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(54) Title: POLYPEPTIDES WITH XYLANASE ACTIVITY

(57) Abstract: Polypeptides with xylanase activity modified to increase bran solubilisation and/or xylanase activity. The modification comprises at least an amino acid modification in position 110 and may have further modifications of one or more amino acids in position 11, 12, 13, 34, 54, 77, 81, 99, 104, 113, 114, 118, 122, 141, 154, 159, 162, 164, 166 or 175 wherein the positions are determined as the position corresponding the position of *Bacillus subtilis* xylanase (SEQ ID NO 1) The polypeptides have at least 88% identity with SEQ ID NO 1 or 75% identity to a sequence selected from SEQ ID NO 2-22.



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## Polypeptides with xylanase activity

### FIELD OF THE INVENTION

The present invention relates to polypeptides with xylanase activity and uses thereof. The present invention also relates to method of modifying polypeptides with xylanase activity to affect, preferably to increase, xylanase activity and/or bran solubility.

### BACKGROUND OF THE INVENTION

For many years, endo- $\beta$ -1,4-xylanases (EC 3.2.1.8) (referred to herein as xylanases) have been used for the modification of complex carbohydrates derived from plant cell wall material. It is well known in the art that the functionality of different xylanases (derived from different micro organisms or plants) differs enormously. Based on structural and genetic information, xylanases have been classified into different Glycoside Hydrolase families (GH's) (Henrissat, 1991; Coutinho and Henrissat, 1999). Until recently, all known and characterized xylanases were belonging to the families GH10 or GH11. Recent work has identified numerous other types of xylanases belonging to the families GH5, GH7, GH8 and GH43 (Coutinho and Henrissat, 1999; Collins *et al.*, 2005). Until now the GH11 family differs from all other GH's, being the only family solely consisting of xylan specific xylanases. The structure of the GH11 xylanases can be described as a  $\beta$ -Jelly roll structure (see Figure 1, discussed herein).

US 6,682,923 relates to xylanase activity proteins and nucleic acids.

Comprehensive studies characterising the functionality of xylanases have been done on well characterised and pure substrates (Kormelink *et al.*, 1992). These studies show that different xylanases have different specific requirements with respect to substitution of the xylose backbone of the arabinoxylan (AX). Some xylanases require three un-substituted xylose residues to hydrolyse the xylose backbone; others require only one or two. The reasons for these differences in

specificity are thought to be due to the three dimensional structure within the catalytic domains, which in turn is dependent on the primary structure of the xylanase, i.e. the amino acid sequence. However, the translation of these differences in the amino acid sequences into differences in the functionality of the xylanases, has up until now not been documented when the xylanase acts in a complex environment, such as plant material.

The xylanase substrates found in wheat (wheat flour), have traditionally been divided into two fractions: The water un-extractable AX (WU-AX) and the water extractable AX (WE-AX). The WU-AX:WE-AX ratio is approx. 70:30 in wheat flour. There have been numerous explanations as to why there are two different fractions of AX. The older literature (D'Appolonia and MacArthur (1976) and Montgomery and Smith (1955)) describes quite high differences in the substitution degree between WE-AX and WU-AX. The highest degree of substitution was found in WE-AX. This was used to explain why some of the AX was extractable. The high degree of substitution made the polymer soluble, compared to a lower substitution degree, which would cause hydrogen bonding between polymers and consequently precipitation.

The difference between the functionality of different xylanases has been thought to be due to differences in xylanase specificity and thereby their preference for the WU-AX or the WE-AX substrates.

However, more recent literature does not find the same huge differences between the substitution degree of the WE-AX and the WU-AX. Hence other parameters than the xylanases substrate specificity might be of importance. These parameters may be the xylanases preference for WE-AX versus WU-AX, determined by other means than classical substrate specificity. This parameter can be found described in literature as substrate selectivity.

In some applications (e.g. bakery) it is desirable to produce high molecular weight (HMW) soluble polymers from the WU-AX fraction. Such polymers have been correlated to a volume increase in bread making (Rouau, 1993; Rouau *et al.*, 1994 and Courtin *et al.*, 1999).

In other applications it is desirable to modify both the WU-AX and WE-AX, solubilising the WU-AX, making the molecular weight lower, reducing their hydrocolloid effect, produce arabinoxylan oligosaccharides, giving access to further degradation of other cell wall components (such as in crackers

5 production, flour separation, feed application, Bio-ethanol production, Prebiotics, etc.).

All the above mentioned characteristics of xylanases used in various applications are directed to the xylanases performance and are of great importance to achieve the functionality needed. However, selection of xylanases having the

10 right characteristics for a certain application or engineering known xylanases to achieve it, often results in a less efficient xylanase molecule, e.g., a molecule with low catalytic activity (i.e., specific activity characterised by the molecules units/mg xylanase protein). Since these molecules are to be used in commercial applications it is therefore of great importance to have as high a catalytic

15 activity as possible. Improvement of this characteristic will be of more and more importance to achieve commercial application of these enzymes in the future, due to the increased use of agricultural by-products such as cereal bran or the use in cellulosic bio-ethanol production.

#### SUMMARY OF THE INVENTION

20 The present invention is predicated on the surprising finding that it is possible – by modifying a polypeptide with xylanase activity at position 110 compared to the *B. subtilis* xylanase polypeptide sequence shown as SEQ ID No. 1 to increase the bran solubilisation and/or xylanase activity of the enzyme.

Thus, it has been shown by the inventors of the present invention that is

25 possible to produce xylanase polypeptides having increased xylanase activity and/or bran solubilisation. This will, for example, make it feasible to hydrolyse the hemicellulosic fraction during cereal processing related to cellulosic bioethanol production, or allow a reduction in the amount of xylanase required in a number of applications such as animal feed, starch liquefaction, bakery, flour

30 separation (wetmilling), production of prebiotics, and paper and pulp production.

In a first aspect, the present invention relates to a polypeptide having xylanase activity and comprising an amino acid sequence having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from SEQ ID No. 2-22, and which polypeptide has an amino acid  
5 modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a second aspect, the present invention relates to a polypeptide having xylanase activity and comprising an amino acid sequence having at least 88%  
10 identity with SEQ ID No. 1 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a third aspect, the present invention relates to a polypeptide having xylanase  
15 activity and comprising an amino acid sequence having at least 75% identity with SEQ ID No. 2 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a further aspect, the present invention relates to a polypeptide having  
20 xylanase activity and comprising an amino acid sequence having at least 75% identity with SEQ ID No. 3 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID  
25 No. 1 by alignment.

In a further aspect, the present invention relates to a method of identifying a polypeptide according to the invention, said method comprising:

(i) preparing a polypeptide having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from SEQ ID

No. 2-22, and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment;

(ii) comparing the bran solubilisation and/or xylanase activity of said polypeptide with the bran solubilisation and/or xylanase activity of the amino acid sequence selected among SEQ ID NOs: 1-22 with which is has the highest percentage of identity; and

(iii) selecting the polypeptide if it has improved bran solubilisation and/or improved xylanase activity compared to the amino acid sequence selected among SEQ ID NOs: 1-22 with which is has the highest percentage of identity.

In a further aspect, the present invention relates to a method of preparing a polypeptide according to the invention, said method comprising expressing a nucleotide sequence encoding said polypeptide; and optionally isolating and/or purifying the polypeptide after expression.

In some embodiments the polypeptide is prepared by modifying either a polypeptide amino acid sequence at position 110 or a codon that encodes an amino acid residue at position 110 in a nucleotide sequence encoding a polypeptide amino acid sequence, wherein position 110 is determined with reference to the *B. subtilis* xylanase sequence shown as SEQ ID No. 1.

In a further aspect, the present invention relates to a nucleotide sequence encoding a polypeptide according to the invention.

In a further aspect, the present invention relates to a vector comprising the nucleotide sequence encoding a polypeptide according to the invention.

In a further aspect, the present invention relates to a cell that has been transformed with the nucleotide sequence encoding a polypeptide according to the invention or the vector comprising the nucleotide sequence encoding a polypeptide according to the invention.

In a further aspect, the present invention relates to a host organism that has been transformed with the nucleotide sequence encoding a polypeptide according to the invention or the vector comprising the nucleotide sequence encoding a polypeptide according to the invention.

- 5 In a further aspect, the present invention relates to a composition comprising the polypeptide according to the invention.

In a further aspect, the present invention relates to a composition comprising a polypeptide identified according to the methods of the invention

- 10 In a further aspect, the present invention relates to a composition comprising a polypeptide prepared according to the invention.

In a further aspect, the present invention relates to a composition comprising the nucleotide sequence encoding a polypeptide according to the invention.

- 15 In a further aspect, the present invention relates to a composition comprising the vector comprising the nucleotide sequence encoding a polypeptide according to the invention

In a further aspect, the present invention relates to a composition comprising the cell that has been transformed with the nucleotide sequence encoding a polypeptide according to the invention.

- 20 In a further aspect, the present invention relates to a composition comprising the vector comprising the nucleotide sequence encoding a polypeptide according to the invention

- 25 In a further aspect, the present invention relates to a composition comprising the organism that has been transformed with the nucleotide sequence encoding a polypeptide according to the invention or the vector comprising the nucleotide sequence encoding a polypeptide according to the invention admixed with a non toxic component.

In a further aspect, the present invention relates to a dough comprising the polypeptide according to the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention.

In a further aspect, the present invention relates to a bakery product comprising the polypeptide according to the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention or a dough according to the invention.

In a further aspect, the present invention relates to an animal feed comprising the polypeptide according to the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention.

In a further aspect, the present invention relates to a cleaning composition comprising xylanase. In some embodiments, the cleaning compositions are laundry detergent compositions, while in other embodiments the cleaning compositions are dishwashing detergents. In some further embodiments, the dishwashing detergents are automatic dishwashing detergents. In some additional embodiments, the xylanase-containing cleaning compositions further comprise one or more additional enzymes. In some embodiments, the additional enzymes are selected from hemicellulases, cellulases, peroxidases, proteases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, pectate lyases, mannanases, keratinases, reductases, oxidases, phenoloxidases,



lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases,  $\beta$ -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and amylases, or mixtures thereof. In some embodiments, a combination of enzymes finds use (i.e., a "cocktail").

- 5 In a further aspect, the present invention relates to a method of degrading or modifying a plant cell wall which method comprises contacting said plant cell wall with the polypeptide according to the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to  
10 the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention.

- In a further aspect, the present invention relates to a method of processing a plant material which method comprises contacting said plant material with the  
15 polypeptide according to any one of the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to  
20 the invention.

- In a further aspect, the present invention relates to the use of the polypeptide according to the invention or a polypeptide identified according to the invention or a polypeptide prepared according to the invention or the nucleotide sequence according to the invention or the vector according to the invention or the cell  
25 according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention in a method of modifying plant materials.

- In a further aspect, the present invention relates to the use of the polypeptide according to the invention or a polypeptide identified according to the invention  
30 or a polypeptide prepared according to the invention or the nucleotide sequence

according to the invention or the vector according to the invention or the cell according to the invention or the organism according to the invention admixed with a non toxic component or a composition according to the invention in any one or more of: baking, processing cereals, starch liquefaction, production of  
5 Bio-ethanol from cellulosic material, animal feed, in processing wood, enhancing the bleaching of wood pulp.

In a further aspect, the present invention relates to a polypeptide or fragment thereof substantially as hereinbefore described with reference to the Examples and drawings.

10 In a further aspect, the present invention relates to a method substantially as hereinbefore described with reference to the Examples and drawings.

In a further aspect, the present invention relates to a composition substantially as hereinbefore described with reference to the Examples and drawings.

15 In a further aspect, the present invention relates to the use substantially as hereinbefore described with reference to the Examples and drawings.

#### LEGENDS TO THE FIGURE

Reference shall be made herein to the following Figure.

Figure 1 shows the *Bacillus subtilis* XynA variant xylanase (T110A) (black), the *Trichoderma reesei* Xyn2 variant xylanase (T120A) (dark grey) and the  
20 *Thermomyces lanuginosus* XynA variant xylanase (T120A) (light grey) superimposed. The residues mutated, T110 and T120 respectively are highlighted.

Figure 2 shows a multiple sequence alignment of SEQ ID NO:1-22 in the AlignX program (part of the vectorNTI suite) with default parameters for multiple  
25 alignment (Gap opening penalty: 10 og Gap extension penalty 0.05). Numbers on the left of the sequence represent the SEQ ID NOs.

## DETAILED DISCLOSURE OF THE INVENTION

Xylanase enzymes have been reported from nearly 100 different organisms, including plants, fungi and bacteria. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The

5 glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases,  $\beta$ -glucanases, cellulases, and other carbohydrases, are classified based on such properties as the sequence of amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al., 1991, Microbiol. Reviews 55: 303-315).

10 In one aspect, the present invention relates to a polypeptide having xylanase activity and comprising at least three, such as five, six, seven, eight, nine or ten amino acid substitutions relative to any one amino acid sequence of SEQ ID NO:1-22, and which polypeptide has an amino acid substitution in position 110, wherein said position 110 is determined as the position corresponding to position  
15 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In one aspect, the present invention relates to a polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from 2-22 and which polypeptide has an  
20 amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

The position of a particular amino acid within a polypeptide according to the present invention is determined by alignment of the amino acid sequence of said  
25 polypeptide with SEQ ID No. 1 using the a standard sequence alignment tool such as a by alignment of two sequences using the Smith-Waterman algorithm, or with the CLUSTALW2 algorithms, wherein the sequences are said to be aligned when the alignment score is highest. Alignment scores may be calculated according to the methods described by Wilbur, W. J. and Lipman, D.  
30 J. (1983) Rapid similarity searches of nucleic acid and protein data banks. Proc.

Natl. Acad. Sci. USA, 80: 726-730. Preferably default parameters are used in the ClustalW2 (1.82) algorithm: Protein Gap Open Penalty = 10.0; Protein Gap Extension Penalty = 0.2; Protein matrix = Gonnet; Protein/DNA ENDGAP = -1; Protein/DNA GAPDIST = 4.

- 5 Preferably a position of a particular amino acid within a polypeptide according to the present invention is determined by alignment of the amino acid sequence of the polypeptide with SEQ ID No. 1 using the AlignX program (part of the vectorNTI suite) with default parameters for multiple alignment (Gap opening penalty: 10 og Gap extension penalty 0.05). For some embodiments according  
10 to the present invention, alignment may be made by using figure 2 as described herein.

In a further aspect, the present invention relates to a polypeptide having xylanase activity and having an amino acid sequence having at least 88% identity with SEQ ID No. 1 and which polypeptide has an amino acid modification  
15 in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a further aspect, the present invention relates to a polypeptide having xylanase activity and having at least 75% identity with SEQ ID No. 2 and which  
20 polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a further aspect, the present invention relates to a polypeptide having xylanase activity and having at least 75% identity with SEQ ID No. 3 and which  
25 polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a further aspect, the present invention relates to a polypeptide having xylanase activity and having at least 75% identity with SEQ ID No. 4 and which

polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

In a further aspect, the present invention relates to a polypeptide having  
5 xylanase activity and comprising an amino acid sequence having at least 75% identity with SEQ ID No. 1, and which polypeptide has an amino acid substitution in position 110 to any one different amino acid residue selected from the group consisting of: glutamic acid, tryptophan, alanine and cysteine, wherein said position 110 is determined as the position corresponding to position  
10 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.

Unless otherwise stated the term "Sequence identity" for amino acids as used herein refers to the sequence identity calculated as  $(n_{ref} - n_{dif}) \cdot 100 / n_{ref}$ , wherein  $n_{dif}$  is the total number of non-identical residues in the two sequences when aligned and wherein  $n_{ref}$  is the number of residues in one of the sequences.  
15 Hence, the amino acid sequence ASTDYWQNWT will have a sequence identity of 80% with the sequence ASTGYWQAWT ( $n_{dif}=2$  and  $n_{ref}=10$ ).

In some embodiments the sequence identity is determined by conventional methods, e.g., Smith and Waterman, 1981, Adv. Appl. Math. 2:482, by the search for similarity method of Pearson & Lipman, 1988, Proc. Natl. Acad. Sci.  
20 USA 85:2444, using the CLUSTAL W algorithm of Thompson et al., 1994, Nucleic Acids Res 22:467380, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group). The BLAST algorithm (Altschul et al., 1990, Mol. Biol. 215:403-10) for which software may be obtained through the National  
25 Center for Biotechnology Information [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) may also be used. When using any of the aforementioned algorithms, the default parameters for "Window" length, gap penalty, etc., are used.

The term "modification" as used herein means any chemical modification to any one amino acid or to the amino acid sequence of the polypeptide selected from  
30 SEQ ID NO: 1-22, as well as genetic manipulation of the DNA encoding that

polypeptide. The modification can be substitutions, deletions and/or insertions of one or more amino acids as well as replacements of one or more amino acid side chains. In some embodiments, the polypeptides have xylanase activity only have amino acid substitutions relative to SEQ ID No.1-22.

- 5 It is to be understood that "modification" in a given polypeptide is relative to the polypeptide selected from SEQ ID NO: 1-22 with the highest percentage sequence identity to this given polypeptide.

The terminology for amino acid substitutions used in this description is as follows. The first letter represents the amino acid naturally present at a position  
10 of a particular sequence. The following number represents the position relative to SEQ ID No. 1. The second letter represents the different amino acid substituting for the natural amino acid. An example is D11F/R122D/T110A, wherein the aspartic acid at position 11 of SEQ ID NO:1 is replaced by a phenylalanine and the arginine at position 122 of SEQ ID NO:1 is replaced by an  
15 aspartic acid, and the threonine at position 110 is replaced by an alanine, all three mutations being in the same polypeptide having xylanase activity.

Apart from the amino acid modifications in the polypeptides with xylanase activity according to the invention, the polypeptides may have further amino acid modifications of a minor nature, that is conservative amino acid  
20 substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another  
25 function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

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  
30 (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 H.

Neurath and R. L. Hill, 1979, In, *The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala to Ser, Val to Ile, Asp to Glu, Thr to Ser, Ala to Gly, Ala to Thr, Ser to Asn, Ala to Val, Ser to Gly, Tyr to Phe, Ala to Pro, Lys to Arg, Asp to Asn, Leu to Ile, Leu to Val, Ala to Glu, and Asp to Gly.

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-/V-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

The term "host organism", as used herein, includes any cell type which is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide encoding the polypeptides of the present invention.

For the present purposes, a xylanase means a protein or a polypeptide having xylanase activity.

The phrase "a polypeptide having xylanase activity" as used herein refers to any protein or polypeptide that has activity in a xylanase assay such as described herein.

Xylanase activity can be measured using any assay, in which a substrate is employed that includes 1,4-beta-D-xylosidic endo-linkages in xylans. The pH

and the temperature used in the assay are to be adapted to the xylanase in question. Examples of suitable pH values are 4, 5, 6, 7, 8, 9, 10 or 11. Examples of suitable temperatures are 30, 35, 37, 40, 45, 50, 55, 60, 65, 70 or 80°C.

Different types of substrates are available for the determination of xylanase

5 activity e.g. Xylazyme tablets (crosslinked, dyed xylan substrate, Megazyme, Bray, Ireland).

Preferably, xylanase activity is measured using the following assay.

Xylanase assay (Endo- $\beta$ -1,4-Xylanase activity)

10 Samples were diluted in citric acid (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx.  $OD_{590} = 0.7$  in this assay. Three different dilutions of the sample were pre-incubated for 5 minutes at 40°C. At time = 5 minutes, 1 Xylazyme tablet (crosslinked, dyed xylan substrate, Megazyme, Bray, Ireland) was added to the enzyme solution in a reaction volume of 1ml. At time = 15 minutes the reaction was terminated by adding 10 ml of 2% TRIS/NaOH, pH 12. Blanks were prepared using 1000 $\mu$ l buffer instead of enzyme solution. 15 The reaction mixture was centrifuged (1500 x g, 10 minutes, 20°C) and the OD of the supernatant was measured at 590 nm. One xylanase unit (XU) is defined as the xylanase activity increasing  $OD_{590}$  with 0.025 per minute.

