(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 November 2006 (09.11.2006)

PCT

(10) International Publication Number
WO 2006/117432 A1

(51) International Patent Classification:
C12N 9/42 (2006.01) *C11D 3/386* (2006.01)
C12N 15/62 (2006.01) *D06M 16/00* (2006.01)
C12N 15/63 (2006.01) *A23K 1/165* (2006.01)
C12N 15/80 (2006.01)

(21) International Application Number:
PCT/FI2006/050161

(22) International Filing Date: 25 April 2006 (25.04.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
20055202 29 April 2005 (29.04.2005) FI
11/119,526 29 April 2005 (29.04.2005) US

(71) Applicant *(for all designated States except US)*: **AB ENZYMES OY** [FI/FI]; Tykkimäentie 15, FI-05200 Rajamäki (FI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **VEHMAANPERÄ, Jari** [FI/FI]; Lepsämäntie 359, FI-01800 Klaukkala (FI). **PURANEN, Terhi** [FI/FI]; Orastie 5, FI-01900 Nurmijärvi (FI). **VALTAKARI, Leena** [FI/FI]; Puistokuja 10 B, FI-05200 Rajamäki (FI). **KALLIO, Jarno** [FI/FI]; Muorinpolku 8 A 12, FI-04400 Järvenpää (FI). **ALAPURANEN, Marika** [FI/FI]; c/o Roal Oy, Tykkimäentie 15, FI-05200 Rajamäki (FI). **PALOHEIMO, Marja** [FI/FI]; Riekkopolku 5, FI-01450 Vantaa (FI). **OJAPALO, Pentti** [FI/FI]; Daalintie 202, FI-04360 Tuusula (FI).

(74) Agent: **KOLSTER OY AB**; Iso Roobertinkatu 23, P.O. Box 148, FI-00121 Helsinki (FI).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** IMPROVED CELLULASES

(57) **Abstract:** The present invention provides novel cellulase fusion proteins, preparations of cellulase fusion proteins and compositions of cellulase fusion proteins. The present invention further provides cellulase expression vectors, host cells expressing cellulase and methods for preparing such vectors and cells. Uses of cellulases, cellulase preparations and cellulase compositions in the textile, detergent, pulp and paper industries are also provided.

WO 2006/117432 A1

IMPROVED CELLULASES

Field of the Invention

The present invention relates to novel cellulase fusion proteins, preparations and compositions containing these cellulase fusion proteins, expression vectors, host cells and methods for their preparation and uses of the cellulases, preparations and compositions in the textile, detergent and pulp and paper industries.

Background of the Invention

Cellulose is a linear polysaccharide of glucose residues connected by β-1,4 linkages. In nature, cellulose is usually associated with lignin together with hemicelluloses, such as xylans and glucomannans. Cellulolytic enzymes hydrolyze cellulose and are produced by a wide variety of bacteria and fungi. Cellulases are industrially important enzymes with a current annual market value of about 190 million US $. In the textile industry, cellulases are used in denim finishing to create a fashionable stone washed appearance in denim cloths in a biostoning process, and they are also used, for instance, to clean fuzz and prevent formation of pills on the surface of cotton garments. In detergent industry cellulases are used to brighten colors and to prevent graying and pilling of garments. Cellulases are further used in food industry and animal feed manufacturing, and they have a great potential in the pulp and paper industry, for instance, in deinking to release ink from fiber surfaces and in improving pulp drainage. The wide spectrum of industrial uses for cellulases has established a need for commercial cellulase products containing different cellulase components and functioning optimally in different pH and temperature ranges.

The practical use of cellulases is hampered by the nature of the known cellulases, which are often mixtures of cellulases having a variety of activities and substrate specificities. For this reason, efforts have been made to obtain cellulases having only the desired activities. The unique properties of each cellulase make some more suitable for certain purposes than others. While the enzymes differ in a number of ways, one of the most important differences is the pH optimum. Neutral cellulases are most active in the pH range 6-8 and alkaline cellulases in the pH range 7.5-10, whereas acid cellulases, having the pH optimum at pH 4.5-5.5, show very low activity levels at higher

pH values. Neutral and acid cellulases are especially useful in the textile industry. In fabric treatment cellulases attack the chains of cellulose molecules that form the cotton fibers, thereby affecting the characteristics of the fabric.

