T92V,I,A,S; A83E; Q149E; L34F,I,V; R340T,N; S332P; T9D; S76W,V,I,K,R,T; N331F,C; M310I,V,L; D324N; G498A,D; D395G and D366H.

9. The variant according to claim 8, wherein the variant comprises one or more substitutions selected from the group consisting of: Q68H; S123P; R156Y,F; K118A; G200P,E; K129T,A; Q137E; H193T; T92V and N331F.

10. The variant according to any one of claims 1 to 4, wherein the variant comprises one or more of the following combinations of substitutions:
Q68H;
Q68H+R156Y;
Q68H+K118R+R156F;
Q68H+R156Y+H193T;
Q68H+R156F+G200P+N331F;
Q68H+T92V+K118A+R156Y;
Q68H+K129T+R156K+G200P+N331F ;
Q68H+T92V+Q137E+R156Y+G200P+N331F ;
Q68H+T92V+K118A+Q137E+R156Y+N331F ;
Q68H+T92V+R156Y+G200P+M310V+N331F ;
Q68H+K118A+K129A+R156Y+G200P+N331F;
Q68H+T92V+K118A+K129A+R156Y+G200P+N331F ;
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A+K129A+R156Y+H193T+D366H;
Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H;
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+N331F ;
Q68H+T92V+K118A+S123P,T+K129A+Q137E+R156Y+G200P+N331F; or
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+N331F.

11. The variant according to any one of claims 1 to 10, wherein the total number of alterations in the variant is two, preferably three, even more preferably four, even more preferably five, even more preferably six, even more preferably seven, even more preferably eight, even more preferably nine, and most preferably ten.

12. The variant according to any one of claims 1 to 11, wherein the variant comprises an amino acid sequence having at least 75% identity to the amino acid sequence of the parent xyloglucanase of SEQ ID NO: 3.

13. The variant according to any one of claims 1 to 12, wherein the variant has improved chemical stability compared to the parent xyloglucanase.

14. The variant according to claim 13, wherein the improved chemical stability results in improved detergent stability.

15. An isolated polynucleotide sequence encoding the variant according to any one of claims 1 to 12.

16. The polynucleotide sequence according to claim 15, wherein the polynucleotide sequence is prepared by mutagenesis of a parent polynucleotide sequence corresponding to SEQ ID NO:1 or a polynucleotide sequence having at least 75% identity to the polynucleotide sequence of SEQ ID NO:1.

17. A recombinant host cell comprising an expression vector comprising the polynucleotide sequence according to claim 15 or claim 16.

18. A method for producing a variant of a parent xyloglucanase, wherein said variant has xyloglucanase activity, said method comprising:

a) cultivating the host cell of claim 17 under conditions suitable for the expression of the variant; and

b) recovering the variant from the cultivation medium.

19. A method for improving the detergent stability of a xyloglucanase, comprising altering of the position 68, which position corresponds to a position in amino acid sequence SEQ ID NO:3 and wherein

a) the alteration(s) are

i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or

ii) deletion of the amino acid which occupies the position, and/or

iii) a substitution of the amino acid which occupies the position with a different amino acid;

b) the parent xyloglucanase is a family 44 xyloglucanase;

c) the variant has xyloglucanase activity, and

d) wherein the parent xyloglucanase is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 5 and SEQ ID NO: 7 or a xyloglucanase having at least 75% identity to the amino acid sequence of SEQ ID NO:3.

20. The method according to claim 19, wherein the parent xyloglucanase is further altered in one or more positions selected from the group consisting of position number 123, 156, 118, 200, 129, 137, 193, 92, 83, 149, 34, 340, 332, 9, 76, 331, 310, 324, 498, 395, 366, 1, 374, 7, 140, 8, 14, 21, 211, 37, 45, 13, 78, 87, 436,101, 104, 111, 306, 117, 119, 414, 139, 268, 142, 159, 164, 102, 168, 176, 180, 482, 183, 202, 206, 217, 4, 222, 19, 224, 228, 232, 2, 240, 244, 5, 247, 249, 328, 252, 259, 406, 267, 269, 275, 179, 166, 278, 281, 288, 298, 301, 18, 302, 165, 80, 303, 316, 169, 322, 120, 146, 342, 348, 147, 353, 380, 468, 382, 383, 38, 384, 389, 391, 10, 392, 396, 177, 397, 399, 409, 237, 413, 253, 415, 418, 40, 443, 445, 148, 449, 225, 450, 454, 3, 455, 456, 299, 461, 470, 204, 476, 488, 347, and 507, which positions corresponds to positions in amino acid sequence SEQ ID NO:3 and wherein the alteration(s) are

i) an insertion of an amino acid downstream of the amino acid which occupies the position, and/or

ii) deletion of the amino acid which occupies the position, and/or

iii) a substitution of the amino acid which occupies the position with a different amino acid.

