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- (71) Applicant (for all designated States except US):
NOVOZYMES A/S [DK/DK]; Krogshøjvej 36, DK-2880
Bagsværd (DK).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): DUFFNER, Fiona
[IE/DK]; Stockholmsgade 13, st. tv., DK-2100 Copen-
hagen (DK). SJØHOLM, Carsten [DK/DK]; Allerødvej
17, DK-3450 Allerød (DK).
- (74) Common Representative: NOVOZYMES A/S; Patents,
Krogshøjvej 36, DK-2880 Bagsværd (DK).
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(54) Title: FAMILY 74 XYLOGLUCANASES

(57) Abstract: Xyloglucanases belonging to family 74 of glycosyl hydrolases are derived from the genus *Jonesia*, especially from the strain *Jonesia sp.*, DSM 14140. It is contemplated that the xyloglucanases exhibit high performance in conventional detergent compositions.

FAMILY 74 XYLOGLUCANASES

The present invention relates to xyloglucanases belonging to family 74 of glycosyl hydrolases, preferably to enzymes exhibiting xyloglucanase activity as their major enzymatic activity in the neutral and alkaline pH ranges; to an enzyme preparation comprising the enzyme of the invention; to a method of producing such enzymes; and to methods for using such enzymes in the textile, detergent and cellulose fiber processing industries.

BACKGROUND OF THE INVENTION

Xyloglucanase activity has not yet been included in the classification of enzymes provided by the Enzyme Nomenclature (1992). Hitherto, this enzymatic activity has simply been classified as glucanase activity and has often been believed to be identical to cellulolytic activity (EC 3.2.1.4), i.e. activity against β -1,4-glycosidic linkages in cellulose or cellulose derivative substrates, or at least to be a side activity in enzymes having cellulolytic activity. However, a true xyloglucanase is a true xyloglucan specific enzyme capable of catalyzing the solubilisation of xyloglucan to xyloglucan oligosaccharides but which does not exhibit substantial cellulolytic activity, e.g. activity against the conventionally used cellulose-like substrates CMC (carboxymethylcellulose), HE cellulose and Avicel (microcrystalline cellulose). A xyloglucanase cleaves the beta-1,4-glycosidic linkages in the backbone of xyloglucan.

Xyloglucanase activity is disclosed in Vincken, J.P., Beldman, G., and Voragen, A.G.J. Substrate-specificity of endoglucanases - what determines xyloglucanase activity. *Carbohydrate Research* 298(4):299-310, 1997, wherein three different endoglucanases EndoI, EndoV and EndoVI from *Trichoderma viride* (similar to *T. reesei*) are characterized. EndoI, EndoV and EndoVI belongs to family 5, 7 and 12 of glycosyl hydrolases, respectively, see Henrissat, B. (1991), A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.*, **280**:309-316, and

Henrissat, B., and A. Bairoch (1993), New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.*, **293**:781-788.

International Patent Publication WO 94/14953 discloses a
5 family 12 xyloglucanase (EG II) cloned from the fungus
Aspergillus aculeatus and expressed in the fungus *Aspergillus
oryzae*.

International Patent Publication WO 99/02663 discloses
xyloglucanases cloned from *Bacillus licheniformis* (family 12)
10 and *Bacillus agaradhaerens* (family 5) and expressed in *Bacillus
subtilis*.

International Patent Application PCT/DK01/00132 discloses
xyloglucanases cloned from *Paenibacillus pabuli* (family 5) and
expressed in *Bacillus subtilis*.

15 International Patent Application PCT/DK01/00116 discloses
xyloglucanases cloned from *Paenibacillus polymyxa* (family 44)
and expressed in *Bacillus subtilis*.

It is an object of the present invention to provide an
enzyme with a high xyloglucanase activity at an alkaline pH and
20 exhibiting excellent performance in conventional detergent
compositions.

SUMMARY OF THE INVENTION

The inventors have now found enzymes having substantial
25 xyloglucanase activity, which enzymes belong to family 74 of
glycosyl hydrolases. It is contemplated that these xyloglucanase
enzymes perform excellent in conventional detergent
compositions, especially in liquid detergent compositions.

The inventors have succeeded in cloning and expressing a
30 family 74 xyloglucanase derived from the marine organism
Jonesia.

Accordingly, in a first aspect the invention relates to a
polypeptide exhibiting xyloglucanase activity as its major
enzymatic activity, which enzyme belongs to family 74 of
35 glycosyl hydrolases and is endogenous to a bacterium,
particularly an alkalophilic bacterium, and more particularly a
bacterial strain belonging to the genus *Jonesia*.

In a further aspect, the invention relates to a family 74

xyloglucanase selected from one of (a) a polypeptide encoded by the DNA sequence of positions 72-2820 of SEQ ID NO: 1; (b) a polypeptide produced by culturing a cell comprising the sequence of positions 72-2820 of SEQ ID NO: 1 under conditions wherein the DNA sequence is expressed; (c) a xyloglucanase enzyme having a sequence of at least 60% identity to positions 24-940 of SEQ ID NO: 2 when identity is determined by the Clustal method with a PAM250 residue table and the default settings of the Megalign program in the Lasergene package using a GAP creation penalty of 3 and Ktuple of 1; and (d) a polypeptide encoded by a DNA sequence that hybridizes to the DNA sequence of SEQ ID NO: 1 under medium stringency conditions, wherein the medium stringency conditions comprise hybridization in 5xSSC at 45°C and washing in 2xSSC at 60°C; and to an isolated polynucleotide molecule encoding a polypeptide having xyloglucanase activity which polynucleotide molecule hybridizes to a denatured double-stranded DNA probe under medium stringency conditions, wherein the probe is selected from the group consisting of DNA probes comprising the sequence shown in positions 72-2820 of SEQ ID NO:1, and DNA probes comprising a subsequence of positions 72-2820 of SEQ ID NO:1, the subsequence having a length of at least about 100 base pairs.

In further aspects, the invention provides an expression vector comprising a DNA segment which is e.g. a polynucleotide molecule of the invention; a cell comprising the DNA segment or the expression vector; and a method of producing a exhibiting xyloglucanase enzyme, which method comprises culturing the cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

In yet another aspect the invention provides an isolated xyloglucanase enzyme being free from homologous impurities.

The novel enzyme of the present invention is useful for the treatment of cellulosic material, especially cellulose-containing fiber, yarn, woven or non-woven fabric. The treatment can be carried out during the processing of cellulosic material into a material ready for garment manufacture or fabric manufacture, e.g. in the desizing or scouring step; or during industrial or household laundering of such fabric or garment.

Accordingly, in further aspects the present invention relates to an enzyme preparation comprising a xyloglucanase enzyme of the invention and conventional fillers or stabilizers; a detergent composition comprising a xyloglucanase enzyme having
5 substantial xyloglucanase activity in the neutral or alkaline range; and to use of the enzyme of the invention for the treatment of cellulose-containing fibers, yarn, woven or non-woven fabric.

The present invention has now made it possible to use a
10 xyloglucanase in detergent compositions, preferably liquid laundry detergent compositions, for removing or bleaching certain soils or stains present on laundry, especially soils and spots resulting from xyloglucan-containing food, plants, and the like. Further, it is contemplated that treatment with detergent
15 compositions comprising the novel enzyme can prevent binding of certain soils to the xyloglucan left on the cellulosic material.

DETAILED DESCRIPTION OF THE INVENTION

20 MICROBIAL SOURCES

The xyloglucanase of the invention may be obtained from a bacterium belonging to a strain of the genus *Jonesia*, in particular a strain of *Jonesia sp.*

In a preferred embodiment, the xyloglucanase of this
25 invention is obtained from the strain *Jonesia sp.*, DSM 14140. It is at present contemplated that a DNA sequence encoding an enzyme having similar functional properties as the xyloglucanase from with *Jonesia sp.*, DSM 14140 and having an amino acid sequence identity of at least 60% to this xyloglucanase may be
30 obtained from other strains belonging to the genus *Jonesia*.

The strain *Jonesia sp.* was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutsche Sammlung von Mikroorganismen
35 und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 23 February 2001 under the deposition number DSM 14140.

DEFINITIONS

For the purpose of the present invention the term "obtained from", "derived from", "obtainable from" or "derivable from", as used herein in connection with a specific source, 5 means that the enzyme is produced or can be produced by the specific source, or by a cell in which a gene from the source have been inserted.

In the present context, the term "enzyme preparation" is intended to mean either be a conventional enzymatic fermentation 10 product, possibly isolated and purified, from a single species of a microorganism, such preparation usually comprising a number of different enzymatic activities; or a mixture of monocomponent enzymes, preferably enzymes derived from bacterial or fungal species by using conventional recombinant techniques, which 15 enzymes have been fermented and possibly isolated and purified separately and which may originate from different species, preferably fungal or bacterial species; or the fermentation product of a microorganism which acts as a host cell for expression of a recombinant xyloglucanase, but which 20 microorganism simultaneously produces other enzymes, e.g. other xyloglucanases, proteases, or cellulases, being naturally occurring fermentation products of the microorganism, i.e. the enzyme complex conventionally produced by the corresponding naturally occurring microorganism.

In the present context the term "expression vector" denotes 25 a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and may 30 optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both. The expression vector of the invention may be any expression vector 35 that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which the vector is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector that exists as an

extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together
5 with the chromosome(s) into which it has been integrated.

The term "recombinant expressed" or "recombinantly expressed" used herein in connection with expression of a polypeptide or protein is defined according to the standard definition in the art. Using an expression vector as described
10 immediately above generally performs recombinant expression of a protein.

The term "isolated", when applied to a polynucleotide molecule, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous
15 or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of
20 other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985). The
25 term "an isolated polynucleotide" may alternatively be termed "a cloned polynucleotide".

When applied to a protein/polypeptide, the term "isolated" indicates that the protein is found in a condition other than its native environment. In a preferred form, the isolated
30 protein is substantially free of other proteins, particularly other homologous proteins (*i.e.* "homologous impurities" (see below)). It is preferred to provide the protein in a greater than 40% pure form, more preferably greater than 60% pure form.

Even more preferably it is preferred to provide the protein
35 in a highly purified form, *i.e.* greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by SDS-PAGE.

The term "isolated protein/polypeptide may alternatively be termed "purified protein/polypeptide".

The term "homologous impurities" means any impurity (e.g. another polypeptide than the polypeptide of the invention), which
5 originate from the homologous cell where the polypeptide of the invention is originally obtained.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription
10 initiates in the promoter and proceeds through the coding segment to the terminator

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and
15 DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

The term "complements of polynucleotide molecules" denotes polynucleotide molecules having a complementary base sequence
20 and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as
25 compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "promoter" denotes a portion of a gene containing
30 DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a
35 component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger peptide is commonly cleaved to remove

the secretory peptide during transit through the secretory pathway.

FAMILY 74

- 5 The xyloglucanase according to the invention has been found to show a 49% homology with a glycoside hydrolase from *Streptomyces* (GenBank/GenPept/SwissProt/AL031515/CAA20642.1/O86727) belonging to the glycoside hydrolase family 74 presently containing 6 members (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>).
- 10 For further reference regarding classification of glycosyl hydrolases see also Coutinho, P.M. and Henrissat, B. (1999) In "Recent Advances in Carbohydrate Bioengineering", H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., The Royal Society of Chemistry, Cambridge, pp. 3-12; and Coutinho, P.M. and
- 15 Henrissat, B. (1999) In "Genetics, 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; Henrissat B., Bairoch A., *Biochem. J.* 316:695-696(1996) "Updating the sequence-based classification of
- 20 glycosyl hydrolases"; Davies G., Henrissat B., *Structure* 3:853-859(1995) "Structures and mechanisms of glycosyl hydrolases".

POLYNUCLEOTIDES

Within preferred embodiments of the invention an isolated

25 polynucleotide of the invention will hybridize to similar sized regions of SEQ ID NO: 1 or a sequence complementary thereto, under at least medium stringency conditions.

In particular polynucleotides of the invention will hybridize to a denatured double-stranded DNA probe comprising

30 either the full sequence shown in SEQ ID NO: 1 or the sequence shown in positions 72-2820 of SEQ ID NO: 1 or any probe comprising a subsequence of SEQ ID NO:1 having a length of at least about 100 base pairs under at least medium stringency conditions, but preferably at high stringency conditions as

35 described in detail below. Suitable experimental conditions for determining hybridization at medium or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involve pre-soaking of the filter containing the DNA fragments or RNA to

hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. 132:6-13), ³²P-dCTP-labeled (specific activity higher than 1 x 10⁹ cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), and even more preferably at least 75°C (very high stringency).

15 Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using an x-ray film.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. DNA and RNA encoding genes of interest can be cloned in Gene Banks or DNA libraries by means of methods known in the art.

Polynucleotides encoding polypeptides having xyloglucanase activity of the invention are then identified and isolated by, for example, hybridization or PCR.

25 The present invention further provides counterpart polypeptides and polynucleotides from different bacterial strains (orthologs or paralogs). Of particular interest are xyloglucanase polypeptides from alkalophilic bacterial strains, including species of *Jonesia*. Of special interest are xyloglucanase peptides from strains which are very closely related to the strain *Jonesia sp.*, DSM 14140.

