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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/42

A1

(11) International Publication Number: **WO 98/08940**

(43) International Publication Date: 5 March 1998 (05.03.98)

(21) International Application Number: PCT/DK97/00348

(22) International Filing Date: 26 August 1997 (26.08.97)

(30) Priority Data:

0893/96	26 August 1996 (26.08.96)	DK
1015/96	17 September 1996 (17.09.96)	DK

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A NOVEL ENDOGLUCANASE

Thr	Arg	Xaa	Xaa	Asp	Cys	Cys	Xaa	Xaa	Xaa	Cys	Xaa	Trp	Xaa	Xaa
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

(a)

Ala	Xaa	Gly	Xaa	Xaa	Ala
1	2	3	4	5	6

(b)

(57) Abstract

An enzyme preparation consisting essentially of an endo-β-1,4-glucanase (EC 3.2.1.4) derived from the bacterial genera *Cellvibrio mixtus* or *Cellvibrio gilvus* may be produced by recombinant techniques using a cloned DNA sequence encoding the enzyme, e.g. SEQ ID NO:1. The endo-β-1,4-glucanase which has two conserved regions, a first amino acid sequence consisting of 15 amino acid residues having sequence (a) and a second amino acid sequence consisting of 6 amino acid residues having sequence (b) is useful in industrial application conventionally using cellulolytic enzymes.

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A NOVEL ENDOGLUCANASE

FIELD OF INVENTION

The present invention relates to an enzyme with cellulolytic activity, especially an endoglucanase; a cloned DNA sequence encoding the enzyme with cellulolytic activity; a method for providing a gene encoding such an enzyme; a method of producing the enzyme; an enzyme composition comprising the enzyme with cellulolytic activity; and the use of said enzyme and enzyme composition for a number of industrial applications.

BACKGROUND OF THE INVENTION

Cellulose is a polymer of glucose linked by β-1,4-glucosidic bonds. Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which result in the formation of insoluble cellulose microfibrils. Microbial hydrolysis of cellulose to glucose involves the following three major classes of cellulases: (i) endoglucanases (EC 3.2.1.4) which cleave β-1,4-glucosidic links randomly throughout cellulose molecules; (ii) cellobiohydrolases (EC 3.2.1.91) which digest cellulose from the nonreducing end, releasing cellobiose; and (iii) β-glucosidases (EC 3.2.1.21) which hydrolyse cellobiose and low-molecular-mass cellodextrins to release glucose. Cellulases are produced by many microorganisms and are often present in multiple forms. Recognition of the economic significance of the enzymatic degradation of cellulose has promoted an extensive search for microbial cellulases which can be used industrially. As a result, the enzymatic properties and the primary structures of a large number of cellulase have been investigated. On the basis of the results of a hydrophobic cluster analysis of the amino acid sequence of the catalytic domain, these cellulases have been placed into different families of glycosyl hydrolases; fungal and bacterial glycosyl hydrolases have been grouped into 35 families (Henrissat et. al. (1991), (1993)). Most cellulases consist of a cellulose-binding domain (CBD) and a catlytic

domain (CAD) separated by a linker which may be rich in proline and hydroxy amino residues. Another classification of cellulases has been established on the basis of the similarity of their CBDs (Gilkes et al. (1991)) giving five families of glycosyl hydrolases (I-V).

Cellulases are synthesized by a large number of microorganisms which include fungi, actinomycetes, myxobacteria and true bacteria but also by plants. Especially endo-β-1,4-glucanases of a wide variety of specificities have been identified. Many bacterial endoglucanases have been described (Henrissat (1993); Gilbert et al.,(1993)), and endoglucanases of a wide variety of specificities have been identified. *Cellvibrio mixtus* is described as a cellulolytic bacterium (Blackall, L.L. et al. (1985) Journal of Applied Bacteriology **59**:81-97). Many bacterial endoglucanases have been described (Henrissat, B. and Bairoch, A. (1993) Biochem J. **293**:781-788; Gilbert, H.J. and Hazlewood, G.P. (1993) J. Gen. Microbiol. **139**:187-194). The cloning of an endoglucanase from *Pseudomonas fluorescens* subsp. *cellulosa* was described by Gilbert, H.J. et al. in Mol. Microbiol., 4, (1990), p.759-767. However, Millward-Sadler, S.J. et al., Biochem. J. 312, p. 39-48, (1995) describes that probing with previously cloned *Pseudomonas* cellulase and hemicellulase genes (xynA, xynB, xynC, xynD, celA, celB, celC and celD) did not reveal any homologous sequences in *Cellvibrio mixtus* genomic DNA. US patent 4,908,311 discloses a crude cellulase enzyme obtained from cultivation of *Cellvibrio gilvus*, ATCC 13127.

A very important industrial use of cellulolytic enzymes is the use for treatment of cellulosic textile or fabric, e.g. as ingredients in detergent compositions or fabric softener compositions, for bio-polishing of new fabric (garment finishing), and for obtaining a "stone-washed" look of cellulose-containing fabric, especially denim, and several methods for such treatment have been suggested, e.g. in GB-A-1 368 599, EP-A-0 307 564 and EP-A-0 435 876, WO 91/17243, WO 91/10732, WO 91/17244, PCT/DK95/000108 and PCT/DK95/00132. Another important industrial use of cellulolytic enzymes is the use for treatment of paper pulp, e.g. for improving the

drainage or for deinking of recycled paper.

It is also known that cellulases may or may not have a cellulose binding domain (a CBD). The CBD enhances the binding of the enzyme to a cellulose-containing fiber and increases the efficacy of the catalytic active part of the enzyme.

There is an ever existing need for providing novel cellulase enzyme preparations which may be used for applications where cellulase, preferably an endoglucanase, activity is desirable.

The object of the present invention is to provide novel enzyme compositions having substantial cellulolytic activity at acid, neutral or alkaline conditions and improved performance in paper pulp processing, textile treatment, laundry processes or in animal feed; preferably novel cellulases, more preferably well-performing endoglucanases, which can be produced by recombinant techniques.

SUMMARY OF THE INVENTION

The inventors have now succeeded in cloning and characterizing DNA sequences from certain bacterial species of the genus *Cellvibrio* which encode an enzyme exhibiting cellulolytic activity, thereby making it possible to prepare a mono-component bacterial cellulase enzyme composition with desirable properties.

Further, it has been found that these enzymes are endo-β-1,4-glucanases (EC 3.2.1.4) which exhibit good performance in many industrial applications, these endoglucanases possessing two conserved regions consisting of 15 and 6 amino acid residues, respectively.

Accordingly, in a first aspect the present invention relates to a bacterial enzyme preparation consisting essentially of an enzyme having cellulolytic activity and comprising a first amino acid sequence consisting of 15 amino acid residues having the following sequence

Thr Arg Xaa Xaa Asp Cys Cys Xaa Xaa Xaa Cys Xaa Trp Xaa Xaa

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

and a second amino acid sequence consisting of 6 amino acid residues having the following sequence

Ala Xaa Gly Xaa Xaa Ala
1 2 3 4 5 6

wherein, in position 3 of the first sequence, the amino acid is Trp, Tyr or Phe; in position 4 of the first sequence, the amino acid is Trp, Tyr or Phe; in position 8 of the first sequence, the amino acid is Arg, Lys or His; in position 9, 10, 12 and 14, respectively, of the first sequence, the amino acid is any of the 20 naturally occurring amino acid residues; in position 15 of the first sequence, the amino acid is any of the 20 naturally occurring amino acid residues except Ala; in position 4 of the second sequence, the amino acid is Phe or Tyr; and in position 2 and 5, respectively, of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues.

More specifically, in a second aspect the invention relates to an enzyme preparation consisting essentially of an enzyme having cellulolytic activity and obtained or being obtainable from a bacterial strain belonging to the genus *Cellvibrio,* preferably to the group consisting of the species *Cellvibrio mixtus* and *Cellvibrio gilvus,* more preferably to the group consisting of the strains *Cellvibrio mixtus*, DSM 11683, *Cellvibrio mixtus*, DSM 11684, *Cellvibrio mixtus*, DSM 11685, *Cellvibrio mixtus*, ACM 2601, *Cellvibrio mixtus*, DSM 1523, and *Cellvibrio gilvus*, DSM 11686, which enzyme comprises an amino acid sequence selected from the group consisting of the sequences

Thr Arg Xaa Phe Asp Cys Cys
1 2 3 4 5 6 7 ;

Thr Arg Xaa Tyr Asp Cys Cys
1 2 3 4 5 6 7 ; and

Thr Arg Xaa Trp Asp Cys Cys
1 2 3 4 5 6 7

wherein, in position 3, the amino acid is Trp, Tyr or Phe.

In a third aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in *Escherichia coli* DSM 11143, or

b) the DNA sequence shown in SEQ ID NO 1, or

c) an analogue of the DNA sequence which

i) is at least 75% homologous with the DNA sequence shown in SEQ ID NO 1, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 1, or

iii) encodes a polypeptide which is at least 75% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 1, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA seqence shown in SEQ ID NO 1.

In a fourth aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11683, or

b) the DNA sequence shown in SEQ ID NO 3, or

c) an analogue of the DNA sequence which

i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 3, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 3, or

iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 3, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 3.

In a fifth aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11685, or

b) the DNA sequence shown in SEQ ID NO 5, or

c) an analogue of the DNA sequence which

i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 5, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 5, or

iii) encodes a polypeptide which is at least 85% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 5, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 5.

In a sixth aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11685, or

b) the DNA sequence shown in SEQ ID NO 7, or

c) an analogue of the DNA sequence which

i) is at least 75% homologous with the DNA sequence shown in SEQ ID NO 7, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 7, or

iii) encodes a polypeptide which is at least 80% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 7, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 7.

In a seventh aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11684, or

b) the DNA sequence shown in SEQ ID NO 9, or

c) an analogue of the DNA sequence which

i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 9, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 9, or

iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 9, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 9.

In a eighth aspect, the invention relates to a cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio gilvus,* DSM 11686, or

b) the DNA sequence shown in SEQ ID NO 11, or

c) an analogue of the DNA sequence which

i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 11, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 11, or

iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 11, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 11.

The cloned first DNA sequence of the invention may further comprise a DNA sequence encoding one, two or more cellulose-binding domains (CBDs), each cellulose-binding domain and enzyme core (catalytically active domain, CAD) of the enzyme encoded by the DNA sequence preferably being operably linked.

In further aspects the invention provides an expression vector harbouring the cloned DNA sequence of the invention, a cell comprising the cloned DNA sequence or the expression vector and a method of producing an enzyme exhibiting cellulolytic activity, 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 enzyme exhibiting cellulolytic activity, characterized by (i) being free from homologous impurities, and (ii) the enzyme is produced by the method described above.

The invention further relates to an isolated enzyme exhibiting cellulolytic activity, preferably an endoglucanase, which is a polypeptide comprising a full or partial amino acid sequence as shown in any of the SEQ ID Nos. 2, 4, 6, 8, 10 or 12.

Further, the present invention relates to an enzyme or an enzyme composition and the use of such an enzyme or an enzyme composition of the invention for various industrial applications.

The invention also relates to an isolated substantially pure biological culture of the *Escherichia coli* strain DSM No. 11143 harbouring a cellulase encoding DNA sequence (the cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in *Escherichia coli* DSM 11143) derived from a strain of the bacterial *Cellvibrio mixtus*, or any mutant of said *E.coli* strain.

DETAILED DESCRIPTION OF THE INVENTION

In the present context, the term "the 20 naturally occurring amino acid residues" denotes the 20 amino acid residues usually found in proteins and conventionally known as alanine (Ala or A), valine (Val or V), leucine (Leu or L), isoleucine (Ile or I), proline (Pro or P), phenylalanine (Phe or F), tryptophan (Trp or W), methionine (Met or M), glycine (Gly or G), serine (Ser or S), threonine (Thr or T), cysteine (Cys or C), tyrosine (Tyr or Y), asparagine (Asn or N), glutamine (Gln or Q), aspartic acid (Asp or D), glutamic acid (Glu or E), lysine (Lys or K), arginine (Arg or R), and histidine (His or H).

In preferred embodiments of the present invention, the cellulase enzyme possesses in position 9 of the first conserved amino acid sequence an amino acid residue selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of proline and threonine; and/or, in position 10 of the first sequence, an amino acid residue selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, histidine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably serine or histidine; and/or, in position 12 of the first sequence, an amino acid residue selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of alanine and glycine; and/or, in position 14 of the first sequence, an amino acid residue selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, preferably from the group consisting of asparagine, proline, threonine, serine, alanine, glutamic acid and aspartic acid; and/or, in position 2 of the second

sequence, an amino acid residue selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, preferably from the group consisting of tyrosine, leucine, and phenylalanine, more preferably tyrosine. A specific example is an enzyme having, in the first sequence, tyrosine in position 3; or tryptophan in position 4; or lysine in position 8.

The cellulase encoding DNA sequence harboured in *E. coli* DSM 11143 is believed to comprise the sequence presented in SEQ ID No 1. Accordingly, in this specification and claims, whenever reference is made to the DNA sequence disclosed in SEQ ID NO 1 such reference is also intended to include the corresponding part of the DNA sequence cloned into plasmid pSJ1678 present in DSM 11143.

In the present context the expression "a cloned DNA sequence", either partial or complete, refers to a DNA sequence cloned by standard cloning procedure used in genetic engineering to relocate a segment of DNA from its natural location to a different site where it will be reproduced. The cloning process involves excision and isolation of the desired DNA segment, insertion of the piece of DNA into the vector molecule and incorporation of the recombinant vector into a cell where multiple copies or clones of the DNA segment will be replicated.

The "cloned DNA sequence" of the invention may alternatively be termed "DNA construct" or "isolated DNA sequence".

The DNA sequence may be of genomic, cDNA, or synthetic origin or any combinations of these.

The cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in *Escherichia coli* DSM 11143 and/or an analogue DNA sequence of the invention may be cloned from a strain of the bacterial species *Cellvibrio mixtus*, preferably the strain DSM 1523, producing the enzyme with cellulase, preferably endo-β-1,4-glucanase, activity, or another or related organism as described further below.