21. The method according to claim 19 or claim 20, wherein the variant comprises one or more of the following combinations of alterations:

K13A+Q68H+T92V+K118A+Q137E+R156Y+G200P;

D33V+Q68H+N168H+V450I;

Q68H,M,N;

Q68H+G200P+N331F;

Q68H+K118A+K129A+R156Y+G200P+N331F;

Q68H+K118A+R156V+G200P+N331F;

Q68H+K118A+R156Y+H193T+D366H;

Q68H+K118R+R156F,Y;

Q68H+K118R+R156Y+G200P;
Q68H+K118S+R156F+G200P+G274D+N331F;
Q68H+K129A,T+R156K+G200P+N331F;
Q68H+R156F,V,Y+G200P+N331F;
Q68H+R156Y;
Q68H+R156Y+H193T;
Q68H+R156Y+H193T+D366H;
Q68H+R156Y+H193T+G200P+M310V;
Q68H+S76W+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92A,D,I,S,V,Y+K118A+K129A+R156Y+G200P+N331F;
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Q68H+T92V+G200P+M310V;
Q68H+T92V+G200P+M310V+N331F;
Q68H+T92V+K118A+K129A+Q137E+R156Y+G200P+A224P+N331F;
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Q68H+T92V+K118A+K129A+Q137E+R156Y+H193T+D366H;
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Q68H+T92V+K118A+Q137E+R156Y+G200P+D324N;
Q68H+T92V+K118A+Q137E+R156Y+G200P+K470T;
Q68H+T92V+K118A+Q137E+R156Y+G200P+M310L;
Q68H+T92V+K118A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118A,R+R156Y,F;
Q68H+T92V+K118A+S123P,T+K129A+Q137E+R156Y+G200P+N331F;
Q68H+T92V+K118R+R156Y+H193T+D366H;
Q68H+T92V+R156F+G200P+M310V+S484C;
Q68H+T92V+R156F,V,Y+G200P+M310V;
Q68H+T92V+R156F,V,Y+G200P+M310V+N331F;
Q68H+T92V+R156F,Y+H193T;

Q68H+T92V+R156F,Y+H193T+D366H;
Q68H+T92V+R156F,Y+H193T+G200P+M310V;
Q68H+T92V+R156Y.

22. A formulation comprising the variant according to any one of claims 1 to 14.

23. The formulation according to claim 22, wherein the formulation is a liquid formulation.

24. A variant produced by the method according to claim 18.

25. The variant according to claim 1 or claim 24; the polynucleotide sequence according to claim 15; the recombinant host cell according to claim 17; the method according to any one of claims 18 or claim 19; or the formulation according to claim 22, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.