20 The substrate (cross-linked and dyed arabinoxylan extracted from wheat) used in the above assay is a good approximate to the corresponding substrate in commercial applications.

Enzymes can furthermore be classified on the basis of the handbook Enzyme Nomenclature from NC-IUBMB, 1992), see also the ENZYME site at the internet: <http://www.expasy.ch/enzyme/>. ENZYME is a repository of information relative 25 to the nomenclature of enzymes. It is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB) and it describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided (Bairoch A. The ENZYME database, 2000, Nucleic Acids Res 28:304-305). This IUB-MB



Enzyme nomenclature is based on their substrate specificity and occasionally on their molecular mechanism; such a classification does not reflect the structural features of these enzymes.

In one aspect of the invention, the xylanase is an enzyme classified as EC

5 3.2.1.8. The official name is endo-1,4-beta-xylanase. The systematic name is 1,4-beta-D-xylan xylanohydrolase. Other names may be used, such as endo-(1-4)-beta-xylanase; (1-4)-beta-xylan 4-xylanohydrolase; endo-1,4-xylanase; xylanase; beta-1,4-xylanase; endo-1,4-xylanase; endo-beta-1,4-xylanase; endo-1,4-beta-D-xylanase; 1,4-beta-xylan xylanohydrolase; beta-xylanase; 10 beta-1,4-xylan xylanohydrolase; endo-1,4-beta-xylanase; beta-D-xylanase. The reaction catalyzed is the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans.

Another classification of certain glycoside hydrolase enzymes, such as endoglucanase, xylanase, galactanase, mannanase, dextranase and alpha-  
15 galactosidase, in families based on amino acid sequence similarities has been proposed a few years ago. They currently fall into 90 different families: See the CAZy(ModO) internet site (Coutinho, P.M. & Henrissat, B. (1999) Carbohydrate-Active Enzymes server at: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> (corresponding papers: Coutinho, P.M. & Henrissat, B. (1999) Carbohydrate-  
20 active enzymes: an integrated database approach. In "Recent Advances in Carbohydrate Bioengineering", HJ. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., The Royal Society of Chemistry, Cambridge, pp. 3-12; Coutinho, P.M. & Henrissat, B. (1999) The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In "Genetics,  
25 Biochemistry and Ecology of Cellulose Degradation"., K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23).

In one aspect of the invention, the xylanase of the invention is a xylanase of Glycoside Hydrolyase (GH) Family 11. The term "of Glycoside Hydrolyase (GH)  
30 Family 11" means that the xylanase in question is or can be classified in the GH family 11.

It is to be understood that protein similarity searches (like ProteinBlast at e.g. [http://toolkit.tuebingen.mpg.de/prot\\_blast](http://toolkit.tuebingen.mpg.de/prot_blast) ) may not necessarily determine whether an unknown sequence actually falls under the term of a GH11 xylanase family member. Proteins sequences found using a blast search might have

5 relatively high identity/homology and still not be actual xylanases, and furthermore, not be xylanases belonging to GH11. Alternatively, protein sequences may have a relatively low primary amino acid sequence identity and still be a GH11 xylanase family member. To determine whether an unknown protein sequence actually is a xylanase protein within the GH11 family, the

10 evaluation will have to be done, not only on sequence similarity, but also on 3D – structure similarity, since the classification within GH-families rely on the 3D fold. A software that will predict the 3D fold of an unknown protein sequence is HHpred (<http://toolkit.tuebingen.mpg.de/hhpred>). The power of this software for protein structure prediction relies on identifying homologous sequences with

15 known structure to be used as template. This works so well because structures diverge much more slowly than primary sequences. Proteins of the same family may have very similar structures even when their sequences have diverged beyond recognition.

20 In practice, an unknown sequence can be pasted into the software (<http://toolkit.tuebingen.mpg.de/hhpred> ) in FASTA format. Having done this, the search can be submitted. The output of the search will show a list of sequences with known 3D structures. To confirm that the unknown sequence indeed is a GH11 xylanase, GH11 xylanases should be found within the list of

25 homologues having a probability of > 90. Not all proteins identified as homologues will be characterised as GH11 xylanases, but some will. The latter proteins are proteins with a known structure and biochemically characterisation identifying them as xylanases. The former have not been biochemically characterised as GH11 xylanases. Several references describes this protocol

30 such as Söding J. (2005) Protein homology detection by HMM-HMM comparison. *Bioinformatics* 21, 951-960 (doi:10.1093/bioinformatics/bti125) and Söding J, Biegert A, and Lupas AN. (2005) The HHpred interactive server for protein

homology detection and structure prediction. Nucleic Acids Research 33, W244--W248 (Web Server issue) (doi:10.1093/nar/gki40).

According to the Cazy(ModO) site, Family 11 glycoside hydrolases can be characterised as follows:

Known Activities: xylanase (EC 3.2.1.8)

Mechanism: Retaining

Catalytic Nucleophile/Base : Glu (experimental)

Catalytic Proton Donor: Glu (experimental)

3D Structure Status: Fold:  $\beta$ -jelly roll

Clan: GH-C

As used herein, "Clan C" refers to groupings of families which share a common three-dimensional fold and identical catalytic machinery (see, for example, Henrissat, B. and Bairoch, A. , (1996) Biochem. J. ,316, 695-696).

As used herein, "Family 11" refers to a family of enzymes as established by Henrissat and Bairoch (1993) Biochem J.,293,781-788 (see, also, Henrissat and Davies (1997) Current Opinion in Structural Biol. 1997, &:637-644). Common features for family 11 members include high genetic homology, a size of about 20 kDa and a double displacement catalytic mechanism (see Tenkanen et al., 1992; Wakarchuk et al., 1994). The structure of the family 11 xylanases includes two large  $\beta$ -sheets made of  $\beta$ -strands and  $\alpha$ - helices.

Family 11 xylanases include but are not limited to the following: *Aspergillus niger* XynA, *Aspergillus kawachii* XynC, *Aspergillus tubigensis* XynA, *Bacillus circulans* XynA, *Bacillus pumilus* XynA, *Bacillus subtilis* XynA, *Neocallimastix patriciarum* XynA, *Streptomyces lividans* XynB, *Streptomyces lividans* XynC, *Streptomyces therinoviolaceus* XynII, *Thermomonospora fusca* XynA, *Trichoderma harzianum* Xyn, *Trichoderma reesei* XynI, *Trichoderma reesei* XynII, *Trichoderma viride* Xyn.

As used herein, "wild-type" refers to a sequence or a protein that is native or naturally occurring.

In another particular embodiment, the xylanase of the invention is derived from a bacterial xylanase, such as from a bacterium of (i) the phylum of Firmicutes; (ii) the class of Bacilli; (iii) the order of Bacillales; (iv) the family of Paenibacillaceae; or (v) the genus of Paenibacillus; such as from a bacterium of (vi) the species of Paenibacillus pabuli, Paenibacillus polymyxa, or Paenibacillus sp.; such as from (vii) strains of Paenibacillus pabuli, or Paenibacillus polymyxa.

The expression "xylanase derived from a bacterial xylanase" as used herein above includes any wild-type xylanase isolated from the bacterium in question, as well as variants or fragments thereof which retain xylanase activity.

In a further particular embodiment the xylanase of the invention is derived from a fungal xylanase.

The above definition of "derived from" (in the context of bacterial xylanases) is applicable by analogy also to fungal xylanases.

Examples of fungal xylanases of family 11 glycoside hydrolase are those which can be derived from the following fungal genera: Aspergillus, Aureobasidium, Emericella, Fusarium, Gaeumannomyces, Humicola, Lentinula, Magnaporthe, Neocallimastix, Nocardiosis, Orpinomyces, Paecilomyces, Penicillium, Pichia, Schizophyllum, Talaromyces, Thermomyces, Trichoderma.

Fungal xylanases include yeast and filamentous fungal xylanases. In some embodiments, the xylanase is derived from a fungus of (i) the phylum of Ascomycota; (ii) the class of Pezizomycotina; (iii) the order of Eurotiomycetes; (iv) the sub-order of Eurotiales; (v) the family of Trichocomaceae, such as the mitosporic Trichocomaceae; or from a fungus of (vi) the genus Aspergillus; such as from (vii) strains of Aspergillus niger. It will be understood that the definition of the aforementioned species includes both the perfect and imperfect states,

and other taxonomic equivalents e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

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

Questions relating to taxonomy can be solved by consulting a taxonomy data base, such as the NCBI Taxonomy Browser which is available at the following internet site: <http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/>.

- 15 However, preferably reference is to the following handbooks: Dictionary of the Fungi, 9th edition, edited by Kirk, P.M., P. F. Cannon, J. C. David & J.A. Stalpers, CAB Publishing, 2001; and Bergey's Manual of Systematic Bacteriology, Second edition (2005).

- 20 The present invention relates to modification(s) at certain amino acid position(s). These position(s) are listed with reference to the *B. subtilis* amino acid sequence shown as SEQ ID No. 1. In the present invention, the polypeptides with xylanase activity have a modification at least in position 110 compared to the *B. subtilis* sequence shown as SEQ ID No. 1. Equivalent  
25 positions in other family 11 xylanases may be found by aligning other Family 11 xylanases with SEQ ID No. 1 and determining which amino acid aligns with the specific amino acid of SEQ ID No. 1 (e.g., see Example 5). Such alignment and use of one sequence as a first reference is simply a matter of routine for one of ordinary skill in the art.

- 30 In one aspect, a variant xylanase according to the invention has an improved bran solubilisation activity which is higher than what may be obtained by use of the corresponding wild-type xylanase, or any one xylanase comprising an amino

acid sequence selected from SEQ ID No. 2-22 as measured in a "bran solubilisation assay".

In one aspect, the xylanase according to the invention has an improved bran solubilisation activity as a result of the modification in position 110.

- 5 Suitably, xylanase bran solubilising activity may be measured using the bran solubilising assay provided herein. Thus, polypeptides having increased xylanase activity and/or increased bran solubilising activity may be provided. The requirement for specificity towards the WU-AX is increasingly more and more important, since many applications are using elevated concentration of
- 10 cereal bran. The bread making industry increases the bran concentration in many products, due to health and nutritional issues, the feed industry incorporates increasing amount of bran material (fibre, Distillers Dried Grains with Solubles (DDGS)) due to the use of cereal in Bioethanol production, for example. It is therefore advantageous to provide new xylanases with increased
- 15 specificity, and hence efficacy in solubilising this bran material.

#### Bran solubilisation assay

Preferably, bran solubility is measured using the following assay.

- A suspension of wheat bran in (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0 is prepared to an concentration of 1,33% bran (w/w). From this
- 20 suspension, aliquots of 750 µl are transferred into eppendorph tubes under stirring. Each substrate tube is pre-heated for 5 minutes at 40°C. Hereto, 250 µl enzyme solution is added, making the end concentration of substrate 1%. Three dilutions (in duplicate) are made from each xylanases, with increasing enzyme concentration (0,33; 1,0 and 3,0 µg xylanase/gram bran) to each time
- 25 of determination (0, 30, 60 and 240 minutes). As blank, a heat denaturated solution of the xylanase is used. The reaction is terminated to the given times, by transferring the tubes to a incubator set at 95°C. Heat denaturated samples are kept at 4°C until all enzyme reactions are terminated. When all enzyme reactions are terminated, Eppendorph tubes are centrifuged to obtain a clear

supernatant. The enzymes capability to solubilise bran is expressed as the increase in reducing end groups as determined using PAHBAH (Lever, 1972).

Since side activities, such as amylase activity, may interfere with the above assay, bran solubilisation assay should only be carried out on purified xylanase samples (see Ex. 2).

In one aspect, the xylanase according to the invention has a reduced sensitivity to a xylanase inhibitor as compared to any one wild type xylanase, or any one xylanase comprising an amino acid sequence selected from SEQ ID No. 2-22.

In a further aspect, the polypeptide having xylanase activity according to the invention has a reduced sensitivity to a xylanase inhibitor as a result of the modification in position 110 in combination with one or more modification(s) at any one or more of amino acid positions: 11, 12, 13, 34, 54, 77, 81, 99, 104, 113, 114, 118, 122, 141, 154, 159, 162, 164, 166 and 175.

The inhibitor may be an inhibitor found naturally in plant tissues.

As used herein, the term "xylanase inhibitor" refers to a compound, typically a protein, whose role is to control the depolymerisation of complex carbohydrates, such as arabinoxylan, found in plant cell walls. These xylanase inhibitors are capable of reducing the activity of naturally occurring xylanase enzymes as well as those of fungal or bacterial origin. Although the presence of xylanase inhibitors have been reported in cereal seeds (see for example McLauchlan et al 1999a; Rouau and Suget 1998).

McLauchlan et al (1999a) disclose the isolation and characterisation of a protein from wheat that binds to and inhibits two family-11 xylanases. Likewise, WO 98/49278 demonstrates the effect of a wheat flour extract on the activity of a group of microbial xylanases all of which are classified as family 11 xylanases. Debyser et al. (1999) also disclose that endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, which are both members of the family 11 xylanases were inhibited by a wheat xylanase inhibitor called TAXI. McLauchlan et al (1999b)

teach that extracts from commercial flours such as wheat, barley, rye and maize are capable of inhibiting both family 10 and 11 xylanases.

The xylanase inhibitor may be any suitable xylanase inhibitor. By way of example, the xylanase inhibitor may be the inhibitor described in WO-A-

5 98/49278 and/or the xylanase inhibitor described by Rouau, X. and Surget, A. (1998), McLauchlan, R., et al. (1999) and/or the xylanase inhibitor described in UK patent application number 9828599.2 (filed 23 December 1998), UK patent application number 9907805.7 (filed 6 April 1999) and UK patent application number 9908645.6 (filed 15 April 1999).

10 The inhibitors described in the prior art may also be used in assays to determine the sensitivity of a variant polypeptide of the invention to xylanase inhibitors. They may also be used as described below to modulate the functionality of a xylanase.

#### Xylanase inhibitor assay

15 Preferably, xylanase inhibition activity is measured using the following assay.

100  $\mu$ l inhibitor preparation (containing various concentrations of xylanase inhibitor (for quantification see Xylanase inhibitor quantification below)), 250  $\mu$ l xylanase solution (containing 12 XU xylanase/ml) and 650  $\mu$ l buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen phosphate buffer, 1% BSA (Sigma-Aldrich, USA), pH 5.0) was mixed. The mixture was thermostated for 5 minutes at 20 40.0°C. At time = 5 minutes one Xylazyme tablet was added. At time = 15 minutes reaction was terminated by adding 10 ml 2% TRIS/NaOH, pH 12. The reaction mixture was centrifuged (1500 x g, 10 minutes, 20° C) and the supernatant measured at 590 nm. The xylanase inhibition was calculated as 25 residual activity in %, compared to the blank.

The endogenous endo- $\beta$ -1,4-xylanase inhibitor used is obtainable from wheat flour. The inhibitor is a di-peptide, having a MW of about 40 kDa (as measured by SDS-PAGE or mass spectrometry) and a pI of about 8 to about 9.5.



Sequence analysis to date has revealed that the inhibitor has the sequence presented as SEQ ID No. 24 or is highly homologous thereto.

A method to quantify the inhibitor concentration in a give inhibitor preparation can be found in Ex. 3

- 5     Blanks were prepared the same way, but substituting the inhibitor solution with water.

The present invention also relates to nucleotide sequence encoding a polypeptide according to the invention comprising a nucleotide sequence operably linked to one or more control sequences that direct the expression of  
10     the coding sequence in a suitable host cell under conditions compatible with the control sequences. A polynucleotide encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide's sequence prior to its  
15     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.

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 sequence  
20     contains transcriptional control sequences which mediate the expression of the polypeptide. 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.

25     Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase

gene {amyM), *Bacillus amyloliquefaciens* alpha-amylase gene {amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the National Academy of Sciences USA 75: 3727-3731), as well as the tac promoter  
 5 (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242: 74-94; and in Sambrook et al., 1989, supra.

Examples of suitable promoters for directing the transcription of the nucleic acid  
 10 constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase {glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae*  
 15 alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Thchoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I,  
 20 *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the  
 25 promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase); and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae*  
 30 galactokinase (GAL1 ), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3- phosphate dehydrogenase (ADH1 , ADH2/GAP), *Saccharomyces cerevisiae* those phosphate isomerase (TPI),

Saccharomyces cerevisiae metallothionine (CUP1 ), and Saccharomyces cerevisiae 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos et al., 1992, Yeast 8: 423-488.

5 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.

10 Terminators for filamentous fungal host cells may be obtained from the genes for Aspergillus oryzae TKA amylase, Aspergillus niger glucoamylase, Aspergillus nidulans anthranilate synthase, Aspergillus niger alpha-glucosidase, and Fusarium oxysporum trypsin-like protease. Terminators for yeast host cells may be obtained from the genes for Saccharomyces cerevisiae enolase, Saccharomyces cerevisiae cytochrome C (CYC1 ), and Saccharomyces cerevisiae  
15 glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, supra.

The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence  
20 encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention.

Leaders for filamentous fungal host cells may be obtained from the genes for Aspergillus oryzae TKA amylase and Aspergillus nidulans triose phosphate isomerase.

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

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the 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  
5 in the host cell of choice may be used in the present invention.

Polyadenylation sequences for filamentous fungal host cells may be obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin- like protease, and *Aspergillus n/geralpha*-glucosidase.

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

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  
15 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  
20 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  
25 pathway of a host cell of choice, i.e., secreted into a culture medium, may be used in the present invention.

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

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

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

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

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

25 Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

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. Regulatory systems in prokaryotic systems include the lac, tec, and tip operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA  
5 alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the  
10 metallothionein genes which are amplified with heavy metals. In these cases, the nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

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

25 The recombinant expression vector may be any vector (e.g., a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about 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  
30 plasmids.

The vector may be an autonomously replicating vector, i.e., a vector which 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 which permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which 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), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. In some embodiments the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus* are used in an *Aspergillus* cell.

The vectors of the present invention preferably contain an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome. For integration into the host cell genome, the vector may rely on the polynucleotide's sequence

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

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate in vivo.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB1 10, pE194, pTA1060, and pAM $\beta$ i permitting replication in *Bacillus*.

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

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



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

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

The present invention also relates to recombinant host cells, comprising a polynucleotide encoding the polypeptide of the present invention, which are advantageously used in the recombinant production of the polypeptides. A  
15 vector comprising a polynucleotide encoding the polypeptide 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 term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during  
20 replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be a unicellular microorganism, e.g., a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Useful unicellular microorganisms are bacterial cells such as gram positive bacteria including, but not limited to, a  
25 *Bacillus* cell, e.g., *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, e.g., *Streptomyces lividans* and *Streptomyces murinus*, or  
30 gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In one aspect, the bacterial host cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus*

stearothermophilus, or *Bacillus subtilis* cell. In another aspect, 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  
5 cells (see, e.g., Young and Spizizen, 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 Thome, 1987, *Journal of Bacteriology* 169: 5771-5278).

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

In one aspect, 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,  
15 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, supra, page 171 ) and all mitosporic fungi (Hawksworth et al., 1995, supra). In another aspect, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi  
20 Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner, F. A., Passmore, S. M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980).

- In an even further aspect, the yeast host cell is a *Candida*, *Hansenula*,  
25 *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

- In one particular aspect, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* cell. In another aspect, the yeast host cell is a *Kluyveromyces lactis*  
30 cell. In another aspect, the yeast host cell is a *Yarrowia lipolytica* cell.