Species homologues of a polypeptide with xyloglucanase activity of the invention can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a DNA sequence of the present invention can be cloned using chromosomal DNA obtained from a cell type that expresses the protein. Suitable sources of DNA can be identified by probing

Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from chromosomal DNA of a positive cell line. A DNA sequence of the invention encoding an polypeptide having xyloglucanase activity can then be isolated
5 by a variety of methods, such as by probing with probes designed from the sequences disclosed in the present specification and claims or with one or more sets of degenerate probes based on the disclosed sequences. A DNA sequence of the invention can also be cloned using the polymerase chain reaction, or PCR
10 (Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the DNA library can be used to transform or transfect host cells, and expression of the DNA of interest can be detected with an antibody (monoclonal or polyclonal) raised against the
15 xyloglucanase cloned from *Jonesia sp.*, e.g. from *Jonesia sp.*, DSM 14140, expressed and purified as described in Materials and Methods and the Examples, or by an activity test relating to a polypeptide having xyloglucanase activity.

20 POLYPEPTIDES

The sequence of amino acids nos. 24-940 of SEQ ID NO: 2 is a mature xyloglucanase sequence including the catalytic active domain. The present invention also provides xyloglucanase polypeptides that are substantially homologous to the
25 polypeptide of amino acids nos. 24-940 of SEQ ID NO: 2 and species homologs (paralogs or orthologs) thereof. The term "substantially homologous" is used herein to denote polypeptides having 60%, preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%,
30 more preferably at least 90%, more preferably at least 95%, more preferably at least 97%, and even more preferably at least 99%, sequence identity to the sequence shown in amino acids nos. 24-940 of SEQ ID NO: 2 or their orthologs or paralogs. Such polypeptides will most preferably be 98% or more identical to
35 the sequence shown in amino acids nos. 24-940 of SEQ ID NO: 2 or its orthologs or paralogs. Percent sequence identity is determined by conventional methods, by the Clustal method (Thompson, J.D., Higgins, D.G., and Gibson, T.J., (1994), Nucleic

Acids Research 22, 4673-4680) with the default settings of the Megalign program in the Lasergene package (DNASTart Inc., 1228 South Park Street, Madison, Wisconsin 53715). The settings for multiple alignment are: GAP penalty of 10, and GAP length
5 penalty 10; while the pair-wise alignment parameters are GAP penalty of 3 and Ktuple of 1.

Sequence identity of polynucleotide molecules is determined by the Clustal method (Thompson, J.D., Higgins, D.G., and Gibson, T.J., (1994), Nucleic Acids Research 22, 4673-4680) with
10 the default settings of the Megalign program in the Lasergene package (DNASTart Inc., 1228 South Park Street, Madison, Wisconsin 53715). The settings for multiple alignment are: GAP penalty of 10, and GAP length penalty 10; while the pair-wise alignment parameters are GAP penalty of 5 and Ktuple of 2.

15 Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 1) and other substitutions that do not significantly affect the
20 folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates
25 purification (an affinity tag), such as a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. DNAs encoding affinity tags
30 are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ; New England Biolabs, Beverly, MA).

However, even though the changes described above preferably are of a minor nature, such changes may also be of a larger nature such as fusion of larger polypeptides of up to 300 amino
35 acids or more both as amino- or carboxyl-terminal extensions to a polypeptide of the invention having xyloglucanase activity.

Table 1

Conservative amino acid substitutions

	Basic:	arginine
		lysine
		histidine
5	Acidic:	glutamic acid
		aspartic acid
	Polar:	glutamine
		asparagine
	Hydrophobic:	leucine
10		isoleucine
		valine
	Aromatic:	phenylalanine
		tryptophan
		tyrosine
15	Small:	glycine
		alanine
		serine
		threonine
		methionine
20		

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-*N*-methyl lysine, 2-aminoisobutyric acid, isovaline and α -methyl serine) may be substituted for amino acid residues of a polypeptide according to the invention. 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, or preferably, are commercially available, and include pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Essential amino acids in the xyloglucanase polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-

1085, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e.* xyloglucanase activity) to identify amino acid residues
5 that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-4708, 1996. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography,
10 electron diffraction or photo affinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-312, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can
15 also be inferred from analysis of homologies with polypeptides, which are related to a polypeptide according to the invention.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis, recombination and/or shuffling followed by a relevant screening procedure, such as
20 those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988), Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-2156, 1989), WO95/17413, or WO 95/22625. Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, or recombination/shuffling of
25 different mutations (WO95/17413, WO95/22625), followed by selecting for functional a polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-
30 10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Mutagenesis/shuffling methods as disclosed above can be
35 combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using

modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

5 Using the methods discussed above, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to residues 24 to 940 of SEQ ID NO: 2 and retain the xyloglucanase activity of the wild-type protein.

10 The xyloglucanase enzyme of the invention may, in addition to the enzyme core comprising the catalytically domain, also comprise a cellulose binding domain (CBD), the cellulose binding domain and enzyme core (the catalytically active domain) of the enzyme being operably linked. The cellulose-binding domain (CBD)
15 may exist as an integral part the encoded enzyme, or a CBD from another origin may be introduced into the xyloglucanase thus creating an enzyme hybrid. In this context, the term "cellulose-binding domain" is intended to be understood as defined by Peter Tomme et al. "Cellulose-Binding Domains: Classification and
20 Properties" in "Enzymatic Degradation of Insoluble Carbohydrates", John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618, 1996. This definition classifies more than 120 cellulose-binding domains into 10 families (I-X), and demonstrates that CBDs are found in various enzymes such as
25 cellulases, xylanases, mannanases, arabinofuranosidases, acetyl esterases and chitinases. CBDs have also been found in algae, e.g. the red alga *Porphyra purpurea* as a non-hydrolytic polysaccharide-binding protein, see Tomme et al., *op.cit.* However, most of the CBDs are from cellulases and xylanases,
30 CBDs are found at the N and C termini of proteins or are internal. Enzyme hybrids are known in the art, see e.g. WO 90/00609 and WO 95/16782, and may be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or
35 without a linker, to a DNA sequence encoding the xyloglucanase and growing the host cell to express the fused gene. Enzyme hybrids may be described by the following formula:

CBD - MR - X

wherein CBD is the N-terminal or the C-terminal region of an amino acid sequence corresponding to at least the cellulose-binding domain; MR is the middle region (the linker), and may be a bond, or a short linking group preferably of from about 2 to about 100 carbon atoms, more preferably of from 2 to 40 carbon atoms; or is preferably from about 2 to about 100 amino acids, more preferably of from 2 to 40 amino acids; and X is an N-terminal or C-terminal region of a polypeptide encoded by the polynucleotide molecule of the invention.

10

IMMUNOLOGICAL CROSS-REACTIVITY

Polyclonal antibodies, especially monospecific polyclonal antibodies, to be used in determining immunological cross-reactivity may be prepared by use of a purified xyloglucanolytic enzyme. More specifically, antiserum against the xyloglucanase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immuno-electrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation $((\text{NH}_4)_2 \text{SO}_4)$, followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immuno-electrophoresis (N. Axelsen et al., *supra*, Chapters 3 and 4), or by rocket immuno-electrophoresis (N. Axelsen et al., Chapter 2).

RECOMBINANT EXPRESSION VECTORS

A recombinant vector comprising a DNA construct encoding the enzyme of the invention may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously

replicating vector, i.e. a vector that exists as an extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is
5 integrated into the host cell genome in part or in its entirety and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the enzyme of the invention is operably
10 linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g.
15 transcription initiates in a promoter and proceeds through the DNA sequence coding for the enzyme.

The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or
20 heterologous to the host cell.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* alpha-amylase gene,
25 the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylosidase gene, or the phage Lambda P_R or P_L promoters or the *E. coli* lac, trp or tac promoters.

The DNA sequence encoding the enzyme of the invention may also, if necessary, be operably connected to a suitable
30 terminator.

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, e.g. a
35 gene the product of which complements a defect in the host cell, or a gene encoding resistance to e.g. antibiotics like kanamycin, chloramphenicol, erythromycin, tetracycline, spectinomycine, or the like, or resistance to heavy metals or

herbicides.

To direct an enzyme of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre
5 sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the enzyme in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the enzyme. The secretory signal sequence may be that
10 normally associated with the enzyme or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present enzyme, the promoter and optionally the terminator and/or secretory signal sequence, respectively, or to assemble
15 these sequences by suitable PCR amplification schemes, and to insert them into suitable vectors containing the information necessary for replication or integration, are well known to persons skilled in the art (cf., for instance, Sambrook et al.,
op.cit.).

20

HOST CELLS

The cloned DNA molecule introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in
25 nature, it will typically be operably connected to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its natural environment. The term "homologous" is intended to include a DNA sequence encoding an enzyme native to the host organism in
30 question. The term "heterologous" is intended to include a DNA sequence not expressed by the host cell in nature. Thus, the DNA sequence may be from another organism, or it may be a synthetic sequence.

The host cell into which the cloned DNA molecule or the
35 recombinant vector of the invention is introduced may be any cell, which is capable of producing the desired enzyme and includes bacteria, yeast, fungi and higher eukaryotic cells.

Examples of bacterial host cells which on cultivation are

contemplated as being capable of producing the enzyme of the invention are gram-positive bacteria such as a strain of *Bacillus*, in particular *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus lautus*, *Bacillus* 5 *lentus*, *Bacillus licheniformis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus megatherium*, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus thuringiensis*, a strain of *Lactobacillus*, a strain of *Streptococcus*, a strain of *Streptomyces*, in particular *Streptomyces lividans* and 10 *Streptomyces murinus*, or the host cell may be a gram-negative bacterium such as a strain of *Escherichia coli*.

The transformation of the bacteria may be effected by protoplast transformation, electroporation, conjugation, or by using competent cells in a manner known per se (cf. e.g. 15 *Sambrook et al., supra*).

When expressing the enzyme in bacteria such as *Escherichia coli*, the enzyme may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be directed to the periplasmic space by a bacterial secretion 20 sequence. In the former case, the cells are lysed and the granules are recovered and denatured after which the enzyme is refolded by diluting the denaturing agent. In the latter case, the enzyme may be recovered from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to 25 release the contents of the periplasmic space and recovering the enzyme.

When expressing the enzyme in gram-positive bacteria such as a strain of *Bacillus* or a strain of *Streptomyces*, the enzyme may be retained in the cytoplasm, or may be directed to the 30 extra cellular medium by a bacterial secretion sequence.

Examples of a fungal host cell which on cultivation may be capable of producing the enzyme of the invention is e.g. a strain of *Aspergillus* or *Fusarium*, in particular *Aspergillus awamori*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus* 35 *oryzae*, and *Fusarium oxysporum*, and a strain of *Trichoderma*, preferably *Trichoderma harzianum*, *Trichoderma reesei* and *Trichoderma viride*.

Fungal cells may be transformed by a process involving

protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. The use of a strain of *Aspergillus* as a host cell is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

Examples of a host cell of yeast origin which on cultivation may be capable of producing the enzyme of the invention is e.g. a strain of *Hansenula sp.*, a strain of *Kluyveromyces sp.*, in particular *Kluyveromyces lactis* and *Kluyveromyces marci*, a strain of *Pichia sp.*, a strain of *Saccharomyces*, in particular *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* and *Saccharomyces uvarum*, a strain of *Schizosaccharomyces sp.*, in particular *Schizosaccharomyces pombe*, and a strain of *Yarrowia sp.*, in particular *Yarrowia lipolytica*.

Examples of a host cell of plant origin which on cultivation may be capable of producing the enzyme of the invention is e.g. a plant cell of *Solanum tuberosum* or *Nicotiana tabacum*.

METHOD OF PRODUCING A XYLOGLUCANOLYTIC ENZYME

In another aspect, the present invention also relates to a method of producing the enzyme preparation of the invention, the method comprising culturing a microorganism capable of producing the xyloglucanase under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

Culturing may be carried out using conventional fermentation techniques, e.g. culturing in shake flasks or fermentors with agitation to ensure sufficient aeration on a growth medium inducing production of the xyloglucanase enzyme. The growth medium may contain a conventional N-source such as peptone, yeast extract or casamino acids, a reduced amount of a conventional C-source such as dextrose or sucrose, and an inducer such as xyloglucan or composite plant substrates such as cereal bran (e.g. wheat bran or rice husk). The recovery may be carried out using conventional techniques, e.g. separation of bio-mass and supernatant by centrifugation or filtration, recovery of the supernatant or disruption of cells if the enzyme

of interest is intracellular, perhaps followed by further purification as described in EP 0 406 314 or by crystallization as described in WO 97/15660.

Further, the present invention provides a method of
5 producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

10 When an expression vector comprising a DNA sequence encoding the enzyme is transformed into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme of the invention.

Thereby it is possible to make a highly purified or
15 monocomponent xyloglucanolytic composition, characterized in being free from homologous impurities.