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as SEQ ID No. 1 or the DNA sequence obtainable from the plasmid present in *Escherichia coli* DSM 11143, *e.g* being a sub-sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the cellulase encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence (*i.e.* a variant of the cellulase of the invention).

When carrying out nucleotide substitutions, amino acid changes are preferably of a minor nature, i.e. conservative amino acid substitutions which do not significantly affect the folding or the enzymatic activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see *e.g*. Ford et al., (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the cloned DNA sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known

in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. *e.g.* Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (*i.e.* cellulolytic) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (cf. *e.g.* de Vos et al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

The endoglucanase encoded by the DNA sequence of the DNA construct of the invention may comprise a cellulose binding domain (CBD) existing as an integral part of the encoded enzyme, or a CBD from another origin may be introduced into the endoglucanase thus creating an enzyme hybride.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 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 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, 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 without a linker, to a DNA sequence encoding

the endoglucanase 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 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 DNA sequence of the invention.

The DNA sequence of the present invention can be cloned from the strain *Escherichia coli* DSM No. 11143 using standard methods *e.g.* as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be cloned by any general method involving

- cloning, in suitable vectors, a DNA library from any organism, e.g. *Cellvibrio* mixtus or *Cellvibrio gilvus*, expected to produce the endoglucanase of interest,
- transforming suitable host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any cellulolytic activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

Alternatively, the DNA encoding a cellulase of the invention may, in accordance with well-known procedures, conveniently be cloned from a suitable source, such as any of the below mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of the DNA sequence disclosed in any of the appended DNA sequence listings SEQ ID Nos. 1, 3, 5, 7, 9, and 11, e.g. the primers

disclosed below under Material and Methods. For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequence presented as SEQ ID No. 1 or any suitable subsequence thereof, or based on the amino acid sequence shown in SEQ ID No. 2.

The DNA sequence of SEQ ID No. 5 encoding a cellulase of the invention may conveniently be cloned from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*. In a preferred embodiment, the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, DSM 11685.

The DNA sequence of SEQ ID No. 7 encoding a cellulase of the invention may conveniently be cloned from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*. In a preferred embodiment, the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, ACM 2601.

The DNA sequence of SEQ ID No. 9 encoding a cellulase of the invention may conveniently be cloned from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*. In a preferred embodiment, the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, DSM 11684.

The DNA sequence of SEQ ID No. 11 encoding a cellulase of the invention may conveniently be cloned from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*. In a preferred embodiment, the DNA sequence is obtained from a strain of *Cellvibrio gilvus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio gilvus*, DSM 11686.

Homology of (partial) DNA sequences

A homology search with the DNA sequence presented as SEQ ID No. 1 and the derived amino acid sequence shown in SEQ ID No. 2 against nucleotide and protein databases was performed. The homology search showed that the most closely related endoglucanase was an endoglucanase from *Pseudomonas fluorescens ssp. cellulosa*, N.C.I.M.B. 10462 (GenBank acc. no. X52615, P18126), to which gene the DNA sequence shown in SEQ ID NO 1 shows 73% identity, and to which the corresponding amino acid sequence (SEQ ID No. 2) shows 70% identity.

Correspondingly, the DNA sequence shown in SEQ ID NO 3 shows 76% identity, and to which the corresponding amino acid sequence (SEQ ID No. 4) shows 84% identity; the DNA sequence shown in SEQ ID NO 5 shows 75% identity, and to which the corresponding amino acid sequence (SEQ ID No. 6) shows 83% identity; the DNA sequence shown in SEQ ID NO 7 shows 72% identity, and to which the corresponding amino acid sequence (SEQ ID No. 8) shows 74% identity; the DNA sequence shown in SEQ ID NO 9 shows 76% identity, and to which the corresponding amino acid sequence (SEQ ID No. 10) shows 85% identity; and the DNA sequence shown in SEQ ID NO 11 shows 76% identity, and to which the corresponding amino acid sequence (SEQ ID No. 12) shows 84% identity.

This demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

The DNA sequence homology referred to herein is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as using FASTA of the GCG package using the following settings: Scoring matrix: GenRunData:blosum50.cmp, Variable pamfactor used Gap creation penalty: 12, Gap extension penalty: 2. provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using FASTA with the above settings for DNA sequence comparison, the DNA sequence of the invention exhibiting a degree of identity of

at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the DNA sequence shown in SEQ ID No. 1.

Hybridization

The hybridization referred to above is intended to indicate that the analogous (partial) DNA sequence hybridizes to an oligonucleotide probe corresponding to the DNA sequence shown in SEQ ID NO 1, 3, 5, 7, 8, and 11, respectively, under certain specified conditions which are described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 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 random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at preferably at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at least 70°C, especially at least 75°C.

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

Homology to amino acid sequences

The protein or polypeptide homology referred to herein is determined as the degree of identity between the two proteins indicating a derivation of the first protein from the second. The homology may suitably be determined by means of computer programs known in the art such as using FASTA of the GCG package using the following settings: Scoring matrix:

GenRunData:blosum50.cmp, Variable pamfactor used Gap creation penalty: 12, Gap extension penalty: 2. provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using FASTA with the above settings for protein comparison, the polypeptide encoded by an analogous (partial) DNA sequence exhibits a degree of identity of at least 75%, preferably at least 80%, more preferably of at least 85%, more preferably at least 90%, more preferably at least 95%, especially at least 97% with the polypeptide encoded by the DNA sequence shown in SEQ ID No. 1, e.g. with the amino acid sequence SEQ ID NO 2.

Immunological cross-reactivity

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified cellulolytic enzyme. More specifically, antiserum against the endoglucanase 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 Immunoelectrophoresis, 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 ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Microbial Sources

The taxonomy applied below is in accordance with the Entrez browser NCBI taxonomy version 3.3, (updated 13.12.95).

For the purpose of the present invention the term

"obtained from" or "obtainable from", as used herein in connection with a specific source, 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 has been inserted.

The cellulase of the invention is obtained from a bacterium, in particular a gram-negative or purple bacterium, especially from the gamma subdivision, in particular the genus *Cellvibrio*. In a preferred embodiment, the cellulase of the invention is obtained from the strain *Cellvibrio mixtus* or *Cellvibrio gilvus*.

An isolate of a strain of *Cellvibrio mixtus* from which a cellulase of the invention can be derived is publicly available from strain collections, e.g. from Deutsche Sammlung von Mikroorganismen, DSM 1523; American Type Culture Collection, ATCC 12120; NCIB 8634, UQM 1224; or Australian Collection of Microorganisms (University of Queensland, QLD 4072, Australia), ACM 2601. Further, isolates of a strain of *Cellvibrio mixtus* have been 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 und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 18 August 1997 under the deposition numbers DSM 11683, DSM 11684, and DSM 11685, respectively; and an isolate of a strain of *Cellvibrio gilvus* has been 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 und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 18 August 1997 under the deposition number DSM 11686.

Further, isolates of *Pseudomonas fluorescens* and *Pseudomonas cepacia* have been 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 und Zellkulturen GmbH, Mascheroder Weg 1b, D-

38124 Braunschweig, Federal Republic of Germany, on 18 August 1997 under the deposition numbers DSM 11681 and DSM 11682, respectively, cf. the examples.

The full 16S rDNA sequences of *Cellvibrio mixtus* and *Pseudomononas cellulosa* were determined. The sequences were compared to the ribosomal database and Phylogenetic dendrograms with the closest relatives were constructed (Bonnie L. Maidak, Niels Larsen, Michael J. McCaughey, Ross Overbeek, Gary J. Olsen, Karl Fogel, James Blandy and Carl R. Woese, Nucleic Acids Research, 1994, Vol. 22, No. 17, p. 3485-3487, The Ribosomal Database Project).

The closest relative to *Cellvibrio mixtus* was a *Pseudomonas* sp. incorrectly described as *Flavobacterium lutescens*. The next relatives were *Pseudomonas aeroginosa*, *Pseudomonas flavescens* and *Pseudomonas cellulosa*. However, *Cellvibrio mixtus* is definitely a new species within the *Pseudomonas sensu* strictu group.

Further, the plasmid pSJ1678 comprising the DNA sequence encoding the endoglucanase of the invention has been transformed into a strain of the *Escherichia coli* which 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 und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 12 September 1996 under the deposition number DSM 11143. Likewise, the plasmid MB275-2 (pBLUESCRIPT II KS minus containing an insert of approximately 400 basepairs, cf. example 1A) comprising a partial DNA sequence (corresponding to the positions 865-1260 of SEQ ID NO: 1) partially encoding the endoglucanase of the invention has been transformed into a strain of the *Escherichia coli* which was deposited 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 und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany, on 22 August 1996 under the deposition number DSM 11120.

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 which exists as an extrachromosomal 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 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 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. 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 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, the *Bacillus subtilis* alkaline protease gen, 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 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 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 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 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 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.).

Host cells

The DNA sequence encoding the present enzyme 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 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 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 DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of producing the present enzyme and includes bacteria, yeast, fungi and higher eukaryotic cells.

Examples of bacterial host cells which, on cultivation, are capable of producing the enzyme of the invention are gram-positive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megatherium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Echerichia 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. Sambrook et al., supra).

When expressing the enzyme in bacteria such as *E. 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 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 release the contents of the periplasmic space and recovering the enzyme.

When expressing the enzyme in gram-positive bacteria such as *Bacillus* or *Streptomyces* strains, the enzyme may be retained in the cytoplasm, or may be directed to the extracellular medium by a bacterial secretion sequence. In the latter case, the enzyme may be recovered from the medium as described below.

Method of producing a cellulolytic enzyme

The present invention provides a method of 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.

As defined herein, an isolated polypeptide (e.g. an enzyme) is a polypeptide which is essentially free of other polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

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

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 monocomponent cellulolytic composition, characterized in being free from homologous impurities.

In this context homologous impurities mean any impurities (e.g. other polypeptides than the enzyme of the invention) which originate from the homologous cell, from which the enzyme of the invention is originally obtained.

In the present invention the homologous host cell may be a strain of *Cellvibrio mixtus* or *Cellvibrio gilvus*. Useful examples are the strains *Cellvibrio mixtus,* DSM 1523, ACM 2601, DSM 11683, DSM 11684, DSM 11685, and *Cellvibrio gilvus,* DSM 11686. The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed cellulolytic enzyme may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or

filtration, 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.

Enzyme

The isolated cellulase enzyme of the present invention is:

(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in Escherichia coli DSM 11143, or

(b) a polypeptide produced by *Cellvibrio mixtus,* DSM 1523, which is encodable by the cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in Escherichia coli DSM 11143, or

(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 2, or

(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 2 which analogue:

(i) is at least 75% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

Alternatively, the enzyme is (a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 3, or (b) a polypeptide produced by *Cellvibrio mixtus,* DSM11683, or (c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 4, or (d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 4 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

Other examples are enzymes which are (a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 6, or (b) a polypeptide produced by *Cellvibrio mixtus,* DSM11685, or (c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 6, or (d) a

polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 4 which analogue is at least 85% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form; or (a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 8, or (b) a polypeptide produced by *Cellvibrio mixtus,* ACM 2601, or (c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 8, or (d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 8 which analogue is at least 80% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form; or (a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 10, or (b) a polypeptide produced by *Cellvibrio mixtus,* DSM11684, or (c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 10, or (d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 10 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form; or (a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 12, or (b) a polypeptide produced by *Cellvibrio gilvus,* DSM11686, or (c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 12, or (d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 12 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

Enzyme compositions

In a still further aspect, the present invention relates to an enzyme composition comprising an enzyme exhibiting cellulolytic activity as described above.

The enzyme composition of the invention may, in addition to the cellulase of the invention, comprise one or more other

enzyme types, for instance hemi-cellulase such as xylanase and mannanase, other cellulase components, chitinase, lipase, esterase, pectinase, cutinase, phytase, oxidoreductase, protease, or amylase.

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

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 on the basis of methods known in the art.

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

Uses

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 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 fabric surface.

The term "soaking liquor" denotes an 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 process.

The term "washing liquor" denotes an 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 an aqueous liquor in which laundry is immersed and treated, conventionally immediately 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 invention may be conventional washable laundry. Preferably, the major part of the laundry is sewn or unsewn fabrics, including knits, wovens, denims, yarns, and toweling, made from cotton, cotton blends or natural or manmade cellulosics (e.g. originating from xylan-containing cellulose fibers such as from wood pulp) or blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fibers, lyocell).

DETERGENT DISCLOSURE AND EXAMPLES

Surfactant system

The detergent compositions according to the present invention comprise a surfactant system, wherein the surfactant can be selected from nonionic and/or anionic and/or cationic and/or ampholytic and/or zwitterionic and/or semi-polar 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 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 inven-tion comprise as a surfactant one or more of the nonionic and/or anionic surfactants described herein.

Polyethylene, polypropylene, and polybutylene oxide conden-sates of alkyl phenols are suitable for use as the nonionic surfactant of the surfactant systems of the present inven-tion, with the polyethylene oxide condensates being pre-ferred. 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 alkylene 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 nonionic 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 alkylphenol alkoxylates (e.g., alkyl phenol ethoxylates).

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 nonionic surfactant of the nonionic 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 nonionic surfactants of this type include Tergitol™ 15-S-9 (The condensation product of C_{11}-C_{15} linear alcohol with 9 moles ethylene oxide), Tergitol™ 24-L-

6 NMW (the condensation product of C_{12}-C_{14} 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 C_{14}-C_{15} linear alcohol with 9 moles of ethylene oxide), Neodol™ 23-3 (the condensation product of C_{12}-C_{13} linear alcohol with 3.0 moles of ethylene oxide), Neodol™ 45-7 (the condensation product of C_{14}-C_{15} linear alcohol with 7 moles of ethylene oxide), Neodol™ 45-5 (the condensation product of C_{14}-C_{15} linear alcohol with 5 moles of ethylene oxide) marketed by Shell Chemical Company, Kyro™ EOB (the condensation product of C_{13}-C_{15} alcohol with 9 moles ethylene oxide), marketed by The Procter & Gamble Company, and Genapol LA 050 (the condensation product of C_{12}-C_{14} 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 nonionic surfactant of the surfactant systems of the present invention are alkylpolysaccharides 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 alkylpolyglycosides have the formula

$$R^2O(C_nH_{2n}O)_t(\text{glycosyl})_x$$

wherein R^2 is selected from the group consisting of alkyl,

alkylphenyl, 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 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 hydrophobic base formed by the condensation of propylene oxide with propylene glycol are also suitable for use as the additional nonionic surfactant systems of the present invention. The hydrophobic portion of these compounds will preferably have a molecular weight from about 1500 to about 1800 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 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 nonionic surfactant of the nonionic 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 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 to about 11,000. Examples of this type of nonionic surfactant include certain of the commercially available Tetronic™ compounds, marketed by BASF.