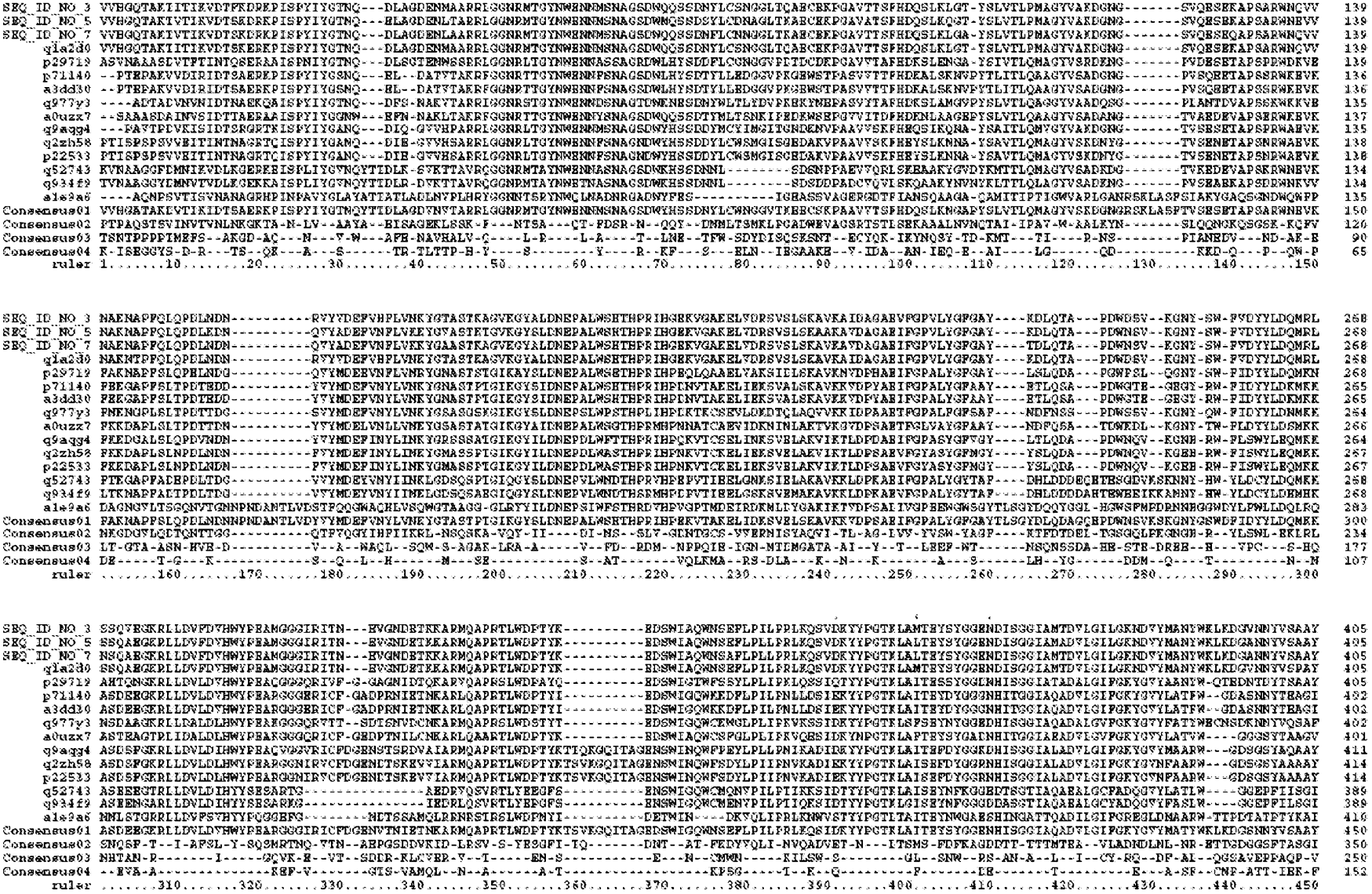

Fig. 1

Fig. 1 Continued

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Gly Pro Val Leu Tyr Gly Phe Gly Ala Tyr Lys Asp Leu Gln Thr Ala
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Met Gln Ala Pro Arg Thr Leu Trp Asp Pro Thr Tyr Lys Glu Asp Ser
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Trp Ile Ala Gln Trp Asn Ser Glu Phe Leu Pro Ile Leu Pro Arg Leu
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Ala Gly Val Lys Gly Tyr Ala Leu Asp Asn Glu Pro Ala Leu Trp Ser
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His Thr His Pro Arg Ile His Gly Glu Lys Val Gly Ala Lys Glu Leu
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Val Asp Arg Ser Val Ser Leu Ser Lys Ala Val Lys Ala Ile Asp Ala
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Gly Ala Glu Val Phe Gly Pro Val Leu Tyr Gly Phe Gly Ala Tyr Lys
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Asp Leu Gln Thr Ala Pro Asp Trp Asp Ser Val Lys Gly Asn Tyr Ser
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Trp Phe Val Asp Tyr Tyr Leu Asp Gln Met Arg Leu Ser Ser Gln Val
            260                 265                 270

Glu Gly Lys Arg Leu Leu Asp Val Phe Asp Val His Trp Tyr Pro Glu
        275                 280                 285

Ala Met Gly Gly Gly Ile Arg Ile Thr Asn Glu Val Gly Asn Asp Glu
    290                 295                 300
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Thr Lys Lys Ala Arg Met Gln Ala Pro Arg Thr Leu Trp Asp Pro Thr
305                 310                 315                 320