In the present invention, the homologous host cell may be a strain of *Jonesia sp.*

The medium used to culture the transformed host cells may
20 be any conventional medium suitable for growing the host cells in question. The expressed xyloglucanolytic enzyme may conveniently be secreted into the culture medium and may be recovered there from by well-known procedures including separating the cells from the medium by centrifugation or filtration,
25 precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

The present invention also relates to a transgenic plant,
30 plant part or plant cell which has been transformed with a DNA sequence encoding the xyloglucanase of the invention so as to express and produce this enzyme in recoverable quantities. The enzyme may be recovered from the plant or plant part.

The transgenic plant can be dicotyledonous or
35 monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as *Agrostis*, and cereals, e.g. wheat, oats, rye, barley,

rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. In the present context, also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the enzyme of the invention may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the enzyme of the invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the enzyme of the invention in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, e.g. based on when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the enzyme of the invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or

plant part such as seeds or leaves. Regulatory sequences are e.g. described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV promoter may be used (Franck et al., 1980. Cell 21: 285-294). Organ-specific
5 promoters may e.g. be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin,
10 globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promoter from a
15 seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998)), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, e.g. as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such
20 as the rbcs promoter from rice or tomato (Kyoizuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993)), the chlorella virus adenine methyltransferase gene promoter (Mittra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al.,
25 Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993)).

A promoter enhancer element may be used to achieve higher
30 expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

35 The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome

according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and electroporation (Gasser et al, Science, 244, 5 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992. Plant Mol. 10 Biol. 19: 15-38), however it can also be used for transforming monocots, although other transformation methods are generally preferred for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the 15 transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by 20 Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well known in 25 the art.

ENZYME COMPOSITIONS

In a still further aspect, the present invention relates to an enzyme composition comprising the xyloglucanase of the 30 invention.

The enzyme composition of the invention may, in addition to the xyloglucanase of the invention, comprise one or more other enzyme types, for instance hemicellulase such as xylanase and mannanase, cellulase or endo- β -1,4-glucanase components, 35 chitinase, lipase, esterase, pectinase, cutinase, phytase, oxidoreductase (peroxidase, haloperoxidase, oxidase, laccase), protease, amylase, reductase, phenoloxidase, ligninase, pullulanase, pectate lyase, pectin acetyl esterase,

polygalacturonase, rhamnogalacturonase, pectin lyase, pectin methylesterase, cellobiohydrolase, transglutaminase; or mixtures thereof.

The enzyme composition may be prepared in accordance with 5 methods known in the art and may be in the form of a liquid or a dry composition. For instance, the enzyme composition may be in granulate or microgranulate form. The enzyme to be included in the composition may be stabilized in accordance with methods known in the art.

10 Xyloglucanases have potential uses in a lot of different industries and applications. Examples are given below of preferred uses of the enzyme composition of the invention. The dosage of the enzyme composition of the invention and other conditions under which the composition is used may be determined 15 based on methods known in the art.

The xyloglucanase or xyloglucanase composition according to the invention may be useful for at least one of the following purposes.

20 USES

Use in the detergent industry

During washing and wearing, dyestuff from dyed fabrics or garment will conventionally bleed from the fabric, which then looks faded and worn. Removal of surface fibers from the fabric 25 will partly restore the original colours and looks of the fabric. By the term "colour clarification", as used herein, is meant the partly restoration of the initial colours of fabric or garment throughout multiple washing cycles.

The term "de-pilling" denotes removing of pills from the 30 fabric surface.

The term "soaking liquor" denotes aqueous liquor in which laundry may be immersed prior to being subjected to a conventional washing process. The soaking liquor may contain one or more ingredients conventionally used in a washing or laundering 35 process.

The term "washing liquor" denotes aqueous liquor in which laundry is subjected to a washing process, i.e. usually a combined chemical and mechanical action either manually or in a

washing machine. Conventionally, the washing liquor is an aqueous solution of a powder or liquid detergent composition.

The term "rinsing liquor" denotes aqueous liquor in which laundry is immersed and treated, conventionally immediately
5 after being subjected to a washing process, in order to rinse the laundry, i.e. essentially remove the detergent solution from the laundry. The rinsing liquor may contain a fabric conditioning or softening composition.

The laundry subjected to the method of the present inven-
10 tion may be conventional washable laundry. Preferably, the major part of the laundry is sewn or unsown fabrics, including knits, wovens, denims, yarns, and towelling, made from cotton, cotton blends or natural or manmade cellulose (e.g. originating from xylan-containing cellulose fibers such as from wood pulp) or
15 blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers,
20 polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fibers, lyocell).

DETERGENT DISCLOSURE AND EXAMPLES

25 **Surfactant system**

The detergent compositions according to the present invention comprise a surfactant system, wherein the surfactant can be selected from non-ionic and/or anionic and/or cationic and/or ampholytic and/or zwitterionic and/or semi-polar
30 surfactants.

The surfactant is typically present at a level from 0.1% to 60% by weight.

The surfactant is preferably formulated to be compatible with enzyme components present in the composition. In liquid or
35 gel compositions the surfactant is most preferably formulated in such a way that it promotes, or at least does not degrade, the stability of any enzyme in these compositions.

Preferred systems to be used according to the present

invention comprise as a surfactant one or more of the non-ionic and/or anionic surfactants described herein.

Polyethylene, polypropylene, and polybutylene oxide condensates of alkyl phenols are suitable for use as the non-ionic surfactant of the surfactant systems of the present invention, with the polyethylene oxide condensates being preferred. These compounds include the condensation products of alkyl phenols having an alkyl group containing from about 6 to about 14 carbon atoms, preferably from about 8 to about 14 carbon atoms, in either a straight chain or branched-chain configuration with the ethylene oxide. In a preferred embodiment, the ethylene oxide is present in an amount equal to from about 2 to about 25 moles, more preferably from about 3 to about 15 moles, of ethylene oxide per mole of alkyl phenol. Commercially available non-ionic surfactants of this type include Igepal™ CO-630, marketed by the GAF Corporation; and Triton™ X-45, X-114, X-100 and X-102, all marketed by the Rohm & Haas Company. These surfactants are commonly referred to as alkyl phenol alkoxyates (e.g., alkyl phenol ethoxyates).

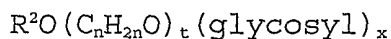
The condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide are suitable for use as the non-ionic surfactant of the non-ionic surfactant systems of the present invention. The alkyl chain of the aliphatic alcohol can either be straight or branched, primary or secondary, and generally contains from about 8 to about 22 carbon atoms. Preferred are the condensation products of alcohols having an alkyl group containing from about 8 to about 20 carbon atoms, more preferably from about 10 to about 18 carbon atoms, with from about 2 to about 10 moles of ethylene oxide per mole of alcohol. About 2 to about 7 moles of ethylene oxide and most preferably from 2 to 5 moles of ethylene oxide per mole of alcohol are present in said condensation products. Examples of commercially available non-ionic surfactants of this type include Tergitol™ 15-S-9 (The condensation product of C₁₁-C₁₅ linear alcohol with 9 moles ethylene oxide), Tergitol™ 24-L-6 NMW (the condensation product of C₁₂-C₁₄ primary alcohol with 6 moles ethylene oxide with a narrow molecular weight distribution), both marketed by Union Carbide Corporation;

Neodol™ 45-9 (the condensation product of C₁₄-C₁₅ linear alcohol with 9 moles of ethylene oxide), Neodol™ 23-3 (the condensation product of C₁₂-C₁₃ linear alcohol with 3.0 moles of ethylene oxide), Neodol™ 45-7 (the condensation product of C₁₄-C₁₅ linear alcohol with 7 moles of ethylene oxide), Neodol™ 45-5 (the condensation product of C₁₄-C₁₅ linear alcohol with 5 moles of ethylene oxide) marketed by Shell Chemical Company, Kyro™ EOB (the condensation product of C₁₃-C₁₅ alcohol with 9 moles ethylene oxide), marketed by The Procter & Gamble Company, and Genapol LA 050 (the condensation product of C₁₂-C₁₄ alcohol with 5 moles of ethylene oxide) marketed by Hoechst. Preferred range of HLB in these products is from 8-11 and most preferred from 8-10.

Also useful as the non-ionic surfactant of the surfactant systems of the present invention are alkyl polysaccharides disclosed in US 4,565,647, having a hydrophobic group containing from about 6 to about 30 carbon atoms, preferably from about 10 to about 16 carbon atoms and a polysaccharide, e.g. a polyglycoside, hydrophilic group containing from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7 saccharide units. Any reducing saccharide containing 5 or 6 carbon atoms can be used, e.g., glucose, galactose and galactosyl moieties can be substituted for the glucosyl moieties (optionally the hydrophobic group is attached at the 2-, 3-, 4-, etc. positions thus giving a glucose or galactose as opposed to a glucoside or galactoside). The intersaccharide bonds can be, e.g., between the one position of the additional saccharide units and the 2-, 3-, 4-, and/or 6-positions on the preceding saccharide units.

The preferred alkyl polyglycosides have the formula

30



wherein R² is selected from the group consisting of alkyl, alkyl phenyl, hydroxyalkyl, hydroxyalkylphenyl, and mixtures thereof in which the alkyl groups contain from about 10 to about 18, preferably from about 12 to about 14, carbon atoms; n is 2 or 3, preferably 2; t is from 0 to about 10, preferably 0; and x is from about 1.3 to about 10, preferably from about 1.3 to about

3, most preferably from about 1.3 to about 2.7. The glycosyl is preferably derived from glucose. To prepare these compounds, the alcohol or alkylpolyethoxy alcohol is formed first and then reacted with glucose, or a source of glucose, to form the
5 glucoside (attachment at the 1-position). The additional glycosyl units can then be attached between their 1-position and the preceding glycosyl units 2-, 3-, 4-, and/or 6-position, preferably predominantly the 2-position.

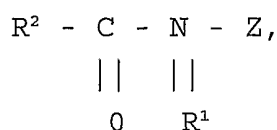
The condensation products of ethylene oxide with a
10 hydrophobic base formed by the condensation of propylene oxide with propylene glycol are also suitable for use as the additional non-ionic surfactant systems of the present invention. The hydrophobic portion of these compounds will preferably have a molecular weight from about 1500 to about 1800
15 and will exhibit water insolubility. The addition of polyoxyethylene moieties to this hydrophobic portion tends to increase the water solubility of the molecule as a whole, and the liquid character of the product is retained up to the point where the polyoxyethylene content is about 50% of the total
20 weight of the condensation product, which corresponds to condensation with up to about 40 moles of ethylene oxide. Examples of compounds of this type include certain of the commercially available Pluronic™ surfactants, marketed by BASF.

Also suitable for use as the non-ionic surfactant of the
25 non-ionic surfactant system of the present invention, are the condensation products of ethylene oxide with the product resulting from the reaction of propylene oxide and ethylenediamine. The hydrophobic moiety of these products consists of the reaction product of ethylenediamine and excess
30 propylene oxide, and generally has a molecular weight of from about 2500 to about 3000. This hydrophobic moiety is condensed with ethylene oxide to the extent that the condensation product contains from about 40% to about 80% by weight of polyoxyethylene and has a molecular weight of from about 5,000
35 to about 11,000. Examples of this type of non-ionic surfactant include certain of the commercially available Tetronic™ compounds, marketed by BASF.

Preferred for use as the non-ionic surfactant of the

surfactant systems of the present invention are polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide, alkyl polysaccharides, and mixtures hereof. Most preferred are C₈-C₁₄ alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C₈-C₁₈ alcohol ethoxylates (preferably C₁₀ avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Highly preferred non-ionic surfactants are polyhydroxy fatty acid amide surfactants of the formula



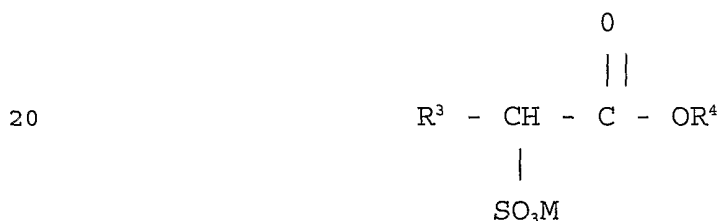
wherein R¹ is H, or R¹ is C₁₋₄ hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl or a mixture thereof, R² is C₅₋₃₁ hydrocarbyl, and Z is a polyhydroxy hydrocarbyl having a linear hydrocarbyl chain with at least 3 hydroxyls directly connected to the chain, or an alkoxyated derivative thereof. Preferably, R¹ is methyl, R² is straight C₁₁₋₁₅ alkyl or C₁₆₋₁₈ alkyl or alkenyl chain such as coconut alkyl or mixtures thereof, and Z is derived from a reducing sugar such as glucose, fructose, maltose or lactose, in a reductive amination reaction.