In another aspect, 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., 1995, supra). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

In another aspect, the filamentous fungal host cell is an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

In another aspect, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another aspect, 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 sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In another aspect, the filamentous fungal host cell is a *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Coprinus cinereus*, *Coriolus hirsutus*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell. Fungal cells

may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 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, J.N. and Simon, M.I., editors, Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, Journal of Bacteriology 153: 163; and Hinnen et al., 1978, Proceedings of the National Academy of Sciences USA 75: 1920.

The present invention also relates to methods for producing a polypeptide of the present invention, comprising: (a) cultivating a cell, which in its wild-type form is capable of producing the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. Preferably, the cell is of the genus *Aspergillus* and more preferably *Aspergillus fumigatus*.

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

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and 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 into the medium, it can be recovered from cell lysates.

5 The polypeptides may be detected using methods known in the art that are specific for the 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 polypeptide as described herein.

10 The resulting polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The polypeptides of the present invention may be purified by a variety of procedures known in the art  
15 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  
20 substantially pure polypeptides.

In one aspect, the amino acid modification in position 110 is an amino acid substitution.

In one aspect, the amino acid modification in position 110 is an amino acid deletion.

25 In one aspect, the amino acid modification in position 110 is an amino acid insertion.

In some embodiments, the sequence identity is measured relative to SEQ ID No. 1, wherein the amino acid sequence according to SEQ ID No. 1 further comprises a signal peptide sequence, such as its natural signal peptide sequence.

- 5 In some embodiments, the polypeptide according to the invention has at least 90, 92 or 95% identity with SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with the sequence with which is has the highest percentage of identity selected from SEQ ID No. 2-22.

- 10 In some embodiments, the polypeptide according to the invention has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 2.

In some embodiments, the polypeptide according to the invention has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 3.

- 15 In some embodiments, the polypeptide according to the invention has a  $\beta$ -jelly roll fold.

In some embodiments according to the invention the amino acid modification in position 110 is an amino acid substitution.

- 20 In some embodiments according to the invention the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

- 25 In some embodiments according to the invention the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: alanine, arginine, asparagine, cysteine, glutamic

acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

In some embodiments according to the invention the amino acid modification in position 110 is a substitution to any one different amino acid residue selected  
5 from the group consisting of: glutamic acid, tryptophan, alanine and cysteine.

In some embodiments according to the invention the amino acid modification in position 110 is a substitution to alanine.

In some embodiments, the polypeptide according to the invention has a total number of amino acids of less than 250, such as less than 240, such as less than  
10 230, such as less than 220, such as less than 210, such as less than 200 amino acids, such as in the range of 160 to 240, such as in the range of 160 to 220 amino acids.

In some embodiments, the polypeptide according to the invention comprises one or more modification(s) at any one or more of amino acid positions: 11, 12, 13,  
15 34, 54, 77, 81, 99, 104, 113, 114, 118, 122, 141, 154, 159, 162, 164, 166 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises one or more amino acid substitutions selected from the group consisting of: 11F,  
20 12F, 54Q, 54W, 122D, 113A, 13Y, 113D, 175L, 122F, 34K, 99Y, 104W, 141Q, 154R, 159D, 175K, 81I, 166F, 162E, 162D, 164F, 114D, 114Y, 114F, 118V, 175K, 77L, 77M, 77S, 77V, and 77Y, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

25 In some embodiments, the polypeptide according to the invention comprises one or more amino acid substitutions selected from the group consisting of: D11F, G12F, N54Q, R122D, Y113A, G13Y, Y113D, N141Q, Q175L, R122F, G34K, K99Y, T104W, K154R, N159D, Q175K, V81I, Y166F, S162E, S162D, W164F, N114D,

N114Y, N114F, I118V, I77L, I77M, I77S, I77V, and I77Y, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

5 In some embodiments, the polypeptide according to the invention comprises one or more modification(s) at any one or more of amino acid positions: 13, 99, 104, 113, 122, 154, 159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

10 In some embodiments, the polypeptide according to the invention comprises substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

15 In some embodiments, the polypeptide according to the invention further comprises one or more modification(s) at any one or more of amino acid positions: 114 and 166, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

20 In some embodiments, the polypeptide according to the invention further comprises one or more substitution(s) at any one or more of amino acid positions: 114 and 166, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

25 In some embodiments, the polypeptide according to the invention comprises substitution(s) in at least at four of the following amino acid positions: 13, 99, 104, 113, 114, 122, 154, 159, 166, and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises substitution(s) at the amino acid positions: 13, 99, 104, 113, 114, 122, 154,



159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159,  
5 166 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159,  
10 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises one or more amino acid substitutions selected from the group consisting of: 13Y, 99Y, 104W, 110A, 113D, 114D, 114F, 122F, 154R, 159D, 166F, 175K, and 175L,  
15 the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention has at least five, six, seven, eight, nine or ten amino acid substitutions compared to the sequence selected among SEQ ID No. 1-22 with which it has the highest identity.

20 In some embodiments, the polypeptide according to the invention has at least nine or ten amino acid substitutions.

In some embodiments, the polypeptide according to the invention comprises one or more amino acid substitutions selected from the group consisting of:

- 25 a. D11F, R122D and T110A;  
b. D11F, R122D, T110A and Y113A;  
c. G13Y, T110A, Y113D, R122D and Q175L;  
d. G13Y, T110A, Y113D, R122F and Q175L;  
e. G13Y, G34K, T110A, Y113D, R122D and Q175L;

- f. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175K;
- g. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Q175 and V81I;
- h. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Y166F and Q175L;
- 5 i. G13Y, T110A, Y113D, R122D, K154R, N159D and Q175L;
- j. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- k. G13Y, T110A, Y113D, R122D, S162E and Q175L;
- l. G13Y, T110A, Y113D, R122D, S162D and Q175L;
- 10 m. G13Y, T110A, Y113D, R122D, W164F and Q175L;
- n. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175L;
- o. G13Y, K99Y, T104W, T110A, Y113D, N114Y, R122F, K154R, N159D and Q175L;
- 15 p. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D and Q175L;
- q. G13Y, K99Y, T104W, T110A, Y113D, I118V, R122F, K154R, N159D and Q175L;
- r. G13Y, K99Y, T104W, T110A, Y113D, N114Y, R122F, K154R, N159D and Q175K;
- 20 s. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175K;
- t. G13Y, I77L, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- 25 u. G13Y, I77M, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- v. G13Y, I77S, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- w. G13Y, I77V, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- 30 x. G13Y, I77Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- y. G13Y, K99Y, T104W, T110A, Y113D, R122F, N141Q, K154R, N159D, Q175L;
- z. G13Y, N54Q, K99Y, T104W, T110A, Y113D, R122F, N141Q, K154R, N159D, Q175;
- 35 aa. G13Y, N54W, K99Y, T104W, T110A, Y113D, R122F, 141Q, K154R, N159D, 175L;
- bb. G13Y, N54Q, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D, Q175L;
- cc. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, 141Q, K154R, N159D, Q175L; and
- 40 dd. G13Y, 54Q, K99Y, T104W, T110A, Y113D, N114F, R122F, 141Q, K154R, N159D, Q175L;

the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention comprises one  
5 or more amino acid substitutions selected from the group consisting of:

- a. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175K;
- b. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Y166F and Q175L;
- 10 c. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- d. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175L;
- e. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D and Q175L;
- 15

the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention has bran  
20 solubilisation activity.

In some embodiments, the polypeptide according to the invention is in isolated form.

The term "isolated" as used herein means that the polypeptide is at least substantially free from at least one other component with which the sequence is  
25 naturally associated in nature.

In some embodiments according to the invention the amino acid modification in position 110 is not T110D.

In some embodiments the polypeptide having xylanase activity does not have an aspartic acid in position 110.

In some embodiments, the polypeptide according to the invention has improved xylanase activity compared to the *B. subtilis* amino acid sequence shown as SEQ ID No. 1 as measured in a xylanase activity assay.

5 In some embodiments, the polypeptide according to the invention has improved xylanase activity as a result of the modification in position 110.

In some embodiments, the polypeptide according to the invention has improved bran solubilisation activity compared to the *B. subtilis* amino acid sequence shown as SEQ ID No. 1 as measured in a bran solubilisation activity assay.

10 In some embodiments, the polypeptide according to the invention has improved bran solubilisation activity as a result of the modification in position 110.

In some embodiments, the polypeptide according to the invention has reduced sensitivity to a xylanase inhibitor.

15 In some embodiments, the polypeptide according to the invention has an amino acid sequence comprising modifications at positions selected from the list consisting of:

- a) 13/110/113/122/154/159/175;
- b) 13/99/104/110/113/122/154/159/166/175;
- c) 13/99/104/110/113/114/122/154/159/175;
- d) 13/110/113/122/175;
- 20 e) 13/99/104/110/113/122/154/159/175;
- f) 13/99/104/110/113/122/154/159/175;
- g) 13/99/104/110/113/114/122/154/159/175;
- h) 13/99/104/110/113/114/122/154/159/175;
- i) 13/99/104/110/113/114/122/154/159/175;
- 25 j) 13/99/104/110/113/114/122/154/159/175;

- k) 13/77/99/104/110/113/122/154/159/175;
- l) 13/81/99/104/110/113/122/154/159/175;
- m) 13/110/113/122/164/175;
- n) 13/110/113/122/162/175;
- 5 o) 13/110/113/122/175;
- p) 11/122/110/113;
- q) 13/77/99/104/110/113/122/154/159/175;
- r) 11/122/110;
- s) 13/34/110/113/122/175;
- 10 t) 13/77/99/104/110/113/122/154/159/175;
- u) 13/77/99/104/110/113/122/154/159/175;
- v) 13/99/104/110/113/118/122/154/159/175;
- w) 13/110/113/122/162/175;
- x) 13/77/99/104/110/113/122/154/159/175;
- 15 y) 13/99/104/110/113/122/141/154/159/175;
- z) 13/54/99/104/110/113/122/141/154/159/175;
- aa) 13/54/99/104/110/113/122/141/154/159/175;
- bb) 13/54/99/104/110/113/114/122/154/159/175;
- cc) 13/99/104/110/113/114/122/141/154/159/175; and
- 20 dd) 13/54/99/104/110/113/114/122/141/154/159/175;

the position(s) being determined as the corresponding position of *subtilis* amino acid sequence shown as SEQ ID No. 1.

- 25 In some embodiments, the polypeptide according to the invention has an amino acid sequence comprising amino acid substitutions selected from the list consisting of:

- a) 13Y/110A/113D/122D/154R/159D/175L;
- b) 13Y/99Y/104W/110A/113D/122F/154R/159D/166F/175L;
- c) 13Y/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- d) 13Y/110A/113D/122F/175L;
- 5 e) 13Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- f) 13Y/99Y/104W/110A/113D/122F/154R/159D/175K;
- g) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175K;
- h) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175L;
- i) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175L;
- 10 j) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175K;
- k) 13Y/77L/99Y/104W/110A/113D/122F/154R/159D/175L;
- l) 13Y/81I/99Y/104W/110A/113D/122F/154R/159D/175L;
- m) 13Y/110A/113D/122D/164F/175L;
- n) 13Y/110A/113D/122D/162D/175L;
- 15 o) 13Y/110A/113D/122D/175L;
- p) 11F/122D/110A/113A;
- q) 13Y/77Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- r) 11F/122D/110A;
- s) 13Y/34K/110A/113D/122D/175L;
- 20 t) 13Y/77V/99Y/104W/110A/113D/122F/154R/159D/175L;
- u) 13Y/77M/99Y/104W/110A/113D/122F/154R/159D/175L;
- v) 13Y/99Y/104W/110A/113D/118V/122F/154R/159D/175L;
- w) 13Y/110A/113D/122D/162E/175L;
- x) 13Y/77S/99Y/104W/110A/113D/122F/154R/159D/175L;
- 25 y) 13Y/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- z) 13Y/54Q/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;

- aa) 13Y/54W/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- bb) 13Y/54Q/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- cc) 13Y/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L; and
- dd) 13Y/54Q/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L;

5 the position(s) being determined as the corresponding position of *subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention has an amino acid sequence of SEQ ID No. 1 comprising amino acid substitutions selected

10 from the list consisting of

- a) G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L;
- b) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L;
- c) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;
- d) G13Y/T110A/Y113D/R122F/Q175L;
- 15 e) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- f) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K;
- g) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175K;
- h) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L;
- i) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L;
- 20 j) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175K;
- k) G13Y/I77L/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- l) G13Y/V81I/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- m) G13Y/T110A/Y113D/R122D/W164F/Q175L;
- n) G13Y/T110A/Y113D/R122D/S162D/Q175L;
- 25 o) G13Y/T110A/Y113D/R122D/Q175L;
- p) D11F/R122D/T110A/Y113A;

- q) G13Y/I77Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- r) D11F/R122D/T110A;
- s) G13Y/G34K/T110A/Y113D/R122D/Q175L;
- t) G13Y/I77V/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- 5 u) G13Y/I77M/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- v) G13Y/K99Y/T104W/T110A/Y113D/I118V/R122F/K154R/N159D/Q175L;
- w) G13Y/T110A/Y113D/R122D/S162E/Q175L; and
- x) G13Y/I77S/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L
- y) G13Y/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;
- 10 z) G13Y/N54Q/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;
- aa) G13Y/N54W/K99Y/T104W/T110A/Y113D/R122F/141Q/K154R/N159D/175L;
- bb) G13Y/N54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;
- 15 cc) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q175L; and
- dd) G13Y/54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q175L.

20

In some embodiments, the polypeptide according to the invention has an amino acid sequence, which consists of amino acid substitutions selected from the list consisting of:

- a) 13Y/110A/113D/122D/154R/159D/175L;
- 25 b) 13Y/99Y/104W/110A/113D/122F/154R/159D/166F/175L;
- c) 13Y/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- d) 13Y/110A/113D/122F/175L;



- e) 13Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- f) 13Y/99Y/104W/110A/113D/122F/154R/159D/175K;
- g) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175K;
- h) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175L;
- 5 i) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175L;
- j) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175K;
- k) 13Y/77L/99Y/104W/110A/113D/122F/154R/159D/175L;
- l) 13Y/81I/99Y/104W/110A/113D/122F/154R/159D/175L;
- m) 13Y/110A/113D/122D/164F/175L;
- 10 n) 13Y/110A/113D/122D/162D/175L;
- o) 13Y/110A/113D/122D/175L;
- p) 11F/122D/110A/113A;
- q) 13Y/77Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- r) 11F/122D/110A;
- 15 s) 13Y/34K/110A/113D/122D/175L;
- t) 13Y/77V/99Y/104W/110A/113D/122F/154R/159D/175L;
- u) 13Y/77M/99Y/104W/110A/113D/122F/154R/159D/175L;
- v) 13Y/99Y/104W/110A/113D/118V/122F/154R/159D/175L;
- w) 13Y/110A/113D/122D/162E/175L;
- 20 x) 13Y/77S/99Y/104W/110A/113D/122F/154R/159D/175L;
- y) 13Y/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- z) 13Y/54Q/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- aa) 13Y/54W/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- bb) 13Y/54Q/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- 25 cc) 13Y/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L; and
- dd) 13Y/54Q/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L,

the position(s) being determined as the corresponding position of *subtilis* amino acid sequence shown as SEQ ID No. 1.

In some embodiments, the polypeptide according to the invention has an amino acid sequence of SEQ ID No. 1, which consists of amino acid substitutions selected from the list consisting of

- a) G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L;
- b) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L;
- c) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;
- 10 d) G13Y/T110A/Y113D/R122F/Q175L;
- e) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- f) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K;
- g) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175K;
- h) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L;
- 15 i) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L;
- j) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175K;
- k) G13Y/I77L/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- l) G13Y/V81I/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- m) G13Y/T110A/Y113D/R122D/W164F/Q175L;
- 20 n) G13Y/T110A/Y113D/R122D/S162D/Q175L;
- o) G13Y/T110A/Y113D/R122D/Q175L;
- p) D11F/R122D/T110A/Y113A;
- q) G13Y/I77Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- r) D11F/R122D/T110A;
- 25 s) G13Y/G34K/T110A/Y113D/R122D/Q175L;
- t) G13Y/I77V/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;

- u) G13Y/I77M/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- v) G13Y/K99Y/T104W/T110A/Y113D/I118V/R122F/K154R/N159D/Q175L;
- w) G13Y/T110A/Y113D/R122D/S162E/Q175L; and
- x) G13Y/I77S/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L
- 5 y) G13Y/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;
- z) G13Y/N54Q/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;
- aa) G13Y/N54W/K99Y/T104W/T110A/Y113D/R122F/141Q/K154R/N159D/175L;
- 10 bb) G13Y/N54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;
- cc) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q175L; and
- dd) G13Y/54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q175L.
- 15

In some embodiments, the polypeptide according to the invention is not SEQ ID No. 25.

20 In some embodiments, the polypeptide according to the invention does not have a sequence selected from the list consisting of:

- SEQ ID No. 57 of International Patent Application WO0238746;
- SEQ ID No. 62 of International Patent Application WO0238746;
- SEQ ID No. 59 of International Patent Application WO0238746;
- SEQ ID No. 53 of International Patent Application WO0238746;
- 25 SEQ ID No. 65 of International Patent Application WO0238746;
- SEQ ID No. 56 of International Patent Application WO0238746;
- SEQ ID No. 64 of International Patent Application WO0238746;
- SEQ ID No. 52 of International Patent Application WO0238746;
- SEQ ID No. 63 of International Patent Application WO0238746;

SEQ ID No. 61 of International Patent Application WO0238746;  
SEQ ID No. 60 of International Patent Application WO0238746;  
SEQ ID No. 58 of International Patent Application WO0238746;  
SEQ ID No. 55 of International Patent Application WO0238746;  
5 SEQ ID No. 12 of International Patent Application WO0238746;  
SEQ ID No. 11 of International Patent Application WO0238746;  
SEQ ID No. 21 of International Patent Application WO0068396; and  
SEQ ID No. 22 of International Patent Application WO0068396.

- 10 In some embodiments of the present invention, the amino acid sequence is used for large scale applications.

Preferably the amino acid sequence is produced in a quantity of from 1 g per litre to about 100 g per litre of the total cell culture volume after cultivation of the host organism.

- 15 The present invention also relates to a composition comprising amino acid sequences and/or nucleotide sequences encoding a xylanase as described herein.

The composition of the present invention can lead to improved aroma, flavour, mildness, consistency, texture, body, mouth feel, firmness, viscosity, gel

- 20 fracture, structure and/or organoleptic properties and nutrition of products for consumption containing said composition. Furthermore, the composition of the present invention can also be used in combination with other components of products for consumption to deliver said improvements.

Although it is preferred that the composition of the present invention is used to  
25 improve the aroma, flavour, mildness, consistency, texture, body, mouth feel, firmness, viscosity, gel fracture, structure, smoothness of the surface and/or organoleptic properties and nutrition of products for consumption containing said composition – the present invention also covers using the composition of the present invention as a component of pharmaceutical combinations with other  
30 components to deliver medical or physiological benefit to the consumer.

Accordingly, the composition of the present invention may be used in combination with other components.

Examples of other components include one or more of: thickeners, gelling agents, emulsifiers, binders, crystal modifiers, sweeteners (including artificial  
5 sweeteners), rheology modifiers, stabilisers, anti-oxidants, dyes, enzymes, carriers, vehicles, excipients, diluents, lubricating agents, flavouring agents, colouring matter, suspending agents, disintegrants, granulation binders etc. These other components may be natural. These other components may be prepared by use of chemical and/or enzymatic techniques.