Preferred for use as the nonionic 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 ethyleneoxide, alkylpolysaccharides, and mixtures hereof. Most preferred are C_8-C_{14} alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C_8-C_{18} alcohol ethoxylates (preferably C_{10} avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Highly preferred nonionic surfactants are polyhydroxy fatty acid amide surfactants of the formula

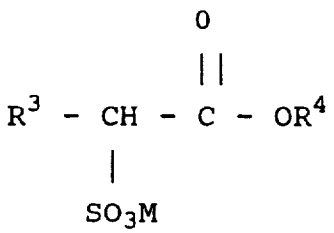
$$R^2 - C(=O) - N(R^1) - Z,$$

wherein R^1 is H, or R^1 is C_{1-4} hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl or a mixture thereof, R^2 is C_{5-31} hydrocarbyl, and Z is a polyhydroxyhydrocarbyl having a linear hydrocarbyl chain with at least 3 hydroxyls directly connected to the chain, or an alkoxylated derivative thereof. Preferably, R^1 is methyl, R^2 is straight C_{11-15} alkyl or C_{16-18} 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 alkoxylated sulfate surfactants. Examples hereof are water soluble salts or acids of the formula $RO(A)_mSO3M$ wherein R is an unsubstituted C_{10}-C-$_{24}$ alkyl or hydroxyalkyl group having a C_{10}-C_{24} alkyl component, preferably a C_{12}-C_{20} alkyl or hydroxyalkyl, more preferably C_{12}-C_{18} 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 ethoxylated sulfates as well as alkyl propoxylated 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, diethylamine, triethylamine, mixtures thereof, and the like. Exemplary surfactants are C_{12}-C_{18} alkyl polyethoxylate (1.0) sulfate (C_{12}-$C_{18}E(1.0)M$), C_{12}-C_{18} alkyl polyethoxylate (2.25) sulfate (C_{12}-$C_{18}(2.25)M$, and C_{12}-C_{18} alkyl polyethoxylate (3.0) sulfate (C_{12}-$C_{18}E(3.0)M$), and C_{12}-C_{18} alkyl polyethoxylate (4.0) sulfate (C_{12}-$C_{18}E(4.0)M$), wherein M is conveniently selected from sodium and potassium. Suitable anionic surfactants to be used are alkyl ester sulfonate surfactants including linear esters of C_8-C_{20} carboxylic acids (i.e., fatty acids) which are sulfonated with gaseous SO_3 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.

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



wherein R^3 is a C_8-C_{20} hydrocarbyl, preferably an alkyl, or combination thereof, R^4 is a C_1-C_6 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, such as monoethanolamine, diethonolamine,

and triethanolamine. Preferably, R^3 is C_{10}-C_{16} alkyl, and R^4 is methyl, ethyl or isopropyl. Especially preferred are the methyl ester sulfonates wherein R^3 is C_{10}-C_{16} alkyl.

Other suitable anionic surfactants include the alkyl sulfate surfactants which are water soluble salts or acids of the formula $ROSO_3M$ wherein R preferably is a C_{10}-C_{24} hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C_{10}-C_{20} alkyl component, more preferably a C_{12}-C_{18} 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 C_{12}-C_{16} are preferred for lower wash temperatures (e.g. below about 50°C) and C_{16}-C_{18} alkyl chains are preferred for higher wash temperatures (e.g. above about 50°C).

Other anionic surfactants useful for detersive purposes can also be included in the laundry detergent compositions of the present invention. Theses can include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C_8-C_{22} primary or secondary alkanesulfonates, C_8-C_{24} 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, C_8-C_{24} alkylpolyglycolethersulfates (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 C_{12}-C_{18} monoesters) and diesters of sulfosuccinates (especially saturated and unsaturated C_6-C_{12} 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 $RO(CH_2CH_2O)_k-CH_2COO-M+$ wherein R is a C_8-C_{22} 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, Perrry 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 detersive 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:

$$[R^2(OR^3)_y][R^4(OR^3)_y]_2R^5N+X-$$

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 form 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:

$$R_1R_2R_3R_4N^+X^- \quad (i)$$

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 $-(C_2H_{40})_xH$ 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.

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 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;

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;

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

$$CH_2\text{-}CH_2\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{12\text{-}14}\ \text{alkyl}$$

and $R_2R_3R_4$ are methyl).

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

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 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, 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 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 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 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.

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 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.

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

$$R^3(OR^4)xN(R^5)2 \rightarrow O$$

wherein R^3 is an alkyl, hydroxyalkyl, or alkyl phenyl group or mixtures thereof containing from about 8 to about 22 carbon atoms; R^4 is an alkylene or hydroxyalkylene group containing from about 2 to about 3 carbon atoms or mixtures thereof; x is from 0 to about 3: and each R^5 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 R^5 groups can be attached to each other, e.g., through an oxygen or nitrogen

atom, to form a ring structure.

These amine oxide surfactants in particular include C_{10}-C_{18} alkyl dimethyl amine oxides and C_8-C_{12} alkoxy ethyl dihydroxy ethyl amine oxides.

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.

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, 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 ($Na_2Si_2O_5$).

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, (ethylenedioxy) diacetic acid, maleic acid, diglycollic acid, tartaric acid, tartronic acid and fumaric acid, as well as the ether carboxylates described in German Offenlenschrift 2,446,686, and 2,446,487, US 3,935,257 and the sulfinyl carboxylates described in Belgian Patent No.

840,623. 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 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, 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, 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 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-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 silicate (SKS-6), and a water-soluble carboxylate chelating agent such as citric acid.

A suitable chelant for inclusion in the detergent composi-ions in accordance with the invention is

ethylenediamine-N,N'-disuccinic acid (EDDS) or the alkali metal, alkaline earth 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 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.

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 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 form 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 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%.

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.

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. 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, 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 those sold under the trade names Alcalase, Savinase, Primase, Durazym, and Esperase by Novo Nordisk A/S (Denmark), 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 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.

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 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 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. stearothermophilus 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 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.

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 Lipase™, Luma fast™ and Lipomax™ (Genencor), Lipolase™ and Lipolase Ultra™ (Novo Nordisk 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, a-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™ (available from Novo Nordisk A/S) and Rapidase™ and Maxamyl P™ (available from 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. 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 and the endoglucanase of the present invention.

Commercially available cellulases include Celluzyme™ produced by a strain of Humicola insolens, (Novo Nordisk A/S), and KAC-500(B)™ (Kao Corporation).

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, 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 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 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.

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 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 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, 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 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 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 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 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 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-oxoperoxycaproic 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 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 normally added at 0.5-10% by weight of the finished product, preferably 1-5% by weight.

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

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 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.

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 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 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 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. Theses 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 form 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 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 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 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 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 form 0.75% to 8%, most preferably from 1% to 6% by weight of the 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-triazin-6 ylamino)stilbene-2-sulphonate, disodium 4,4' -bis-(2-anilino-4-(N-methyl-N-2-hydroxyethylamino)-s-triazin-6-ylamino)stilbene-2,2' - disulphonate, di-sodium 4,4' -bis-(4-phenyl-2,1,3-triazol-2-yl)-stilbene-2,2' disulphonate, di-sodium 4,4'bis(2-anilino-4-(1-methyl-2-hydroxyethylamino)-s-triazin-6-ylami-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 polycarboxylate 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:

$(CH_3(PEG)_{43})_{0.75}(POH)_{0.25}[T\text{-}PO)_{2.8}(T\text{-}PEG)_{0.4}]T(POH)_{0.25}((PEG)_{43}CH_3)_{0.75}$

where PEG is $-(OC_2H_4)O-$, 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.

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 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 C_{12}-C_{14} quaternary ammonium salts are disclosed in EP-B-0 026 528 and di-long-chain amides as 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% 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, 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, 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

The detergent compositions according to the present invention may also comprise from 0.001% to 10%, preferably from 0.01% to 2%, more preferably form 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, polyvinyloxazolidones and polyvinylimidazoles or mixtures thereof.

Addition of such polymers also enhances the performance of the enzymes according 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. form 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:

LAS: Sodium linear C_{12} alkyl benzene sulphonate

TAS: Sodium tallow alkyl sulphate

XYAS: Sodium C_{1X} - C_{1Y} alkyl sulfate

SS: Secondary soap surfactant of formula 2-butyl octanoic acid

25EY: A C_{12} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

45EY: A C_{14} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

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

Nonionic: C_{13} - C_{15} 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 LF404 by BASF Gmbh

CFAA: C_{12} - C_{14} alkyl N-methyl glucamide

TFAA: C_{16} - C_{18} alkyl N-methyl glucamide

Silicate: Amorphous Sodium Silicate (SiO_2:Na_2O ratio = 2.0)

NaSKS-6: Crystalline layered silicate of formula d-$Na_2Si_2O_5$

Carbonate: Anhydrous sodium carbonate

Phosphate: Sodium tripolyphosphate

MA/AA: Copolymer of 1:4 maleic/acrylic acid, average 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 $Na_{12}(AlO_2SiO_2)_{12}.\ 27H_2O$ 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, empirical formula $NaBO_2.H_2O_2$

PB4: Anhydrous sodium perborate tetrahydrate

Percarbonate: Anhydrous sodium percarbonate bleach of empirical formula $2Na_2CO_3.3H_2O_2$

TAED: Tetraacetyl ethylene diamine

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 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

Sulphate: Anhydrous sodium sulphate

HMWPEO: High molecular weight polyethylene oxide

TAE 25: Tallow alcohol ethoxylate (25)

Detergent Example I

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
Sodium nitrilotriacetate	5.0
Enzyme of the invention	0.1
PVP	0.5
TAED	3.0
Boric acid	4.0
Perborate	18.0
Phenol sulphonate	0.1
Minors	Up to 100

Detergent Example II

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

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
45AS	3.1	10.0
45E7	4.0	-
25E3S	-	3.0
68E11	1.8	-
25E5	-	8.0
Citrate	15.0	7.0
Carbonate	-	10
Citric acid	2.5	3.0
Zeolite A	32.1	25.0
Na-SKS-6	-	9.0
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
PVP	0.5	-
Poly (4-vinylpyridine)-N-Oxide/copolymer of vinyl-imidazole and vinyl-pyrrolidone	-	0.2
Perborate	1.0	-
Phenol sulfonate	0.2	-
Water/Minors	Up to 100%	

Detergent Example IV

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	-

68AS	1.3	-
45E7	4.0	-
25E3	-	5.0
Coco-alkyl-dimethyl hydroxy-ethyl ammonium chloride	1.4	1.0
Citrate	5.0	3.0
Na-SKS-6	-	11.0
Zeolite A	15.0	15.0
MA/AA	4.0	4.0
DETPMP	0.4	0.4
Perborate	15.0	-
Percarbonate	-	15.0
TAED	5.0	5.0
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
Granular suds suppressor	1.0	4.0
CMC	0.2	0.1
Water/Minors	Up to 100%	

Detergent Example V

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

	I	II
LAS acid form	-	25.0
Citric acid	5.0	2.0
25AS acid form	8.0	-
25AE2S acid form	3.0	-
25AE7	8.0	-
CFAA	5	-
DETPMP	1.0	1.0
Fatty acid	8	-
Oleic acid	-	1.0
Ethanol	4.0	6.0

Propanediol	2.0	6.0
Enzyme of the invention	0.10	0.05
Coco-alkyl dimethyl hydroxy ethyl ammonium chloride	-	3.0
Smectite clay	-	5.0
PVP	2.0	-
Water / Minors	Up to 100%	

Textile applications

In another embodiment, the present invention relates to use of the endoglucanase of the invention in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. The most important effects of Bio-Polishing can be characterized by less fuzz and pilling, increased gloss/luster, improved fabric handle, increased durable softness and altered water absorbency. Bio-Polishing usually takes place in the wet processing of the manufacture of knitted and woven fabrics. Wet processing comprises such steps as e.g. desizing, scouring, bleaching, washing, dying/printing and finishing. During each of these steps, the fabric is more or less subjected to mechanical action. In general, after the textiles have been knitted or woven, the fabric proceeds to a desizing stage, followed by a scouring stage, etc. Desizing is the act of removing size from textiles. Prior to weaving on mechanical looms, warp yarns are often coated with size starch or starch derivatives in order to increase their tensile strength. After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. It is known that in order to achieve the effects of Bio-Polishing, a combination of cellulolytic and mechanical action is required. It is also known that "super-softness" is achievable when the treatment with a cellulase is combined with a conventional treatment with softening agents. It is contemplated that use of the endoglucanase of the invention for bio-polishing of

cellulosic fabrics is advantageous, e.g. a more thorough polishing can be achieved. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

Stone-washing

It is known to provide a "stone-washed" look (localized abrasion of the colour) in dyed fabric, especially in denim fabric or jeans, either by washing the denim or jeans made from such fabric in the presence of pumice stones to provide the desired localized lightening of the colour of the fabric or by treating the fabric enzymatically, in particular with cellulolytic enzymes. The treatment with an endoglucanase of the present invention may be carried out either alone such as disclosed in US 4,832,864, together with a smaller amount of pumice than required in the traditional process, or together with perlite such as disclosed in WO 95/09225.

Pulp and paper applications

In the papermaking pulp industry, the endoglucanase of the present invention may be applied advantageously e.g. as follows:

- For debarking: pretreatment with the endoglucanase may degrade the cambium layer prior to debarking in mechanical drums resulting in advantageous energy savings.