In textile industry "stone washed" look or an abraded look has been denim producers' interest in recent years. Traditional stone washing with pumice stones reduces the strength of fabric and burdens the laundering apparatuses. The trend has been towards enzymatic denim finishing processes and cellulases have replaced or are being used together with pumice stones to give the fabric its desired "worn" look. Controlled enzyme treatment results in less damage to the garments and machines and eliminates the need for disposal of stones.

Cellulases applied in denim treatment are usually divided into two main groups: acid and neutral cellulases. Acid cellulases typically operate at pH 4.5 – 5.5 and the neutral cellulases in the range of pH 6-8. Acid cellulases used in biostoning mainly originate from *Trichoderma reesei* (sexual form *Hypocrea jecorina*) and the neutral cellulases come from a variety of fungi, including genera of *Melanocarpus, Humicola, Thielavia, Myceliophthora, Fusarium, Acremonium,* and *Chrysosporium* (Haakana *et al*. 2004). *T. reesei* enzymes include, e.g., cellulases from the glycoside family 5 (endoglucanase II, EGII), family 7 (cellobiohydrolase I, CBHI) and family 12 (endoglucanase III, EGIII; Ward *et al.* 1993), and the neutral cellulases, most often endoglucanases, from family 45 and family 7 (Henrissat, 1991; Henrissat and Bairoch, 1993).

Cellulases comprise a catalytic domain/core (CD) expressing cellulase activity. In addition to the catalytic domain the cellulase molecule may comprise one or more cellulose binding domains (CBDs), also named as carbohydrate binding domains/modules (CBD/CBM), which can be located either at the N- or C-terminus of the catalytic domain. CBDs have carbohydrate-binding activity and they mediate the binding of the cellulase to crystalline cellulose but have little or no effect on cellulase hydrolytic activity of the enzyme on soluble substrates. These two domains are typically connected via a flexible and highly glycosylated linker region.

Cellulases that attack primarily on the surface of the fiber are especially useful in stone washing of denim dyed with Indigo dye, as the dye is located on the surface of the fiber. When used to treat cotton fabric, neutral cellulases generally require a longer washing time than the acid cellulases. However, neutral cellulases have less aggressive action on cotton than acid

cellulases, and do not affect on the strength of the fabric as much as acid cellulases. Neutral cellulases have a broader pH profile and thus the pH increase that occurs during biostoning has little effect on the activity of neutral cellulase enzymes. However, since cellulase treatments also have undesirable effects, such as fiber damage and strength loss, a suitable balance between the desired and unwanted effects has to be sought.

WO97/14804, which is incorporated herein by reference, discloses three novel neutral cellulases of *Melanocarpus* origin, which are especially useful in the textile and detergent industry. Specifically a 20 kDa endoglucanase (Cel45A), a 50 kDa endoglucanase (Cel7A), and a 50 kDa cellobiohydrolase (Cel7B) are described. These cellulases designated herein as "20K-cellulase", "50K-cellulase", and "50K cellulase B", respectively, are derived from *Melanocarpus albomyces* and show good stone washing effects.

Since there is an existing demand, especially in the textile and detergent industry, for further improved cellulases, it has been suggested that improvements in cellulases could be obtained by forming fusion proteins. Also in WO97/14804 fusion protein constructs of 20K-cellulase, 50K-cellulase, and 50K cellulase B with, for instance, *Trichoderma reesei* cellulase, hemicellulase or mannase or functional domains thereof, are generally suggested. Further, in order to create new properties for the disclosed cellulases, fusions of the disclosed cellulases with domains, such as cellulose binding domain (CBD), preferably with its linker, are suggested. However, no specific examples are given, nor are described the new properties aimed to.

Cellulase fusion proteins are additionally known, for instance, from WO96/29397, which discloses endoglucanases formed by a fusion between endoglucanases from *Myceliophthora thermophila*, from *Macrophomina phaseolina* and from *Crinipellis scabella* and the CBD/linker from *Humicola insolens*. Said endoglucanases in their natural form do not have a CBD/linker.