Tyr Lys Glu Asp Ser Trp Ile Ala Gln Trp Asn Ser Glu Phe Leu Pro
                325                 330                 335

Ile Leu Pro Arg Leu Lys Gln Ser Val Asp Lys Tyr Tyr Pro Gly Thr
            340                 345                 350

Lys Leu Ala Met Thr Glu Tyr Ser Tyr Gly Gly Glu Asn Asp Ile Ser
        355                 360                 365

Gly Gly Ile Ala Met Thr Asp Val Leu Gly Ile Leu Gly Lys Asn Asp
    370                 375                 380

Val Tyr Met Ala Asn Tyr Trp Lys Leu Lys Asp Gly Val Asn Asn Tyr
385                 390                 395                 400

Val Ser Ala Ala Tyr Lys Leu Tyr Arg Asn Tyr Asp Gly Lys Asn Ser
                405                 410                 415

Thr Phe Gly Asp Thr Ser Val Ser Ala Gln Thr Ser Asp Ile Val Asn
            420                 425                 430

Ser Ser Val His Ala Ser Val Thr Asn Ala Ser Asp Lys Glu Leu His
        435                 440                 445

Leu Val Val Met Asn Lys Ser Met Asp Ser Ala Phe Asp Ala Gln Phe
    450                 455                 460

Asp Leu Ser Gly Ala Lys Thr Tyr Ile Ser Gly Lys Val Trp Gly Phe
465                 470                 475                 480

Asp Lys Asn Ser Ser Gln Ile Lys Glu Ala Ala Pro Ile Thr Gln Ile
                485                 490                 495

Ser Gly Asn Arg Phe Thr Tyr Thr Val Pro Pro Leu Thr Ala Tyr His
            500                 505                 510