Highly preferred anionic surfactants include alkyl alkoxyated sulfate surfactants. Examples hereof are water soluble salts or acids of the formula RO(A)_mSO₃M wherein R is an unsubstituted C₁₀-C₂₄ alkyl or hydroxyalkyl group having a C₁₀-C₂₄ alkyl component, preferably a C₁₂-C₂₀ alkyl or hydroxyalkyl, more preferably C₁₂-C₁₈ alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxyated sulfates as well as alkyl propoxyated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and those derived from alkylamines

such as ethylamine, diethyl amine, triethylamine, mixtures thereof, and the like. Exemplary surfactants are C₁₂-C₁₈ alkyl polyethoxylate (1.0) sulfate (C₁₂-C₁₈E(1.0)M), C₁₂-C₁₈ alkyl polyethoxylate (2.25) sulfate (C₁₂-C₁₈(2.25)M), and C₁₂-C₁₈ alkyl
 5 polyethoxylate (3.0) sulfate (C₁₂-C₁₈E(3.0)M), and C₁₂-C₁₈ alkyl polyethoxylate (4.0) sulfate (C₁₂-C₁₈E(4.0)M), wherein M is conveniently selected from sodium and potassium.

Suitable anionic surfactants to be used are alkyl ester sulfonate surfactants including linear esters of C₈-C₂₀
 10 carboxylic acids (i.e., fatty acids), which are, sulfonated with gaseous SO₃ according to "The Journal of the American Oil Chemists Society", 52 (1975), pp. 323-329. Suitable starting materials would include natural fatty substances as derived from tallow, palm oil, etc.

15 The preferred alkyl ester sulfonate surfactant, especially for laundry applications, comprise alkyl ester sulfonate surfactants of the structural formula:



wherein R³ is a C₈-C₂₀ hydrocarbyl, preferably an alkyl, or
 25 combination thereof, R⁴ is a C₁-C₆ hydrocarbyl, preferably an alkyl, or combination thereof, and M is a cation, which forms a water-soluble salt with the alkyl ester sulfonate. Suitable salt-forming cations include metals such as sodium, potassium, and lithium, and substituted or unsubstituted ammonium cations,
 30 such as monoethanolamine, diethanolamine, and triethanolamine. Preferably, R³ is C₁₀-C₁₆ alkyl, and R⁴ is methyl, ethyl or isopropyl. Especially preferred are the methyl ester sulfonates wherein R³ is C₁₀-C₁₆ alkyl.

Other suitable anionic surfactants include the alkyl
 35 sulfate surfactants which are water soluble salts or acids of the formula ROSO₃M wherein R preferably is a C₁₀-C₂₄ hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C₁₀-C₂₀ alkyl component, more preferably a C₁₂-C₁₈ alkyl or hydroxyalkyl, and M is

H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium (e.g. methyl-, dimethyl-, and trimethyl ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and quaternary ammonium cations derived from alkylamines such as ethylamine, diethylamine, triethylamine, and mixtures thereof, and the like). Typically, alkyl chains of C₁₂-C₁₆ are preferred for lower wash temperatures (e.g. below about 50°C) and C₁₆-C₁₈ alkyl chains are preferred for higher wash temperatures (e.g. above about 50°C).

Other anionic surfactants useful for deterative purposes can also be included in the laundry detergent compositions of the present invention. These can include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C₈-C₂₂ primary or secondary alkanesulfonates, C₈-C₂₄ olefinsulfonates, sulfonated polycarboxylic acids prepared by sulfonation of the pyrolyzed product of alkaline earth metal citrates, e.g., as described in British patent specification No. 1,082,179, C₈-C₂₄ alkylpolyglycoethersulfates (containing up to 10 moles of ethylene oxide); alkyl glycerol sulfonates, fatty acyl glycerol sulfonates, fatty oleyl glycerol sulfates, alkyl phenol ethylene oxide ether sulfates, paraffin sulfonates, alkyl phosphates, isethionates such as the acyl isethionates, N-acyl taurates, alkyl succinamates and sulfosuccinates, monoesters of sulfosuccinates (especially saturated and unsaturated C₁₂-C₁₈ monoesters) and diesters of sulfosuccinates (especially saturated and unsaturated C₆-C₁₂ diesters), acyl sarcosinates, sulfates of alkylpolysaccharides such as the sulfates of alkylpolyglucoside (the nonionic nonsulfated compounds being described below), branched primary alkyl sulfates, and alkyl polyethoxy carboxylates such as those of the formula RO(CH₂CH₂O)_k-CH₂COO-M⁺ wherein R is a C₈-C₂₂ alkyl, k is an integer from 1 to 10, and M is a soluble salt forming cation. Resin acids and hydrogenated resin acids are also suitable, such as rosin, hydrogenated rosin, and resin acids and hydrogenated resin acids present in or derived from tall oil.

Alkylbenzene sulfonates are highly preferred. Especially

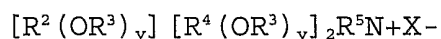
preferred are linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group preferably contains from 10 to 18 carbon atoms.

Further examples are described in "Surface Active Agents and Detergents" (Vol. I and II by Schwartz, Perry and Berch). A variety of such surfactants are also generally disclosed in US 3,929,678, (Column 23, line 58 through Column 29, line 23, herein incorporated by reference).

When included therein, the laundry detergent compositions of the present invention typically comprise from about 1% to about 40%, preferably from about 3% to about 20% by weight of such anionic surfactants.

The laundry detergent compositions of the present invention may also contain cationic, ampholytic, zwitterionic, and semi-polar surfactants, as well as the nonionic and/or anionic surfactants other than those already described herein.

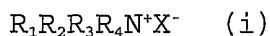
Cationic deterative surfactants suitable for use in the laundry detergent compositions of the present invention are those having one long-chain hydrocarbyl group. Examples of such cationic surfactants include the ammonium surfactants such as alkyltrimethylammonium halogenides, and those surfactants having the formula:



wherein R^2 is an alkyl or alkyl benzyl group having from about 8 to about 18 carbon atoms in the alkyl chain, each R^3 is selected from the group consisting of $-CH_2CH_2-$, $-CH_2CH(CH_3)-$, $-CH_2CH(CH_2OH)-$, $-CH_2CH_2CH_2-$, and mixtures thereof; each R^4 is selected from the group consisting of C_1-C_4 alkyl, C_1-C_4 hydroxyalkyl, benzyl ring structures formed by joining the two R^4 groups, $-CH_2CHOHCHOHCOR^6CHOHCH_2OH$, wherein R^6 is any hexose or hexose polymer having a molecular weight less than about 1000, and hydrogen when y is not 0; R^5 is the same as R^4 or is an alkyl chain, wherein the total number of carbon atoms or R^2 plus R^5 is not more than about 18; each y is from 0 to about 10, and the sum of the y values is from 0 to about 15; and X is any compatible anion.

Highly preferred cationic surfactants are the water

soluble quaternary ammonium compounds useful in the present composition having the formula:



5

wherein R_1 is C_8 - C_{16} alkyl, each of R_2 , R_3 and R_4 is independently C_1 - C_4 alkyl, C_1 - C_4 hydroxy alkyl, benzyl, and $-(C_2H_4)_xH$ where x has a value from 2 to 5, and X is an anion. Not more than one of R_2 , R_3 or R_4 should be benzyl.

10 The preferred alkyl chain length for R_1 is C_{12} - C_{15} , particularly where the alkyl group is a mixture of chain lengths derived from coconut or palm kernel fat or is derived synthetically by olefin build up or OXO alcohols synthesis.

Preferred groups for R_2R_3 and R_4 are methyl and
15 hydroxyethyl groups and the anion X may be selected from halide, methosulphate, acetate and phosphate ions.

Examples of suitable quaternary ammonium compounds of formulae (i) for use herein are:

coconut trimethyl ammonium chloride or bromide;
20 coconut methyl dihydroxyethyl ammonium chloride or bromide;
decyl triethyl ammonium chloride;
decyl dimethyl hydroxyethyl ammonium chloride or bromide;
 C_{12-15} dimethyl hydroxyethyl ammonium chloride or bromide;
coconut dimethyl hydroxyethyl ammonium chloride or bromide;
25 myristyl trimethyl ammonium methyl sulphate;
lauryl dimethyl benzyl ammonium chloride or bromide;
lauryl dimethyl (ethenoxy)₄ ammonium chloride or bromide;
choline esters (compounds of formula (i) wherein R_1 is

30 $CH_2-CH_2-O-C-C_{12-14}$ alkyl and $R_2R_3R_4$ are methyl).

$$\begin{array}{c} || \\ O \end{array}$$

di-alkyl imidazolines [compounds of formula (i)].

35 Other cationic surfactants useful herein are also described in US 4,228,044 and in EP 000 224.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about

25%, preferably from about 1% to about 8% by weight of such cationic surfactants.

Ampholytic surfactants are also suitable for use in the laundry detergent compositions of the present invention. These 5 surfactants can be broadly described as aliphatic derivatives of secondary or tertiary amines, or aliphatic derivatives of heterocyclic secondary and tertiary amines in which the aliphatic radical can be straight- or branched-chain. One of the aliphatic substituents contains at least about 8 carbon atoms, 10 typically from about 8 to about 18 carbon atoms, and at least one contains an anionic water-solubilizing group, e.g. carboxy, sulfonate, sulfate. See US 3,929,678 (column 19, lines 18-35) for examples of ampholytic surfactants.

When included therein, the laundry detergent compositions 15 of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such ampholytic surfactants.

Zwitterionic surfactants are also suitable for use in laundry detergent compositions. These surfactants can be broadly 20 described as derivatives of secondary and tertiary amines, derivatives of heterocyclic secondary and tertiary amines, or derivatives of quaternary ammonium, quaternary phosphonium or tertiary sulfonium compounds. See US 3,929,678 (column 19, line 38 through column 22, line 48) for examples of zwitterionic 25 surfactants.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such zwitterionic surfactants.

30 Semi-polar nonionic surfactants are a special category of nonionic surfactants which include water-soluble amine oxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 35 3 carbon atoms; watersoluble phosphine oxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon

atoms; and water-soluble sulfoxides containing one alkyl moiety from about 10 to about 18 carbon atoms and a moiety selected from the group consisting of alkyl and hydroxyalkyl moieties of from about 1 to about 3 carbon atoms.

5 Semi-polar nonionic detergent surfactants include the amine oxide surfactants having the formula:



wherein R³ is an alkyl, hydroxyalkyl, or alkyl phenyl group or mixtures thereof containing from about 8 to about 22 carbon atoms; R⁴ is an alkylene or hydroxyalkylene group containing
 15 from about 2 to about 3 carbon atoms or mixtures thereof; x is from 0 to about 3; and each R⁵ is an alkyl or hydroxyalkyl group containing from about 1 to about 3 carbon atoms or a polyethylene oxide group containing from about 1 to about 3 ethylene oxide groups. The R⁵ groups can be attached to each
 20 other, e.g., through an oxygen or nitrogen atom, to form a ring structure.

These amine oxide surfactants in particular include C₁₀-C₁₈ alkyl dimethyl amine oxides and C₈-C₁₂ alkoxy ethyl dihydroxy ethyl amine oxides.

25 When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such semi-polar nonionic surfactants.

30 **Builder system**

The compositions according to the present invention may further comprise a builder system. Any conventional builder system is suitable for use herein including aluminosilicate materials, silicates, polycarboxylates and fatty acids,
 35 materials such as ethylenediamine tetraacetate, metal ion sequestrants such as aminopolyphosphonates, particularly ethylenediamine tetramethylene phosphonic acid and diethylene triamine pentamethylenephosphonic acid. Though less preferred

for obvious environmental reasons, phosphate builders can also be used herein.

Suitable builders can be an inorganic ion exchange material, commonly an inorganic hydrated aluminosilicate material, more particularly a hydrated synthetic zeolite such as hydrated zeolite A, X, B, HS or MAP.

Another suitable inorganic builder material is layered silicate, e.g. SKS-6 (Hoechst). SKS-6 is a crystalline layered silicate consisting of sodium silicate ($\text{Na}_2\text{Si}_2\text{O}_5$).

10 Suitable polycarboxylates containing one carboxy group include lactic acid, glycolic acid and ether derivatives thereof as disclosed in Belgian Patent Nos. 831,368, 821,369 and 821,370. Polycarboxylates containing two carboxy groups include the water-soluble salts of succinic acid, malonic acid, 15 (ethylenedioxy) diacetic acid, maleic acid, diglycollic acid, tartaric acid, tartronic acid and fumaric acid, as well as the ether carboxylates described in German Offenle-enschrift 2,446,686, and 2,446,487, US 3,935,257 and the sulfinyl carboxylates described in Belgian Patent No. 840,623.

20 Polycarboxylates containing three carboxy groups include, in particular, water-soluble citrates, aconitrates and citraconates as well as succinate derivatives such as the carboxymethyloxysuccinates described in British Patent No. 1,379,241, lactoxysuccinates described in Netherlands 25 Application 7205873, and the oxypolycarboxylate materials such as 2-oxa-1,1,3-propane tricarboxylates described in British Patent No. 1,387,447.

Polycarboxylates containing four carboxy groups include oxydisuccinates disclosed in British Patent No. 1,261,829, 30 1,1,2,2,-ethane tetracarboxylates, 1,1,3,3-propane tetracarboxylates containing sulfo substituents include the sulfosuccinate derivatives disclosed in British Patent Nos. 1,398,421 and 1,398,422 and in US 3,936,448, and the sulfonated pyrolysed citrates described in British Patent No. 1,082,179, 35 while polycarboxylates containing phosphone substituents are disclosed in British Patent No. 1,439,000.