- For defibration: treatment of a material containing cellulosic fibers with the endoglucanase prior to refining or beating may result in reduction of the energy consumption due to the hydrolysing effect of the cellulase on the interfibre surfaces. Use of the endoglucanase may result in improved energy savings as compared to the use of known enzymes, since it is believed that the enzyme composition of the invention may possess a higher ability to penetrate fibre walls.

- For fibre modification, i.e. improvement of fibre properties where partial hydrolysis across the fibre wall is needed which requires deeper penetrating enzymes (e.g. in order to make coarse fibers more flexible). Deep treatment of fibers has so far not been possible for high yield pulps e.g.

mechanical pulps or mixtures of recycled pulps. This has been ascribed to the nature of the fibre wall structure that prevents the passage of enzyme molecules due to physical restriction of the pore matrix of the fibre wall. It is contemplated that the present endoglucanase is capable of penetrating into the fibre wall.

- For drainage improvement. The drainability of papermaking pulps may be improved by treatment of the pulp with hydrolysing enzymes, e.g. cellulases. Use of the present endoglucanase may be more effective, e.g. result in a higher degree of loosening bundles of strongly hydrated micro-fibrils in the fines fraction (consisting of fibre debris) that limits the rate of drainage by blocking hollow spaces between fibers and in the wire mesh of the paper machine. The Canadian standard freeness (CSF) increases and the Schopper-Riegler drainage index decreases when pulp in subjected to cellulase treatment, see e.g. US patent 4,923,565; TAPPI T227, SCAN C19:65.ence.

- For inter fibre bonding. Hydrolytic enzymes are applied in the manufacture of papermaking pulps for improving the inter fibre bonding. The enzymes rinse the fibre surfaces for impurities e.g. cellulosic debris, thus enhancing the area of exposed cellulose with attachment to the fibre wall, thus improving the fibre-to-fibre hydrogen binding capacity. This process is also referred to as dehornification. Paper and board produced with a cellulase containing enzyme preparation may have an improved strength or a reduced grammage, a smoother surface and an improved printability.

- For enzymatic deinking. Partial hydrolysis of recycled paper during or upon pulping by use of hydrolysing enzymes such as cellulases are known to facilitate the removal and agglomeration of ink particles. Use of the present endoglucanse may give a more effective loosening of ink from the surface structure due to a better penetration of the enzyme molecules into the fibrillar matrix of the fibre wall, thus softening the surface whereby ink particles are effectively loosened. The agglomeration of loosened ink particles are also improved, due to a more efficient hydrolysis of

cellulosic fragments found attached to ink particles originating from the fibres.

The treatment of lignocellulosic pulp may, e.g., be performed as described in WO 91/14819, WO 91/14822, WO 92/17573 and WO 92/18688.

Degradation of plant material

In yet another embodiment, the present invention relates to use of the endoglucanase and/or enzyme preparation according to the invention for degradation of plant material e.g. cell walls.

It is contemplated that the novel endoglucanase and/or enzyme preparation of the invention is useful in the preparation of wine, fruit or vegetable juice in order to increase yield. Endoglucanases according to the invention may also be applied for enzymatic hydrolysis of various plant cell-wall derived materials or waste materials, e.g. agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, and the like. The plant material may be degraded in order to improve different kinds of processing, facilitate purification or extraction of other components like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

MATERIALS AND METHODS

Deposited organisms:

Cellvibrio mixtus, DSM 1523, DSM 11683, DSM 11684, DSM 11685, ACM 2601, and *Cellvibrio gilvus,* DSM 11686, comprise the cellulase encoding DNA sequence of the invention.

Escherichia coli, DSM 11143, containing the plasmid comprising a DNA sequence encoding the cellulolytic enzyme of the invention, in the cloning vector pSJ1678.

Escherichia coli, DSM 11120, containing the plasmid

comprising a DNA sequence partially encoding the core region of the cellulolytic enzyme of the invention, in the cloning vector pBluescriptII KS.

Pseudomonas cellulosa, NCIMB 10462, comprising one of the cellulase encoding DNA sequences cloned.

Pseudomonas fluorescens, DSM 11681, which is used as donor for expressing one of the cloned cellulases.

Pseudomonas cepacia, DSM 11682, which is used as donor for expressing one of the cloned cellulases.

Other strains:

E. coli: *E. coli* XL1-Blue (Stratagene, USA). Cells of *E. coli* SJ2 (Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol., 172, 4315-4321), were prepared for and transformed by electroporation using a Gene Pulser™ electroporator from BIO-RAD as described by the supplier.

A. oryzae: strain JaL228 (Danish patent application DK 1024/96).

Plasmids:

pBluescript II KS- (Stratagene, U.S.A.), and pSJ1678 (see WO 94/19454).

pCaHj418: For use in the constructions below the pCaHj418 was constructed by inserting the 43K gene isolated from pCaHj201 (WO 94/07998) as a BamH I, Sal I fragment inro pHD 414 (WO 94/07998) digested with BamH I and Xho I. The two fragments were ligated and introduced into *E. coli* XLI-blue by electroporation. A positive clone was isolated and was designated CaHj418.

pToC202: ((I_{666}+ I_Q table 2) Tove Christensen in: The Genus Aspergillus, Ed. K.A. Powell et al., Plenum Press NY (1994)).

Media

TE-buffer and TY and LB agar (as described in Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

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 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.

Isolation of the DNA sequence encoding the cellulolytic enzyme of the invention:

The DNA sequence, comprising the DNA sequence shown in SEQ ID No. 1, encoding the endoglucanase of the invention, can be obtained from the deposited organism *E. coli*, DSM 11143, by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Similarly, a DNA sequence corresponding to the nucleotides 889-1236 shown in SEQ ID No. 1 can be obtained from the deposited organism *E. coli*, DSM 11120.

PCR primers for molecular screening of cellulases of the present invention:

The two degenerate, deoxyinosine-containing oligonucleotide primers (sense and antisense) are:

sense,

5'-GCTGTCCGTGAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}{}^{A}/_{C}$C-3'

antisense,

5'-CGCGTGGATC CT$^{C}/_{T}{}^{A}/_{G}$AAI$^{A}/_{G}{}^{C}/_{T}$I CCIA^{C}/G/$_{A}$ICCIC CICCIGG -3'

The I's in the above corresponds to deoxyinosines, restriction sites BamHI and HindIII are underlined.

In vitro amplification of genomic DNA.

Approximately 100 to 200 ng of genomic DNA was PCR amplified in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin) containing 200 μM of each dNTP, 2.5 units of AmpliTaq polymerase (Perkin-Elmer, Cetus, USA) and 100 pmol of each degenerate primer:

sense,
5'-GCTGTCCGTGAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}^{A}/_{C}$C-3'
antisense1,
5'-CGCGTGGATC CT$^{C}/_{T}^{A}/_{G}$AAI$^{A}/_{G}^{C}/_{T}$I CCIA^{C}/G/$_{A}$ICCIC CICCIGG -3'

The I's in the above corresponds to deoxyinosines, restriction sites BamHI and HindIII are underlined.

The PCR reactions were performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 1 min followed by 40 cycles of PCR performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. 10μl aliquots of the amplification product was analyzed by electrophoresis in 1.5 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

Direct sequencing of the PCR products obtained from DSM 11120

80 μl aliquots of the PCR product were purified using the QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The nucleotide sequences of the amplified PCR fragments were determined directly on the purified PCR products by the dideoxy chain-termination method, using 50-150 ng template, the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labeled terminators and 5 pmol of the sense primer:
5'-GCTGTCCGTGAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}^{A}/_{C}$C-3'.

In another reaction the nucleotide sequence was

determined using the antisense primer:
5'-CGCGTGGATC CT$^{C}/_{T}{}^{A}/_{G}$AAI$^{A}/_{G}{}^{C}/_{T}$I CCIA^{C}/G/$_{A}$ICCIC CICCIGG -3'

Analysis of the sequence data were performed according to Devereux et al., (1984) Nucleic Acids Res. 12, p.387-395. The DNA sequence is shown in positions 865-1260 of SEQ ID No. 1, and the derived amino acid sequence is shown in positions 289-420 of SEQ ID NO:2.

Cloning by polymerase chain reaction (PCR):

Subcloning of PCR fragments

25 µl aliquots of the PCR products generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with HindIII and BamHI, electrophoresed in 0.8% low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragments were excised from the gels, and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to *BamHI, HindIII* digested pBluescriptII KS- and the ligation mixture was used to transform *E. coli* SJ2.

Cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (5-Bromo-4-chloro-3-indolyl alpha-D-galactopyranoside, 50 µg/ml).

Identification and charaterization of positive clones.

The transformed cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (50 µg/ml) and incubated at 37°C over night. Next day white colonies were rescued by restreaking these onto fresh LB-ampicillin agar plates and incubated at 37°C over night. The next day single colonies of each clone were transferred to liquid LB medium containing ampicillin (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. 5µl samples of the

plasmids were digested with *BamHI* and *HindIII*. The digestions were checked by gelelectrophoresis on a 1.5 % agarose gel (NuSieve, FMC). The appearence of a DNA fragment of about 0.4 kb indicated a positive clone.

Nucleotide sequencing the cloned DNA fragment.

Qiagen purified plasmid DNA was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and the primer Reverse or the primer Forward.

Reverse:
5'-GTT TTC CCA GTC ACG AC-3'

Forward:
5'-GCG GAT AAC AAT TTC ACA CAG G-3'

using an Applied Biosystems 373A automated sequencer according to the manufacturers instructions. Analysis of the sequence data was performed according to Devereux *et al.* (1984) Nucleic Acids Research,12, p. 387-395.

Hybridization conditions (to be used in evaluating property ii) of the DNA construct of the invention):

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 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 random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* **132**:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at preferably at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at leasty

70°C, especially at least 75°C.

The nucleotide probe to be used in the hybridization is the DNA sequence shown in SEQ ID No. 1.

Immunological cross-reactivity:

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified endoglucanase. More specifically, antiserum against the endoglucanase 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 Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Homology of endoglucanase encoding DNA sequences.

The DNA sequence homology referred to below is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as using FASTA of the GCG package using the following settings: Scoring matrix: GenRunData:blosum50.cmp, Variable pamfactor used Gap creation penalty: 12, Gap extension penalty: 2. provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453).

Homology of endoglucanase protein sequence.

The protein homology referred to below is determined as the degree of identity between the two proteins indicating a derivation of the first protein from the second. The homology may suitably be determined by means of computer programs known in the art such as using FASTA of the GCG package using the following settings: Scoring matrix: GenRunData:blosum50.cmp, Variable pamfactor used Gap creation penalty: 12, Gap extension penalty: 2. provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453).

The following non-limiting examples illustrates the invention.

EXAMPLE 1

A. Cloning and sequencing of a DNA fragment corresponding to a partial nucleotide sequence of an endoglucanase from *Cellvibrio mixtus,* **DSM 1523, ATCC 12120, NCIB 8634**

Preparation of genomic DNA from *Cellvibrio mixtus* DSM 1523, PCR amplification of genomic DNA, cloning of PCR fragment, screening and DNA sequencing was performed as described in Materials and Methods. One positive transformant isolated was MB275-2, containing the plasmid designated is pMB275-2. PMB275-2 is pBLUESCRIPT II KS minus containing an insert of approximately 400 basepairs. This insert was DNA sequenced, and revealed the presence of the partial sequence of an endoglucanase-encoding gene. The nucleotide sequence corresponds to the positions 865-1260 of SEQ ID NO: 1.

The DNA corresponding to part of the endoglucanase gene is obtainable from the plasmid obtainable from the strain deposited as DSM 11120.

B. Cloning and expression of an endoglucanase from *Cellvibrio mixtus,* **DSM 1523, ATCC 12120, NCIB 8634.**

Genomic DNA preparation:

Strain *Cellvibrio mixtus,* DSM1523, was propagated on TY-agar medium supplemented with 2% soluble starch at 25 °C for 3-4 days. Cells were harvested, and genomic DNA isolated by the method described by Pitcher et al. (Pitcher, D. G., Saunders, N. A., Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol., 8, 151-156).

Genomic library construction:

Genomic DNA was partially digested with restriction enzyme Sau3A, and size-fractionated by electrophoresis on a 0.7 % agarose gel. Fragments between 2 and 7 kb in size were isolated by electrophoresis onto DEAE-cellulose paper (Dretzen, G., Bellard, M., Sassone-Corsi, P., Chambon, P. (1981) A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem., 112, 295-298).

Isolated DNA fragments were ligated to BamHI digested pSJ1678 plasmid DNA, and the ligation mixture was used to transform *E. coli* SJ2.

Cells were plated on LB agar plates containing 0.1% CMC (Sodium-Carboxy-Methyl-Cellulose, Aqualon, France) and 9 µg/ml Chloramphenicol and incubated overnight at 37°C.

Identification of positive clones by colony hybridization

A DNA library in *E. coli*, constructed as described above, was screened by colony hybridization (Sambrook, 1989) using the corresponding nick translation ^{32}P-labelled PCR product (obtained as described above) as probe. The hybridization was carried out in 2 x SSC (Sambrook, 1989), 5 x Denhardt's solution (Sambrook, 1989), 0.5 % (w/v) SDS, 100 mg/ml denatured salmon sperm DNA for 20 h at 65°C followed by washes in 5 x SSC at 25°C (2 x 15 min), 2 x SSC, 0.5 % SDS at 65°C (30 min), 0.2 x SSC, 0.5 % SDS at 65°C (30 min) and finally in 5 x SSC (2 x 15 min) at 25°C. Positive clones were characterized as described below.

Identification of positive clones by activity:

Cells were plated on LB agar plates containing 0.1% CMC (Carboxy-methyl-cellulose) and 9 μg/ml Chloramphenicol and incubated overnight at 37°C. The transformants were subsequently replica plated onto the same type of plates, and these new plates were incubated 8 hours or overnight at 37°C.