10 As used herein the term "thickener or gelling agent" as used herein refers to a product that prevents separation by slowing or preventing the movement of particles, either droplets of immiscible liquids, air or insoluble solids. Thickening occurs when individual hydrated molecules cause an increase in viscosity, slowing the separation. Gelation occurs when the hydrated molecules link to  
15 form a three-dimensional network that traps the particles, thereby immobilizing them.

The term "stabiliser" as used here is defined as an ingredient or combination of ingredients that keeps a product (e.g. a food product) from changing over time.

The term "emulsifier" as used herein refers to an ingredient (e.g. a food product  
20 ingredient) that prevents the separation of emulsions. Emulsions are two immiscible substances, one present in droplet form, contained within the other. Emulsions can consist of oil-in-water, where the droplet or dispersed phase is oil and the continuous phase is water; or water-in-oil, where the water becomes the dispersed phase and the continuous phase is oil. Foams, which are gas-in-  
25 liquid, and suspensions, which are solid-in-liquid, can also be stabilised through the use of emulsifiers. Aeration can occur in a three phase system where air is entrapped by liquid oil then stabilised by agglomerated fat crystals stabilised with an emulsifier. Emulsifiers have a polar group with an affinity for water (hydrophilic) and a non-polar group which is attracted to oil (lipophilic). They  
30 are absorbed at the interfaces of the two substances, providing an interfacial

film acting to stabilise the emulsion. The hydrophilic/lipophilic properties of emulsifiers are affected by the structure of the molecule. These properties are identified by the hydrophilic/lipophilic balance (HLB) value. Low HLB values indicate greater lipophilic tendencies which are used to stabilise water-in-oil emulsions. High HLB values are assigned to hydrophilic emulsifiers, typically used in oil-in-water emulsions. These values are derived from simple systems. Because foods often contain other ingredients that affect the emulsification properties, the HLB values may not always be a reliable guide for emulsifier selection.

- 10 As used herein the term "binder" refers to an ingredient (e.g. a food ingredient) that binds the product together through a physical or chemical reaction. During "elation" for instance, water is absorbed, providing a binding effect. However, binders can absorb other liquids, such as oils, holding them within the product. In the context of the present invention binders would typically be used in solid or low-moisture products for instance baking products: pastries, doughnuts, bread and others.

- 20 The term "crystal modifier" as used herein refers to an ingredient (e.g. a food ingredient) that affects the crystallisation of either fat or water. Stabilisation of ice crystals is important for two reasons. The first is directly related to the product stability from a separation standpoint. The more freeze/thaw cycles a product encounters, the larger the ice crystals become. These large crystals can break down product structure, either naturally occurring, as in the case of cell walls, or that which is created by "elation". Because the water is no longer held in place, the product may exhibit syneresis, or weeping, after thawing.

- 25 Secondly, in the case of a product which is consumed frozen, these large crystals result in an undesirable, gritty mouth feel.

"Carriers" or "vehicles" mean materials suitable for compound administration and include any such material known in the art such as, for example, any liquid, gel, solvent, liquid diluent, solubilizer, or the like, which is non-toxic and which

does not interact with any components of the composition in a deleterious manner.

Examples of nutritionally acceptable carriers include, for example, water, salt solutions, alcohol, silicone, waxes, petroleum jelly, vegetable oils, polyethylene glycols, propylene glycol, liposomes, sugars, gelatin, lactose, amylose, magnesium stearate, talc, surfactants, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, and the like.

Examples of excipients include one or more of: microcrystalline cellulose and other celluloses, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate, glycine, starch, milk sugar and high molecular weight polyethylene glycols.

Examples of disintegrants include one or more of: starch (preferably corn, potato or tapioca starch), sodium starch glycollate, croscarmellose sodium and certain complex silicates.

Examples of granulation binders include one or more of: polyvinylpyrrolidone, hydroxypropylmethylcellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, maltose, gelatin and acacia.

Examples of lubricating agents include one or more of: magnesium stearate, stearic acid, glyceryl behenate and talc.

Examples of diluents include one or more of: water, ethanol, propylene glycol and glycerin, and combinations thereof.

The other components may be used simultaneously (e.g. when they are in admixture together or even when they are delivered by different routes) or sequentially (e.g. they may be delivered by different routes).

As used herein the term "component suitable for animal or human consumption" means a compound which is or can be added to the composition of the present invention as a supplement which may be of nutritional benefit, a fibre substitute or have a generally beneficial effect to the consumer. The ingredients can be used in a wide variety of products that require gelling, texturising, stabilising, suspending, film-forming and structuring, retention of juiciness, without adding unnecessary viscosity. Preferably, the ingredients will be able to improve the shelf life and stability of the viable culture.

By way of example, the components may be prebiotics such as alginate, xanthan, pectin, locust bean gum (LBG), inulin, guar gum, galacto-oligosaccharide (GOS), fructo-oligosaccharide (FOS), lactosucrose, soybean oligosaccharides, palatinose, isomalto-oligosaccharides, gluco-oligosaccharides and xylo-oligosaccharides.

The composition of the present invention may be used as – or in the preparation of - a food. Here, the term "food" is used in a broad sense – and covers food for humans as well as food for animals (i.e. a feed). In a preferred aspect, the food is for human consumption.

The food may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

When used as – or in the preparation of - a food – such as functional food - the composition of the present invention may be used in conjunction with one or more of: a nutritionally acceptable carrier, a nutritionally acceptable diluent, a nutritionally acceptable excipient, a nutritionally acceptable adjuvant, a nutritionally active ingredient.

The composition of the present invention may be used as a food ingredient.

As used herein the term "food ingredient" includes a formulation which is or can be added to functional foods or foodstuffs as a nutritional supplement and/or fiber supplement. The term food ingredient as used here also refers to



formulations which can be used at low levels in a wide variety of products that require gelling, texturising, stabilising, suspending, film-forming and structuring, retention of juiciness and improved mouthfeel, without adding viscosity.

5 The food ingredient may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

The composition of the present invention may be – or may be added to – food supplements.

The composition of the present invention may be – or may be added to – functional foods.

10 As used herein, the term “functional food” means food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a further beneficial effect to consumer.

15 Accordingly, functional foods are ordinary foods that have components or ingredients (such as those described herein) incorporated into them that impart to the food a specific functional – e.g. medical or physiological benefit – other than a purely nutritional effect.

Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

20 Some functional foods are nutraceuticals. Here, the term “nutraceutical” means a food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a therapeutic (or other beneficial) effect to the consumer. Nutraceuticals cross the traditional dividing lines between foods and medicine.

25 Surveys have suggested that consumers place the most emphasis on functional food claims relating to heart disease. Preventing cancer is another aspect of

nutrition which interests consumers a great deal, but interestingly this is the area that consumers feel they can exert least control over. In fact, according to the World Health Organization, at least 35% of cancer cases are diet-related. Furthermore claims relating to osteoporosis, gut health and obesity effects are also key factors that are likely to incite functional food purchase and drive market development.

The composition of the present invention can be used in the preparation of food products such as one or more of: jams, marmalades, jellies, dairy products (such as milk or cheese), meat products, poultry products, fish products and bakery products.

By way of example, the composition of the present invention can be used as ingredients to soft drinks, a fruit juice or a beverage comprising whey protein, health teas, cocoa drinks, milk drinks and lactic acid bacteria drinks, yoghurt and drinking yoghurt, cheese, ice cream, water ices and desserts, confectionery, biscuits cakes and cake mixes, snack foods, breakfast cereals, instant noodles and cup noodles, instant soups and cup soups, balanced foods and drinks, sweeteners, texture improved snack bars, fibre bars, bake stable fruit fillings, care glaze, chocolate bakery filling, cheese cake flavoured filling, fruit flavoured cake filling, cake and doughnut icing, heat stable bakery filling, instant bakery filling creams, filing for cookies, ready-to-use bakery filling, reduced calorie filling, adult nutritional beverage, acidified soy/juice beverage, aseptic/retorted chocolate drink, bar mixes, beverage powders, calcium fortified soy/plaim and chocolate milk, calcium fortified coffee beverage.

A composition according to the present invention can further be used as an ingredient in food products such as American cheese sauce, anti-caking agent for grated & shredded cheese, chip dip, cream cheese, dry blended whip topping fat free sour cream, freeze/thaw dairy whipping cream, freeze/thaw stable whipped tipping, low fat & lite natural cheddar cheese, low fat Swiss style yoghurt, aerated frozen desserts, and novelty bars, hard pack ice cream, label friendly, improved economics & indulgence of hard pack ice cream, low fat ice

cream: soft serve, barbecue sauce, cheese dip sauce, cottage cheese dressing, dry mix Alfredo sauce, mix cheese sauce, dry mix tomato sauce and others.

For certain aspects, preferably the foodstuff is a beverage.

For certain aspects, preferably the foodstuff is a bakery product - such as bread,  
5 Danish pastry, biscuits or cookies.

The present invention also provides a method of preparing a food or a food ingredient, the method comprising xylanase produced by the process of the present invention or the composition according to the present invention with another food ingredient. The method for preparing or a food ingredient is also  
10 another aspect of the present invention.

In a general sense, a polypeptide having xylanase activity of the invention may be used to solubilise and/or degrade insoluble plant cell wall material containing arabinoxylan, alter, for example reduce, the viscosity derived from the presence of hemicellulose or arabinoxylan in a solution or system comprising plant cell  
15 wall material. Typically said plant cell wall materials will comprise one or more xylanase inhibitors.

Specifically, a polypeptide having xylanase activity of the invention may be used in processing plant materials for use as foodstuffs, such as animal feed, in starch production, in baking, in production of Bio-ethanol from cellulosic material and in  
20 the processing of wood pulp to make paper.

A polypeptide having xylanase activity of the invention may be used to process plant materials such as cereals that are used in foodstuffs including animal feed. As used herein, the term "cereal" means any kind of grain used for food and/or any grass producing this grain such as but not limited to any one of wheat,  
25 milled wheat, barley, maize, sorghum, rye, oats, triticale and rice or combinations thereof. In one preferred embodiment, the cereal is a wheat cereal.

The xylan in the food and/or feed supplement is modified by contacting the xylan with the polypeptide having xylanase activity of the present invention.

As used herein, the term "contacting" includes but is not limited to spraying, coating, impregnating or layering the food and/or feed supplement with the polypeptide having xylanase activity of the present invention.

In one embodiment, the food and/or feed supplement of the present invention may be prepared by mixing the polypeptide having xylanase activity directly with a food and/or feed supplement. By way of example, the polypeptide having xylanase activity may be contacted (for example, by spraying) onto a cereal-based food and/or feed supplement such as milled wheat, maize or soya flour.

It is also possible to incorporate the polypeptide having xylanase activity it into a second (and different) food and/or feed or drinking water which is then added to the food and/or feed supplement of the present invention. Accordingly, it is not essential that the polypeptide having xylanase activity provided by the present invention is incorporated into the cereal-based food and/or feed supplement itself, although such incorporation forms a particularly preferred aspect of the present invention.

In one embodiment of the present invention, the food and/or feed supplement may be combined with other food and/or feed components to produce a cereal-based food and/or feed. Such other food and/or feed components may include one or more other (preferably thermostable) enzyme supplements, vitamin food and/or feed supplements, mineral food and/or feed supplements and amino acid food and/or feed supplements. The resulting (combined) food and/or feed supplement comprising possibly several different types of compounds can then be mixed in an appropriate amount with the other food and/or feed components such as cereal and protein supplements to form a human food and/or an animal feed.

In one preferred embodiment, the food and/or feed supplement of the present invention can be prepared by mixing different enzymes having the appropriate

activities to produce an enzyme mix. By way of example, a cereal-based food and/or feed supplement formed from e.g. milled wheat or maize may be contacted (e.g. by spraying) either simultaneously or sequentially with the xylanase enzyme and other enzymes having appropriate activities. These enzymes may include but are not limited to any one or more of an amylase, a glucoamylase, a mannanase, a galactosidase, a phytase, a lipase, a phospholipase, , a galactolipase, a glucanase, an-arabinofuranosidase, a ferulyl esterase, a pectinase, a protease, a glucose oxidase, a hexose oxidase and a xylanase. Enzymes having the desired activities may for instance be mixed with the xylanase of the present invention either before contacting these enzymes with a cereal-based food and/or feed supplement or alternatively such enzymes may be contacted simultaneously or sequentially on such a cereal based supplement. The food and/or feed supplement is then in turn mixed with a cereal-based food and/or feed to prepare the final food and/or feed. It is also possible to formulate the food and/or feed supplement as a solution of the individual enzyme activities and then mix this solution with a food and/or feed material prior to processing the food and/or feed supplement into pellets or as a mash.

The present invention provides the use of a polypeptide having xylanase activity of the invention in a process for preparing a foodstuff. Typical bakery (baked) products in accordance with the present invention include bread - such as loaves, rolls, buns, pizza bases etc. - pretzels, tortillas, cakes, cookies, biscuits, crackers etc. The preparation of foodstuffs such as bakery products is well known in the art. Dough production, for example, is described in example 4. The use of a polypeptide having xylanase activity of the invention to alter the baking performance is described in example 4.

A polypeptide having xylanase activity of the invention may also be used in starch production from plant materials derived from cereals and tubers, such as potatoes.

A polypeptide having xylanase activity of the invention may also be used in processing wood pulp, for example in the preparation of paper.

## Processing of cellulosic material for bio-ethanol production

A polypeptide having xylanase activity of the invention may also be used in the hydrolysis of cellulosic plant material for production of sugars fermentable to bio-ethanol.

- 5 In some particular embodiments the polypeptide having xylanase activity according to the invention has an optimal xylanase activity at dough processing temperatures, such as in the range of about 20 to about 40°C. In some embodiments the polypeptide having xylanase activity according to the invention are inactivated during a baking process.
- 10 In some alternative embodiments the polypeptide having xylanase activity according to the invention has increased thermostability and/or temperature optimum as compared to the corresponding wild type enzyme to retain activity after heat treatment. Both characteristics are known to persons skilled in the art.

## 15 **EXAMPLES**

### **Example 1 – Site-directed mutagenesis of xylanases and expression**

- Specific mutants of the *Bacillus subtilis* xylanase were obtained using a construct comprising the ribosome binding site from pET24a (ctagaaataatTTTgtttaactttaagaaggagatatacat) fused to the wild type xylanase
- 20 gene without signal sequence (atggctagcacagactactggcaa-----tggtaa) was transferred to the vector pCRBlunt (InVitrogen, Carlsbad, CA, USA). This resulted in constitutive expression of xylanase in TOP10 cells (InVitrogen) after transformation with the constructed vector, provided that the orientation of the gene is in a "clockwise" direction. Site directed mutation in the gene was then
  - 25 obtained by the use of the "QuickChange" mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturers protocol. Mutants were verified by sequencing. Sufficient production of the verified mutants was obtained by growing the transformed TOP10 cells in 1 L scale.

## Example 2 – Bran solubilisation studies of xylanase mutants

We used wheat bran as substrate to evaluate the specific activity of the xylanase variants since this is used in commercial applications.

### Bran substrate:

- 5 By means of example, bran could be wheat bran obtained from dry milling of wheat using a lab scale Chopin CD Auto Mill (Chopin Technologies, France), using the setting and conditions provided by the supplier, for milling wheat into wheat flour and bran. The obtained bran fraction may be used as substrate in the bran solubilisation assay. In this Example wheat was used as the cereal  
10 source.

### Bran solubilisation assay:

- A suspension of wheat bran in (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0 is prepared to an concentration of 1,33% bran (w/w). From this suspension, aliquots of 750 l are transferred into eppendorph tubes under  
15 stirring. Each substrate tube is pre-heated for 5 minutttes at 40°C. Hereto, 250 µl enzyme solution is added, making the end concentration of substrate 1%. Three dilutions (in duplicate) are made from each xylanases, with increasing enzyme concentration (0,33; 1,0 and 3,0 µg xylanase/gram bran) to each time of determination (0, 30, 60 and 240 minutttes). As blank, a heat denaturated  
20 solution of the xylanase is used. The reaction is terminated to the given times, by transferring the tubes to an incubator set at 95°C. Heat denaturated samples are kept at 4°C until all enzyme reactions are terminated. When all enzyme reactions are terminated, Eppendorph tubes are centrifuged to obtain a clear supernatant. The enzymes capability to solubilise bran is expressed as  
25 OD<sub>410</sub> increase, determined by the increase in reducing end groups using PAHBAH reagens (Lever, 1972).

In short, reducing end groups are reacted with PAHBAH forming a colored reaction product, which can be quantified at OD OD<sub>410</sub>.

The above bran solubilisation assay is sensitive to side activity of enzymes active on residual starch in the bran substrate.

*Bacillus subtilis* Xylanase Purification protocol:

*E. coli* TOP10 cells having expressed the xylanase were harvested by  
5 centrifugation (20 minutes, 3500 x g, 20° C) and resuspended in 50 mM Tris, 2  
mM EDTA, pH 7.4. Cells were opened by addition of 1 mg/ml lysozyme (ICN  
Biomedicals, Costa Mesa, CA, US, cat. No. 100831), stirring of the slurry for 2  
hours at ambient temperature, freezing and thawing followed by sonication. pH  
was adjusted to 4.0 using 1M HCl followed by centrifugation (20 minutes, 3500 x  
10 g, 20°C). The supernatant containing the xylanase was desalted using  
disposable PD-10 desalting columns (Amersham Bioscience, Sweden)  
equilibrated in and eluted with 50mM sodium acetate, pH 4.5. The desalted  
sample was loaded onto a 10 ml SOURCE 15S column (Amersham Bioscience,  
Sweden) pre-equilibrated with 50mM sodium acetate, pH 4.5. The column was  
15 then washed with equilibration buffer and eluted with a linear NaCl gradient  
(50mM sodium acetate, 0 - 0.35M NaCl, pH 4.5). Fractions containing xylanase  
activity were pooled and used for further analysis.