Ile Val Leu Thr Thr Gly Asn Asp Thr Ser Pro Val
        515                 520
```

<210> 4
<211> 1590
<212> DNA

<213> Paenibacillus polymyxa

<400> 4
gtagttcacg gtcaaacggc aaagactgtt accattaaag tcgatacatc caaggatcgt 60

aagcctatta gcccttatat ttacggtacg aatcaggagt tggcaggcga tgagaatctg 120

actgccagac gacttggtgg caatcgaatg accggatata actgggaaaa caatatgtcc 180

aatgcaggaa gcgactggat gcagtccagc gatagctatt tatgcgacaa cgccggattg 240

acaaaagccg aatgtgaaaa gccaggtgcg gtggcaacct cgtttcacga tcaatcgctg 300

aagcagggca catattcttt agtcacactg ccgatggccg gttatgtggc caaggatgga 360

aacggaagtg tgcaggaaag cgaaaaggct ccttccgctc ggtggaatga ggtcgtaaac 420

gctaaaaatg cgccgtttca attgcagcct gatctgaaag acaatcaggt ttatgcggat 480

gaattcgtca acttttagt gaaaaagtac ggcgttgctt caacaaaaac gggcgtgaaa 540

ggatactcgc tcgacaatga acccgctctc tggtcgcata cgcatccgcg cattcatggt 600

gaaaaggtcg gagcgaaaga gttggtagac cggtcggtaa gtttatccaa agccgctaag 660

gcggttgacg cgggtgcgga aatttttggg cccgttcttt acggttttgg cgcctataaa 720

gatcttcaaa ctgcacctga ttggaactct gtaaaaggca actacagctg gttcgtggac 780

tattacctcg atcaaatgcg cctcagctcg caagccgaag gcaagagatt gctggatgtc 840

ttcgatgtac actggtatcc tgaagcgatg ggcggaggca tacgaattac aaatgaggta 900

ggcaacgacg aaacgaagaa agccagaatg caagcgcctc gtactttgtg ggatccgacc 960

tacaaggaag atagctggat cgctcaatgg aacagtgaat tcttgccttt actgcctcga 1020

ttaaagcagt cggtggataa gtattacccg ggaaccaagc tggctttgac tgagtatagc 1080

tatggtggcg aaaatgatat ttccggcggt atcgctatgg ccgatgtgct gggcatcttg 1140

ggcaaaaacg acgtttatat ggcaaactac tggaagttaa aggatggtgc caacaactac 1200

gttagtgccg cttacaagct ttaccgcaat tatgacggaa aaagctctac tttcggtgat 1260

atcagcgttc atgcgcaaac gtcggatatt gttaatagct cggtgcatgc ttccgtaacg 1320

gatgcatcct acaaagaact gcacctcgtt gtcatgaata aaagcatgga cagtgcattc 1380

gacgcccaat ttgatctttc cggcgagacg acttacggtt ccggtaaagt atggggtttc 1440

gacaaaaata gctcgcaaat taaggaagca gcgccaatca cscaaatttc aggcaaccgy 1500

tttacctata cagtaccgcc tttgacggct tatcacatcg tgttgactgc cggcaatgat 1560

acacctgtag aaaatcctga aagctttgcg 1590

<210> 5
<211> 530
<212> PRT

<213> Paenibacillus polymyxa

<400> 5

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Val Val His Gly Gln Thr Ala Lys Thr Val Thr Ile Lys Val Asp Thr
1               5                  10                  15

Ser Lys Asp Arg Lys Pro Ile Ser Pro Tyr Ile Tyr Gly Thr Asn Gln
            20                  25                  30

Glu Leu Ala Gly Asp Glu Asn Leu Thr Ala Arg Arg Leu Gly Gly Asn
        35                  40                  45

Arg Met Thr Gly Tyr Asn Trp Glu Asn Asn Met Ser Asn Ala Gly Ser
    50                  55                  60

Asp Trp Met Gln Ser Ser Asp Ser Tyr Leu Cys Asp Asn Ala Gly Leu
65                  70                  75                  80

Thr Lys Ala Glu Cys Glu Lys Pro Gly Ala Val Ala Thr Ser Phe His
                85                  90                  95

Asp Gln Ser Leu Lys Gln Gly Thr Tyr Ser Leu Val Thr Leu Pro Met
            100                 105                 110

Ala Gly Tyr Val Ala Lys Asp Gly Asn Gly Ser Val Gln Glu Ser Glu
        115                 120                 125

Lys Ala Pro Ser Ala Arg Trp Asn Glu Val Val Asn Ala Lys Asn Ala
    130                 135                 140

Pro Phe Gln Leu Gln Pro Asp Leu Lys Asp Asn Gln Val Tyr Ala Asp
145                 150                 155                 160

Glu Phe Val Asn Phe Leu Val Lys Lys Tyr Gly Val Ala Ser Thr Lys
                165                 170                 175

Thr Gly Val Lys Gly Tyr Ser Leu Asp Asn Glu Pro Ala Leu Trp Ser
            180                 185                 190

His Thr His Pro Arg Ile His Gly Glu Lys Val Gly Ala Lys Glu Leu
        195                 200                 205

Val Asp Arg Ser Val Ser Leu Ser Lys Ala Ala Lys Ala Val Asp Ala
    210                 215                 220

Gly Ala Glu Ile Phe Gly Pro Val Leu Tyr Gly Phe Gly Ala Tyr Lys
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225                 230                 235                 240

Asp Leu Gln Thr Ala Pro Asp Trp Asn Ser Val Lys Gly Asn Tyr Ser
                245                 250                 255

Trp Phe Val Asp Tyr Tyr Leu Asp Gln Met Arg Leu Ser Ser Gln Ala
            260                 265                 270

Glu Gly Lys Arg Leu Leu Asp Val Phe Asp Val His Trp Tyr Pro Glu
        275                 280                 285

Ala Met Gly Gly Gly Ile Arg Ile Thr Asn Glu Val Gly Asn Asp Glu
    290                 295                 300

Thr Lys Lys Ala Arg Met Gln Ala Pro Arg Thr Leu Trp Asp Pro Thr
305                 310                 315                 320

Tyr Lys Glu Asp Ser Trp Ile Ala Gln Trp Asn Ser Glu Phe Leu Pro
                325                 330                 335

Leu Leu Pro Arg Leu Lys Gln Ser Val Asp Lys Tyr Tyr Pro Gly Thr
            340                 345                 350

Lys Leu Ala Leu Thr Glu Tyr Ser Tyr Gly Gly Glu Asn Asp Ile Ser
        355                 360                 365

Gly Gly Ile Ala Met Ala Asp Val Leu Gly Ile Leu Gly Lys Asn Asp
    370                 375                 380

Val Tyr Met Ala Asn Tyr Trp Lys Leu Lys Asp Gly Ala Asn Asn Tyr
385                 390                 395                 400

Val Ser Ala Ala Tyr Lys Leu Tyr Arg Asn Tyr Asp Gly Lys Ser Ser
                405                 410                 415

Thr Phe Gly Asp Ile Ser Val His Ala Gln Thr Ser Asp Ile Val Asn
            420                 425                 430

Ser Ser Val His Ala Ser Val Thr Asp Ala Ser Tyr Lys Glu Leu His
        435                 440                 445

Leu Val Val Met Asn Lys Ser Met Asp Ser Ala Phe Asp Ala Gln Phe
    450                 455                 460

Asp Leu Ser Gly Glu Thr Thr Tyr Gly Ser Gly Lys Val Trp Gly Phe
465                 470                 475                 480
```