The original plates were coloured using 1mg/ml of Congored (SIGMA, USA). The coloring was continued for half an hour with moderate orbital shaking, after which the plates were washed two times 15 minutes using 1 M NaCl.

Yellowish halos appeared at positions where cellulase positive clones were present, from the replica plates these cellulase positive clones were rescued and restreaked onto LB agar plates containing 0.1% CMC and 9 μg/ml Chloramphenicol and incubated overnight at 37°C.

Characterization of positive clones:

From the restreaking plates the endoglucanase positive clones were obtained as single colonies, and plasmids were extracted. Phenotypes were confirmed by retransformation of *E.coli* SJ2, and plasmids characterized by restriction digests. One positive transformant isolated was DSM 11143 containing the plasmid pSJ1678 containing an insert of approximately 5000 base-pairs. This insert was DNA sequenced, and revealed the presence of the sequence of an endoglucanase-encoding gene. The nucleotide sequence is designated SEQ NO 1.

The endoglucanase gene was characterized by DNA sequencing using the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescent labeled terminators and 5 pmol of the sense primer:

5'-GCTGTCCGTGAAGCTTACI$^{A}/_{C}$GITA$^{C}/_{T}$TGGGA$^{C}/_{T}$TG$^{C}/_{T}$TG$^{C}/_{T}$AA$^{A}/_{G}{}^{A}/_{C}$C-3'.

In another reaction the nucleotide sequence was determined using the antisense1 primer:

5'-CGCGTGGATC CT$^{C}/_{T}{}^{A}/_{G}$AAI$^{A}/_{G}{}^{C}/_{T}$I CCIA^{C}/G/$_{A}$ICCIC CICCIGG -3'

The obtained DNA sequence was then used for designing new primers for sequencing, this procedure being repeated until the whole sequence had been obtained.

Analysis of the sequence data was performed according to Devereux *et al.* The sequence corresponds to the DNA sequence shown in SEQ ID No 1.

The DNA corresponding to the endoglucanase gene is obtainable from the plasmid obtainable from the strain deposited as DSM 11143.

The *E.coli* clone DSM 11143 containing the cloned endoglucanase of *Cellvibrio mixtus* was further characterized by DNA sequencing using primer walking and sequencing reactions as described above. The corresponding DNA sequence is listed as SEQ ID No. 1.

SEQ ID No. 2 shows the amino acid sequence (derivable from SEQ ID No. 1. The first 32 amino acid residues correspond to a signal peptide. Amino acid residues nos. 33 to 134 correspond to a cellulose binding domain belonging to the family IIa (Tomme et al.). The amino acid residues nos. 135 to 185 correspond to a Ser rich linker, the amino acid residues nos. 186 to 234 correspond to a cellulose binding domain belonging to the family X. The amino acid residues nos. 235 to 277 correspond to a second Ser rich linker. Amino acid residues nos. 278 to the end of the sequence correspond to the catalytical domain of endoglucanases belonging to the Family 45 of glycosyl hydrolases (Henrissat et al.)

Homology search was performed using the DNA sequence presented as SEQ ID No. 1. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID NO 1 shows 74% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 2. The homology search showed that the most related protein was an endoglucanase to which the protein shown in SEQ ID NO 2 shows 70.4% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 2

Cloning of an endoglucanase gene from *Cellvibrio mixtus*, DSM 11683

Chomosomal DNA from *Cellvibrio mixtus*, DSM 11683, was obtained as described in example 1 and in Materials and methods. This chromosomal DNA was used as a template for PCR and PCR reactions containing the primers sense and antisense1 were performed. The resulting amplified PCR fragment of 0.4 kb was sequenced and the DNA sequence is listed as SEQ ID No. 3.

The corresponding a.a. derived from the SEQ ID No. 3 is listed as SEQ ID No. 4.

Homology search was performed using the DNA sequence presented as SEQ ID No. 3. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID NO 3 shows 76% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 4. The homology search showed that the most related protein was an endoglucanase, to which the protein shown in SEQ ID NO 4 shows 84% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 3

Cloning of an endoglucanase gene from *Cellvibrio mixtus*, DSM 11685

Chomosomal DNA from *Cellvibrio mixtus*, DSM 11685, was obtained as described in example 1 and in Materials and methods. This chromosomal DNA was used as a template for PCR and PCR reactions containing the primers sense and antisense1 were performed. The resulting amplified PCR fragment of 0.4

kb was sequenced and the DNA sequence is listed as SEQ ID No. 5.

The corresponding a.a. derived from the SEQ ID No. 3 is listed as SEQ ID No. 6.

Homology search was performed using the DNA sequence presented as SEQ ID No. 5. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID NO 5 shows 75% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 6. The homology search showed that the most related protein was an endoglucanase, to which the protein shown in SEQ ID NO 6 shows 83% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 4

Cloning of an endoglucanase gene from *Cellvibrio mixtus*, ACM 2601

Chomosomal DNA from *Cellvibrio mixtus*, ACM 2601, was obtained as described in example 1 and in Materials and methods. This chromosomal DNA was used as a template for PCR and PCR reactions containing the primers sense and antisense1 were performed. The resulting amplified PCR fragment of 0.4 kb was sequenced and the DNA sequence is listed as SEQ ID No. 7.

The corresponding a.a. derived from the SEQ ID No. 7 is listed as SEQ ID No. 8.

Homology search was performed using the DNA sequence presented as SEQ ID No. 7. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID

NO 7 shows 72% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 8. The homology search showed that the most related protein was an endoglucanase, to which the protein shown in SEQ ID NO 8 shows 74% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 5

Cloning of an endoglucanase gene from *Cellvibrio mixtus*, DSM 11685

Chomosomal DNA from *Cellvibrio mixtus*, DSM 11685, was obtained as described in example 1 and in Materials and methods. This chromosomal DNA was used as a template for PCR and PCR reactions containing the primers sense and antisense1 were performed. The resulting amplified PCR fragment of 0.4 kb was sequenced and the DNA sequence is listed as SEQ ID No. 3.

The corresponding a.a. derived from the SEQ ID No. 9 is listed as SEQ ID No. 10.

Homology search was performed using the DNA sequence presented as SEQ ID No. 9. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID NO 9 shows 76% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 10. The homology search showed that the most related protein was an endoglucanase, to which the protein shown in SEQ ID NO 10 shows 85% identity.

The low homology identified using the homology search in

the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 6

Cloning of an endoglucanase gene from *Cellvibrio gilvus*, DSM 11686

Chomosomal DNA from *Cellvibrio gilvus*, DSM 11686, was obtained as described in example 1 and in Materials and methods. This chromosomal DNA was used as a template for PCR and PCR reactions containing the primers sense and antisense1 were performed. The resulting amplified PCR fragment of 0.4 kb was sequenced and the DNA sequence is listed as SEQ ID No. 11.

The corresponding a.a. derived from the SEQ ID No. 3 is listed as SEQ ID No. 12.

Homology search was performed using the DNA sequence presented as SEQ ID No. 11. The homology search showed that the most related DNA sequence was a gene encoding an endoglucanase to which gene the DNA sequence shown in SEQ ID NO 11 shows 76% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

Homology search was performed using the protein sequence presented as SEQ ID No. 12. The homology search showed that the most related protein was an endoglucanase, to which the protein shown in SEQ ID NO 12 shows 84% identity.

The low homology identified using the homology search in the above demonstrates that the endoglucanase of the invention is distant from any known endoglucanase.

EXAMPLE 7

Cloning of an endoglucanase from *Pseudomonas cellulosa*, NCIMB 10462.

Preparing *Pseudomonas cellulosa* lysate for PCR:

Pseudomonas cellulosa, NCIMB 10462, was propagated

on LB-agar plates for 24 hours at 30°C. Cells were taken with 10 µl inoculation loop and resuspended in 30 µl of TE buffer. The cells were lysed by heating the sample to 99°C for 5 min, the cell-debris was removed by centrifugation 20.000 g for 5 min at 4°C.

In vitro amplification of genomic DNA:

5 µl of cell lysate was used for PCR amplification in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin) containing 200 µM of each dNTP, 2.5 units of AmpliTaq polymerase (Perkin-Elmer, Cetus, USA) and 100 pmol of each primer:

Cellulosa 1,
5'-GTG TCG CCG CGG CAG CAG TGT GTG AAT ATC GGG TGA CG -3'
Cellulosa 2,
5'-GTG TCG GTG GCG GCC GCG GGT TGA TAA GGA TAG GCT ATG G -3'

Restriction sites SacII and EagI are underlined. The primers were designed using the published DNA sequence present i GenBank under ACCESSION X52615.

The PCR reactions was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 1 min followed by forty cycles of PCR performed using a cycle profile of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Ten-µl aliquots of the amplification product was analyzed by electrophoresis in 0.7 % agarose gels (NuSieve, FMC).

Subcloning of PCR fragments:

45µl aliquots of the PCR products generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25µl of the purified PCR fragment was digested with SacII and EagI, electrophoresed in 0.8 % low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragments were excised from the gels, and purified

using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to SacII and EagI digested pBluescriptII KS- and the ligation mixture was used to transform *E. coli* SJ2.

Cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (5-Bromo-4-chloro-3-indolyl alpha-D-galactopyranoside, 50 µg/ml).

Identification and charaterization of positive clones:

The transformed cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (50 µg/ml) and incubated at 37°C over night. Next day white colonies were rescued by restreaking these onto fresh LB-ampicillin agar plates and incubated at 37°C over night. The next day single colonies of each clone were transferred to liquid LB medium containing ampicillin (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. Five-µl samples of the plasmids were digested with SacII and EagI. The digestions were checked by gelelectrophoresis on a 0.7 % agarose gel (NuSieve, FMC). The appearence of a DNA fragment of about 1.6 kb indicated a positive clone. The clone was designated PSCel45 and the plasmid pPSCel45.

EXAMPLE 8

Construction and characterisation of a hybrid endoglucanase: *Pseudomonas* cel45 core with *Humicola insolens* EG V linker and CBD.

A construction embodying inframe fusions of *Humicola insolens* F45 endoglucanase signal peptide encoding sequence and the *Pseudomonas fluorescens* catalytic domain encoding sequence and the linker CBD (cellulose binding domain) of *Humicola insolens* family 45 encoding sequence was performed as described below. The relevant *Humicola insolens* sequences are disclosed in e.g. WO 91/17243.

The *Pseudomonas fluorescens* family 45 endoglucanase (PsF45) clone PSCel45 described in example 7 and *the H. insolens* family 45 endoglucanase (HiF45) were used as template in the following PCR reactions 96 _C 60'' - 3x(94 _C 30'', 45 _C 45'', 72 _C 60'') - 25x(94 _C 30'', 55 _C 45'', 72 _C 60'') 72 _C 7' performed in 50 ml volume with Pwo polymerase according to the manufactures (Boehringer) protocol

1: Template. pCaHj418 (HiF45)
primer 1: CAACATCACATCAAGCTCTCC
primer 2: GCCATTACAGCCACCGTCGGCGGCAAGGGCC

2: Template: pPsF45
primer 3: GGCCCTTGCCGCCGACGGTGGCTGTAATGGC
primer 4: CCTTTCTCTATTGATCGGCTCC

3: Template: pPsF45
primer 5: GCAGCTCATCGCGCTCC
primer 6: GGATCTGGACGGCGGGACAGGTGTTGC

4: Template: pCaHj418 (HiF45)
primer 7: GCAACACCTGTCCCGCCGTCCAGATCC
primer 8: CCCCATCCTTTAACTATAGCG

The 141 bp PCR product of reaction 1 and the 838 bp product of reaction 2 were mixed in equimolar amounts and joined in a SOE-PCR (sequence overlap extension) reaction with the outsite primers 1 and 4: 25x(94 _C 30'', 55 _C 45'', 72 _C 60'') 72 _C 7'.

The resulting 948 bp PCR product of this reaction was purified via a 1% agarose gel, and cleaved with the restriction endonucleases BamH1 and BspE1. The resulting 771 bp fragment was purified via a 1 % agarose gel.

The 839 bp product of reaction 3 and the 317 bp product of reaction 4 were mixed in equimolar amounts and joined in a SOE-PCR reaction with the outsite primers 5 and 8 as above.

The resulting 1129 bp PCR product of this reaction was purified via a 1% agarose gel, and cleaved with the restriction endonucleases BspE1 and Xba1. The resulting 285bp fragment was purified via a 1 % agarose gel.

The 771 bp BamH1-BspE1 and the 285 bp BspE1-Xba1 fragments were ligated in a reaction including the 4.1 kbp Xba-BamH1 fragment of pCaHj418. The resulting ligation mixture was transformed into *E. coli* XL1-Blue. Upon restriction analysis and DNA sequencing of the plasmids of 4 individual transformants (all identical) one such isolate was transformed into *A. oryzae* strain JaL228 together with the selection plasmid pToC202, selecting for the ability to utilize AMDS as sole nitrogen source (Aspergillus transformation was performed as described in EP 238.023). 10 transformants were analyzed for endoglucanase activity on carboxy-methyl cellulose (CMC), and upon three reisolation steps via spores, *the A. oryzae* strain LaC2812, expressing the hybrid protein composed of P. fluoresence F45 catalytic domain and HiF45 linker CBD, was isolated.

Characterization of clone LaC2812. The Pseudomonas cel45 core with Humicola insolens EG V linker and CBD:

As described above the hybrid enzyme was produced by cloning the gene and transforming it into *Aspergillus oryzae* using a plasmid with the gene inserted between the gene coding for fungal amylase promoter and the gene coding for the AMG terminator from *A. niger* (Christensen, T. Wöldike, H. Boel, E., Mortensen, S. B., Hjortshøj, K., Thim, L. and Hansen, M.T. (1988) High Level Expression of Recombinant Genes in Aspergillus oryzae. Biotechnology 6, 1419-1422).