Alicyclic and heterocyclic polycarboxylates include cyclopentane-cis,cis-cis-tetracarboxylates, cyclopentadienide

pentacarboxylates, 2,3,4,5-tetrahydro-furan - cis, cis, cis-tetracarboxylates, 2,5-tetrahydro-furan-cis, discarboxylates, 2,2,5,5,-tetrahydrofuran - tetracarboxylates, 1,2,3,4,5,6-hexane - hexacarboxylates and carboxymethyl derivatives of polyhydric
5 alcohols such as sorbitol, mannitol and xylitol. Aromatic polycarboxylates include mellitic acid, pyromellitic acid and the phthalic acid derivatives disclosed in British Patent No. 1,425,343.

Of the above, the preferred polycarboxylates are hydroxy-
10 carboxylates containing up to three carboxy groups per molecule, more particularly citrates.

Preferred builder systems for use in the present compositions include a mixture of a water-insoluble aluminosilicate builder such as zeolite A or of a layered
15 silicate (SKS-6), and a water-soluble carboxylate chelating agent such as citric acid.

A suitable chelant for inclusion in the detergent compositions in accordance with the invention is ethylenediamine-N,N'-disuccinic acid (EDDS) or the alkali metal, alkaline earth
20 metal, ammonium, or substituted ammonium salts thereof, or mixtures thereof. Preferred EDDS compounds are the free acid form and the sodium or magnesium salt thereof. Examples of such preferred sodium salts of EDDS include Na_2EDDS and Na_4EDDS . Examples of such preferred magnesium salts of EDDS include
25 MgEDDS and Mg_2EDDS . The magnesium salts are the most preferred for inclusion in compositions in accordance with the invention.

Preferred builder systems include a mixture of a water-insoluble aluminosilicate builder such as zeolite A, and a water soluble carboxylate chelating agent such as citric acid.

30 Other builder materials that can form part of the builder system for use in granular compositions include inorganic materials such as alkali metal carbonates, bicarbonates, silicates, and organic materials such as the organic phosphonates, amino polyalkylene phosphonates and amino
35 polycarboxylates.

Other suitable water-soluble organic salts are the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals

separated from each other by not more than two carbon atoms.

Polymers of this type are disclosed in GB-A-1,596,756. Examples of such salts are polyacrylates of MW 2000-5000 and their copolymers with maleic anhydride, such copolymers having a
5 molecular weight of from 20,000 to 70,000, especially about 40,000.

Detergency builder salts are normally included in amounts of from 5% to 80% by weight of the composition. Preferred levels of builder for liquid detergents are from 5% to 30%.

10

Enzymes

Preferred detergent compositions, in addition to the enzyme preparation of the invention, comprise other enzyme(s) which provides cleaning performance and/or fabric care benefits.

15 Such enzymes include proteases, lipases, cutinases, amylases, cellulases, peroxidases, oxidases (e.g. laccases).

Proteases: Any protease suitable for use in alkaline solutions can be used. Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred.
20 Chemically or genetically modified mutants are included. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg,
25 subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium protease described in WO 89/06270.

Preferred commercially available protease enzymes include
30 those sold under the trade names Alcalase, Savinase, Primase, Durazym, and Esperase by Novozymes A/S, those sold under the tradename Maxatase, Maxacal, Maxapem, Properase, Purafect and Purafect OXP by Genencor International, and those sold under the tradename Opticlean and Optimase by Solvay Enzymes. Protease
35 enzymes may be incorporated into the compositions in accordance with the invention at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the

composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

5 Lipases: Any lipase suitable for use in alkaline solutions can be used. Suitable lipases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included.

Examples of useful lipases include a Humicola lanuginosa
10 lipase, e.g., as described in EP 258 068 and EP 305 216, a Rhizomucor miehei lipase, e.g., as described in EP 238 023, a Candida lipase, such as a C. antarctica lipase, e.g., the C. antarctica lipase A or B described in EP 214 761, a Pseudomonas
15 lipase such as a P. alcaligenes and P. pseudoalcaligenes lipase, e.g., as described in EP 218 272, a P. cepacia lipase, e.g., as described in EP 331 376, a P. stutzeri lipase, e.g., as disclosed in GB 1,372,034, a P. fluorescens lipase, a Bacillus
lipase, e.g., a B. subtilis lipase (Dartois et al., (1993), Biochemica et Biophysica acta 1131, 253-260), a B. stearo-
20 thermophilus lipase (JP 64/744992) and a B. pumilus lipase (WO 91/16422).

Furthermore, a number of cloned lipases may be useful, including the Penicillium camembertii lipase described by Yamaguchi et al., (1991), Gene 103, 61-67), the Geotricum
25 candidum lipase (Schimada, Y. et al., (1989), J. Biochem., 106, 383-388), and various Rhizopus lipases such as a R. delemar lipase (Hass, M.J et al., (1991), Gene 109, 117-113), a R. niveus lipase (Kugimiya et al., (1992), Biosci. Biotech. Biochem. 56, 716-719) and a R. oryzae lipase.

30 Other types of lipolytic enzymes such as cutinases may also be useful, e.g., a cutinase derived from Pseudomonas mendocina as described in WO 88/09367, or a cutinase derived from Fusarium solani pisi (e.g. described in WO 90/09446).

Especially suitable lipases are lipases such as M1
35 Lipase™, Luma fast™ and Lipomax™ (Genencor), Lipolase™ and Lipolase Ultra™ (Novozymes A/S), and Lipase P "Amano" (Amano Pharmaceutical Co. Ltd.).

The lipases are normally incorporated in the detergent

composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Amylases: Any amylase (a and/or b) suitable for use in alkaline solutions can be used. Suitable amylases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Amylases include, for example, α -amylases obtained from a special strain of *B. licheniformis*, described in more detail in GB 1,296,839. Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (Novozymes A/S) and Rapidase™ and Maxamyl P™ (Genencor).

The amylases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Cellulases: Any cellulase suitable for use in alkaline solutions can be used. Suitable cellulases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Suitable cellulases are disclosed in US 4,435,307 which discloses fungal cellulases produced from *Humicola insolens*, in WO 96/34108 and WO 96/34092 which disclose bacterial alkalophilic cellulases (BCE 103) from *Bacillus*, and in WO 94/21801, US 5,475,101 and US 5,419,778 which disclose EG III cellulases from *Trichoderma*. Especially suitable cellulases are the cellulases having colour care benefits. Examples of such cellulases are cellulases described in European patent application No. 0 495 257. Commercially available cellulases include Celluzyme™ and Carezyme™ produced by a strain of *Humicola insolens* (Novozymes A/S), KAC-500(B)™ (Kao Corporation), and Puradax™ (Genencor International).

Cellulases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, 5 more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Peroxidases/Oxidases :Peroxidase enzymes are used in 10 combination with hydrogen peroxide or a source thereof (e.g. a percarbonate, perborate or persulfate). Oxidase enzymes are used in combination with oxygen. Both types of enzymes are used for "solution bleaching", i.e. to prevent transfer of a textile dye from a dyed fabric to another fabric when said fabrics are 15 washed together in a wash liquor, preferably together with an enhancing agent as described in e.g. WO 94/12621 and WO 95/01426. Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically or genetically modified mutants are included.

20 Peroxidase and/or oxidase enzymes are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 25 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Mixtures of the above mentioned enzymes are encompassed herein, in particular a mixture of a protease, an amylase, a 30 lipase and/or a cellulase.

The enzyme of the invention, or any other enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, 35 preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level from 0.01% to 0.2% of enzyme

protein by weight of the composition.

Bleaching agents

Additional optional detergent ingredients that can be
5 included in the detergent compositions of the present invention
include bleaching agents such as PB1, PB4 and percarbonate with
a particle size of 400-800 microns. These bleaching agent
components can include one or more oxygen bleaching agents and,
depending upon the bleaching agent chosen, one or more bleach
10 activators. When present oxygen bleaching compounds will
typically be present at levels of from about 1% to about 25%. In
general, bleaching compounds are optional added components in
non-liquid formulations, e.g. granular detergents.

The bleaching agent component for use herein can be any of
15 the bleaching agents useful for detergent compositions including
oxygen bleaches as well as others known in the art.

The bleaching agent suitable for the present invention can
be an activated or non-activated bleaching agent.

One category of oxygen bleaching agent that can be used
20 encompasses percarboxylic acid bleaching agents and salts
thereof. Suitable examples of this class of agents include
magnesium monoperoxyphthalate hexahydrate, the magnesium salt of
meta-chloro perbenzoic acid, 4-nonylamino-4-oxoperoxybutyric
acid and diperoxydodecanedioic acid. Such bleaching agents are
25 disclosed in US 4,483,781, US 740,446, EP 0 133 354 and US
4,412,934. Highly preferred bleaching agents also include 6-
nonylamino-6-oxoperoxypropionic acid as described in US 4,634,551.

Another category of bleaching agents that can be used
encompasses the halogen bleaching agents. Examples of hypohalite
30 bleaching agents, for example, include trichloro isocyanuric
acid and the sodium and potassium dichloroisocyanurates and N-
chloro and N-bromo alkane sulphonamides. Such materials are nor-
mally added at 0.5-10% by weight of the finished product,
preferably 1-5% by weight.

35 The hydrogen peroxide releasing agents can be used in
combination with bleach activators such as tetra-
acetylenediamine (TAED), nonanoyloxybenzenesulfonate (NOBS,
described in US 4,412,934), 3,5-trimethyl-

hexsanoloxylbenzenesulfonate (ISONOBS, described in EP 120 591) or pentaacetylglucose (PAG), which are perhydrolyzed to form a peracid as the active bleaching species, leading to improved bleaching effect. In addition, very suitable are the bleach
5 activators C8(6-octanamido-caproyl) oxybenzene-sulfonate, C9(6-nonanamido caproyl) oxybenzenesulfonate and C10 (6-decanamido caproyl) oxybenzenesulfonate or mixtures thereof. Also suitable activators are acylated citrate esters such as disclosed in European Patent Application No. 91870207.7.

10 Useful bleaching agents, including peroxyacids and bleaching systems comprising bleach activators and peroxygen bleaching compounds for use in cleaning compositions according to the invention are described in application USSN 08/136,626.

The hydrogen peroxide may also be present by adding an
15 enzymatic system (i.e. an enzyme and a substrate therefore) which is capable of generation of hydrogen peroxide at the beginning or during the washing and/or rinsing process. Such enzymatic systems are disclosed in European Patent Application EP 0 537 381.

20 Bleaching agents other than oxygen bleaching agents are also known in the art and can be utilized herein. One type of non-oxygen bleaching agent of particular interest includes photoactivated bleaching agents such as the sulfonated zinc and/or aluminium phthalocyanines. These materials can be deposited
25 upon the substrate during the washing process. Upon irradiation with light, in the presence of oxygen, such as by hanging clothes out to dry in the daylight, the sulfonated zinc phthalocyanine is activated and, consequently, the substrate is bleached. Preferred zinc phthalocyanine and a photoactivated
30 bleaching process are described in US 4,033,718. Typically, detergent composition will contain about 0.025% to about 1.25%, by weight, of sulfonated zinc phthalocyanine.

Bleaching agents may also comprise a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds
35 described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

Suds suppressors

Another optional ingredient is a suds suppressor, exemplified by silicones, and silica-silicone mixtures. Silicones can generally be represented by alkylated polysiloxane materials, while silica is normally used in finely divided forms exemplified by silica aerogels and xerogels and hydrophobic silicas of various types. These materials can be incorporated as particulates, in which the suds suppressor is advantageously releasably incorporated in a water-soluble or waterdispersible, substantially non surface-active detergent impermeable carrier. Alternatively the suds suppressor can be dissolved or dispersed in a liquid carrier and applied by spraying on to one or more of the other components.

A preferred silicone suds controlling agent is disclosed in US 3,933,672. Other particularly useful suds suppressors are the self-emulsifying silicone suds suppressors, described in German Patent Application DTOS 2,646,126. An example of such a compound is DC-544, commercially available from Dow Corning, which is a siloxane-glycol copolymer. Especially preferred suds controlling agent are the suds suppressor system comprising a mixture of silicone oils and 2-alkyl-alkanols. Suitable 2-alkyl-alkanols are 2-butyl-octanol which are commercially available under the trade name Isofol 12 R.

Such suds suppressor system are described in European Patent Application EP 0 593 841.

Especially preferred silicone suds controlling agents are described in European Patent Application No. 92201649.8. Said compositions can comprise a silicone/ silica mixture in combination with fumed nonporous silica such as Aerosil^R.

The suds suppressors described above are normally employed at levels of from 0.001% to 2% by weight of the composition, preferably from 0.01% to 1% by weight.

Other components

Other components used in detergent compositions may be employed such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or nonencapsulated perfumes.

Especially suitable encapsulating materials are water soluble capsules which consist of a matrix of polysaccharide and polyhydroxy compounds such as described in GB 1,464,616.