Similar protocols may be adapted to non-*Bacillus subtilis* XynA derived xylanase  
variants having a pI significantly different from *Bacillus subtilis* XynA



**Table 1. Xylanases bran solubilising activity expressed as, maximum optical density, slope index of xylanase mutants, relative optical density and slope compared to the xylanase BS1 (the *Bacillus subtilis* enzyme shown as SEQ ID No. 1) and the xylanase BS3 (*Bacillus subtilis* variant shown as SEQ ID No. 23)**

| Modifications made to SEQ ID No. 1                  | Slope OD/hr | Maximum OD | Relative Slope to BS1 | Relative Max. OD to BS1 | Relative Slope to BS3 | Relative Max. OD to BS3 |
|-----------------------------------------------------|-------------|------------|-----------------------|-------------------------|-----------------------|-------------------------|
| None (BS1)                                          | 0.23        | 0.69       | 100                   | 100                     | 140                   | 140                     |
| D11F/R122D (BS3)                                    | 0,16        | 0,49       | 72                    | 72                      | 100                   | 100                     |
| D11F/R122D/T110A                                    | 0.25        | 0.75       | 110                   | 110                     | 154                   | 154                     |
| D11F/R122D/T110A/Y113A                              | 0.27        | 0.80       | 117                   | 117                     | 163                   | 163                     |
| G13Y/T110A/Y113D/R122D/Q175L                        | 0.27        | 0.82       | 119                   | 119                     | 167                   | 167                     |
| G13Y/T110A/Y113D/R122F/Q175L                        | 0.37        | 1.12       | 164                   | 164                     | 229                   | 229                     |
| G13Y/G34K/T110A/Y113D/R122D/Q175L                   | 0.25        | 0.74       | 108                   | 108                     | 150                   | 150                     |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K | 0.37        | 1.11       | 162                   | 162                     | 226                   | 226                     |
| G13Y/K99Y/T104W                                     |             |            |                       |                         |                       |                         |

|                                                                       |      |      |     |     |     |     |
|-----------------------------------------------------------------------|------|------|-----|-----|-----|-----|
| /T110A/Y113D/R12<br>2F/K154R/N159D/<br>Q175/V81I                      | 0.29 | 0.87 | 128 | 128 | 178 | 178 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/R12<br>2F/K154R/N159D/Y<br>166F/Q175L | 0.39 | 1.18 | 172 | 172 | 241 | 241 |
| G13Y/T110A/Y113<br>D/R122D/K154R/N<br>159D/Q175L                      | 0.40 | 1.19 | 173 | 173 | 242 | 242 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/R12<br>2F/K154R/N159D/<br>Q175L       | 0.37 | 1.11 | 162 | 162 | 226 | 226 |
| G13Y/T110A/Y113<br>D/R122D/S162E/Q<br>175L                            | 0.18 | 0.53 | 78  | 78  | 109 | 109 |
| G13Y/T110A/Y113<br>D/R122D/S162D/Q<br>175L                            | 0.28 | 0.83 | 121 | 121 | 169 | 169 |
| G13Y/T110A/Y113<br>D/R122D/W164F/Q<br>175L                            | 0.28 | 0.83 | 121 | 121 | 169 | 169 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N11<br>4D/R122F/K154R/<br>N159D/Q175L | 0.34 | 1.02 | 149 | 149 | 208 | 208 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N11<br>4Y/R122F/K154R/N<br>159D/Q175L | 0.35 | 1.04 | 152 | 152 | 212 | 212 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N11                                   | 0.38 | 1.15 | 169 | 169 | 235 | 235 |

|                                                                       |      |      |     |     |     |     |
|-----------------------------------------------------------------------|------|------|-----|-----|-----|-----|
| 4F/R122F/K154R/N<br>159D/Q175L                                        |      |      |     |     |     |     |
| G13Y/K99Y/T104W<br>/T110A/Y113D/I11<br>8V/R122F/K154R/N<br>159D/Q175L | 0.21 | 0.63 | 92  | 92  | 128 | 128 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N11<br>4Y/R122F/K154R/N<br>159D/Q175K | 0.33 | 0.98 | 144 | 144 | 201 | 201 |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N11<br>4D/R122F/K154R/<br>N159D/Q175K | 0.35 | 1.05 | 153 | 153 | 214 | 214 |
| G13Y/I77L/K99Y/T<br>104W/T110A/Y113<br>D/R122F/K154R/N<br>159D/Q175L  | 0.31 | 0.93 | 136 | 136 | 190 | 190 |
| G13Y/I77M/K99Y/T<br>104W/T110A/Y113<br>D/R122F/K154R/N<br>159D/Q175L  | 0.22 | 0.67 | 98  | 98  | 137 | 137 |
| G13Y/I77V/K99Y/T<br>104W/T110A/Y113<br>D/R122F/K154R/N<br>159D/Q175L  | 0.23 | 0.69 | 101 | 101 | 141 | 141 |
| G13Y/I77Y/K99Y/T<br>104W/T110A/Y113<br>D/R122F/K154R/N<br>159D/Q175L  | 0.27 | 0.80 | 117 | 117 | 163 | 163 |

### **Example 3 – Testing of xylanase activity and relative inhibition by cereal xylanase inhibitors**

The mutants of Example 2 were tested for xylanase activity and relative sensitivity to a xylanase inhibitor by the protocols presented below and in accordance with the following teachings.

#### Xylanase assay (Endo- $\beta$ -1,4-Xylanase activity)

Samples were diluted in citric acid (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx.  $OD_{590} = 0.7$  in this assay. Three different dilutions of the sample were pre-incubated for 5 minutes at 40°C. At time = 5 minutes, 1 Xylazyme tablet (crosslinked, dyed xylan substrate, Megazyme, Bray, Ireland) was added to the enzyme solution in a reaction volume of 1ml. At time = 15 minutes the reaction was terminated by adding 10 ml of 2% TRIS/NaOH, pH 12. Blanks were prepared using 1000 $\mu$ l buffer instead of enzyme solution. The reaction mixture was centrifuged (1500 x g, 10 minutes, 20°C) and the OD of the supernatant was measured at 590 nm. One xylanase unit (XU) is defined as the xylanase activity increasing  $OD_{590}$  with 0.025 per minute.

Specific activity determination:

Optical density at 280 nm of the purified samples was measured for determining xylanase protein concentration. A theoretically calculated, specific  $OD_{280}$  (Gasteiger *et al.*, 2003) of 0.25 units/mg x ml was used for the specific activity calculation of the *Bacillus subtilis* XynA derived variants. Xylanase activity was determined as described above.

#### Xylanase inhibitor assay

100  $\mu$ l inhibitor preparation (containing various concentrations of xylanase inhibitor (for quantification see Xylanase inhibitor quantification below)), 250  $\mu$ l

xylanase solution (containing 12 XU xylanase/ml) and 650 µl buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen phosphate buffer, 1% BSA (Sigma-Aldrich, USA), pH 5.0) was mixed. The mixture was thermostated for 5 minutes at 40.0°C. At time = 5 minutes one Xylazyme tablet (crosslinked, dyed xylan substrate, Megazyme, Bray, Ireland) was added. At time = 15 minutes reaction was terminated by adding 10 ml 2% TRIS/NaOH, pH 12. The reaction mixture was centrifuged (1500 x g, 10 minutes, 20° C) and the supernatant measured at 590 nm. The xylanase inhibition was calculated as residual activity in %, compared to the blank. Blanks were prepared the same way, but substituting the inhibitor solution with water.

Xylanase inhibitor quantification:

*1 XIU (Xylanase Inhibitor Unit) is defined as the amount of inhibitor that decreases 1 XU of the Bacillus subtilis XynA xylanase (Seq ID No 1) to 0.5 XU under the conditions described below.*

250µl xylanase solution containing 12 XU/ml, approx. 100µl xylanase inhibitor solution and McIlvaine buffer, pH 5, to reach a reaction volume of 1000µl is pre-incubated for 5 minutes at 40°C. At t = 5 minutes, 1 Xylazyme tablet is added to the reaction mixture. At t = 15 minutes the reaction is terminated, by addition of 10 ml 2% TRIS/NaOH, pH 12. The solution is filtered and the absorbance of the supernatant is measured at 590 nm. By choosing several different concentrations of inhibitor in the above assay, it is possible to create a plot of OD versus inhibitor concentration. Using the slope (a) and intercept (b) from this plot and the concentration of the xylanase it is possible to calculate the amount of XIU in a given inhibitor solution (equation 1).

Equation 1 
$$XIU = ((b/2)/-a)/x$$

X = Xylanase units (XU) in the assay

Inhibitor preparation:

A crude inhibitor preparation (containing both TAXI and XIP, hereafter referred to as inhibitor preparation) was prepared from 1 kg wheat (*Triticum aestivum*) flour. The inhibitor preparation was extracted from the flour using water in a 1:3 ratio (w/w) followed by centrifugation (3500 x g, 20 minutes, 4 °C). The  
5 extract was kept at 65 °C for 40 minutes, centrifuged (3500 x g, 20 minutes, 4 °C) and desalted using disposable PD-10 desalting columns (Amersham Bioscience, Sweden) pre-equilibrated with 20mM sodium phosphate buffer, pH 7. TAXI concentration in the inhibitor preparation was determined by as described above. The protocol for purification and quantification of TAXI is  
10 described elsewhere (Sibbesen and Sørensen, 2001). By mean of example only, the TAXI in the preparation could be SEQ ID No. 24 or a sequence having 90 % identity thereto.

**Table 2 – Xylanase activity (XU/mg) and xylanase inhibitor sensitivity of mutants indicated as residual xylanase activity at increasing xylanase inhibitor concentrations (XIU/ml assay).**

| Modifications made to SEQ ID No. 1                        | Specific Activity XU/mg | % Activity @ 5.6 XIU/ml | % Activity @ 33.5 XIU/ml | % Activity @ 50 XIU/ml |
|-----------------------------------------------------------|-------------------------|-------------------------|--------------------------|------------------------|
| None (BS1)                                                | 23,000                  | 29                      |                          |                        |
| D11F R122D (BS3)                                          | 8,400                   | 100                     | 100                      | 95                     |
| D11F/R122D/T110A                                          | 14,992                  | 98                      | 100                      |                        |
| D11F/R122D/T110A/Y113A                                    | 13,362                  | 99                      |                          |                        |
| G13Y/T110A/Y113D/R122D/Q175L                              | 59,571                  |                         | 50                       |                        |
| G13Y/T110A/Y113D/R122F/Q175L                              | 53,855                  |                         |                          | 65                     |
| G13Y/G34K/T110A/Y113D/R122D/Q175L                         | 15,876                  |                         |                          | 97                     |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K       | 47,975                  |                         |                          | 72                     |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175/V81I   | 41,791                  |                         |                          | 46                     |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L | 53,331                  |                         |                          | 68                     |
| G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L                  | 54,924                  |                         |                          | 32                     |
| G13Y/K99Y/T104W/T110A/Y113D/R122                          | 54,811                  |                         |                          | 64                     |

|                                                           |        |  |  |    |
|-----------------------------------------------------------|--------|--|--|----|
| F/K154R/N159D/Q175L                                       |        |  |  |    |
| G13Y/T110A/Y113D/R122D/S162E/Q175L                        | 55,249 |  |  | 44 |
| G13Y/T110A/Y113D/R122D/S162D/Q175L                        | 52,735 |  |  | 40 |
| G13Y/T110A/Y113D/R122D/W164F/Q175L                        | 51,884 |  |  | 29 |
| G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L | 47,445 |  |  | 79 |
| G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L | 46,263 |  |  | 78 |
| G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L | 42,077 |  |  | 79 |
| G13Y/K99Y/T104W/T110A/Y113D/I118V/R122F/K154R/N159D/Q175L | 27,363 |  |  | 79 |
| G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175K | 35,906 |  |  | 84 |
| G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175K | 46,939 |  |  | 79 |
| G13Y/I77L/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L  | 48,177 |  |  | 75 |
| G13Y/I77M/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L  | 28,412 |  |  | 46 |
| G13Y/I77S/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L  | 12,003 |  |  | 20 |
| G13Y/I77V/K99Y/T104W/T110A/Y113D/                         | 35,907 |  |  | 45 |



|                                                                      |        |  |  |    |
|----------------------------------------------------------------------|--------|--|--|----|
| R122F/K154R/N159<br>D/Q175L                                          |        |  |  |    |
| G13Y/I77Y/K99Y/T1<br>04W/T110A/Y113D/<br>R122F/K154R/N159<br>D/Q175L | 33,236 |  |  | 37 |

#### Example 4 – Baking performance of mutants

Baking was done using a scale-down of the Danish Roll recipe (Table 3), using either wheat flour or wheat whole meal flour.

#### 5 Table 3 -. Recipe used for production of bread.

| Ingredients | Mini skala |
|-------------|------------|
|             | ml or g    |
| Flour       | 50         |
| Dry yeast   | 1          |
| Salt        | 0.8        |
| Sugar       | 0.8        |
|             | 400BU -    |
| Water       | 2%         |

Note: Water is the water absorption @ 400BU determined by Farinograph analysis of flour (i.e, 400 bakers absorbance - water added according to water absorbtion determination using a Brabender Farinograph, Brabender,

10 Germany). If enzymes are added to the dough, they are added as liquid solution and by substitution of the same amount of water.

#### Dough making and baking

The flour and dry ingredients were mixed for one minute in a 50 gram Farinograph (Brabender, Duisburg, Germany), hereafter water was added and  
15 mixing was continued for another five minutes.

After mixing, four dough lumps were weighed out, each containing 10-grams of flour. These were moulded into bread using a hand moulder. Loaves were put into baking pans and placed in a sealed container (with a lid) and left to rest at room temperature for 10 minutes. Hereafter, breads were proofed at 34°C, 85%  
5 relative humidity (RH) , for 45 minutes and finally baked at 230°C for five minutes in a Bago oven (Bago-line, Fåborg, Denmark).

The breads were cooled for 20 minutes before evaluation (weighing, volume measurement, crumb and crust evaluation).

**Table 4 – Baking performance of mutants – bread volume (ml/g) and relative volume increase compared to control (no enzyme added) and BS3 (SEQ ID No.1 with the modifications D11F and R122D) which show superior baking performance compared to the *Bacillus sub. XynA* wildtype xylanase (SEQ ID No. 1).**

| Modifications made to Seq ID No 1                         | Bread vol @ 0,04mg/kg flour | Relative vol. Increase vs control, % | Relative volume increase vs. BS3, % |
|-----------------------------------------------------------|-----------------------------|--------------------------------------|-------------------------------------|
| G13Y/G34K/T110A/Y113D/R122D/Q175L                         | 4,22                        | 41,89                                | 20                                  |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K       | 2,90                        | 21,93                                | 7,54                                |
| G13Y/V81I/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L  | 2,80                        | 18,02                                | 4,09                                |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L | 2,82                        | 18,86                                | 4,83                                |
| G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L                  | 2,81                        | 18,08                                | 4,15                                |
| G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L       | 2,89                        | 21,74                                | 7,24                                |
| G13Y/T110A/Y113D/R122D/S162E/Q175L                        | 2,75                        | 13,55                                | 1,99                                |
| G13Y/T110A/Y113D/R122D/S162D/Q175L                        | 2,82                        | 15,54                                | 4,48                                |
| G13Y/T110A/Y113D/R122D/W164F/Q175L                        | 2,78                        | 14,02                                | 3,10                                |
| G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L | 2,81                        | 16,44                                | 4,11                                |
| G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L | 2,73                        | 13,26                                | 1,26                                |

|                                                                       |      |       |        |
|-----------------------------------------------------------------------|------|-------|--------|
| G13Y/K99Y/T104W<br>/T110A/Y113D/N114F<br>/R122F/K154R/N159D<br>/Q175L | 2,80 | 16,22 | 3,74   |
| G13Y/K99Y/T104W<br>/T110A/Y113D/I118V<br>/R122F/K154R/N159D<br>/Q175L | 2,83 | 17,58 | 4,95   |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N114Y<br>/R122F/K154R/N159D<br>/Q175K | 2,89 | 20,65 | 7,34   |
| G13Y/K99Y/T104W<br>/T110A/Y113D/N114D<br>/R122F/K154R/N159D<br>/Q175K | 2,77 | 14,73 | 2,63   |
| G13Y/I77L/K99Y<br>/T104W/T110A/Y113D<br>/R122F/K154R/N159D<br>/Q175L  | 2,81 | 15,08 | 4,32   |
| G13Y/I77M/K99Y<br>/T104W/T110A/Y113D<br>/R122F/K154R/N159D<br>/Q175L  | 2,70 | 12,43 | 0,17   |
| G13Y/I77S/K99Y<br>/T104W/T110A/Y113D<br>/R122F/K154R/N159D<br>/Q175L  | 2,53 | 5,40  | (6,09) |
| G13Y/I77V/K99Y<br>/T104W/T110A/Y113D<br>/R122F/K154R/N159D<br>/Q175L  | 2,60 | 8,19  | (3,63) |
| G13Y/I77Y/K99Y<br>/T104W/T110A/Y113D<br>/R122F/K154R/N159D<br>/Q175L  | 2,73 | 13,81 | 1,38   |

**Example 5 – Effect of modification to residue 110 in the *Bacillus subtilis* XynA xylanase (SEQ ID NO. 1) or equivalent position in other family 11 xylanases.**

5 Xylanases:

Xylanases mutated in this example are the *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1), a variant of the *Trichoderma reesei* Xyn2 xylanase (SEQ ID No. 2) and the *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID No. 3).

- 10 The residue mutated is T110 in the *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1), the equivalent position, T120, in *Trichoderma reesei* xylanase (SEQ ID No. 2) and the equivalent position, T120, in *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID No. 3). The following mutations were made in *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1): T110H, T110Y, T110S,
- 15 T110R, T110F, T110Q, T110G, T110K, T110L, T110M, T110I, T110T, T110N, T110E, T110W, T110A and T110C. In both *Trichoderma reesei* xylanase (SEQ ID No. 2) and *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID No. 3) the equivalent position, T120, was mutated. The mutations made is to the amino acid alanine.
- 20 Mutations, expression, purification and determination of specific activity of the wildtype xylanases or their variants are carried out as described in Examples 1, 2 and 3. Except for the purification of the *Trichoderma reesei* variant xylanase, the *Thermomyces lanuginosus* XynA wildtype xylanase and their T120A variant, here the purification protocol was modified to reflect their pI.

**Table 5. Specific activity determined as XU/mg xylanase protein of the *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1), the *Bacillus subtilis* XynA variant xylanase (T110A), the *Trichoderma reesei* xylanase (SEQ ID No. 2), the *Trichoderma reesei* Xyn2 variant xylanase (T120A), the *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID NO. 3) and the *Thermomyces lanuginosus* XynA variant xylanase (T120A).**

| Xylanase                                                            | Specific activity, XU/mg |
|---------------------------------------------------------------------|--------------------------|
| <i>Bacillus subtilis</i> XynA wildtype xylanase (SEQ ID No. 1)      | 23,000                   |
| <i>Bacillus subtilis</i> XynA variant xylanase (T110A)              | 25874                    |
| <i>Bacillus subtilis</i> XynA variant xylanase (T110N)              | 23104                    |
| <i>Bacillus subtilis</i> XynA variant xylanase (T110E)              | 24782                    |
| <i>Bacillus subtilis</i> XynA variant xylanase (T110W)              | 25478                    |
| <i>Bacillus subtilis</i> XynA variant xylanase (T110C)              | 26390                    |
| <i>Trichoderma reesei</i> xylanase (SEQ ID No. 2)                   | 17,500                   |
| <i>Trichoderma reesei</i> variant xylanase (T120A)                  | 36797                    |
| <i>Thermomyces lanuginosus</i> XynA wildtype xylanase (SEQ ID No 3) | 31,300                   |
| <i>Thermomyces lanuginosus</i> XynA variant xylanase (T120A)        | 36066                    |

- 10 In all cases, the mutation T110A in the *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1) or the equivalent position (T120) in the *Trichoderma reesei* xylanase (SEQ ID No. 2) or the *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID No 3) results in a significant increased specific activity.

- In *Bacillus subtilis* XynA wildtype xylanase (SEQ ID No. 1) also the mutations  
 15 T110E, T110N, T110W, and T110C results in an increased specific activity.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although  
5 the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be  
10 within the scope of the following claims.

**Example 6 Activity of xylanase variants on water-insoluble substrate versus insoluble substrate.**

Xylanase variants of the BACSU\_XynA and TRIRE\_Xyn2 was generated using site-directed mutagenesis of xylanases and expression in *E. coli*.

15 Assay to determine activity on Water unextractable substrate, WU-AX act. (insoluble substrate):

Samples were diluted in citric acid (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx.  $OD_{590} = 0.7$  in this assay. Three different dilutions of the sample were pre-incubated for 5 minutes at 40°C. At time = 5  
20 minutes, 1 Xylazyme tablet (crosslinked, dyed xylan substrate, Megazyme, Bray, Ireland) was added to the enzyme solution in a reaction volume of 1ml. At time = 15 minutes the reaction was terminated by adding 10 ml of 2% TRIS/NaOH, pH 12. Blanks were prepared using 1000µl buffer instead of enzyme solution. The reaction mixture was centrifuged (1500 x g, 10 minutes, 20°C) and the OD  
25 of the supernatant was measured at 590 nm. One xylanase unit (WU-AX act) is defined as the xylanase activity increasing  $OD_{590}$  with 0.025 per minute.

The substrate (cross-linked and dyed arabinoxylan extracted from wheat) used in the above assay is a good approximate to the corresponding substrate in commercial applications.

The following assay was used to determine activity on Water extractable substrate, WE-AX act (soluble substrate).