Purification of the cellulase with the CBD:

The cellulases with a CBD were purified by exploiting the binding to Avicel. After the extracellular fluid was separated from the production organism. The cellulase was then purified to a high degree using affinity chromatography. 35 g Avicel in a slurry with 20 mM sodium phosphate at pH 7.5

was mixed with the crude spent medium containing about 1 g of protein in total. After incubation at 4° C for 20 min, the Avicel - bound enzyme was packed into a 100 ml column. The column was washed with 200 ml buffer, then washed with 0.5 M NaCl in the same buffer until no more protein eluted, and washed with 500 ml buffer (20 mM Tris pH 8.5). Finally, the pure full- length enzyme was eluted with 0.2 M Tris pH 11.8. The eluted cellulase was adjusted to pH 7.65 using phophoric acid. The purified enzymes all gave a single band on SDS-PAGE with a apparent molecular weight of 48 kda. The activity of the purified enzyme was 43 ECU per A280 giving a specific activity of 63 ECU per mg protein. The molar extinction coefficient was 70490 based on the amino acid composition deduced from the DNA sequence.

Cellulase kinetics using reduced cellodextrins:

This method is described in detail in (Schou et al. (1993) EUROPEAN JOURNAL OF BIOCHEMISTRY Vol. 217 , No. 3 pp. 947-953). The principle is that cellobiose dehydrogenase does not react on reduced cellodextrins, but when the cellulase cleaves the substrate, one of the two products has a reducing group and will be oxidised with the cellobiose dehydrogenase. The dehydrogenase then reduces a coloured substrate, 2,6-dichloroindophenol or cytochrome c. Catalytic activities using red DP6 was done at pH 7.5.

The following result was obtained using reduced DP6 : K_{cat} of 2.2 per sec and K_M of 212 μM.

Apparent kinetic constant determination using phosphoric-acid swollen cellulose (PASC):

PASC stock solution was prepared the following way. 5 g of cellulose (Avicel) was moistened with water, and 150 ml ice cold 85% ortho-phosphoric-acid was added. The suspension was slowly stirred in an ice-bath for 1 h. Then 100 ml ice cold acetone was added while stirring. The slurry was transfered to a Buchner filter with Pyrex sintered disc number 3 and then washed three times with 100 ml ice cold

acetone, sucked as dry as possible after each wash. Finally it was washed twice with 500 ml water, and again sucked as dry as possible after each wash. The PASC was mixed with deionized water to a total volume of 300 ml. It was blended to homogeneity (using an Ultra Turrax Homogenizer) and stored in a refrigerator for up to one month.

Substrate was equilibrated with buffer using the following procedure: 20 g phosphoric-acid swollen cellulose PASC stock solution was centrifuged for 20 min at 5000 rpm, the supernatant was poured off, and the sediment was resuspended in 30 ml of buffer. After 20 min centrifugation at 5000 rpm, the supernatant was decanted, and the sediment was resuspended in buffer to a total of 30 g. This corresponds to a substrate concentration of 10 mg l^{-1}.

To measure kinetic parameters, substrate concentrations from 0.2 mg ml^{-1} to 8 mg ml^{-1} were used. Rates were measured at 8 different substrate concentrations in duplicate. The amount of reducing sugars was determined using the PHBAH method modified from (Lever, M. (1972) A new reaction for colormetric determination of carbohydrates. Anal. Biochem. 47, 273-279.).

The enzyme concentration was calculated using the molar absorbancy. The apparent kinetic constants $K_{M(app.)}$, $V_{max(app.)}$ and $k_{cat(app.)}$ were calculated using the equation for enzyme kinetics in the computer program GraFit (Leatherbarrow, R. J. (1992) Grafit version 3.0 Erithacus Software Ltd. Staines, U.K.)

At pH 8.5 and 40 degrees C the following data was obtained:

K_{cat} of 10 per sec and K_M of 0.6 g per l.

EXAMPLE 9

Construction of a hybrid endoglucanase: *Cellvibrio mixtus* **cel45 core with** *Humicola insolens* **EG V linker and CBD**

A construction embodying inframe fusions of *Humicola insolens* Family45 endoglucanase signal peptide encoding sequence and the *Cellvibrio mixtus* catalytic domain encoding sequence and the linker CBD (cellulose binding domain) of *H. insolens* Family45 encoding sequence is performed as described below.

The *Cellvibrio mixtus* family 45 endoglucanase (CmF45) clone DSM 11143 (plasmid p DSM11143) and *the H. insolens* family 45 endoglucanase (HiF45) (plasmid pCaHj418), are used as templates in the following PCR reactions 96 _C 60'' - 3x(94 _C 30'', 45 _C 45'', 72 _C 60'') - 25x(94 _C 30'', 55 _C 45'', 72 _C 60'') 72 _C 7' performed in 50 ul volume with Pwo polymerase according to the manufactures (Boehringer) protocol

1: Template. pCaHj418 (HiF45)
primer 9: CAACATCACATCAAGCTCTCC
primer 10: CCATCACAACCACCAACGGCGGCAAGGGCC

2: Template: pDSM11143
primer 11: GGCCCTTGCCGCCGTTGGTGGTTGTGATGG
primer 12: GGTTTTAATATCATTTAACGCAC

3: Template: pDSM 11143
primer 13: CAAGTCGGACGAGTCTTACC
primer 14: GGATCTGGACGGCATTACAACTGGTTTTAATATC

4: Template: pCaHj418 (HiF45)
primer 15: GATATTAAAACCAGTTGTAATGCCGTCCAGATCC
primer 16: CCCCATCCTTTAACTATAGCG

The 0.15 kb PCR product of reaction 1 and the 0.8 kb product of reaction 2 are mixed in equimolar amounts and joined in a SOE-PCR (sequence overlap extension) reaction with the outsite primers 9 and 12: 25x(94 _C 30'', 55 _C 45'', 72 _C 60'') 72 _C 7'.

The resulting 1.0 kb PCR product of this reaction is purified via a 1% agarose gel, and cleaved with the restriction endonucleases BamH1 and NdeI. The resulting 0.25 kb fragment is purified via a 2 % agarose gel.

The 0.9 kb product of reaction 3 and the 0.3 kb product of reaction 4 are mixed in equimolar amounts and joined in a SOE-PCR reaction with the outsite primers 13 and 16 as disclosed in example 8.

The resulting 1.2 kb PCR product of this reaction is purified via a 1% agarose gel, and cleaved with the restriction endonucleases Nde1 and Xba1. The resulting 0.8 kb fragment is purified via a 1 % agarose gel.

The 0.25 kb BamH1-Nde1 and the 0.8 kb Nde1-Xba1 fragments are ligated in a reaction including the 4.1 kbp Xba-BamH1 fragment of pCaHj418. The resulting ligation mixture is transformed into *E. coli* XL1-Blue. Upon restriction analysis and DNA sequencing of the plasmids of 4 individual transformants (all identical) one such isolate is transformed into *A. oryzae* strain JaL228 together with the selection plasmid pToC202, selecting for the ability to utilize AMDS as sole nitrogen source (Aspergillus transformation is performed as described in EP 238.023). 10 transformants are analyzed for endoglucanase activity on carboxy-methyl cellulose (CMC), and upon three reisolation steps via spores, the *A. oryzae* strain (Ao:CmF45-HiF45), expressing the hybrid protein composed of *Cellvibrio mixtus* F45 catalytic domain and HiF45 linker CBD, is isolated.

Purification and characterization of the cloned hybrid cellulase CmF45-HiF45 is performed as described in example 8.

EXAMPLE 10

Expression in *Pseudomonas fluorescens* and *Pseudomonas cepacia* of endoglucanase cloned from *Cellvibrio mixtus*

The cloned endoglucanase of *Cellvibrio mixtus* contained on the plasmid pSJ1678 in the *E.coli* clone DSM 11143 could be obtained as a HindIII fragment. This HindIII fragment was subcloned in the broad-host range vector pMFY42. PMFY42 is a

direct derivative of pMFY40 (Fukuda, M and Yano, K (1985) Agric. Biol. Chem. 49(9), 2719-2724). The gene conferring ampicilin resistance of pMFY40 was substituted by a gene conferring Neomycin resistance and thus establishing pMFY42 (personal communication with Professor M. Takagi, Department of Agricultural Chemistry, University of Tokyo, Japan). The ligation mixture of pDSM11143 HindIII fragment with pMFY42 HindIII fragment was introduced into E.coli SJ2 by electroporation. Positive clones conferring resistance to 25 ug/ml of Kanamycin and having activity on CMC (identified by congo red colouring as described above) were choosen. One positive clone was choosen for further work, MB431. Plasmid of MB431 were isolated using the Qiagen Spin Prep kit (Qiagen, GMBH, Germany) as indicated by the manufacturer. This plasmid prep was used to transform Pseudomonas fluorescens and Pseudomonas cepacia.

Transformation of *Pseudomonas fluorescens* **and** *Pseudomonas cepacia*:

The *Pseudomonas fluorescens,* DSM 11681, and the *Pseudomonas cepacia,* DSM 11682, strains used in this example were isolated and characterised as indicated in (Balows, A. et al., (Editors) (1992): The Prokaryotes. A Handbook on the Biology of Bacteria. Ecophysiology, Isolation, Identification, Applications. Springer verlag. Vol. I-IV.)

Both strains were transformed using electroporation according to the following procedure:

Apparatus:
Gene Pulser Apparatus with Pulse Controller (Bio-Rad)
Cuvette: 0.4 cm
Conditions: 25μF, 1000S, 2.5V
Medium:
L8 (1% polypeptone, 0.5% NaCl, 0.5% yeast extract, pH should be adjusted to 8.0 by adding 10% Na_2CO_3 after autoclaving)
L8 selection plate (L8 + 2% agar + 25ug/ml kanamycin + 0.1% CMC)

The full procedure was carried out at room temperature, and the plasmid was absolutely deionized.

1 *Pseudomonas* host strain was inoculated in L8 medium and cultivated at 37ºC overnight.

2 50µl of this seed culture (1% of main culture) was inoculated into 5ml of new L8 medium and cultivated at 37ºC until the OD_{660} reached 0.7.

3 The culture was centrifuged at 5000 g for 3 minutes.

4 The cell pellet was washed twice with 10ml of sterile H_2O and re-suspended with 0.5 ml of sterile H_2O.

5 Qiagen purified plasmid (0.5~1ug) was dissolved in 40µl of sterile H_2O and added to 0.5ml of the cell suspension.

6 The mixed suspension was transferred to a Gene Pulser cuvette and pulsed once.

7 The cells were transferred to a Falcon polypropylene tube, diluted with 2ml of L8 medium and cultivated at 37ºC for 1~2 hours with shaking (around 120 rpm).

8 The cells were spread onto L8 selection plates and incubated at 37ºC for 2-7 days.

Colonies were restreaked on fresh selection plates and clones were checked for being positive on CMC congo-red assay (as described above).

One *Pseudomonas fluorecens* (MB481) and one *Pseudomonas cepacia* (MB483) both expressing the *Cellvibrio mixtus* cellulase were kept for further characterization.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: 45 4444 8888
(H) TELEFAX: 45 4449 3256

(ii) TITLE OF INVENTION: A Novel Endoglucanase

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1584 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAGCTAT TTTCGGGCTG GATTTCAGGT TGGGCAAAAT CGTTATTGCC AGGATGCTGT 60

GTATTGCTGG CGTTATATGG TAATGCCGCA TCTGCGGCGA AATGTGAATA CAGGATTGCG 120

AATGATTGGG GCAATGGTTT TACGGCCACT ATCCGAATCA CCAATGATGG CACCGTTCCT 180

GTATCTGGCT GGTCGATCAA TTGGAATTAC AGCGATGGTT CGCGTGTCAC CAGTAGTTGG 240

AATGCCACGT TGTCCGGTGC TAATCCCTAC ACCGCTGTGC CCTTAAATTG GAATAGCAAT 300

ATTGCGGTGG GTTCGAGTGT CGAATTCGGC GTGCAGGGGA CAAATGGTGG AAGTAAGGCG 360

CAGGTACCAA CAGTAGCCGG TGCTGTTTGT TCCGGTGTAG TTGCTTCAAG TATGGCAGCC 420

TCCAGTGTGG TTCCAGCAAG TTCAAGCGTC AGATCCAGTT CCAGTGCGCC ATCCTCAGTG 480

GCACTGAGTT CCCGTTCATC TTCCAGTGTC AGCATTGTTT CTTCTATTCG CAGTTCCACT 540

GCCACATCTG CGTCGGGACA AGCGTGCAAC TGGTATGGCA CCCTCACACC GCTGTGCGCG 600

ACTACCACCA GCGGTTGGGG TTACGAGAAT GGCAAAAGCT GCGTTGCGGT CGCCACTTGT 660

AGTGCGCAGC CCGCACCCTA TGGTGTTGTC GGTGCGGCAT CGAGCACCGC TTCTTCAATT 720

GTGGCGTCTT CAAGTCGGAC GAGTCTTACC AATTCTTCGT CTTCATCAAC ACCGGGTTCT 780

TCATCGCGCA GTTCATCCAG TGCAATAAGT AGTTCGGCCA GCAGTATTCC TCCTATCGTT 840

GGTGGTTGTG ATGGTTACGC GACGCGCTAT TGGGATTGTT GTAAGCCGCA TTGTGGATGG 900

TCGGGCAATG TGCCTGCGTT AGTTGCACCC TTGCAAAGTT GCGCGGCGAA TAATTCGCGC 960

TTGAGTGATT TGACCTTGCC GAGCAGTTGC GACGGCGGCA ATGCGCATAT GTGTTGGGGA 1020

ATGGCTCCTT TTGCGGTGAG CGATACACTC GCTTATGGTT TTGCTGCCAC ATCCAGTGGC 1080

GATGTCTGCG GCCGCTGTTA TCAATTGCAA TTTACGGGCA GCTCACACAA CTCACCGGGT 1140

GATCCGGGAT CGGCAGCACT CGCCGGTAAA ACTATGATCG TGCAGGCTAC CAATATTGGT 1200

TACGACGTAG GTGGCGGGCA ATTCGATATT CTTGTGCCGG GCGGTGGAGT AGGCGCGTTT 1260

AATGCTTGCT CCGCGCAGTG GGGTGTTTCC AATTCTGAAT TGGGCGCGCA ATACGGTGGA 1320

TTGCTGGCAG CATGTAAACA AGAGCTGGGT TATAACGCGA GCCTTGCGCA ATACAAATCC 1380

TGTTTGACGA ATCGCTGTAA CAGCGTTTTT GGCTCAAGGG GATTAACAGA GTTGCAGCGT 1440

GCCTGTACCT GGTACGCGGA TTGGTTCCAG GCCGCTGATA ACCCCGCACT GAAATACAAA 1500

GAAGTTGCAT GTCCGGCTGA ACTTACATCG CGCTCTGGCA TGAACCGCGG TGCGTTAAAT 1560

GATATTAAAA CCAGTTGTAA TTAA 1584

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 527 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Leu Phe Ser Gly Trp Ile Ser Gly Trp Ala Lys Ser Leu Leu
1 5 10 15