Other suitable water soluble encapsulating materials
5 comprise dextrins derived from ungelatinized starch acid esters of substituted dicarboxylic acids such as described in US 3,455,838. These acid-ester dextrins are, preferably, prepared from such starches as waxy maize, waxy sorghum, sago, tapioca and potato. Suitable examples of said encapsulation materials
10 include N-Lok manufactured by National Starch. The N-Lok encapsulating material consists of a modified maize starch and glucose. The starch is modified by adding monofunctional substituted groups such as octenyl succinic acid anhydride.

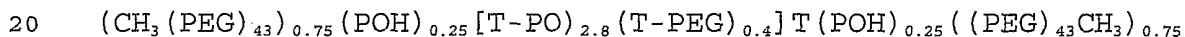
Antiredeposition and soil suspension agents suitable
15 herein include cellulose derivatives such as methylcellulose, carboxymethylcellulose and hydroxyethylcellulose, and homo- or co-polymeric polycarboxylic acids or their salts. Polymers of this type include the polyacrylates and maleic anhydride-acrylic acid copolymers previously mentioned as builders, as well as
20 copolymers of maleic anhydride with ethylene, methylvinyl ether or methacrylic acid, the maleic anhydride constituting at least 20 mole percent of the copolymer. These materials are normally used at levels of from 0.5% to 10% by weight, more preferably from 0.75% to 8%, most preferably from 1% to 6% by weight of the
25 composition.

Preferred optical brighteners are anionic in character, examples of which are disodium 4,4'-bis-(2-diethanolamino-4-anilino -s- triazin-6-ylamino)stilbene-2:2' disulphonate, disodium 4, - 4'-bis-(2-morpholino-4-anilino-s-triazin-6-ylamino-stilbene-2:2' - disulphonate, disodium 4,4' - bis-(2,4-dianilino-s-triazin-6-ylamino)stilbene-2:2' - disulphonate, monosodium 4',4'' - bis-(2,4-dianilino-s-tri-azin-6-ylamino)stilbene-2-sulphonate, disodium 4,4' -bis-(2-anilino-4-(N-methyl-N-2-hydroxyethylamino) -s-triazin-6-ylamino)stilbene-
30 2,2' - disulphonate, di-sodium 4,4' -bis-(4-phenyl-2,1,3-triazol-2-yl) -stilbene-2,2' disulphonate, di-so-dium 4,4'bis(2-anilino-4-(1-methyl-2-hydroxyethylamino) -s-triazin-6-ylami-
35 no)stilbene-2,2'disulphonate, sodium 2(stilbyl-4''-(naphtho-

1',2':4,5)-1,2,3, - triazole-2''-sulphonate and 4,4'-bis(2-sulphostyryl)biphenyl.

Other useful polymeric materials are the polyethylene glycols, particularly those of molecular weight 1000-10000, more particularly 2000 to 8000 and most preferably about 4000. These are used at levels of from 0.20% to 5% more preferably from 0.25% to 2.5% by weight. These polymers and the previously mentioned homo- or co-polymeric poly-carboxylate salts are valuable for improving whiteness maintenance, fabric ash deposition, and cleaning performance on clay, proteinaceous and oxidizable soils in the presence of transition metal impurities.

Soil release agents useful in compositions of the present invention are conventionally copolymers or terpolymers of terephthalic acid with ethylene glycol and/or propylene glycol units in various arrangements. Examples of such polymers are disclosed in US 4,116,885 and 4,711,730 and EP 0 272 033. A particular preferred polymer in accordance with EP 0 272 033 has the formula:



where PEG is $-(OC_2H_4)_n-$, PO is (OC_3H_6O) and T is $(pOOC_6H_4CO)$.

Also very useful are modified polyesters as random copolymers of dimethyl terephthalate, dimethyl sulfoisophthalate, ethylene glycol and 1,2-propanediol, the end groups consisting primarily of sulphobenzoate and secondarily of mono esters of ethylene glycol and/or 1,2-propanediol. The target is to obtain a polymer capped at both end by sulphobenzoate groups, "primarily", in the present context most of said copolymers herein will be endcapped by sulphobenzoate groups. However, some copolymers will be less than fully capped, and therefore their end groups may consist of monoester of ethylene glycol and/or 1,2-propanediol, thereof consist "secondarily" of such species.

The selected polyesters herein contain about 46% by weight of dimethyl terephthalic acid, about 16% by weight of 1,2-propanediol, about 10% by weight ethylene glycol, about 13% by weight of dimethyl sulfobenzoic acid and about 15% by weight of

sulfoisophthalic acid, and have a molecular weight of about 3.000. The polyesters and their method of preparation are described in detail in EP 311 342.

5 Softening agents

Fabric softening agents can also be incorporated into laundry detergent compositions in accordance with the present invention. These agents may be inorganic or organic in type. Inorganic softening agents are exemplified by the smectite clays
10 disclosed in GB-A-1 400898 and in US 5,019,292. Organic fabric softening agents include the water insoluble tertiary amines as disclosed in GB-A1 514 276 and EP 0 011 340 and their combination with mono C₁₂-C₁₄ quaternary ammonium salts are disclosed in EP-B-0 026 528 and di-long-chain amides as
15 disclosed in EP 0 242 919. Other useful organic ingredients of fabric softening systems include high molecular weight polyethylene oxide materials as disclosed in EP 0 299 575 and 0 313 146.

Levels of smectite clay are normally in the range from 5%
20 to 15%, more preferably from 8% to 12% by weight, with the material being added as a dry mixed component to the remainder of the formulation. Organic fabric softening agents such as the water-insoluble tertiary amines or dilong chain amide materials are incorporated at levels of from 0.5% to 5% by weight,
25 normally from 1% to 3% by weight whilst the high molecular weight polyethylene oxide materials and the water soluble cationic materials are added at levels of from 0.1% to 2%, normally from 0.15% to 1.5% by weight. These materials are normally added to the spray dried portion of the composition,
30 although in some instances it may be more convenient to add them as a dry mixed particulate, or spray them as molten liquid on to other solid components of the composition.

Polymeric dye-transfer inhibiting agents

35 The detergent compositions according to the present invention may also comprise from 0.001% to 10%, preferably from 0.01% to 2%, more preferably from 0.05% to 1% by weight of polymeric dye-transfer inhibiting agents. Said polymeric dye-

transfer inhibiting agents are normally incorporated into detergent compositions in order to inhibit the transfer of dyes from colored fabrics onto fabrics washed therewith. These polymers have the ability of complexing or adsorbing the fugitive dyes washed out of dyed fabrics before the dyes have the opportunity to become attached to other articles in the wash.

Especially suitable polymeric dye-transfer inhibiting agents are polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinylpyrrolidone polymers, polyvinylloxazolidones and polyvinylimidazoles or mixtures thereof.

Addition of such polymers also enhances the performance of the enzymes according to the invention.

The detergent composition according to the invention can be in liquid, paste, gels, bars or granular forms.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591.

Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. from 550 to 950 g/l; in such case, the granular detergent compositions according to the present invention will contain a lower amount of "Inorganic filler salt", compared to conventional granular detergents; typical filler salts are alkaline earth metal salts of sulphates and chlorides, typically sodium sulphate; "Compact" detergent typically comprise not more than 10% filler salt. The liquid compositions according to the

present invention can also be in "concentrated form", in such case, the liquid detergent compositions according to the present invention will contain a lower amount of water, compared to conventional liquid detergents. Typically, the water content of the concentrated liquid detergent is less than 30%, more preferably less than 20%, most preferably less than 10% by weight of the detergent compositions.

The compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The following examples are meant to exemplify compositions for the present invention, but are not necessarily meant to limit or otherwise define the scope of the invention. In the detergent compositions, the abbreviated component identifications have the following meanings:

20

LAS: Sodium linear C₁₂ alkyl benzene sulphonate

TAS: Sodium tallow alkyl sulphate

XYAS: Sodium C_{1x} - C_{1y} alkyl sulfate

SS: Secondary soap surfactant of formula 2-butyl

25 octanoic acid

25EY: A C₁₂ - C₁₅ predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

45EY: A C₁₄ - C₁₅ predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

30 XYEZS: C_{1x} - C_{1y} sodium alkyl sulfate condensed with an average of Z moles of ethylene oxide per mole

Nonionic: C₁₃ - C₁₅ mixed ethoxylated/propoxylated fatty alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5 sold under the tradename Plurafax

35 LF404 by BASF GmbH

CFAA: C₁₂ - C₁₄ alkyl N-methyl glucamide

TFAA: C₁₆ - C₁₈ alkyl N-methyl glucamide

Silicate: Amorphous Sodium Silicate (SiO₂:Na₂O ratio = 2.0)

- NaSKS-6: Crystalline layered silicate of formula $d\text{-Na}_2\text{Si}_2\text{O}_5$
Carbonate: Anhydrous sodium carbonate
Phosphate: Sodium tripolyphosphate
MA/AA: Copolymer of 1:4 maleic/acrylic acid, average
5 molecular weight about 80,000
Polyacrylate: Polyacrylate homopolymer with an average
molecular weight of 8,000 sold under the tradename PA30 by BASF
GmbH
Zeolite A: Hydrated Sodium Aluminosilicate of formula
10 $\text{Na}_{12}(\text{AlO}_2\text{SiO}_2)_{12} \cdot 27\text{H}_2\text{O}$ having a primary particle size in the range
from 1 to 10 micrometers
Citrate: Tri-sodium citrate dihydrate
Citric: Citric Acid
Perborate: Anhydrous sodium perborate monohydrate bleach,
15 empirical formula $\text{NaBO}_2 \cdot \text{H}_2\text{O}_2$
PB4: Anhydrous sodium perborate tetrahydrate
Percarbonate: Anhydrous sodium percarbonate bleach of
empirical formula $2\text{Na}_2\text{CO}_3 \cdot 3\text{H}_2\text{O}_2$
TAED: Tetraacetyl ethylene diamine
20 CMC: Sodium carboxymethyl cellulose
DETPMP: Diethylene triamine penta (methylene phosphonic
acid), marketed by Monsanto under the Tradename Dequest 2060
PVP: Polyvinylpyrrolidone polymer
EDDS: Ethylenediamine-N, N'-disuccinic acid, [S,S] isomer
25 in the form of the sodium salt
Suds Suppressor: 25% paraffin wax Mpt 50°C, 17% hydrophobic
silica, 58% paraffin oil
Granular Suds suppressor: 12% Silicone/silica, 18% stearyl
alcohol, 70% starch in granular form
30 Sulphate: Anhydrous sodium sulphate
HMWPEO: High molecular weight polyethylene oxide
TAE 25: Tallow alcohol ethoxylate (25)

Detergent Example I

35

A granular fabric cleaning composition in accordance with the invention may be prepared as follows:

	Sodium linear C ₁₂ alkyl benzene sulfonate	6.5
	Sodium sulfate	15.0
	Zeolite A	26.0
5	Sodium nitrilotriacetate	5.0
	Enzyme of the invention	0.1
	PVP	0.5
	TAED	3.0
	Boric acid	4.0
10	Perborate	18.0
	Phenol sulphonate	0.1
	Minors	Up to 100

Detergent Example II

15 A compact granular fabric cleaning composition (density 800 g/l) in accord with the invention may be prepared as follows:

	45AS	8.0
	25E3S	2.0
20	25E5	3.0
	25E3	3.0
	TFAA	2.5
	Zeolite A	17.0
	NaSKS-6	12.0
25	Citric acid	3.0
	Carbonate	7.0
	MA/AA	5.0
	CMC	0.4
	Enzyme of the invention	0.1
30	TAED	6.0
	Percarbonate	22.0
	EDDS	0.3
	Granular suds suppressor	3.5
	water/minors	Up to 100%

35

Detergent Example III

Granular fabric cleaning compositions in accordance with the invention which are especially useful in the laundering of

coloured fabrics were prepared as follows:

	LAS	10.7	-
	TAS	2.4	-
	TFAA	-	4.0
5	45AS	3.1	10.0
	45E7	4.0	-
	25E3S	-	3.0
	68E11	1.8	-
	25E5	-	8.0
10	Citrate	15.0	7.0
	Carbonate	-	10
	Citric acid	2.5	3.0
	Zeolite A	32.1	25.0
	Na-SKS-6	-	9.0
15	MA/AA	5.0	5.0
	DETPMP	0.2	0.8
	Enzyme of the invention	0.10	0.05
	Silicate	2.5	-
	Sulphate	5.2	3.0
20	PVP	0.5	-
	Poly (4-vinylpyridine)-N-Oxide/copolymer of vinylimidazole and vinylpyrrolidone	-	0.2
25	Perborate	1.0	-
	Phenol sulfonate	0.2	-
	Water/Minors		Up to 100%

Detergent Example IV

30 Granular fabric cleaning compositions in accordance with the invention which provide "Softening through the wash" capability may be prepared as follows:

	45AS	-	10.0
	LAS	7.6	-
35	68AS	1.3	-
	45E7	4.0	-
	25E3	-	5.0
	Coco-alkyl-dimethyl hydroxy-	1.4	1.0

	ethyl ammonium chloride		
	Citrate	5.0	3.0
	Na-SKS-6	-	11.0
	Zeolite A	15.0	15.0
5	MA/AA	4.0	4.0
	DETPMP	0.4	0.4
	Perborate	15.0	-
	Percarbonate	-	15.0
	TAED	5.0	5.0
10	Smectite clay	10.0	10.0
	HMWPEO	-	0.1
	Enzyme of the invention	0.10	0.05
	Silicate	3.0	5.0
	Carbonate	10.0	10.0
15	Granular suds suppressor	1.0	4.0
	CMC	0.2	0.1
	Water/Minors	Up to 100%	

Detergent Example V

20 Heavy duty liquid fabric cleaning compositions in accordance with the invention may be prepared as follows:

	I	II	
	LAS acid form	-	25.0
25	Citric acid	5.0	2.0
	25AS acid form	8.0	-
	25AE2S acid form	3.0	-
	25AE7	8.0	-
	CFAA	5	-
30	DETPMP	1.0	1.0
	Fatty acid	8	-
	Oleic acid	-	1.0
	Ethanol	4.0	6.0
	Propanediol	2.0	6.0
35	Enzyme of the invention	0.10	0.05
	Coco-alkyl dimethyl hydroxy ethyl ammonium chloride	-	3.0

54

Smectite clay	-	5.0
PVP	2.0	-
Water / Minors	Up to 100%	

5 THE XYLOGLUCAN SUBSTRATE

In addition to the aforesaid about xyloglucan it should be noted that xyloglucan from tamarind seeds supplied by Megazyme, Ireland has a complex branched structure with glucose, xylose, galactose and arabinose in the ratio of 45:36:16:3. Accordingly,
10 it is strongly believed that an enzyme showing catalytic activity on this xyloglucan also has catalytic activity on other xyloglucan structures from different sources (angiosperms or gymnosperms).