The method used is a modified version of the method described by Lever (Lever, M. Analytical Biochemistry. 47, 273-279, 1972). Soluble wheat arabinoxylan (medium viscosity, obtainable from Megazyme, Bray, Ireland) was used as substrate in a buffersystem containing 50mM NaOAc, pH 5. Substrate concentration was 0.5%. Xylanase activity was measured by quantifying the formation of reducing ends using PAHBAH reagents. The amount of reducing ends formed and hereby the xylanase activity was determined from a xylose standard curve. Here referred to as WE-AX act.

Backbones used for developing new variants:

Table 6 show xylanase variants backbones used. Y5 corresponds to SEQ ID NO. 2.

| ID       | Variant                                                              |
|----------|----------------------------------------------------------------------|
| #154     | BACSU_XynA-G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L       |
| #160     | BACSU_XynA-G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L |
| Y5       | TRIIE_Xyn2-T2C/T28C/K58R/+191D                                       |
| Y5-T120A | TRIIE_Xyn2-T2C/T28C/K58R/T120A/+191D                                 |

The mutations introduced and the results obtained are illustrated in table 7

Table 7. Mutations introduced and results obtained. The backbones used are in bold.

| <b>Mutant</b>   | <b>WU-AX act</b> | <b>WE-AX act</b> | <b>WU-AX/WE-AX</b> |
|-----------------|------------------|------------------|--------------------|
| #154/N141Q      | 1.965            | 13               | 146                |
| #154/N54Q/N141Q | 1.611            | 10               | 159                |
| #160/N54Q       | 1.203            | 7                | 161                |



|                 |               |              |            |
|-----------------|---------------|--------------|------------|
| #160/N141Q      | 1.785         | 10           | 175        |
| #154/N54W/N141Q | 824           | 7            | 118        |
| #160/N54W/N141Q | 1.005         | 6            | 169        |
| Y5/S63W         | 918           | 25           | 36         |
| <b>Y5</b>       | <b>35.550</b> | <b>1.487</b> | <b>24</b>  |
| <b>#154</b>     | <b>10.350</b> | <b>106</b>   | <b>98</b>  |
| <b>#160</b>     | <b>5.400</b>  | <b>34</b>    | <b>157</b> |

SEQUENCE LISTING (amino acids in bold are the amino acid, which corresponds to T110 of SEQ ID No.1):

The amino acid sequence of the mature *Bacillus subtilis* wildtype xylanase (SEQ ID No 1):

ASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSNTGNFVVGKGWTTGSPFRT  
 INYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTGTYKGTVKSDGGTYDIY**TT**  
 10 TRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQV  
 MA TEGYQSSGSSNVTW

The amino acid sequence of the mature *Trichoderma reesei* xylanase (SEQ ID No 2), also referred to herein as Y5:

QCIQPGTGYNNGYFYSYWNDGHGGVTYCNGPGGQFSVNWSNSGNFVGGKGWQPGTKN  
 15 RVINFSGSYNPNGNSYLSVYGWSRNPLIEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIY  
 RTQRVNQPSIIGTATFYQYWSVRRNHRSSGSVNTANHFNAWAQQGLTLGTMDYQIVAVE  
 GYFSSGSASITVSD

The amino acid sequence of the mature *Thermomyces lanuginosus* XynA wildtype xylanase (SEQ ID No. 3):

QTPNSEGWHDGYYYSWWSDGGAQATYTNLEGGTYEISWGDGGNLVGGKGWNPGLNA  
RAIHFEFVYQPNGNSYLAVYGWTRNPLVEYYIVENFGTYDPSSGATDLGTVECDGSIYRLG  
KTTTRVNAPSIDGTQTFDQYWSVRQDKRTSGTVQTGCHFDAWARAGLNVNGDHYYQIVA  
TEGYFSSGYARITVADVG

- 5 The amino acid sequence of the mature *Streptomyces viridosporus* xylanase (Seq ID No 4):

WTDAQGTVSMDLGSGGTYSTQWRNTGNFVAGKGWSTGGRKTVNYSGT FNPSGNAYLT  
LYGWTTGPLIEYYIVDNWGT YRPTGKYKGT VTS DGGTYDIYKTTTRYNAPSIEGKTFDQYW  
SVRQSKRTGGTITSGNHFDAWARNGMNLGNHNYMIMATEGYQSSGSSTITV

- 10 Seq ID No 5 (gi|139868|sp|P18429.1|XYNA BACSU RecName: Full=Endo-1,4-beta-xylanase A; Short=Xylanase A; AltName: Full=1,4-beta-D-xylan xylanohydrolase A):

MFKFKKNFLVGLS AALMSISLFSATASAASTDY WQNWTDGGGIVNAVNGSGGNYSVNW  
SNTGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSP LIEYYVDSWGTYR  
15 PTGTYKGT VKSDGGTYDIYTTTRYNAPSIDGDR TTFTQYWSVRQSKRPTGSNATITFSNH  
VNAWKSHGMNLGSNWAYQVMATEGYQSSGSSNVTW

Seq ID No 6 (gi|2302074|emb|CAA03092.1| unnamed protein product [unidentified]):

- 20 MRQKKLTLILAFLVCFALTLP AEIIQAQIVTDNSIGNHDGYDYEFWKDSGGSGTMILNHGG  
TFSAQWNNVNNILFRKGKKFNETQTHQOVGNMSINYGANFQPNGNAYLCVYGWTV DPLV  
EYYIVDSWGNWRPPGATPKGTITVDGGTYDIYETLRVNQPSIKGIATFKQYWSVRRSKRT  
SGTISVSNHFWENLGMNMGKMYEVALTVEGYQSSGSANVYSNTLRINGNPLSTISND  
ESITLDKNN

Seq ID No 7 (gi|167246404|gb|ABZ24364.1| Sequence 5 from patent US 7314743):

- 25 MVSFTSLLAASPPSRASCRPA AEVESVAVEKRQTIQPGTGYNNGYFYSYWNDGHGGV TYT  
NGPGGQFSVNWSNSGNFVGGKGWQPGTKNKVINFSGSYNPNNGNSYLSVYGWSRNPLIE  
YYIVENFGTYNPSTGATKLGEVTS DGSVYDIYRTQRVNQPSIIGTATFYQYWSVRRNHRSS  
GSVNTANHFNAWAQOGLTLGTM DYQIVAVEGYFSSGSASITVS

Seq ID No 8 (gi|5969551|gb|AAE10889.1| Sequence 2 from patent US 5817500):

MVGFTPVALAALAATGALAFPAGNATELEKRQTPNSEGWHDGYYYSWWSGDGGAQATYT  
NLEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEY  
 5 YIVENFGTYDPSSGATDLGTVECDGSIYRLGK**T**TRVNAPSIDGTQTFDQYWSVRQDKRTS  
GTVQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSSGYARITVADVG

Seq ID No 9 (gi|76059070|emb|CAJ30753.1| unnamed protein product [Paenibacillus pabuli]):

MFKFGKKLLTVVLAASMSFGVFAATTGATDYWQNWTDGGGTVNAVNGSGGNYSVNWQ  
 10 NTGNFVVGKGWTYGTPNRVVNYNAGVFSPSGNGYLTfyGWTRNALIEYYVVDNWGTYRP  
TGTYKGTVTSDGGTYDIYT**T**MRYNQPSIDGYSTFPQYWSVRQSKRPIGVNSQITFQNHVN  
AWASKGMYLGNSWSYQVMATEGYQSSGSSNVTWV

Seq ID No 10 (gi|74197761|emb|CAJ29666.1| unnamed protein product [Bacillus halodurans]):

MFKFVTKVLTVVIAATISFCLSAVPASANTYWQYWTDGGGTVNATNGPGGNYSVTWRDT  
 15 GNFVVGKGWEIGSPNRTIHYNAGVWEPNGNGYLTLYGWTRNQLIEYYVVDNWGTYRPTG  
THRGTVVSDGGTYDIYT**T**MRYNAPSIDGTQTFQFWSVRQSKRPTGNNVSITFSNHVNA  
WRNAGMNLGSSWSYQVLATEGYQSSGRSNVTWV

Seq ID No 11 (gi|4756811|emb|CAB42305.1| unnamed protein product [unidentified]):

MRQKKLTFILAFLVCFALTLP AEIIQAQIVTDNSIGNHDGYDYEFWKDSGGSGTMILNHGG  
 20 TFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQPNGNAYLCVYGWTVDPDV  
EYYIVDSWGNWRPPGATPKGTITVDGGTYDIYE**T**LRVNQPSIKGIATFKQYWSVRRSKRT  
SGTISVSNHFRAWENLGMNMGKMYEVALTVEGYQSSGSANVYSNTLRINGNPLSTISND  
 25 KSITLDKNN

Seq ID No 12 (gi|2293951|emb|CAA02246.1| unnamed protein product [Bacillus subtilis] Bacillus subtilis: (US 5306633)):

MFKFKKKFLVGLTAAFMISMFSATASAAGTDYWQNWTDGGGTVNAVNGSGGNYSVNW  
SNTGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDVSWGTYR

PTGTYKGTVKSDGGTYDIYTTTRYNAPSIDGDNTTFTQYWSVRQSKRPTGSNAAITFSNH  
VNAWKSHGMNLGSNWAYQVLATEGYKSSGSSNVTW

Seq ID No 13 (gi|42688917|gb|AAS31735.1| Sequence 14 from patent US  
6682923):

5 MNLRKLRLLFVMCIGLTLILTAVPAHARTITNNEMGNHSGYDYELWKDYGNTSMTLNNGG  
AFSAGWNNIGNALFRKGKKFDSTRTHHQLGNISINYNASFNPGGNSYLCVYGWTQSPLAE  
YYIVDSWGTYPRTGAYKGSFYADGGTYDIYETTRVNQPSIIGIATFKQYWSVRQTKRTSGT  
VSVSAHFRKWESLGMMPMGKMYETAFTVEGYQSSGSANVMTNQLFIGN

Seq ID No 14 (gi|10040204|emb|CAC07798.1| unnamed protein product  
 10 [Penicillium funiculosum]):  
MKLFLAAIVLCATAATAFPSELAQRAAGDLSKRQSITTSQTGTNNGYYSFWTNNGGGEVTY  
TNGDNGEYSVTWVDCGDFTSKGKWNPNANAQTVTYSGEFNPSGNAYLAVYGWTTDPLVE  
YYILESYGTYNPSSGLTSLGQVTSDDGGTYDIYSTQRVNQPISIEGTSTFNQYWSVRTEKRVG  
GTVTTANHFAAWKALGLEMGTYNYMIVSTEGYESSGSSTITVS

15 Seq ID No 15 (gi|2302074|emb|CAA03092.1| unnamed protein product  
[unidentified]):  
QIVTDNSIGNHDGYDYEFWKDSGGSGTMILNHGGTFSAQWNNVNNILFRKGKKFNETQT  
HQQVGNMSINYGANFQPNGNAYLCVYGWTVDPDPLVEYYIVDSWGNWRPPGATPKGTITVD  
GGTYDIYETLRVNQPSIKGIATFKQYWSVRRSKRTSGTISVSNHFWENLGMNMGKMY  
 20 EVALTVEGYQSSGSANVYSNTRLRINGNPLSTISNDESITLTKNN

Seq ID No 16 (gi|167246404|gb|ABZ24364.1| Sequence 5 from patent  
US7314743):  
QTIQPGTGYNNGYFYSYWNDGHGGVITYTNGPGGQFSVNWSNSGNFVGGKGWQPGTKN  
KVINFGSGSYNPNGNSYLSVYGWSRNPPIEYYIVENFGTYNPSTGATKLGEVTSDDGSVDIY  
 25 RTQRVNQPISIIGTATFYQYWSVRRNHRSSGSVNTANHFNAWAQQGLTLGTMDYQIVAVE  
GYFSSGSASITVS

Seq ID No 17 (gi|76059070|emb|CAJ30753.1| unnamed protein product  
[Paenibacillus pabuli]):  
TDYWQNWTDGGGTVNAVNGSGGNYSVNWQNTGNFVVGKGWYGTGPNRVVNYNAGVF

SPSGNGYLTFYGWTRNALIEYYVVDNWGTYRPTGTYKGTVTSDGGTYDIYTTMRYNQPSI  
DGYSTFPQYWSVRQSKRPIGVNSQITFQNHVNAWASKGMYLGNSWSYQVMATEGYQSS  
GSSNVTWV

Seq ID No 18 (gi|74197761|emb|CAJ29666.1| unnamed protein product

5 [Bacillus halodurans]):

NTYWQYWTDGGGTVNATNGPGGNYSVTWRDTGNFVVGKGWEIGSPNRTIHYNAGVWE  
PSGNGYLTLYGWTRNQLIEYYVVDNWGTYRPTGTHRGTVVSDGGTYDIYTTMRYNAPSID  
GTQTFQQFWSVRQSKRPTGNNVSITFSNHVNAWRNAGMNLGSSWSYQVLATEGYQSSG  
RSNVTWV

10 Seq ID No 19 (gi|4756811|emb|CAB42305.1| unnamed protein product):

QIVTDNSIGNHDGYDYEFWKDSGGSGTMILNHGGTFSAQWNNVNNILFRKGKKFNETQT  
HQQVGNMSINYGANFQPNGNAYLCVYGWTVDPLEYYIVDSWGNWRPPGATPKGTITVD  
GGTYDIYETLRVNQPSIKGIATFKQYWSVRRSKRTSGTISVSNHFRAWENLGMNMGKMY  
EVALTVEGYQSSGSANVYSNTLRINGNPLSTISNDKSITLDKNN

15 Seq ID No 20 (gi|2293951|emb|CAA02246.1| unnamed protein product [Bacillus  
subtilis] Bacillus subtilis: (US5306633)):

AGTDYWQNWTDGGGTVNAVNGSGGNYSVNWSNTGNFVVGKGWTTGSPFERTINYNAGV  
WAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTGTYKGTVKSDGGTYDIYTTTRYNAPS  
IDGDNTTFTQYWSVRQSKRPTGSNAAITFSNHVNAWKSHGMNLGSNWAYQVLATEGYK

20 SSGSSNVTWV

Seq ID No 21 (gi|42688917|gb|AAS31735.1| Sequence 14 from patent  
US6682923):

RTITNNEMGNHSGYDYELWKDYGNTSMTLNNGGAFSAGWNNIGNALFRKGKKFDSTRT  
HHQLGNISINYNASFNPGGNSYLCVYGWTSPLAEYYIVDSWGTYRPTGAYKGSFYADGG  
 25 TYDIYETTRVNQPSIIGIATFKQYWSVRQTKRTSGTVSVSAHFRKWESLGMMPMGKMYETA  
FTVEGYQSSGSANVMTNQLFIGN

Seq ID No 22 (gi|10040204|emb|CAC07798.1| unnamed protein product  
[Penicillium funiculosum]):

AFPSELAQRAAGDLSKRQSITTSQTGTNNGYYYYSFWTNNGGGEVITYTNGDNGEYSVTWVD  
CGDFTSGKGWNPANAQTVTYSGEFNPSGNAYLAVYGWTTDPLVEYYILES YGTYNPSSGL  
TSLGQVTS DGGTYDIYSTQRVNQPSIEGTSTFNQYWSVRTEKRVGGTVTTANHFAAWKA  
LGLEMGTYNYMIVSTEGYESSGSSTITVS

- 5 SEQ ID No 23 shows the amino acid sequence of the mature *Bacillus subtilis* xylanase variant, BS3 (wildtype with D11F/R122D mutations):

ASTDYWQNWTFGGGIVNAVNGSGGNYSVNWSNTGNFVVGKGWTTGSPFRTINYNAGV  
 WAPNGNGYLTLYGWTRSPLIEYYVDSWGTYRPTGTYKGTVKSDGGTYDIYT**T**TRYNAPS  
 IDGDDTTFTQYWSVRQSKRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQVMATEGYQ  
 10 SSGSSNVTWV

Seq ID No 24 shows the sequence of the mature wheat xylanase inhibitor sequence:

MPPVLLLVLAA SLVALPSCQSLPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDG  
 GQPPAEIPCSSPTCLLANAYPAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAGSLSHTRF  
 15 VANTTDGSKPVSKVNVGVLAACAPSKLLASLPRGSTGVAGLANSGLALPAQVASAQKVAN  
 RFLCLPTGGPGVAIFGGGPVPWPQFTQSMPTPLVTKGGSPAHYISARSIVVGDRVPVP  
 EGALATGGVMLSTRLPYVLLRPDVYRPLMDAFTKALAAQHANGAPVARAVEAVAPFGVCY  
 DTKTLGNNLGGYAVPNVQLGLDGGSDWTMTGKNSMVDVKQGTACVAFVEMKGVAAGD  
 GRAPAVILGGAQMEDFVLD FDM EKKRLGFSRLPHFTGCGGL

- 20 Seq ID No 25 (sequence 11 of US 6,682,923):

ASTDWWENWTIGGGIVNAVNGSGGNYSVNWSNTGNFDVAKGWTTGSPFRTINYNAGV  
 WAPNGWGELELYGWTRSPLIEYLVDSWGTYRPTGTYKGTVKSDGGTYDIYT**D**TRYNYP  
 SEDGDRTTMTQYSSVRQSKRPTGSNATITFTNHVNAWKSHGMNLGSNWAYQDMATEGY  
 QSSGSSNVTWV

25

Embodiments of the invention:

1. A polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from SEQ ID No. 2-22, and which polypeptide has an amino acid modification in  
5 position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
2. A polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 88% identity with SEQ ID  
10 No. 1 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
3. A polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 75% identity with SEQ ID  
15 No. 2 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
4. A polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 75% identity with SEQ ID  
20 No. 3 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
5. The polypeptide according to embodiment 2, wherein said polypeptide has at least 90, 92 or 95% identity with SEQ ID No. 1.
- 25 6. The polypeptide according to any one of the embodiments 1 and 3-4, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with the sequence with which is has the highest percentage of identity selected from SEQ ID No. 2-22.

7. The polypeptide according embodiment 3, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 2.

8. The polypeptide according embodiment 4, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 3.

5 9. The polypeptide according to any one of the embodiments 1-6 having a  $\beta$ -jelly roll fold.

10. The polypeptide according to any one of the embodiments 1-9, wherein the amino acid modification in position 110 is an amino acid substitution.

10 11. The polypeptide according to any one of the embodiments 1-10, wherein the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

15 12. The polypeptide according to any one of the embodiments 1-11, wherein the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: glutamic acid, tryptophan, alanine and cysteine.

20 13. The polypeptide according to embodiment 12, wherein the amino acid modification in position 110 is a substitution to alanine.

14. The polypeptide according to any one of the embodiments 1-13 having a total number of amino acids of less than 250, such as less than 240, such as less than 230, such as less than 220, such as less than 210, such as less than 200 amino acids, such as in the range of 160 to 240, such as in the range of 160  
25 to 220 amino acids.



15. The polypeptide according to any one of the embodiments 1-14, comprising one or more modification(s) at any one or more of amino acid positions: 11, 12, 13, 34, 54, 77, 81, 99, 104, 113, 114, 118, 122, 141, 154, 159, 162, 164, 166 and 175, the position(s) being determined as the corresponding position of *B.*

5 *subtilis* amino acid sequence shown as SEQ ID No. 1.