Pro Gly Cys Cys Val Leu Leu Ala Leu Tyr Gly Asn Ala Ala Ser Ala
20 25 30

Ala Lys Cys Glu Tyr Arg Ile Ala Asn Asp Trp Gly Asn Gly Phe Thr
35 40 45

Ala Thr Ile Arg Ile Thr Asn Asp Gly Thr Val Pro Val Ser Gly Trp
50 55 60

Ser Ile Asn Trp Asn Tyr Ser Asp Gly Ser Arg Val Thr Ser Ser Trp
65 70 75 80

Asn Ala Thr Leu Ser Gly Ala Asn Pro Tyr Thr Ala Val Pro Leu Asn
85 90 95

Trp Asn Ser Asn Ile Ala Val Gly Ser Ser Val Glu Phe Gly Val Gln
100 105 110

Gly Thr Asn Gly Gly Ser Lys Ala Gln Val Pro Thr Val Ala Gly Ala
115 120 125

Val Cys Ser Gly Val Val Ala Ser Ser Met Ala Ala Ser Ser Val Val
130 135 140

Pro Ala Ser Ser Ser Val Arg Ser Ser Ser Ser Ala Pro Ser Ser Val
145 150 155 160

Ala Leu Ser Ser Arg Ser Ser Ser Ser Val Ser Ile Val Ser Ser Ile
165 170 175

Arg Ser Ser Thr Ala Thr Ser Ala Ser Gly Gln Ala Cys Asn Trp Tyr
180 185 190

Gly Thr Leu Thr Pro Leu Cys Ala Thr Thr Thr Ser Gly Trp Gly Tyr
195 200 205

Glu Asn Gly Lys Ser Cys Val Ala Val Ala Thr Cys Ser Ala Gln Pro
210 215 220

Ala Pro Tyr Gly Val Val Gly Ala Ala Ser Ser Thr Ala Ser Ser Ile
225 230 235 240

Val Ala Ser Ser Ser Arg Thr Ser Leu Thr Asn Ser Ser Ser Ser Ser
245 250 255

Thr Pro Gly Ser Ser Ser Arg Ser Ser Ser Ser Ala Ile Ser Ser Ser
260 265 270

Ala Ser Ser Ile Pro Pro Ile Val Gly Gly Cys Asp Gly Tyr Ala Thr
275 280 285

Arg Tyr Trp Asp Cys Cys Lys Pro His Cys Gly Trp Ser Gly Asn Val
290 295 300

Pro Ala Leu Val Ala Pro Leu Gln Ser Cys Ala Ala Asn Asn Ser Arg
305 310 315 320

Leu Ser Asp Leu Thr Leu Pro Ser Ser Cys Asp Gly Gly Asn Ala His
325 330 335

Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Thr Leu Ala Tyr
340 345 350

Gly Phe Ala Ala Thr Ser Ser Gly Asp Val Cys Gly Arg Cys Tyr Gln
355 360 365

Leu Gln Phe Thr Gly Ser Ser His Asn Ser Pro Gly Asp Pro Gly Ser
370 375 380

Ala Ala Leu Ala Gly Lys Thr Met Ile Val Gln Ala Thr Asn Ile Gly
385 390 395 400

Tyr Asp Val Gly Gly Gly Gln Phe Asp Ile Leu Val Pro Gly Gly Gly
405 410 415

Val Gly Ala Phe Asn Ala Cys Ser Ala Gln Trp Gly Val Ser Asn Ser
420 425 430

Glu Leu Gly Ala Gln Tyr Gly Gly Leu Leu Ala Ala Cys Lys Gln Glu
435 440 445

Leu Gly Tyr Asn Ala Ser Leu Ala Gln Tyr Lys Ser Cys Leu Thr Asn
450 455 460

Arg Cys Asn Ser Val Phe Gly Ser Arg Gly Leu Thr Glu Leu Gln Arg
465 470 475 480

Ala Cys Thr Trp Tyr Ala Asp Trp Phe Gln Ala Ala Asp Asn Pro Ala
485 490 495

Leu Lys Tyr Lys Glu Val Ala Cys Pro Ala Glu Leu Thr Ser Arg Ser
500 505 510

Gly Met Asn Arg Gly Ala Leu Asn Asp Ile Lys Thr Ser Cys Asn
515 520 525

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACGCGGTATT GGGATTGTTG NAAGCCGCAT TGTGGATGGT CGGGCAATGT GCCTGCGTTA 60

GTTGCACCCT TGCAAAGTTG CGCGGCGAAT AATTCGCGCT TGAGTGATTT GACCTTGCCG 120

AGCAGTTGCG ACGGCGGCAA TGCGCATATG TGTTGGGGAA TGGCTCCTTT TGCGGTGAGC 180

GATACACTCG CTTATGGTTT TGCTGCCACA TCCAGTGGCG ATGTCTGCGG CCGCTGTTAT 240

CAATTGCAAT TTACGGGCAG CTCACACAAC TCACCGGGTG ATCCGGGATC GGCAGCACTC 300

GCCGGTAAAA CTATGATCGT GCAGGCTACC AATATTGGTT ACGACGTAGG TGGCGGGCAA 360

TTCGATATTC TTGTGCCCGG CGGCGGCCTC GGCGCCTTC 399

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Thr Arg Tyr Trp Asp Cys Xaa Lys Pro His Cys Gly Trp Ser Gly Asn
1 5 10 15

Val Pro Ala Leu Val Ala Pro Leu Gln Ser Cys Ala Ala Asn Asn Ser
20 25 30

Arg Leu Ser Asp Leu Thr Leu Pro Ser Ser Cys Asp Gly Gly Asn Ala
35 40 45

His Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Thr Leu Ala
50 55 60

Tyr Gly Phe Ala Ala Thr Ser Ser Gly Asp Val Cys Gly Arg Cys Tyr
65 70 75 80

Gln Leu Gln Phe Thr Gly Ser Ser His Asn Ser Pro Gly Asp Pro Gly
85 90 95

Ser Ala Ala Leu Ala Gly Lys Thr Met Ile Val Gln Ala Thr Asn Ile
100 105 110

Gly Tyr Asp Val Gly Gly Gly Gln Phe Asp Ile Leu Val Pro Gly Gly
115 120 125

Gly Leu Gly Ala Phe
130

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACGCGGTATT GGGATTGTTG NAAGCCGCAT TGTGGATGGT CGGGCAATGT GCCGGCATTG 60
GTTGCGCCTT TGCAAAGTTG TGCGGCGAAT AATTCGCGCT TGAGTGATTT AACCTTGCCG 120
AGTAGTTGCG ATGGTGGCAA TGCACACATG TGTTGGGGCA TGGCACCGTT TGCGGTGAGT 180
GATACACTCG CTTACGGCTT TGCTGCTACA TCCAGTGGCG ACGTATGTGG TCGCTGTTAT 240
CAATTGCAAT TTACGGGCAG CTCACACAAC TCACCGGGTG ATCCAGGCTC GGCGGCGCTC 300
GCCGGTAAAA CCATGATCGT ACAAGCTACC AACATTGGTT ACGACGTAGG TGGCGGGCAG 360
TTCGATATTC TCGTACCCGG CGGCGGCCTC GGCGNCTTC 399

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Thr Arg Tyr Trp Asp Cys Xaa Lys Pro His Cys Gly Trp Ser Gly Asn
1 5 10 15

Val Pro Ala Leu Val Ala Pro Leu Gln Ser Cys Ala Ala Asn Asn Ser
20 25 30

Arg Leu Ser Asp Leu Thr Leu Pro Ser Ser Cys Asp Gly Gly Asn Ala
35 40 45

His Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Thr Leu Ala
50 55 60

Tyr Gly Phe Ala Ala Thr Ser Ser Gly Asp Val Cys Gly Arg Cys Tyr
65 70 75 80

Gln Leu Gln Phe Thr Gly Ser Ser His Asn Ser Pro Gly Asp Pro Gly
85 90 95

Ser Ala Ala Leu Ala Gly Lys Thr Met Ile Val Gln Ala Thr Asn Ile
100 105 110

Gly Tyr Asp Val Gly Gly Gly Gln Phe Asp Ile Leu Val Pro Gly Gly

115 120 125

Gly Leu Gly Xaa Phe
130

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 405 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACGCGGTATT GGGATTGTTG NAAGCCACAT TGCAGTTGGA CTGGCAATGT GCCTTCCGTT 60

GTTAATCCGC TACCGGCATG TGGCAGCAAC AATTCGCGTT TAACCGATGT GAATGCGGGC 120

AGTGCATGTG GTAATGGCGG TGGCAGTGCG CACATGTGTT GGGGCATGGC ACCATTTGCG 180

GTGAGCGATA AATTAGCTTA CGGCTATGCG GCTACGGCGA GTGGCGATGT GTGCGGCCGT 240

TGTTATCAAT TGGAATTCAC GGGGCAATCC CACAACTCAC CGGGTGATCC GGGTTCGTCA 300

GCGCTCGCCG GAAAAGTGAT GATCGTCCAG GCAACGAATA TCGGTTACGA CGTGGGTGGT 360

GGCCAATTCC ATATTCTGGT TCCCGGCGGC GGCCTCGGCG NCTTC 405

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 135 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Thr Arg Tyr Trp Asp Cys Xaa Lys Pro His Cys Ser Trp Thr Ala Asn
1 5 10 15

Val Pro Ser Val Val Asn Pro Leu Pro Ala Cys Gly Ser Asn Asn Ser
20 25 30

Arg Leu Thr Asp Val Asn Ala Gly Ser Ala Cys Gly Asn Gly Gly Gly
35 40 45

Ser Ala His Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Lys
50 55 60

Leu Ala Tyr Gly Tyr Ala Ala Thr Ala Ser Gly Asp Val Cys Gly Arg
65 70 75 80

Cys Tyr Gln Leu Glu Phe Thr Gly Gln Ser His Asn Ser Pro Gly Asp
85 90 95

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

Asn Ile Gly Tyr Asp Val Gly Gly Gly Gln Phe His Ile Leu Val Pro
115 120 125

Gly Gly Gly Leu Gly Xaa Phe
130 135

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACGCGGTATT GGGATTGTTG NAAGCCGCAT TGTGGTTGGT CAGGCAATGT GCCCTCATTG 60

GTTACACCGC TGCAAAGTTG TGCTGCCAAT AACACGCGCC TAAGTGATCT GACCTTGCCC 120

AGCAGCTGTG ATGGCGGTAA TGCGCATATG TGTTGGGGAA TGGCGCCCTT TGCAGTAAGC 180

GACACATTGG CGTATGGCTT TGCGGCAACG TCCAATGGCG ATGTATGTGG CCGCTGTTAT 240

CAATTGCAAT TTACCGGCAG CTCACACAAT TCTCCAGGTG ATCCGGGATC GGCCGCGCTG 300

GCAGGTAAAA CTATGATCGT GCAGGCCACC AATATTGGTT ATGACGTCGG CGGTGGACAG 360

TTCGATATTT TAGTACCGGG CGGYGGAGTC GGTGCGTTT 399

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Thr Arg Tyr Trp Asp Cys Xaa Lys Pro His Cys Gly Trp Ser Gly Asn
1 5 10 15

Val Pro Ser Leu Val Thr Pro Leu Gln Ser Cys Ala Ala Asn Asn Thr
20 25 30

Arg Leu Ser Asp Leu Thr Leu Pro Ser Ser Cys Asp Gly Gly Asn Ala
35 40 45

His Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Thr Leu Ala
50 55 60

Tyr Gly Phe Ala Ala Thr Ser Asn Gly Asp Val Cys Gly Arg Cys Tyr
65 70 75 80

Gln Leu Gln Phe Thr Gly Ser Ser His Asn Ser Pro Gly Asp Pro Gly
85 90 95

Ser Ala Ala Leu Ala Gly Lys Thr Met Ile Val Gln Ala Thr Asn Ile
100 105 110

Gly Tyr Asp Val Gly Gly Gly Gln Phe Asp Ile Leu Val Pro Gly Gly
115 120 125

Gly Val Gly Ala Phe
130

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ACGCGGTACT GGGACTGTTG CAAGCCGCAT TGTGGTTGGT CAGGCAATGT GCCCTCATTG 60

GTTACACCGC TGCAAAGTTG TGCTGCCAAT AACACGCGCC TAAGTGATCT GACCTTGCCC 120

AGCAGCTGTG ATGGCGGTAA TGCGCATATG TGTTGGGGAA TGGCTCCCTT TGCAGTAAGC 180

GACACATTGG CGTATGGCTT TGCGGCAACA TCCAATGGCG ATGTATGTGG CCGCTGTTAT 240

CAATTGCAAT TTACCGGCAG CTCACACAAT TCTCCAGGTG ATCCGGGATC GGCCGCACTG 300

GCAGGTAAAA CTATGATCGT GCAGGCCACC AATATTGGTT ATGACGTCGG CGGCGGACAA 360

TTCGATATTC TAGTAGCCGG CGGCGGCCTC GGCGCCTTC 399

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Thr Arg Tyr Trp Asp Cys Cys Lys Pro His Cys Gly Trp Ser Gly Asn
1 5 10 15