EP 663 950 discloses cellulase variants, especially *Humicola insolens* 43 kDa cellulase variants, wherein the cellulase may include a linking region from another microorganism species, for instance for providing improved properties, such as improved resistance to anionic surfactants, to oxidation or to bleaching agents.

However, there is a continuous need for improved cellulases that also are less harmful to the fiber in textile industry and in other fields, where

cellulases traditionally are used. In particular, there is a continuous need for more efficient cellulases to improve the process economics.

The present invention aims to meet this need.

Brief description of the invention

An object of the present invention is to provide novel cellulase fusion proteins having improved hydrolytic properties for use in textile industry, especially in stone washing denim, and for use in detergent compositions as well as in other fields. The novel cellulase fusion proteins of the invention are active at neutral and alkaline pH values, they have highly improved washing performance in textile biofinishing and biostoning applications and in detergent applications, and yet they do not compromise the strength of fabrics. With the improved efficiency of the cellulase fusion proteins of the invention, the manufacturing process of the enzymes is significantly more economical. Additional advantages are achieved also in terms of logistics and the storage of the enzyme products, when smaller amounts of the enzyme product are needed.

A further object of the present invention is to provide polynucleotides encoding the novel cellulase fusion proteins of the present inventions.

A further object of the present invention is to provide novel expression plasmids or vectors containing such polynucleotides, useful for the production of the novel cellulase fusion proteins of the present invention, and novel hosts transformed with said expression plasmids.

A further object of the present invention is to provide enzyme preparations, which contain one or more novel cellulase fusion proteins having improved hydrolytic properties.

A still further object of the present invention is to provide methods of using the enzyme preparations and the cellulase fusion proteins for finishing of textiles, especially for biostoning of denim.

A still further object of the present invention is to provide means for the use of the enzyme preparations of the invention in detergent compositions.

The present invention relates to a novel cellulase fusion protein comprising

A. an optionally modified first amino acid sequence of a cellulase core derived from one species, and

B. an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species,

wherein a junction region has been introduced between said first amino acid sequence and said second amino acid sequence, whereby a stable fusion protein is obtained.

Preferably, the junction region has the following general formula:

$^{1}A - ^{2}B - ^{3}C - ^{4}D - ^{5}E - ^{6}F$

wherein

^{1}A is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{1}A is Gly or Val, most preferably Gly;

^{2}B is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, Phe, Val, Glu, Asp, Gln, and Asn; preferably ^{2}B is Pro, Gln, or Glu;

^{3}C is selected from a group consisting of Gly, Ala, Lys, Leu, Pro, Ile, Val, Ser, and Thr; preferably ^{3}C is Ile;

^{4}D is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{4}D is Gly or Pro;

^{5}E is selected from a group consisting of Ser, Pro and Thr; preferably ^{5}E is Ser; and

^{6}F is selected from a group consisting of Ser, Thr or is absent, preferably ^{6}F is Ser or is absent; wherein ^{1}A is attached at the C-terminal amino acid sequence of the cellulase core and ^{6}F is attached at the N-terminal amino acid sequence of the linker and/or domain (CBD).

The present invention further relates to an expression vector comprising a first polynucleotide sequence encoding an optionally modified first amino acid sequence of a cellulase core derived from one species, and a second polynucleotide sequence encoding an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species, and a polynucleotide encoding a junction region connecting said first and second polynucleotide sequences, said polynucleotide sequences encoding the respective amino acid sequences of the cellulase fusion proteins of the invention.

The present invention further relates to novel hosts transformed with the vectors of the invention, especially hosts that are capable of high level expression of the cellulase fusion protein of the invention.

The present invention further relates to an enzyme preparation, which contains one or more cellulase fusion proteins of the invention.

The present invention further relates to a method for using the enzyme preparations of the invention for the finishing of textiles, especially for biostoning of denim.

The present invention further relates to the use of the enzyme preparations of the invention in detergent compositions.

Drawings

Fig. 1 is the schematic map of the plasmid pALK1480.

Fig. 2 is the schematic map of the plasmid pALK492.