16. The polypeptide according to any one of the embodiments 1-15, comprising one or more amino acid substitutions selected from the group consisting of: 11F, 12F, 54Q, 54W, 122D, 113A, 13Y, 113D, 141Q, 175L, 122F, 34K, 99Y, 104W, 154R, 159D, 175K, 81I, 166F, 162E, 162D, 164F, 114D, 114Y, 114F, 118V,  
10 175K, 77L, 77M, 77S, 77V, and 77Y, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

17. The polypeptide according to any one of the embodiments 2, 5, 9-16, comprising one or more amino acid substitutions selected from the group  
15 consisting of: D11F, G12F, N54Q, R122D, Y113A, G13Y, Y113D, N141Q, Q175L, R122F, G34K, K99Y, T104W, K154R, N159D, Q175K, V81I, Y166F, S162E, S162D, W164F, N114D, N114Y, N114F, I118V, I77L, I77M, I77S, I77V, and I77Y, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

20 18. The polypeptide according to any one of the embodiments 1-17, comprising one or more modification(s) at any one or more of amino acid positions: 13, 99, 104, 113, 122, 154, 159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

25 19. The polypeptide according to any one of the embodiments 1-18, comprising substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

20. The polypeptide according to any one of the embodiments 18-19, further comprising one or more modification(s) at any one or more of amino acid positions: 114 and 166, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

5 21. The polypeptide according to any one of the embodiments 18-19, further comprising one or more substitution(s) at any one or more of amino acid positions: 114 and 166, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

10 22. The polypeptide according to any one of the embodiments 18-19, comprising substitution(s) in at least at four of the following amino acid positions: 13, 99, 104, 113, 114, 122, 154, 159, 166, and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

15 23. The polypeptide according to any one of the embodiments 18-22, comprising substitution(s) at the amino acid positions: 13, 99, 104, 113, 114, 122, 154, 159 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

20 24. The polypeptide according to any one of the embodiments 18-22, comprising substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159, 166 and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

25 25 The polypeptide according to any one of the embodiments 18-20, comprising substitution(s) at the amino acid positions: 13, 99, 104, 113, 122, 154, 159, and 175, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

26. The polypeptide according to any one of the embodiments 22-25, comprising one or more amino acid substitutions selected from the group consisting of: 13Y, 99Y, 104W, 110A, 113D, 114D, 114F, 122F, 154R, 159D, 166F, 175K, and 175L,

the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

27. The polypeptide according to any one of the embodiments 1-26, wherein the amino acid sequence of said polypeptide has at least five, six, seven, eight, nine or ten amino acid substitutions compared to the sequence selected among SEQ ID No. 1-22 with which it has the highest identity.

28. The polypeptide according to embodiment 27, wherein the amino acid sequence of said polypeptide has at least nine or ten amino acid substitutions.

29. The polypeptide according to any one of the embodiments 1-28, comprising one or more amino acid substitutions selected from the group consisting of:

- a. D11F, R122D and T110A;
- b. D11F, R122D, T110A and Y113A;
- c. G13Y, T110A, Y113D, R122D and Q175L;
- d. G13Y, T110A, Y113D, R122F and Q175L;
- 15 e. G13Y, G34K, T110A, Y113D, R122D and Q175L;
- f. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175K;
- g. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Q175 and V81I;
- h. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Y166F and Q175L;
- 20 i. G13Y, T110A, Y113D, R122D, K154R, N159D and Q175L;
- j. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
- k. G13Y, T110A, Y113D, R122D, S162E and Q175L;
- l. G13Y, T110A, Y113D, R122D, S162D and Q175L;
- m. G13Y, T110A, Y113D, R122D, W164F and Q175L;
- 25 n. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175L;
- o. G13Y, K99Y, T104W, T110A, Y113D, N114Y, R122F, K154R, N159D and Q175L;
- p. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D and Q175L;
- 30 q. G13Y, K99Y, T104W, T110A, Y113D, I118V, R122F, K154R, N159D and Q175L;
- r. G13Y, K99Y, T104W, T110A, Y113D, N114Y, R122F, K154R, N159D and Q175K;
- 35 s. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175K;
- t. G13Y, I77L, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;

- u. G13Y, I77M, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
  - v. G13Y, I77S, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
  - 5 w. G13Y, I77V, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
  - x. G13Y, I77Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L
  - y. G13Y, K99Y, T104W, T110A, Y113D, R122F, N141Q, K154R, N159D, and Q175L;
  - 10 z. G13Y, N54Q, K99Y, T104W, T110A, Y113D, R122F, N141Q, K154R, N159D, and Q175;
  - aa.G13Y, N54W, K99Y, T104W, T110A, Y113D, R122F, 141Q, K154R, N159D, and 175L;
  - bb.G13Y, N54Q, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D,
  - 15 and Q175L;
  - cc. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, 141Q, K154R, N159D, and Q175L;
  - dd.G13Y, 54Q, K99Y, T104W, T110A, Y113D, N114F, R122F, 141Q, K154R, N159D, and Q175L;
  - 20 the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.
30. The polypeptide according to any one of the embodiments 1-29, comprising one or more amino acid substitutions selected from the group consisting of:
- 25 a. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175K;
  - b. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D, Y166F and Q175L;
  - c. G13Y, K99Y, T104W, T110A, Y113D, R122F, K154R, N159D and Q175L;
  - 30 d. G13Y, K99Y, T104W, T110A, Y113D, N114D, R122F, K154R, N159D and Q175L;
  - e. G13Y, K99Y, T104W, T110A, Y113D, N114F, R122F, K154R, N159D and Q175L;
  - 35 the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

31. The polypeptide according to any one of the embodiments 1-30 having bran solubilisation activity.

32. The polypeptide according to any one of the embodiments 1-31 in isolated form.

5 33. The polypeptide according to any one of the embodiments 1-32, wherein the amino acid modification in position 110 is not T110D.

34. The polypeptide according to any one of embodiments 1-33 having an improved xylanase activity compared to the *B. subtilis* amino acid sequence shown as SEQ ID No. 1 as measured in a xylanase activity assay.

10 35. The polypeptide according to any one of embodiments 1-34 having an improved xylanase activity as a result of the modification in position 110.

36. The polypeptide according to any one of embodiments 1-35 having an improved bran solubilisation activity compared to the *B. subtilis* amino acid sequence shown as SEQ ID No. 1 as measured in a bran solubilisation activity  
15 assay.

37. The polypeptide according to any one of embodiments 1-36 having an improved bran solubilisation activity as a result of the modification in position 110.

38. The polypeptide according to any one of embodiments 1-37 having a  
20 reduced sensitivity to a xylanase inhibitor.

39. The polypeptide according to any one of embodiments 1-38, wherein said polypeptide has an amino acid sequence comprising modifications at positions selected from the list consisting of:

a) 13/110/113/122/154/159/175;

25 b) 13/99/104/110/113/122/154/159/166/175;

- c) 13/99/104/110/113/114/122/154/159/175;
- d) 13/110/113/122/175;
- e) 13/99/104/110/113/122/154/159/175;
- f) 13/99/104/110/113/122/154/159/175;
- 5 g) 13/99/104/110/113/114/122/154/159/175;
- h) 13/99/104/110/113/114/122/154/159/175;
- i) 13/99/104/110/113/114/122/154/159/175;
- j) 13/99/104/110/113/114/122/154/159/175;
- k) 13/77/99/104/110/113/122/154/159/175;
- 10 l) 13/81/99/104/110/113/122/154/159/175;
- m) 13/110/113/122/164/175;
- n) 13/110/113/122/162/175;
- o) 13/110/113/122/175;
- p) 11/122/110/113;
- 15 q) 13/77/99/104/110/113/122/154/159/175;
- r) 11/122/110;
- s) 13/34/110/113/122/175;
- t) 13/77/99/104/110/113/122/154/159/175;
- u) 13/77/99/104/110/113/122/154/159/175;
- 20 v) 13/99/104/110/113/118/122/154/159/175;
- w) 13/110/113/122/162/175;
- x) 13/77/99/104/110/113/122/154/159/175;
- y) 13/99/104/110/113/122/141/154/159/175;
- z) 13/54/99/104/110/113/122/141/154/159/175;
- 25 aa) 13/54/99/104/110/113/122/141/154/159/175;
- bb) 13/54/99/104/110/113/114/122/154/159/175;

cc) 13/99/104/110/113/114/122/141/154/159/175; and

dd) 13/54/99/104/110/113/114/122/141/154/159/175,

the position(s) being determined as the corresponding position of *subtilis* amino acid sequence shown as SEQ ID No. 1.

5

40. The polypeptide according to any one of embodiments 1-39, wherein said polypeptide has an amino acid sequence comprising amino acid substitutions selected from the list consisting of:

- a) 13Y/110A/113D/122D/154R/159D/175L;
- 10 b) 13Y/99Y/104W/110A/113D/122F/154R/159D/166F/175L;
- c) 13Y/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- d) 13Y/110A/113D/122F/175L;
- e) 13Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- f) 13Y/99Y/104W/110A/113D/122F/154R/159D/175K;
- 15 g) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175K;
- h) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175L;
- i) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175L;
- j) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175K;
- k) 13Y/77L/99Y/104W/110A/113D/122F/154R/159D/175L;
- 20 l) 13Y/81I/99Y/104W/110A/113D/122F/154R/159D/175L;
- m) 13Y/110A/113D/122D/164F/175L;
- n) 13Y/110A/113D/122D/162D/175L;
- o) 13Y/110A/113D/122D/175L;
- p) 11F/122D/110A/113A;
- 25 q) 13Y/77Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- r) 11F/122D/110A;

s) 13Y/34K/110A/113D/122D/175L;

t) 13Y/77V/99Y/104W/110A/113D/122F/154R/159D/175L;

u) 13Y/77M/99Y/104W/110A/113D/122F/154R/159D/175L;

v) 13Y/99Y/104W/110A/113D/118V/122F/154R/159D/175L;

5 w) 13Y/110A/113D/122D/162E/175L;

x) 13Y/77S/99Y/104W/110A/113D/122F/154R/159D/175L;

y) 13Y/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;

z) 13Y/54Q/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;

aa) 13Y/54W/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;

10 bb) 13Y/54Q/99Y/104W/110A/113D/114F/122F/154R/159D/175L;

cc) 13Y/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L; and

dd) 13Y/54Q/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L;

the position(s) being determined as the corresponding position of *subtilis* amino acid sequence shown as SEQ ID No. 1.

15

41. The polypeptide according to any one of embodiments 1-40, wherein said polypeptide has an amino acid sequence of SEQ ID No. 1 comprising amino acid substitutions selected from the list consisting of

a) G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L;

20 b) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L;

c) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;

d) G13Y/T110A/Y113D/R122F/Q175L;

e) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;

f) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K;

25 g) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175K;

h) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L;



- i) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L;  
j) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175K;  
k) G13Y/I77L/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;  
l) G13Y/V81I/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;  
5 m) G13Y/T110A/Y113D/R122D/W164F/Q175L;  
n) G13Y/T110A/Y113D/R122D/S162D/Q175L;  
o) G13Y/T110A/Y113D/R122D/Q175L;  
p) D11F/R122D/T110A/Y113A;  
q) G13Y/I77Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;  
10 r) D11F/R122D/T110A;  
s) G13Y/G34K/T110A/Y113D/R122D/Q175L;  
t) G13Y/I77V/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;  
u) G13Y/I77M/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;  
v) G13Y/K99Y/T104W/T110A/Y113D/I118V/R122F/K154R/N159D/Q175L;  
15 w) G13Y/T110A/Y113D/R122D/S162E/Q175L; and  
x) G13Y/I77S/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L  
y) G13Y/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;  
z) G13Y/N54Q/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q1  
75L;  
20 aa) G13Y/N54W/K99Y/T104W/T110A/Y113D/R122F/141Q/K154R/N159D/17  
5L;  
bb) G13Y/N54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q  
175L;  
cc) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q17  
5L; and  
25 dd) G13Y/54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N  
159D/Q175L.

42. The polypeptide according to any one of embodiments 1-41, wherein said polypeptide has an amino acid sequence, which consists of amino acid substitutions selected from the list consisting of:

- a) 13Y/110A/113D/122D/154R/159D/175L;
- 5 b) 13Y/99Y/104W/110A/113D/122F/154R/159D/166F/175L;
- c) 13Y/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- d) 13Y/110A/113D/122F/175L;
- e) 13Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- f) 13Y/99Y/104W/110A/113D/122F/154R/159D/175K;
- 10 g) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175K;
- h) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175L;
- i) 13Y/99Y/104W/110A/113D/114D/122F/154R/159D/175L;
- j) 13Y/99Y/104W/110A/113D/114Y/122F/154R/159D/175K;
- k) 13Y/77L/99Y/104W/110A/113D/122F/154R/159D/175L;
- 15 l) 13Y/81I/99Y/104W/110A/113D/122F/154R/159D/175L;
- m) 13Y/110A/113D/122D/164F/175L;
- n) 13Y/110A/113D/122D/162D/175L;
- o) 13Y/110A/113D/122D/175L;
- p) 11F/122D/110A/113A;
- 20 q) 13Y/77Y/99Y/104W/110A/113D/122F/154R/159D/175L;
- r) 11F/122D/110A;
- s) 13Y/34K/110A/113D/122D/175L;
- t) 13Y/77V/99Y/104W/110A/113D/122F/154R/159D/175L;
- u) 13Y/77M/99Y/104W/110A/113D/122F/154R/159D/175L;
- 25 v) 13Y/99Y/104W/110A/113D/118V/122F/154R/159D/175L;
- w) 13Y/110A/113D/122D/162E/175L; and

- x) 13Y/77S/99Y/104W/110A/113D/122F/154R/159D/175L
- y) 13Y/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- z) 13Y/54Q/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- aa) 13Y/54W/99Y/104W/110A/113D/122F/141Q/154R/159D/175L;
- 5 bb) 13Y/54Q/99Y/104W/110A/113D/114F/122F/154R/159D/175L;
- cc) 13Y/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L; and
- dd) 13Y/54Q/99Y/104W/110A/113D/114F/122F/141Q/154R/159D/175L,

the position(s) being determined as the corresponding position of *subtilis* amino  
 10 acid sequence shown as SEQ ID No. 1.

43. The polypeptide according to any one of embodiments 1-42, wherein said polypeptide has an amino acid sequence of SEQ ID No. 1, which consists of amino acid substitutions selected from the list consisting of

- 15 a) G13Y/T110A/Y113D/R122D/K154R/N159D/Q175L;
- b) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Y166F/Q175L;
- c) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q175L;
- d) G13Y/T110A/Y113D/R122F/Q175L;
- e) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- 20 f) G13Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175K;
- g) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175K;
- h) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175L;
- i) G13Y/K99Y/T104W/T110A/Y113D/N114D/R122F/K154R/N159D/Q175L;
- j) G13Y/K99Y/T104W/T110A/Y113D/N114Y/R122F/K154R/N159D/Q175K;
- 25 k) G13Y/I77L/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- l) G13Y/V81I/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;

- m) G13Y/T110A/Y113D/R122D/W164F/Q175L;
- n) G13Y/T110A/Y113D/R122D/S162D/Q175L;
- o) G13Y/T110A/Y113D/R122D/Q175L;
- p) D11F/R122D/T110A/Y113A;
- 5 q) G13Y/I77Y/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- r) D11F/R122D/T110A;
- s) G13Y/G34K/T110A/Y113D/R122D/Q175L;
- t) G13Y/I77V/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- u) G13Y/I77M/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L;
- 10 v) G13Y/K99Y/T104W/T110A/Y113D/I118V/R122F/K154R/N159D/Q175L;
- w) G13Y/T110A/Y113D/R122D/S162E/Q175L; and
- x) G13Y/I77S/K99Y/T104W/T110A/Y113D/R122F/K154R/N159D/Q175L
- y) G13Y/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q175L;
- z) G13Y/N54Q/K99Y/T104W/T110A/Y113D/R122F/N141Q/K154R/N159D/Q1
- 15 75L;
- aa) G13Y/N54W/K99Y/T104W/T110A/Y113D/R122F/141Q/K154R/N159D/17
- 5L;
- bb) G13Y/N54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/K154R/N159D/Q
- 175L;
- 20 cc) G13Y/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D/Q17
- 5L; and
- dd) G13Y/54Q/K99Y/T104W/T110A/Y113D/N114F/R122F/141Q/K154R/N159D
- /Q175L.

25 44. The polypeptide according to any one of embodiments 1-43, which is not SEQ ID No. 25.

45. A method of identifying a polypeptide according to any one of the embodiments 1-44, said method comprising:

(i) preparing a polypeptide having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from SEQ ID No. 2-22, and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment;

(ii) comparing the bran solubilisation and/or xylanase activity of said polypeptide with the bran solubilisation and/or xylanase activity of the amino acid sequence selected among SEQ ID NOs: 1-22 with which is has the highest percentage of identity; and

(iii) selecting the polypeptide if it has improved bran solubilisation and/or improved xylanase activity compared to the amino acid sequence selected among SEQ ID NOs: 1-22 with which is has the highest percentage of identity.

46. A method of preparing a polypeptide according to any one of embodiments 1- 44, said method comprising expressing a nucleotide sequence encoding said polypeptide; and optionally isolating and/or purifying the polypeptide after expression.

47. The method according to embodiment 46, wherein said polypeptide is prepared by modifying either a polypeptide amino acid sequence at position 110 or a codon that encodes an amino acid residue at position 110 in a nucleotide sequence encoding a polypeptide amino acid sequence, wherein position 110 is determined with reference to the *B. subtilis* xylanase sequence shown as SEQ ID No. 1.

48. A nucleotide sequence encoding a polypeptide according to any one of embodiments 1 to 44.

49. A vector comprising the nucleotide sequence according to embodiment 48.

50. A cell that has been transformed with the nucleotide sequence of embodiment 48 or the vector of embodiment 49.

51. A host organism that has been transformed with the nucleotide sequence of embodiment 48 or the vector of embodiment 49.

52. A composition comprising the polypeptide according to any one of embodiments 1-44 or a polypeptide identified according to any embodiment 45  
5 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component.

53. A dough comprising the polypeptide according to any one of embodiments 1-  
10 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component or a composition according to embodiment  
15 52.

54. A bakery product comprising the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment  
20 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component or a composition according to embodiment 52 or a dough according to embodiment 53.

55. Animal feed comprising the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a  
25 polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component or a composition according to embodiment 52.

56. A cleaning compositions comprising the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47.

57. A method of degrading or modifying a plant cell wall which method  
5 comprises contacting said plant cell wall with the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to  
10 embodiment 51 admixed with a non toxic component or a composition according to embodiment 52.

58. A method of processing a plant material which method comprises contacting said plant material with the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide  
15 prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 47 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component or a composition according to embodiment 52.

20 59. Use of the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to embodiment 48 or the vector according to embodiment 49 or the cell according to embodiment 50 or the organism according to embodiment 51 admixed with a  
25 non toxic component or a composition according to embodiment 52 in a method of modifying plant materials.

60. Use of the polypeptide according to any one of embodiments 1- 44 or a polypeptide identified according to embodiment 45 or a polypeptide prepared according to embodiments 46-47 or the nucleotide sequence according to  
30 embodiment 48 or the vector according to embodiment 49 or the cell according

to embodiment 50 or the organism according to embodiment 51 admixed with a non toxic component or a composition according to embodiment 52 in any one or more independently selected from: baking, processing cereals, starch liquefaction, production of Bio-ethanol from cellulosic material, animal feed, in  
5 processing wood, enhancing the bleaching of wood pulp, and as a cleaning composition.

61. A polypeptide or fragment thereof substantially as hereinbefore described with reference to the Examples and drawings.