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Asp Lys Asn Ser Ser Gln Ile Lys Glu Ala Ala Pro Ile Thr Gln Ile
               485                 490                 495

Ser Gly Asn Arg Phe Thr Tyr Thr Val Pro Pro Leu Thr Ala Tyr His
            500                 505                 510

Ile Val Leu Thr Ala Gly Asn Asp Thr Pro Val Glu Asn Pro Glu Ser
        515                 520                 525

Phe Ala
    530
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<210> 6
<211> 1575
<212> DNA
<213> Paenibacillus polymyxa

<400> 6

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aagcctatta gtccttatat atacggtacg aatcaggatt tggcaggcga tgaaaatctg      120

gctgccagac gacttggtgg caatcgaatg accggataca actgggaaaa taatatgtcc      180

aatgcgggaa gcgattggca gcaatccagc gataactttt tatgcaacaa tggtggcctg      240

acaaaagccg aatgtgaaaa gccgggagca gtgacgactt cgtttcatga tcaatcgctg      300

aagctgggcg cttattcttt agtcacgctg ccgatggccg gttatgtggc caaggatgga      360

aacggaagtg tgcaggaaag cgaacaggct ccttccgctc gttggaatca ggtcgtaaat      420

gccaaaaatg cgccgttcca actacagcct gatctgaatg acaatcaggt atatgcggat      480

gaattcgtca atttttttagt gaaaaagtac ggcgctgctt caacaaaggc gggtgtgaaa      540

ggatatgcgc tcgacaatga acccgctctc tggtcgcata cgcatccgcg cattcatggt      600

gaaaaggtcg gagcgaaaga gttggtagac cggtcggtaa gtttatccaa agctgttaaa      660

gcggttgacg cgggtgcaga aatttttggg ccggttcttt acggttttgg cgcctataca      720

gatcttcaaa ctgcacctga ttggaactct gtaaaaggca actatagctg gttcgtggac      780

tattacctgg atcaaatgcg cctcaactcg caagccgarg gcaagagatt gctggaygta      840

ttcgatgtgc actggtatcc cgaagcgatg ggcggaggca tacgaattac aaatgaggta      900

ggcaatgacg aaacgaagaa agccagaatg caggcgcctc gtactttgtg ggacccgacc      960

tacaaggaag atagctggat cgctcaatgg aacagcgcat tcttgccttt actgcctcga     1020

ttgaagcagt cggtggacaa gtattacccg ggaaccaagc tggctttgac cgagtatagc     1080

tacggcggcg aaaatgatat ttccggcggt attgctatga ccgatgtgct gggcatcttg     1140
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ggcaaaaacg acgtttatat ggcgaactat tggaagttaa aggatggtgc caacaactac 1200