15 MATERIALS AND METHODS

Strains

Jonesia sp., DSM 14140, producing the xyloglucanase of the invention.

20 Other strains

E. coli hosts: XL1-lue MRF- and XL0LR *E. coli* strains were provided by Stratagene Inc. (USA) and used according to the manufacturer's instructions.

25 Plasmids

PBK-CAMV: Stratagene Inc., La Jolla, California, USA.

Bacteriophage ZAP Express: Stratagene Inc., La Jolla, California, USA.

30 Media

TY (Ausubel, F. M. et al. (Eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

LB agar (Ausubel, F. M. et al. (Eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

35 **LBPG** is LB agar supplemented with 0.5% Glucose and 0.05 M potassium phosphate, pH 7.0

AZCL-xyloglucan is added to LBPG-agar to 0.5 %. AZCL-xyloglucan is from Megazyme, Ireland.

BPX media is described in EP 0 506 780 (WO 91/09129).

NZY agar (per litre) 5 g of NaCl, 2 g of MgSO₄, 5 g of yeast extract, 10 g of NZ amine (casein hydrolysate), 15 g of agar; add de-ionized water to 1 litre, adjust pH with NaOH to pH 7.5 and autoclave

NZY broth (per litre) 5 g of NaCl, 2 g of MgSO₄, 5 g of yeast extract, 10 g of NZ amine (casein hydrolysate); add deionized water to 1 litre, adjust pH with NaOH to pH 7.5 and autoclave

NZY Top Agar (per litre) 5 g of NaCl, 2 g of MgSO₄, 5 g of yeast extract, 10 g of NZ amine (casein hydrolysate), 0.7 % (w/v) agarose; add de-ionized water to 1 litre, adjust pH with NaOH to pH 7.5 and autoclave.

15 **Xyloglucanase assay (XyloU)**

The xyloglucanase activity is measured using AZCL-xyloglucan from Megazyme, Ireland, (<http://www.megazyme.com/purchase/index.html>) as substrate.

A solution of 0.2 % of the blue substrate is suspended in a 0.1 M phosphate buffer pH 7.5 under stirring. The solution is distributed under stirring to 1.5 ml Eppendorf tubes (0.75 ml to each), 50 µl enzyme solution is added and they are incubated in an Eppendorp Thermomixer model 5436 for 20 minutes at 40°C with a mixing of 1200 rpm. After incubation the colored solution is separated from the solid by 4 minutes centrifugation at 14,000 rpm and the absorbance of the supernatant is measured at 600 nm.

One XyloU unit is defined as the amount of enzyme resulting in an absorbance of 0.24 in a 1 cm cuvette at 600 nm.

30 **Isolation of *Jonesia sp.*, DSM 14140**

An amount of marine sediment the size of a small pea was distributed on a minimal agar medium at pH 9 with 1% xyloglucan as C source and 0.025% AZCL-xyloglucan for xyloglucanase detection. The plate was incubated at 30°C for 4 days. A yellow colony with xyloglucanase activity was selected and identified as a *Jonesia sp.* and deposited as DSM 14140.

General molecular biology methods

DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab. Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current
5 protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

10

The following examples illustrate the invention.

EXAMPLE 1

Cloning of xyloglucanase encoding genes from *Jonesia*

15

Genomic DNA preparation

The strain *Jonesia sp.*, DSM 14140, was propagated in liquid TY medium. After 16 hours incubation at 30°C and 300 rpm, the cells were harvested, and genomic DNA was isolated by the method
20 describe by Duffner, F., Fitzsimmons, A., Brophy, G., O'Kiely, P, O'Connell, M. *Journal Appl. Bact.* 76: 583-591 (1994)
"Dominance of *Lactobacillus plantarum* strains in grass silage as demonstrated by a novel competition assay".

25 Genomic library construction

A Lambda ZAP library was prepared from genomic DNA of the strain *Jonesia sp.*, DSM 14140. The ZAP Express cloning kit used was with BamHI digested and dephosphorylated arms from Stratagene. Isolated DNA was partially digested with Sau3A and
30 size fractionated on a 1% agarose gel. DNA was excised from the agarose gel between 3 and 10 Kb and purified using Qiaspin DNA fragment purification procedure (Qiagen GmbH). 100 ng of purified, fractionated DNA was ligated with 1 µg of BamHI dephosphorylated ZAPexpress vector arms (4 degrees overnight).
35 Ligation reaction was packaged directly with GigaPackIII Gold according to the manufacturers instructions (Stratagene). Phage libraries were titered with XL1blue mrf⁻ (Stratagene).

Screening for xyloglucanase clones by functional expression in lambda ZAP Express

Approximately 10,000 plaque-forming units (pfu) from the genomic library were plated on NZY-agar plates containing 0.1 % AZCL-xyloglucan (MegaZyme, Ireland), using *E. coli* XL1-Blue MRF' (Stratagene, USA) as a host, followed by incubation of the plates at 37°C for 24 hours. Xyloglucanase-positive lambda clones were identified by the formation of blue hydrolysis halos around the positive phage clones. These were recovered from the screening plates by coring the TOP-agar slices containing the plaques of interest into 500 µl of SM buffer and 20 µl of chloroform. The xyloglucanase-positive lambda ZAP Express clones were plaque-purified by plating an aliquot of the cored phage stock on NZY plates containing 0.1 % AZCL-xyloglucan as above. Single xyloglucanase-positive lambda clones were cored into 500 µl of SM buffer and 20 µl of chloroform, and purified by one more plating round as described above.

Single-clone *in vivo* excision of the phagemids from the xyloglucanase-positive lambda ZAP Express clone

E. coli XL1-Blue cells (Stratagene, La Jolla Ca.) were prepared and resuspended in 10mM MgSO₄ as recommended by Stratagene (La Jolla, USA). 250µl aliquots of the pure phage stocks from the xyloglucanase-positive clone was combined in Falcon 2059 tubes with 200µl of XL1-Blue MRF' cells (OD₆₀₀=1.0) and >10⁶ pfu/ml of the ExAssist M13 helper phage (Stratagene), and the mixture was incubated at 37°C for 15 minutes. 3 ml of NZY broth was added to the tube and the tube was incubated at 37°C for 2.5 hours. The tube was heated at 65°C for 20 minutes to kill the *E. coli* cells and bacteriophage lambda, the phagemids being resistant to heating. The tube was spun at 3000 rpm for 15 minutes to remove cellular debris and the supernatant was decanted into a clean Falcon 2059 tube. Aliquots of the supernatant containing the excised single-stranded phagemids were used to infect 200µl of *E. coli* XL0LR cells (Stratagene, OD₆₀₀=1.0 in 10mM MgSO₄) by incubation at 37°C for 15 minutes. 350µl of NZY broth was added to the cells and the tubes were

incubated for 45 min at 37°C. Aliquots of the cells were plated onto LB kanamycin agar plates and incubated for 24 hours at 37°C. Single colonies were re-streaked onto LB kanamycin agar plates containing 0.1 % AZCL-xyloglucan (MegaZyme, Australia).

5 The xyloglucanase-positive phagemid clones were characterized by the formation of blue hydrolysis halos around the positive colonies. All were identical and called *E.coli* XYG1020. Plasmid DNA was isolated from *E.coli* XYG1020 using Qiapsin plasmid preparation kit (Qiagen) and designated pXYG1020.

10

Nucleotide sequence analysis

The nucleotide sequence of the genomic xyloglucanase clone was determined from both strands by the dideoxy chain-termination method (Sanger, F., Nicklen, S., and Coulson, A. R. 15 (1977) Proc. Natl. Acad. Sci. USA, 74, 5463-5467) using 500 ng of Qiaspin-purified template (Qiagen, USA), the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labelled terminators and 5 pmol of either pBK-CMV polylinker primers (Stratagene, USA) or custom synthetic oligonucleotide 20 primers. In addition clones containing a transposon which had inserted into the xyloglucanase gene were obtained using the transposon tagging kit (New England Biolabs). DNA sequence assembly was performed with the DNA Star package (www.DNASTAR.com).

25 A large open reading frame with an ATG translational start site was identified in the insert of pXYG1020. The DNA sequence of the ORF is attached as SEQ ID NO: 1. The G+C content of the ORF is 55%. This ORF encodes a protein of 940 amino acids with a predicted molecular mass of 99kDa. The predicted protein 30 sequence is attached as SEQ ID NO: 2. A 23 amino acid signal sequence for secretion was predicted using the bioinformatic programme, Signal P (Nielsen, Henrik, Jacob Engelbrecht, Søren Brunak and Gunnar von Heijne: Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage 35 sites. *Protein Engineering* 10, 1-6 (1997)).

This new xyloglucanase belongs to the glycosyl hydrolase family 74.

EXAMPLE 2**Expression of XYG1020 in *E.coli***

SEQ ID NO: 1 was amplified from *Jonesia sp.*, DSM 14140,
5 genomic DNA by PCR and subcloned into an *E.coli* expression
vector, pSE420 (Invitrogen). Colonies were positive on LB agar
plates containing ampicillin and AZCL-xyloglucan confirming that
this sequence encodes the xyloglucanase.

10 **EXAMPLE 3****Heterologous expression of XYG1020 in *Aspergillus oryzae*****Construction of the xyloglucanase expression cassette for
Aspergillus expression**

15 SEQ ID NO:1 was amplified from *Jonesia sp.* DSM 14140 genomic DNA
by PCR using standard procedures using the primers:
GACTAGCAAAGCTTCACCATGCGTTTACGAACCGTGGTGGCC (SEQ ID NO:4)
GCTAGTCCACCTCGAGGTATGACTAACCTCTAGTCAGCTAGCG (SEQ ID NO:5)
and a PTC-200 Peltier Thermal cycler (MJ Research, USA) and the
20 Expand High Fidelity PCR System (Roche Molecular Biochemicals,
Germany) according to the manufacturer's instructions. Thirty
cycles of PCR was performed using a cycle profile of
denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec,
and extension at 72 °C for 5 min. The amplification products
25 were analyzed by electrophoresis in a 1 % agarose gel, and
subsequently the ca. 2.8 kb XYG1020 PCR fragment was extracted
from the gel using the GFX gel band purification kit (Amersham-
Pharmacia Biotech, Uppsala, Sweden) according to the
manufacturer's instructions. Both the purified PCR fragment and
30 *Aspergillus* expression vector pMStr57 were double digested with
HindIII and XhoI and the PCR fragment was ligated to the vector
using standard methods (Sambrook et al. 1989). *E. coli* strain
DH10B (supplied by Stratagene (La Jolla, Ca.)) was transformed
with the ligation mixture, and transformants were screened for
35 presence of the PCR insert in pMStr57 by restriction
endonuclease analysis.

An expression clone thus identified was designated
pXYG1020-AO, and the sequence of the cloned PCR fragment was

determined to ensure that no errors were introduced during amplification.

Transformation of *Aspergillus oryzae*

5 Transformation of *Aspergillus oryzae* is carried out as described by Christensen et al., (1988), *Biotechnology* 6, 1419-1422. A number of *Aspergillus* transformants are obtained which show a positive reaction on medium containing AZCL-xyloglucan confirming expression in the heterologous host.