Val Pro Ser Leu Val Thr Pro Leu Gln Ser Cys Ala Ala Asn Asn Thr
20 25 30

Arg Leu Ser Asp Leu Thr Leu Pro Ser Ser Cys Asp Gly Gly Asn Ala
35 40 45

His Met Cys Trp Gly Met Ala Pro Phe Ala Val Ser Asp Thr Leu Ala
50 55 60

Tyr Gly Phe Ala Ala Thr Ser Asn Gly Asp Val Cys Gly Arg Cys Tyr
65 70 75 80

Gln Leu Gln Phe Thr Gly Ser Ser His Asn Ser Pro Gly Asp Pro Gly

85 90 95

Ser Ala Ala Leu Ala Gly Lys Thr Met Ile Val Gln Ala Thr Asn Ile
100 105 110

Gly Tyr Asp Val Gly Gly Gly Gln Phe Asp Ile Leu Val Ala Gly Gly
115 120 125

Gly Leu Gly Ala Phe
130

Applicant's or agent's file reference number	International applic : No. PCT/DK97/00348

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 19, line 38.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet X ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
22.08.1996	DSM 11120

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Applicant's or agent's file reference number	International applic. No. PCT/DK97/00348

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 9-10.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [ ]

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
12.09.1996	DSM 11143

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

[X] This sheet was received with the international application

Authorized officer

For International Bureau use only

[ ] This sheet was received by the International Bureau on:

Authorized officer

Applicant's or agent's file reference number	International applic No. PCT/DK97/00348

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 27.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet X ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11683

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 27-28.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [ ]

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Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11684

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number	International applic No PCT/DK97/00348

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 28.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet X ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11685

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

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D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 4, line 29-30.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

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Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11686

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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Applicant's or agent's file reference number	International applic No. PCT/DK97/00348

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 19, line 2.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11681

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

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D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 19, line 2.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X] [ ]

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
18.08.1996	DSM 11682

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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CLAIMS

1. A bacterial enzyme preparation consisting essentially of an enzyme having cellulolytic activity and comprising a first amino acid sequence consisting of 15 amino acid residues having the following sequence

Thr	Arg	Xaa	Xaa	Asp	Cys	Cys	Xaa	Xaa	Xaa	Cys	Xaa	Trp	Xaa	Xaa
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

and a second amino acid sequence consisting of 6 amino acid residues having the following sequence

Ala	Xaa	Gly	Xaa	Xaa	Ala
1	2	3	4	5	6

wherein,
in position 3 of the first sequence, the amino acid is Trp, Tyr or Phe;
in position 4 of the first sequence, the amino acid is Trp, Tyr or Phe;
in position 8 of the first sequence, the amino acid is Arg, Lys or His;
in position 9, 10, 12 and 14, respectively, of the first sequence, the amino acid is any of the 20 naturally occurring amino acid residues;
in position 15 of the first sequence, the amino acid is any of the 20 naturally occurring amino acid residues except Ala;
in position 4 of the second sequence, the amino acid is Phe or Tyr; and
in position 2 and 5, respectively, of the second sequence, the amino acid is any of the 20 naturally occurring amino acid residues.

2. The enzyme preparation according to claim 1, wherein the amino acid residue in position 9 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine,

cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of proline and threonine.

3. The enzyme preparation according to claim 1 or 2, wherein the amino acid residue in position 10 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, histidine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably serine or histidine.

4. The enzyme preparation according to any of the claims 1-3, wherein the amino acid residue in position 12 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine and tryptophan, preferably from the group consisting of alanine and glycine.

5. The enzyme preparation according to any of the claims 1-4, wherein the amino acid residue in position 14 of the first sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, preferably from the group consisting of asparagine, proline, threonine, serine, alanine, glutamic acid and aspartic acid.

6. The enzyme preparation according to any of the claims 1-5, wherein the amino acid residue in position 2 of the second sequence is selected from the group consisting of proline, threonine, valine, alanine, leucine, isoleucine, phenylalanine, glycine, cysteine, asparagine, glutamine, tyrosine, serine, methionine, tryptophan, glutamic acid and aspartic acid, preferably from the group consisting of tyrosine, leucine, and phenylalanine, more preferably tyrosine.

7. The enzyme preparation according to any of the claims 1-6, wherein, in the first sequence, the amino acid residue in position 3 is tyrosine; or the amino acid residue in position 4 is tryptophan; or the amino acid residue in position 8 is lysine.

8. An enzyme preparation consisting essentially of an enzyme having cellulolytic activity and obtained or being obtainable from a bacterial strain belonging to the genus *Cellvibrio* which enzyme comprises an amino acid sequence selected from the group consisting of the sequences

Thr Arg Xaa Phe Asp Cys Cys
1 2 3 4 5 6 7 ;

Thr Arg Xaa Tyr Asp Cys Cys
1 2 3 4 5 6 7 ; and

Thr Arg Xaa Trp Asp Cys Cys
1 2 3 4 5 6 7

wherein, in position 3, the amino acid is Trp, Tyr or Phe.

9. The enzyme preparation according to claim 8, wherein the strain belongs to the group consisting of the species *Cellvibrio mixtus* and *Cellvibrio gilvus*.

10. The preparation according to claim 9, wherein the strain belongs to the group consisting of the strains *Cellvibrio mixtus*, DSM 11683, *Cellvibrio mixtus*, DSM 11684, *Cellvibrio mixtus*, DSM 11685, *Cellvibrio mixtus*, ACM 2601, and *Cellvibrio gilvus*, DSM 11686.

11. The enzyme preparation according to any of the claims 1-10 wherein the enzyme further has, in the position in the polypeptide corresponding to position 409 of the SEQ ID No. 2 when subjected to a conventional sequence alignment, the

amino acid residue aspartic acid.

12. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in *Escherichia coli* DSM 11143, or

b) the DNA sequence shown in SEQ ID NO 1, or

c) an analogue of the DNA sequence which

i) is at least 75% homologous with the DNA sequence shown in SEQ ID NO 1, or

ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 1, or

iii) encodes a polypeptide which is at least 75% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 1, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA seqence shown in SEQ ID NO 1.

13. The cloned DNA sequence according to claim 12, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

14. The cloned DNA sequence according to claim 13, in which the DNA sequence is obtained from a strain of *Cellvibrio mixtus*.

15. The cloned DNA sequence according to claim 14, in which the DNA sequence is cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, DSM 1523, ATCC 12120, or NCIB 8634.

16. The cloned DNA sequence according to claim 12, in which the DNA sequence is cloned from *Escherichia coli*, DSM 11143.

17. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus*, DSM 11683, or
b) the DNA sequence shown in SEQ ID NO 3, or
c) an analogue of the DNA sequence which
i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 3, or
ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 3, or
iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 3, or
iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 3.

18. The cloned DNA sequence according to claim 17, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

19. The cloned DNA sequence according to claim 18, in which the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, DSM 11683.

20. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11685, or
b) the DNA sequence shown in SEQ ID NO 5, or
c) an analogue of the DNA sequence which
i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 5, or
ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 5, or
iii) encodes a polypeptide which is at least 85% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 5, or
iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 5.

21. The cloned DNA sequence according to claim 20, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

22. The cloned DNA sequence according to claim 21, in which the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus,* DSM 11685.

23. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus,* DSM 11685, or
b) the DNA sequence shown in SEQ ID NO 7, or
c) an analogue of the DNA sequence which
i) is at least 75% homologous with the DNA sequence shown in SEQ ID NO 7, or
ii) hybridizes with the same nucleotide probe as the DNA

sequence of SEQ ID NO 7, or
iii) encodes a polypeptide which is at least 80% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 7, or
iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 7.

24. The cloned DNA sequence according to claim 23, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

25. The cloned DNA sequence according to claim 24, in which the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, ACM 2601.

26. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio mixtus*, DSM 11684, or
b) the DNA sequence shown in SEQ ID NO 9, or
c) an analogue of the DNA sequence which
i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 9, or
ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 9, or
iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 9, or
iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence

comprising the DNA sequence shown in SEQ ID NO 9.

27. The cloned DNA sequence according to claim 26, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

28. The cloned DNA sequence according to claim 27, in which the DNA sequence is obtained from a strain of *Cellvibrio mixtus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio mixtus*, DSM 11684.

29. A cloned first DNA sequence encoding an enzyme or enzyme core exhibiting cellulolytic activity and comprising a second DNA sequence, which second DNA sequence comprises

a) the cellulase or endoglucanase encoding part of the DNA sequence present in *Cellvibrio gilvus*, DSM 11686, or
b) the DNA sequence shown in SEQ ID NO 11, or
c) an analogue of the DNA sequence which
    i) is at least 80% homologous with the DNA sequence shown in SEQ ID NO 11, or
    ii) hybridizes with the same nucleotide probe as the DNA sequence of SEQ ID NO 11, or
    iii) encodes a polypeptide which is at least 90% homologous with the polypeptide encoded by the DNA sequence of SEQ ID NO 11, or
    iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified cellulolytic enzyme encoded by the DNA sequence comprising the DNA sequence shown in SEQ ID NO 11.

30. The cloned DNA sequence according to claim 29, in which the DNA sequence encoding an enzyme exhibiting cellulolytic activity is obtained from a bacterium, preferably a gram-negative or purple bacterium, more preferably from the gamma subdivision, especially from the genus *Cellvibrio*.

31. The cloned DNA sequence according to claim 30, in which the DNA sequence is obtained from a strain of *Cellvibrio gilvus*, preferably cloned from or produced on the basis of a DNA library of the strain *Cellvibrio gilvus*, DSM 11686.

32. A recombinant expression vector comprising a cloned DNA sequence according to any of the claims 12-31.

33. A cell comprising a cloned DNA sequence according to any of the claims 12-31 or a recombinant expression vector according to claim 32.

34. The cell according to claim 33, which is a prokaryotic cell, in particular a *Bacillus* cell.

35. The cell according to claim 34, which is a strain of *Bacillus subtilis* or *Bacillus lentus*.

36. The cell according to claim 33, which is a strain belonging to the group of strains consisting of *Cellvibrio mixtus* and *Cellvibrio gilvus*, preferably DSM 1523, ACM 2601, 11683, DSM 11684, DSM 11685 and DSM 11686.

37. The cell according to claim 33, which is a strain of *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae*.

38. A method of producing an enzyme exhibiting cellulolytic activity, the method comprising culturing a cell according to any of claims 33-37 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

39. An isolated enzyme exhibiting cellulolytic activity, characterized in (i) being free from homologous impurities and (ii) being produced by the method according to claim 38.

40. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in Escherichia coli DSM 11143, or
(b) a polypeptide produced by *Cellvibrio mixtus,* DSM 1523, which is encodable by the cellulase encoding part of the DNA sequence cloned into plasmid pSJ1678 present in Escherichia coli DSM 11143, or
(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 2, or
(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 2 which analogue:
(i) is at least 75% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

41. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 3, or
(b) a polypeptide produced by *Cellvibrio mixtus,* DSM11683, or
(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 4, or
(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 4 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

42. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 6, or
(b) a polypeptide produced by *Cellvibrio mixtus,* DSM11685, or
(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 6, or

(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 4 which analogue is at least 85% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

43. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 8, or
(b) a polypeptide produced by *Cellvibrio mixtus,* ACM 2601, or
(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 8, or
(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 8 which analogue is at least 80% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

44. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 10, or
(b) a polypeptide produced by *Cellvibrio mixtus,* DSM11684, or
(c) a polypeptide comprising an amino acid sequence as shown in SEQ ID NO 10, or
(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 10 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

45. An isolated enzyme exhibiting cellulolytic activity which is:
(a) a polypeptide encoded by the cellulase encoding part of the DNA sequence shown in SEQ ID NO: 12, or
(b) a polypeptide produced by *Cellvibrio gilvus,* DSM11686, or
(c) a polypeptide comprising an amino acid sequence as shown

in SEQ ID NO 12, or
(d) a polypeptide comprising an analogue of the polypeptide with the amino acid sequence shown in SEQ ID NO 12 which analogue is at least 90% homologous with said polypeptide, or is immunologically reactive with an antibody raised against said polypeptide in purified form.

46. An enzyme composition comprising the enzyme according to any of the claims 39-45.

47. The composition according to claim 46, which additionally comprises one or more enzymes selected from the group consisting of galactanases, xylanases, arabinanases, pectin acetyl esterases, polygalacturonases, rhamnogalacturonases, pectin lyases, pectate lyases, endoglucanases, pectin methylesterases, proteases, lipases, amylases, cutinases, peroxidases, laccases, cellobiohydrolases and transglutaminases.

48. Use of an enzyme according to any of the claims 39-45 or an enzyme composition according to claims 1-11, 46 or 47 in a laundry detergent.

49. An isolated substantially pure biological culture of the deposited strain *Escherichia coli* DSM 11143.

50. Use of the enzyme according to any of the claims 39-45 or the composition according to claims 1-11, 46 or 47 for degradation or modification of plant material, e.g. cell walls; treatment of fabric or textile, preferably for preventing backstaining, for bio-polishing or for "stone-washing" cellulosic fabric; or in the treatment of paper pulp, preferably for debarking, defibration, fibre modification, enzymatic de-inking or drainage improvement.

51. A method of providing colour clarification of laundry, which method comprising treating the laundry with a soaking, washing or rinsing liquor comprising the enzyme according to

any of the claims 39-45 or the enzyme preparation according to claims 1-11, 46 or 47.

52. The cloned first DNA sequence according to any of the claims 12-31 which further comprises a DNA sequence encoding a cellulose-binding domain.

53. The DNA sequence according to claim 52 which further comprises a DNA sequence encoding a cellulose-binding domain (CBD), the cellulose-binding domain and enzyme core (catalytically active domain) of the enzyme encoded by the DNA sequence being operably linked.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00348

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/42
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

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

SE,DK,FI,NO classes as above

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

WPI, MEDLINE, CA, GENBANK/EMBL/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9407998 A1 (NOVO NORDISK A/S), 14 April 1994 (14.04.94), See fig 1a --	1-53
A	Applied and Environmental Microbiology, Volume 36, No 1, 1978, Linda V. Oberkotter et al, "Extracellular Endo-Beta-1,4-Glucanase in Cellvibrio vulgaris" page 205 - page 209 --	1-53
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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International application No.
PCT/DK 97/00348

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