gttagcgccg cttacaagct ttaccgcaat tatgacggaa aaaacgctac tttcggcgat 1260

atcagcgtta atgcgcaaac gtcggatatt gttaatagct cggtgcatgc ttccgtaacg 1320

gatgcatcct acaaagaact gcacctcatt gtcatgaata aaagcatgga cagcgcattc 1380

gacgcccaat tcgatctttc cggcgagacg acttacagtt ccggtaaaat atggggcttc 1440

gataaaaata gctcgcaaat taaggcagta gcgccaatca cgcaaatttc aggcaaccgc 1500

tttacctata cagtaccacc tttgacggct tatcacatcg tgttgactgc cgacaatgat 1560

acacctgtgc cataa 1575

<210> 7
<211> 524
<212> PRT
<213> Paenibacillus polymyxa

<400> 7

Val Val His Gly Gln Thr Ala Lys Thr Val Thr Ile Lys Val Asp Thr
1 5 10 15

Ser Lys Asp Arg Lys Pro Ile Ser Pro Tyr Ile Tyr Gly Thr Asn Gln
20 25 30

Asp Leu Ala Gly Asp Glu Asn Leu Ala Ala Arg Arg Leu Gly Gly Asn
35 40 45

Arg Met Thr Gly Tyr Asn Trp Glu Asn Asn Met Ser Asn Ala Gly Ser
50 55 60

Asp Trp Gln Gln Ser Ser Asp Asn Phe Leu Cys Asn Asn Gly Gly Leu
65 70 75 80

Thr Lys Ala Glu Cys Glu Lys Pro Gly Ala Val Thr Thr Ser Phe His
85 90 95

Asp Gln Ser Leu Lys Leu Gly Ala Tyr Ser Leu Val Thr Leu Pro Met
100 105 110

Ala Gly Tyr Val Ala Lys Asp Gly Asn Gly Ser Val Gln Glu Ser Glu
115 120 125

Gln Ala Pro Ser Ala Arg Trp Asn Gln Val Val Asn Ala Lys Asn Ala
130 135 140

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Pro Phe Gln Leu Gln Pro Asp Leu Asn Asp Asn Gln Val Tyr Ala Asp
145                 150                 155                 160

Glu Phe Val Asn Phe Leu Val Lys Lys Tyr Gly Ala Ala Ser Thr Lys
                165                 170                 175

Ala Gly Val Lys Gly Tyr Ala Leu Asp Asn Glu Pro Ala Leu Trp Ser
            180                 185                 190

His Thr His Pro Arg Ile His Gly Glu Lys Val Gly Ala Lys Glu Leu
        195                 200                 205

Val Asp Arg Ser Val Ser Leu Ser Lys Ala Val Lys Ala Val Asp Ala
    210                 215                 220

Gly Ala Glu Ile Phe Gly Pro Val Leu Tyr Gly Phe Gly Ala Tyr Thr
225                 230                 235                 240

Asp Leu Gln Thr Ala Pro Asp Trp Asn Ser Val Lys Gly Asn Tyr Ser
                245                 250                 255

Trp Phe Val Asp Tyr Tyr Leu Asp Gln Met Arg Leu Asn Ser Gln Ala
            260                 265                 270

Glu Gly Lys Arg Leu Leu Asp Val Phe Asp Val His Trp Tyr Pro Glu
        275                 280                 285

Ala Met Gly Gly Gly Ile Arg Ile Thr Asn Glu Val Gly Asn Asp Glu
    290                 295                 300

Thr Lys Lys Ala Arg Met Gln Ala Pro Arg Thr Leu Trp Asp Pro Thr
305                 310                 315                 320

Tyr Lys Glu Asp Ser Trp Ile Ala Gln Trp Asn Ser Ala Phe Leu Pro
                325                 330                 335

Leu Leu Pro Arg Leu Lys Gln Ser Val Asp Lys Tyr Tyr Pro Gly Thr
            340                 345                 350

Lys Leu Ala Leu Thr Glu Tyr Ser Tyr Gly Gly Glu Asn Asp Ile Ser
        355                 360                 365

Gly Gly Ile Ala Met Thr Asp Val Leu Gly Ile Leu Gly Lys Asn Asp
    370                 375                 380

Val Tyr Met Ala Asn Tyr Trp Lys Leu Lys Asp Gly Ala Asn Asn Tyr
```

385 390 395 400

Val Ser Ala Ala Tyr Lys Leu Tyr Arg Asn Tyr Asp Gly Lys Asn Ala
405 410 415

Thr Phe Gly Asp Ile Ser Val Asn Ala Gln Thr Ser Asp Ile Val Asn
420 425 430

Ser Ser Val His Ala Ser Val Thr Asp Ala Ser Tyr Lys Glu Leu His
435 440 445

Leu Ile Val Met Asn Lys Ser Met Asp Ser Ala Phe Asp Ala Gln Phe
450 455 460

Asp Leu Ser Gly Glu Thr Thr Tyr Ser Ser Gly Lys Ile Trp Gly Phe
465 470 475 480

Asp Lys Asn Ser Ser Gln Ile Lys Ala Val Ala Pro Ile Thr Gln Ile
485 490 495

Ser Gly Asn Arg Phe Thr Tyr Thr Val Pro Pro Leu Thr Ala Tyr His
500 505 510

Ile Val Leu Thr Ala Asp Asn Asp Thr Pro Val Pro
515 520