62. A method substantially as hereinbefore described with reference to the  
10 Examples and drawings.

63. A composition substantially as hereinbefore described with reference to the Examples and drawings.

64. A use substantially as hereinbefore described with reference to the Examples and drawings.

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## CLAIMS

1. A polypeptide having xylanase activity and comprising an amino acid sequence, said amino acid sequence having at least 88% identity with SEQ ID No. 1 or having at least 75% identity with an amino acid sequence selected from  
5 SEQ ID No. 2-22, and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
2. A polypeptide having xylanase activity and comprising an amino acid  
10 sequence, said amino acid sequence having at least 88% identity with SEQ ID No. 1 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
3. A polypeptide having xylanase activity and comprising an amino acid  
15 sequence, said amino acid sequence having at least 75% identity with SEQ ID No. 2 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
4. A polypeptide having xylanase activity and comprising an amino acid  
20 sequence, said amino acid sequence having at least 75% identity with SEQ ID No. 3 and which polypeptide has an amino acid modification in position 110, wherein said position 110 is determined as the position corresponding to position 110 of *B. subtilis* xylanase sequence shown as SEQ ID No. 1 by alignment.
5. The polypeptide according to claim 2, wherein said polypeptide has at least  
25 90, 92 or 95% identity with SEQ ID No. 1.
6. The polypeptide according to any one of the claims 1 and 3-4, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with the

sequence with which is has the highest percentage of identity selected from SEQ ID No. 2-22.

7. The polypeptide according claim 3, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 2.

5 8. The polypeptide according claim 4, wherein said polypeptide has at least 76, 78, 80, 85, 90, 95, 98 or 95% identity with SEQ ID No. 3.

9. The polypeptide according to any one of the claims 1-6 having a  $\beta$ -jelly roll fold.

10 10. The polypeptide according to any one of the claims 1-9, wherein the amino acid modification in position 110 is an amino acid substitution.

11. The polypeptide according to any one of the claims 1-10, wherein the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, 15 leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine and valine.

12. The polypeptide according to any one of the claims 1-11, wherein the amino acid modification in position 110 is a substitution to any one different amino acid residue selected from the group consisting of: glutamic acid, tryptophan, alanine 20 and cysteine.

13. The polypeptide according to claim 12, wherein the amino acid modification in position 110 is a substitution to alanine.

14. The polypeptide according to any one of the claims 1-13, comprising one or more modification(s) at any one or more of amino acid positions: 11, 12, 13, 34, 25 54, 77, 81, 99, 104, 113, 114, 118, 122, 141, 154, 159, 162, 164, 166 and 175,

the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

15. The polypeptide according to any one of the claims 1-14, comprising one or more amino acid substitutions selected from the group consisting of: 11F, 12F,  
5 122D, 113A, 13Y, 54Q, 54W, 113D, 141Q, 175L, 122F, 34K, 99Y, 104W, 154R, 159D, 175K, 81I, 166F, 162E, 162D, 164F, 114D, 114Y, 114F, 118V, 175K, 77L, 77M, 77S, 77V, and 77Y, the position(s) being determined as the corresponding position of *B. subtilis* amino acid sequence shown as SEQ ID No. 1.

**Figure 1**



**Figure 2**

|    |       |                                                                  |     |
|----|-------|------------------------------------------------------------------|-----|
|    |       | 1                                                                | 60  |
| 1  | (1)   | -----ASTDYWQNWT DGGGIVN                                          |     |
| 10 | (1)   | -----MFKFVTKVLT VVIAATISFCLSAVPAS--ANTYWQYWTDGGGTVN              |     |
| 18 | (1)   | -----NTYWQYWTDGGGTVN                                             |     |
| 11 | (1)   | -----MRQKKLTFILAF LVCFALTLP AEIIQAQIVTDNSIGNHDGYDYEFWKDSGGSGT    |     |
| 6  | (1)   | -----MRQKKLTLILAF LVCFALTLP AEIIQAQIVTDNSIGNHDGYDYEFWKDSGGSGT    |     |
| 15 | (1)   | -----QIVTDNSIGNHDGYDYEFWKDSGGSGT                                 |     |
| 19 | (1)   | -----QIVTDNSIGNHDGYDYEFWKDSGGSGT                                 |     |
| 13 | (1)   | -----MNLRLKRLLFV MCIGLT LILTAVPAHARTITNNEMGNHSGYDYLWKDYG-NTS     |     |
| 21 | (1)   | -----RTITNNEMGNHSGYDYLWKDYG-NTS                                  |     |
| 14 | (1)   | MKLFLAAIVLCATAATAFPSELAQRAAGDLSKRQSITTSQTGTNNGYYSFWTNGGGEVT      |     |
| 22 | (1)   | -----AFPSELAQRAAGDLSKRQSITTSQTGTNNGYYSFWTNGGGEVT                 |     |
| 16 | (1)   | -----Q-TIQPGTGYNNGYFY SYWNDGHGGVT                                |     |
| 7  | (1)   | -MVSFTSLLAASPPSRASCRPAAEVESVAVEKQ-TIQPGTGYNNGYFY SYWNDGHGGVT     |     |
| 2  | (1)   | -----Q-CIQPGTGYNNGYFY SYWNDGHGGVT                                |     |
| 3  | (1)   | -----Q-TTPNSEGWH DGYYYSWWS DGGGAQAT                              |     |
| 8  | (1)   | --MVGF TPVALAALAAATGALAFPAGNATELEKQ-TTPNSEGWH DGYYYSWWS DGGGAQAT |     |
| 4  | (1)   | -----WTDAQGTVS                                                   |     |
| 17 | (1)   | -----TDYWQNWT DGGGTVN                                            |     |
| 9  | (1)   | -----MFKFGKKLLTVVLAASMSFGVFAATT--GATDYWQNWT DGGGTVN              |     |
| 12 | (1)   | -----MFKFKKKFLVGLTAAFMSISMFSATASAAGTDYWQNWT DGGGTVN              |     |
| 20 | (1)   | -----AGTDYWQNWT DGGGTVN                                          |     |
| 5  | (1)   | -----MFKFKKNFLVGLSAA LMSISLFSATASAASTDYWQNWT DGGGIVN             |     |
|    |       | 61                                                               | 120 |
| 1  | (18)  | AVNGSGGNYSVNWSNTGNFVVGKG-----WTTGSPFRTINYNAGVWAPNGNGYLTLYG       |     |
| 10 | (44)  | ATNGPGGNYSVTWRDTGNFVVGKG-----WEIGSPNRTIHYNAGVWEPSPNGYLTLYG       |     |
| 18 | (16)  | ATNGPGGNYSVTWRDTGNFVVGKG-----WEIGSPNRTIHYNAGVWEPSPNGYLTLYG       |     |
| 11 | (55)  | MILNHGGTFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQ-PNGNAYLCVYG     |     |
| 6  | (55)  | MILNHGGTFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQ-PNGNAYLCVYG     |     |
| 15 | (28)  | MILNHGGTFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQ-PNGNAYLCVYG     |     |
| 19 | (28)  | MILNHGGTFSAQWNNVNNILFRKGKKFNETQTHQQVGNMSINYGANFQ-PNGNAYLCVYG     |     |
| 13 | (54)  | MTLNNGGAFSAGWNNIGNALFRKGKKFDSTRTHHQLGNISINYNASFN-PGGNSYLCVYG     |     |
| 21 | (27)  | MTLNNGGAFSAGWNNIGNALFRKGKKFDSTRTHHQLGNISINYNASFN-PGGNSYLCVYG     |     |
| 14 | (61)  | YTNGDNGEYSVTWVDCGDFTSKGK-----WNP-ANAQTVTYSGEFN-PSGNAYLAVYG       |     |
| 22 | (45)  | YTNGDNGEYSVTWVDCGDFTSKGK-----WNP-ANAQTVTYSGEFN-PSGNAYLAVYG       |     |
| 16 | (27)  | YTNGPGGQFSVNWSNSGNFVVGKG-----WQPGTKNKVINFGSGSYN-PNGNSYLSVYG      |     |
| 7  | (59)  | YTNGPGGQFSVNWSNSGNFVVGKG-----WQPGTKNKVINFGSGSYN-PNGNSYLSVYG      |     |
| 2  | (27)  | YCNGPGGQFSVNWSNSGNFVVGKG-----WQPGTKNRVINFGSGSYN-PNGNSYLSVYG      |     |
| 3  | (27)  | YTNLEGGTYEISWGDGGNLVGGKG-----WNPGLNARAIHFEGVYQ-PNGNSYLA VYG      |     |
| 8  | (58)  | YTNLEGGTYEISWGDGGNLVGGKG-----WNPGLNARAIHFEGVYQ-PNGNSYLA VYG      |     |
| 4  | (10)  | MDLGGGTYSTQWRNTGNFVAGKG-----WSTGG-RKTVNY-SGTFNPSGNAYLTLYG        |     |
| 17 | (16)  | AVNGSGGNYSVNWQNTGNFVVGKG-----WTYGTPNRVVNYNAGVFSPSNGYLTIFYG       |     |
| 9  | (44)  | AVNGSGGNYSVNWQNTGNFVVGKG-----WTYGTPNRVVNYNAGVFSPSNGYLTIFYG       |     |
| 12 | (46)  | AVNGSGGNYSVNWSNTGNFVVGKG-----WTTGSPFRTINYNAGVWAPNGNGYLTLYG       |     |
| 20 | (18)  | AVNGSGGNYSVNWSNTGNFVVGKG-----WTTGSPFRTINYNAGVWAPNGNGYLTLYG       |     |
| 5  | (46)  | AVNGSGGNYSVNWSNTGNFVVGKG-----WTTGSPFRTINYNAGVWAPNGNGYLTLYG       |     |
|    |       | 121                                                              | 180 |
| 1  | (71)  | WTRSPLEIYYVVD SWGTYRPTG--TYKGT VKSDGGTYDIYTTTRYNAPSIDGDR TTFQY   |     |
| 10 | (97)  | WTRNQLIEYYVVDN WGTYRPTG--THRGTVVSDGGTYDIYTTMRYNAPSIDG-TQTFQQF    |     |
| 18 | (69)  | WTRNQLIEYYVVDN WGTYRPTG--THRGTVVSDGGTYDIYTTMRYNAPSIDG-TQTFQQF    |     |
| 11 | (114) | WTVDP LVEYYIVDSWGNWRPPG-ATPKGTITVDGGTYDIYETLRVNQPSIKG-IATFKQY    |     |
| 6  | (114) | WTVDP LVEYYIVDSWGNWRPPG-ATPKGTITVDGGTYDIYETLRVNQPSIKG-IATFKQY    |     |
| 15 | (87)  | WTVDP LVEYYIVDSWGNWRPPG-ATPKGTITVDGGTYDIYETLRVNQPSIKG-IATFKQY    |     |
| 19 | (87)  | WTVDP LVEYYIVDSWGNWRPPG-ATPKGTITVDGGTYDIYETLRVNQPSIKG-IATFKQY    |     |
| 13 | (113) | WTQSPLAEYYIVDSWGTYRPTG-AY-KGSFYADGGTYDIYETTRVNQPSIIG-IATFKQY     |     |
| 21 | (86)  | WTQSPLAEYYIVDSWGTYRPTG-AY-KGSFYADGGTYDIYETTRVNQPSIIG-IATFKQY     |     |
| 14 | (112) | WTTDPLVEYYI L ESYGTYNPSSGLTSLGQVTS DGGTYDIYSTQRVNQPSIEG-TSTFNQY  |     |
| 22 | (96)  | WTTDPLVEYYI L ESYGTYNPSSGLTSLGQVTS DGGTYDIYSTQRVNQPSIEG-TSTFNQY  |     |

16 (79) WSRNPLIEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVNQPSIIG-TATFYQY  
 7 (111) WSRNPLIEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVNQPSIIG-TATFYQY  
 2 (79) WSRNPLIEYYIVENFGTYNPSTGATKLGEVTSDGSVYDIYRTQRVNQPSIIG-TATFYQY  
 3 (79) WTRNPLVEYYIVENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDG-TQTFDQY  
 8 (110) WTRNPLVEYYIVENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDG-TQTFDQY  
 4 (61) WTTGPLIEYYIVDNWGTYRPTG--KYKGTVTSDDGGTYDIYKTTRYNAPSIEG-TKTFDQY  
 17 (69) WTRNALIEYYVVDNWGTYRPTG--TYKGTVTSDDGGTYDIYTTMRYNQPSIDG-YSTFPQY  
 9 (97) WTRNALIEYYVVDNWGTYRPTG--TYKGTVTSDDGGTYDIYTTMRYNQPSIDG-YSTFPQY  
 12 (99) WTRSPLIEYYVVDNWGTYRPTG--TYKGTVKSDDGGTYDIYTTTRYNAPSIDGDNTTFTQY  
 20 (71) WTRSPLIEYYVVDNWGTYRPTG--TYKGTVKSDDGGTYDIYTTTRYNAPSIDGDNTTFTQY  
 5 (99) WTRSPLIEYYVVDNWGTYRPTG--TYKGTVKSDDGGTYDIYTTTRYNAPSIDGDRTTFTQY  
 181 240  
 1 (129) WSVRQSKRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQVMATEGYQSSGSSNVTWV---  
 10 (154) WSVRQSKRPTGNNVSITFSNHVNAWRNAGMNLGSSWSYQVLATEGYQSSGRSNVTWV---  
 18 (126) WSVRQSKRPTGNNVSITFSNHVNAWRNAGMNLGSSWSYQVLATEGYQSSGRSNVTWV---  
 11 (172) WSVRRSKR---TSGTISVSNHFRAWENLGMNMG-KMYEVALTVEGYQSSGSANVYSNTRLR  
 6 (172) WSVRRSKR---TSGTISVSNHFRAWENLGMNMG-KMYEVALTVEGYQSSGSANVYSNTRLR  
 15 (145) WSVRRSKR---TSGTISVSNHFRAWENLGMNMG-KMYEVALTVEGYQSSGSANVYSNTRLR  
 19 (145) WSVRRSKR---TSGTISVSNHFRAWENLGMNMG-KMYEVALTVEGYQSSGSANVYSNTRLR  
 13 (170) WSVRQTKR---TSGTVSVSAHFRKWESLGMPMG-KMYETAFTVEGYQSSGSANVMTNQLF  
 21 (143) WSVRQTKR---TSGTVSVSAHFRKWESLGMPMG-KMYETAFTVEGYQSSGSANVMTNQLF  
 14 (171) WSVRTEKR---VGGTVTTTANHFAAWKALGLEMG-TYNYMIVSTEGYESSGSSTITVS---  
 22 (155) WSVRTEKR---VGGTVTTTANHFAAWKALGLEMG-TYNYMIVSTEGYESSGSSTITVS---  
 16 (138) WSVRRNHR---SSGSVNTANHFNAWAQQGLTLG-TMDYQIVAVEGYFSSGSASITVS---  
 7 (170) WSVRRNHR---SSGSVNTANHFNAWAQQGLTLG-TMDYQIVAVEGYFSSGSASITVS---  
 2 (138) WSVRRNHR---SSGSVNTANHFNAWAQQGLTLG-TMDYQIVAVEGYFSSGSASITVSD--  
 3 (138) WSVRQDKR---TSGTVQGTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG  
 8 (169) WSVRQDKR---TSGTVQGTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG  
 4 (118) WSVRQSKR---TGGTITSGNHFDAWARNGMNLG-NHNYMIMATEGYQSSGSSSTITV----  
 17 (126) WSVRQSKRPIGVNSQITFQNHVNAWASKGMYLGNSWSYQVMATEGYQSSGSSNVTWV---  
 9 (154) WSVRQSKRPIGVNSQITFQNHVNAWASKGMYLGNSWSYQVMATEGYQSSGSSNVTWV---  
 12 (157) WSVRQSKRPTGSNAAITFSNHVNAWKSHGMNLGSNWAYQVLATEGYKSSGSSNVTWV---  
 20 (129) WSVRQSKRPTGSNAAITFSNHVNAWKSHGMNLGSNWAYQVLATEGYKSSGSSNVTWV---  
 5 (157) WSVRQSKRPTGSNATITFSNHVNAWKSHGMNLGSNWAYQVMATEGYQSSGSSNVTWV---

241 261  
 1 (186) -----  
 10 (211) -----  
 18 (183) -----  
 11 (228) INGNPLSTISNDKSITLDKNN  
 6 (228) INGNPLSTISNDESITLDKNN  
 15 (201) INGNPLSTISNDESITLDKNN  
 19 (201) INGNPLSTISNDKSITLDKNN  
 13 (226) IGN-----  
 21 (199) IGN-----  
 14 (224) -----  
 22 (208) -----  
 16 (191) -----  
 7 (223) -----  
 2 (192) -----  
 3 (195) -----  
 8 (226) -----  
 4 (170) -----  
 17 (183) -----  
 9 (211) -----  
 12 (214) -----  
 20 (186) -----  
 5 (214) -----



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK2009/050351

## A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/24 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Genome Quest, Dgene (STN), EBI secureline

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                 | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------|-----------------------|
| X         | US6682923 B1, (BENTZIEN J., DAHIYAT B.) 27 January 2004<br>See especially SEQ ID NO. 11 and 12     | 1-2, 5-6, 9-11, 14-15 |
| X         | WO0238746 A2, (XENCOR INC [US]) 16 May 2002<br>See especially SEQ ID NO.11-12, 22, 52-53 and 55-65 | 1-2, 5-6, 9-11, 14-15 |
| X         | WO0068396 A2 (XENCOR INC [US]) 16 November 2000<br>See especially SEQ ID NO 21 and 22              | 1-2, 5-6, 9-11, 14-15 |
| X         | Genbank accession number ABX42471.1 (Leschine, S. B. et al) 19 November 2007                       | 1-2, 5-6, 9-11, 14-15 |
| X         | Genbank accession number ABR35180.1 (Copeland, A. et al) 27 June 2007                              | 1-2, 5-6, 9-15        |
| P,X       | Genbank accession number ACT03278.1 (Lucas, S. et al) 02 July 2009                                 | 1-2, 5-6, 9-15        |
| P,X       | NCBI reference sequence YP_003013364.1 (Lucas,S. et al) 07 July 2009                               | 1-2, 5-6, 9-15        |

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

30 January 2010

Date of mailing of the international search report

16 March 2010

Name and mailing address of the ISA/

Nordic Patent Institute, Helgeshoj Allé 81, 2630 Taastrup, Denmark

Facsimile No.

Authorized officer

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK2009/050351

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The international application does not comply with the requirement of unity of invention (Rules 13.1 and 13.3). The claims relate to 3 separate inventions which are not linked by a single inventive concept. The separate inventions are the following:

Invention I. Claims 1-2, 5 (all complete) and 6, 9-15 (all in part), relates to a polypeptide having xylanase activity, comprising an amino acid sequence with at least 88% identity with SEQ ID NO 1 or at least 75% identity with SEQ ID NO 2-22, and which has an amino acid modification i position 110 (corresponding to position 110 of SEQ ID NO 1).

[continued in the supplemental box]

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-2, 5 (all complete) and 6, 9-15 (all in part)

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/DK2009/050351

US6682923 B1 20040127

US2003049654 A1 20030313

WO0068396 A2 20001116

WO0068396 A3 20010308

WO0068396 A9 20020704

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WO0068396 A3 20010308

WO0068396 A9 20020704

US6682923 B1 20040127

JP2002543791T T 20021224

EP1179075 A2 20020213

CA2373135 A1 20001116

AU5132700 A 20001121

## Continuation of Box III:

Invention II. Claims 3, 7 (all complete) and 6, 9-15 (all in part), relates to a polypeptide having xylanase activity, comprising an amino acid sequence with at least 75% identity with SEQ ID NO 2 and which has an amino acid modification in position 110 (corresponding to position 110 of SEQ ID NO 1).

Invention III. Claims 4, 8 (all complete) and 6, 9-15 (all in part), relates to a polypeptide having xylanase activity, comprising an amino acid sequence with at least 75% identity with SEQ ID NO 3 and which has an amino acid modification in position 110 (corresponding to position 110 of SEQ ID NO 1).

The common concept linking inventions I-III is a polypeptide with xylanase activity having an amino acid modification in position 110. However, documents D1- D7 all relate to polypeptide with xylanase activity having an aminoacid modification in position 110.

In absence of any common new and inventive special technical feature, linking the inventions, the three inventions merely constitute alternative polypeptides with xylanase activity, and are not so linked as to form a single general inventive concept.