10

Expression vector pMStr57

The *Aspergillus oryzae* expression plasmid pMStr57 contains an *Aspergillus* expression cassette based on the *Aspergillus niger* neutral amylase II promoter fused to the *Aspergillus*
 15 *nidulans* triose phosphate isomerase non-translated leader sequence (NA2TPI promoter) and the *Aspergillus niger* amyloglycosidase terminator (T_{amg}). Also present on the plasmid is the *Aspergillus* selective marker *amdS* from *Aspergillus*
 20 *nidulans* enabling growth on acetamide as sole nitrogen source. These elements are cloned into the *E. coli* vector pUC19. pMStr57 is constructed from pCaHj483 (WO 98/00529) by switching out the EcoRI-XhoI promoter-bearing fragment for a modified version of the same promoter. The modifications were introduced by site directed mutagenesis to the sequence shown in SEQ ID
 25 NO:3.

Table 2. List of main features in plasmid pMSTR57 (overall size in 6795bp)

FEATURE	Relative Position (nucleotide)
<i>amdS</i> , selective marker	1781-4505 (complementary)
<i>ampR</i> , selective marker	5741-6598 (complementary)
NA2TPI promoter	428-1041
<i>colE1</i> origin	4979
AMG terminator	1083-1780
RESTRICTION ENZYME SITE	
BamHI	1043

EcoRI	423
HindIII	1063
XbaI	1083
XbaI	1780
XbaI	4505
XhoI	1076

EXAMPLE 4**Detergent stability**

5 Agar plugs were taken in triplicate from center of xyloglucanase positive colonies of *Jonesia sp.*, DSM 14140, applied to three sets of plates and embedded in 1% agarose in $\frac{1}{2}$ strength tap water with 0.025% AZCL-xyloglucan as reference and either of the commercial laundry detergents Ariel Futur powder
10 (dosage: 6.7 g/l) or Tide powder (dosage: 1 g/l) added in the test plates. The solidified plates were incubated overnight at 37 °C, and the detergent stability was evaluated by visual comparison of the blue zones around the agar plugs in the detergent plates with the reference. The activity following
15 incubation in the presence of each of the commercial laundry detergents was similar to the activity of the reference, thus demonstrating full stability of the xyloglucanase of the invention in presence of conventional, commercial laundry detergent compositions.

20

EXAMPLE 5**Wash Evaluation of Xyloglucanases****Wash Example I**

25 The following provides general guidelines on how to perform a wash evaluation of detergent compositions containing xyloglucanases.

Preparation of tamarind gum swatches

30 5 l ionized water is heated to 50°C. 10 g Tamarind seed polysaccharide is added slowly during agitation. The suspension is agitated until the polymer is solubilized. Clean cotton

swatches (style 400 from Testfabrics Inc.) are placed in the solution for 30 min, squeezed between two rolls and dried overnight. The swatches are rinsed in an EU wash machine, dried overnight and cut into 5 cm x 5 cm pieces.

5

Preparation of dinginess swatches

Old white cotton fabrics are cut into 5 cm x 5 cm pieces.

Wash in Launder-o-meter

10 Each beaker (500 ml) is added steel balls, 200 ml detergent solution and 6-12 dinginess or tamarind gum swatches. The wash cycle is conducted and the swatches are rinsed in tap water and air dried overnight at room temperature.

15 Iron oxide soiling

The following mixture is prepared and stirred for 30 min:

5,25g Fe₂O₃ (Merck 3924)

800 ml ionised water

42 ml Na₂CO₃ (5g/l)

20 The swatches and iron oxide solution is placed in a Japanese washing machine (NA-F38A1M) using the programme "Large SPEEDY" (42 l water, 3 min wash, 1 rinse, 3 min centrifuging). The swatches are air dried overnight at room temperature.

25 Evaluation

Remission of the swatches is measured at 440 nm using a MacBeth ColorEye 7000 remission spectrophotometer. The results are expressed as Delta remission = $R_{\text{enzyme, after soiling}} - R_{\text{control, after soiling}}$, where R is the remission at 440 nm.

30

Data

The effect on the tamarind gum swatches expresses the effect of the xyloglucanase during wash on tamarind gum containing stains e.g. food stains, whereas the effect on the
35 dinginess swatches expresses the general cleaning effect of the xyloglucanase during wash.

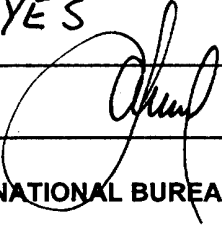
PCT

10153.204-WO

Original (for SUBMISSION) - printed on 26.03.2002 03:30:33 PM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	10153.204-WO
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	4
1-2	line	31-37
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	23 February 2001 (23.02.2001)
1-3-4	Accession Number	DSMZ 14140
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	

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0-5-1	Authorized officer	

CLAIMS

1. A xyloglucanase enzyme belonging to family 74 of glycosyl hydrolases, which enzyme is endogenous to a bacterium.
- 5 2. The xyloglucanase enzyme of claim 1, wherein the bacterium comprises alkalophilic bacteria.
3. The xyloglucanase enzyme according to any of the preceding
10 claims, wherein the bacterium belongs to the genus *Jonesia*.
4. The xyloglucanase enzyme according to any of the preceding claims, wherein the strain is *Jonesia sp.*, DSM 14140.
- 15 5. A xyloglucanase, which is selected from one of
 - (a) a polypeptide encoded by the DNA sequence of positions 72-2820 of SEQ ID NO: 1;
 - (b) a polypeptide produced by culturing a cell comprising the sequence of positions 72-2820 of SEQ ID NO: 1 under conditions
20 wherein the DNA sequence is expressed;
 - (c) a xyloglucanase enzyme having a sequence of at least 60% identity to positions 24-940 of SEQ ID NO: 2 when identity is determined by the Clustal method with a PAM250 residue table and the default settings of the Megalign program in the Lasergene
25 package using a GAP creation penalty of 3 and Ktuple of 1; and
 - (d) a polypeptide encoded by a DNA sequence that hybridizes to the DNA sequence of SEQ ID NO: 1 under medium stringency conditions, wherein the medium stringency conditions comprise hybridization in 5xSSC at 45°C and washing in 2xSSC at 60°C.
- 30 6. The xyloglucanase according to claim 5 which is obtained or obtainable from a bacterium, preferably from a strain belonging to the genus *Jonesia*, preferably from *Jonesia sp.*, DSM 14140.
- 35 7. The xyloglucanase according to any of the claims 5 and 6, wherein the xyloglucanase is comprised in family 74 of glycosyl hydrolases.

8. The xyloglucanase of claim 5, which enzyme is isolated and free from homologous impurities.

9. An enzyme preparation comprising the enzyme according to any
5 of the claims 1-8, and conventional fillers or stabilizers.

10. The preparation according to claim 9 which further comprises one or more enzymes selected from the group consisting of proteases, cellulases (endoglucanases), β -glucanases,
10 hemicellulases, lipases, peroxidases, laccases, α -amylases, glucoamylases, cutinases, pectinases, reductases, oxidases, phenoloxidases, ligninases, pullulanases, pectate lyases, other xyloglucanases not belonging to family 74, xylanases, pectin acetyl esterases, polygalacturonases, rhamnogalacturonases,
15 pectin lyases, other mannanases, pectin methylesterases, cellobiohydrolases, transglutaminases; or mixtures thereof.

11. An isolated polynucleotide molecule encoding a polypeptide having xyloglucanase activity which polynucleotide molecule
20 hybridizes to a denatured double-stranded DNA probe under medium stringency conditions, wherein the probe is selected from the group consisting of DNA probes comprising the sequence shown in positions 72-2820 of SEQ ID NO: 1, and DNA probes comprising a subsequence of positions 72-2820 of SEQ ID NO: 1, the
25 subsequence having a length of at least about 100 base pairs.

12. An expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment selected from the group consisting of (a) polynucleotide
30 molecules encoding a polypeptide having xyloglucanase activity comprising a nucleotide sequence as shown in SEQ ID NO: 1 from nucleotide 72 to nucleotide 2820, (b) polynucleotide molecules encoding a polypeptide having xyloglucanase activity that is at least 60% identical to the amino acid sequence of SEQ ID NO: 2
35 from amino acid residue 24 to amino acid residue 940, and (c) degenerate nucleotide sequences of (a) or (b); and a transcription terminator.

13. A cultured cell into which has been introduced an expression vector according to claim 12, wherein said cell expresses the polypeptide encoded by the DNA segment.

5 14. A method of producing a polypeptide having xyloglucanase activity, which method comprises culturing a cell into which has been introduced an expression vector according to claim 12, whereby said cell expresses a polypeptide encoded by the DNA segment; and recovering the polypeptide.

10

15. A detergent composition comprising the enzyme preparation according to claim 9 or the enzyme according to any of the claims 1-8.

15

16. A process for machine treatment of fabrics which process comprises treating fabric during a washing cycle of a machine washing process with a washing solution containing the enzyme preparation according to claim 9 or the enzyme according to any of the claims 1-8.

20

17. Use of the enzyme preparation according to claim 9 or the enzyme according to any of the claims 1-8 in the textile industry for improving the properties of cellulosic fibers, yarn, woven or non-woven fabric.

25

18. The use according to claim 17, wherein the enzyme preparation or the enzyme is used in a textile scouring process step.

30

19. Use of the enzyme preparation according to claim 9 or the enzyme according to any of the claims 1-8 in the cellulose fiber processing industry for ratting of fibers selected from the group consisting of hemp, jute, flax and linen.

SEQUENCE LISTING

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 Val Asn Ala Asn Thr Leu Tyr Gly Phe Val Asp Gly Lys Phe Tyr His
 590 595 600
 Ser Thr Asn Gly Gly Ala Ser Phe Thr Ala Ser Ala Phe Thr Gly Phe
 605 610 615
 Pro Thr Ser Gly Asn Val Arg Phe Arg Ala Val Pro Gly Arg Gln Gly
 620 625 630
 His Leu Trp Leu Ala Gly Gly Val Ser Gly Ser Thr Tyr Gly Met Trp
 635 640 645
 Arg Ser Thr Asp Gly Gly Lys Asn Trp Thr Lys Val Ser Gly Val Gln
 650 655 660 665
 Glu Gly Asp Ala Val Gly Phe Gly Lys Ala Thr Ser Ser Ser Gly Tyr
 670 675 680
 Pro Val Ile Phe Thr Ser Ala Lys Ile Asp Gly Val Arg Gly Ile Phe
 685 690 695
 Arg Ser Asp Asp Glu Gly Lys Thr Trp Lys Arg Ile Asn Asp Asn His
 700 705 710
 Asn Gln Trp Tyr Trp Thr Gly Ala Ser Ile Thr Gly Asp Pro Asp Val
 715 720 725
 Glu Gly Arg Val Tyr Ile Gly Thr Asn Gly Arg Gly Ile Ile Val Gly
 730 735 740 745
 Asp Ser Ser Thr Pro Pro Pro Pro Gly Gln Asp Ser Gly Ser Ala Ser
 750 755 760
 Gly Asn Thr Gly Ala Thr Pro Thr Pro Glu Thr Pro Asp Ser Asp His

	765		770		775
Asn Thr Gly Gly Ser Thr Asp Thr Gly Glu Thr Ser Glu Pro Gly Thr					
	780		785		790
Gly Ser Gly Ser Gly Thr Ala Pro Asp Ser Gly Asn Thr Ser Pro Gly					
	795		800		805
Asn Thr Ser Ser Cys Lys Val Arg Tyr Ser Thr Thr Asp Trp Gly Ser					
810		815		820	825
Gly Phe Thr Gly Ala Ala Thr Ile Thr Asn Thr Ser Pro Ser Pro Ile					
		830		835	840
Asn Gly Trp Thr Leu Ala Phe Thr Tyr Pro Ser Gly Gln Thr Ile Ser					
		845		850	855
Ser Val Trp Ser Ala Thr Gln Thr Leu Ser Gly Arg Asn Val Val Leu					
	860		865		870
Lys Asn Ser Gly Trp Asn Pro Thr Ile Pro Ala Gly Gly Ser Val Glu					
	875		880		885
Phe Gly Phe Asn Gly Ala Tyr Thr Ser Lys Asn Thr Pro Pro Thr Ser					
890		895		900	905
Phe Ser Leu Asn Gly Val Thr Cys Ala Leu Ala Asp					
		910		915	

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 <212> DNA
 <213> Artificial sequence

<220>
 <223> Modified NA2TPI promoter

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 gatgcaagac caaacggtta aatttccgga gtcaacagca tccaagccca agtccttcac 180
 ggagaaacc cagcgtccac atcacgagcg aaggaccacc tctagggcatc ggacgcacca 240
 tccaattaga agcagcaaag cgaacagcc caagaaaaag gtcggcccgt cggccttttc 300
 tgcaacgctg atcacgggca gcgatccaac caacaccctc cagagtgact aggggcggaa 360
 atttatcggg attaatttcc actcaaccac aatcacagt cgtccccggt aatttaacgg 420
 ctgcagacgg caatttaacg gcttctgcga atcgcttggga ttccccgccc ctggccgtag 480
 agcttaaagt atgtcccttg tcgatgcat gtatcacaac atataaatac tggcaagggg 540
 tgccatgctt ggagtttcca actcaattta cctctatcca cacttctctt ccttctcaa 600
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<212> DNA
<213> Artificial sequence

<220>
<223> PCR primer

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<210> 5
<211> 43
<212> DNA
<213> Artificial sequence

<220>
<223> PCR primer

<400> 5
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