Fig. 3 is the schematic map of the plasmid pALK424.

Fig. 4 is the schematic map of the plasmid pALK1237.

Fig. 5 is the schematic map of the plasmid pALK1241.

Fig. 6 is the schematic map of the plasmid p3SR2.

Fig. 7 is the schematic map of the plasmid pALK1649.

Fig. 8 is the schematic map of the plasmid pALK1694.

Fig. 9A. The expression cassette used in the transformation of *Trichoderma reesei* protoplasts for producing the 20K+CBD fusion proteins. The 20K+CBD gene was under the control of the *cbh1 (cel7A)* promoter (*cbh1* prom) and termination of transcription was ensured by using the *cbh1* terminator sequence (term). The *amd*S gene (amdS) and the *cbh*I 3' flanking region (cbhI 3' flanking) were included. Fig. 9B. Amino acid sequence of a junction point at which *Melanocarpus albomyces* 20K (Cel45A) protein is fused to linker peptide of *Trichoderma reesei* CBHI (Cel7A) followed by the cellulose-binding domain (CBD) in pALK1434 and pALK1435 plasmids. The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid in the CBD region is indicated by superscript numbers.

Fig. 10A. The expression cassette used in the transformation of *Trichoderma reesei* protoplasts for producing the 20K+CBD fusion proteins. The 20K+CBD gene was under the control of the *cbh1 (cel7A)* promoter (*cbh1* prom) and termination of transcription was ensured by using the *cbh1* terminator sequence (term). The *amd*S gene (amdS) and the *cbh*I 3' flanking region (cbhI 3' flanking) were included. Fig. 10B. Amino acid sequence of a junction point at which *Melanocarpus albomyces* 20K (Cel45A) protein is fused to linker peptide of *Trichoderma reesei* CBHI (Cel7A) followed by the cellulose-binding domain (CBD) in pALK1768, pALK1769, pALK1770 and pALK1775 plasmids. The amino acids contained in the linker region are underlined, and the amino

acid sequence of the CBD region is marked by italics. The first amino acid in the CBD region is indicated by superscript numbers.

Fig. 11A. The expression cassette used in the transformation of *Trichoderma reesei* protoplasts for producing the 20K+CBD_{mut} fusion proteins. The 20K+CBD_{mut} gene was under the control of the *cbh1 (cel7A)* promoter (*cbh1* prom) and termination of transcription was ensured by using the *cbh1* terminator sequence (term). The *amdS* gene was included as a transformation marker. Fig. 11B. Amino acid sequence of a junction point at which *Melanocarpus albomyces* 20K (Cel45A) protein is fused to linker peptide of *Trichoderma reesei* CBHI (Cel7A) followed by the cellulose-binding domain (CBD). The amino acid substitutions in the CBD region of the pALK1877 – pALK1880 expression cassettes are also presented. The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid and the tyrosine residues or their substitutions in the CBD region are indicated by superscript numbers.

Fig. 12A Amino acid sequence of the interdomain linker peptide of *T. reesei* CBHI (Cel7A). The amino acids contained in the linker region are underlined. ΔG-444 and ΔG-460 represent the linker deletion of residues 434-444 and 434-460, respectively. Fig. 12B. Amino acid sequence of a junction point at which *Melanocarpus albomyces* 20K (Cel45A) protein is fused to truncated linker peptide of *Trichoderma reesei* CBHI (Cel7A) followed by the intact or mutated cellulose-binding domain (CBD) in the pALK1893, pALK1896, pALK1899 and pALK1952 expression cassettes. The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid and the tyrosine residues or their substitutions in the CBD region are indicated by superscript numbers.

Fig. 13A. The expression cassette used in transformation of *Trichoderma reesei* protoplasts for production of the 50K+CBD fusion protein. The 50K+CBD gene is under control of *T. reesei cbh*I promoter (cbhI prom) and transcription termination is ensured with the addition of the *cbh*I terminator (term). The *amd*S gene (amdS) and the *cbh*I 3' flanking region (cbhI 3') are included. Fig. 13B. Amino acid sequence of the junction point of the *M. albomyces* 50K linked to the *T. reesei* CBHI linker+CBD. The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid in the CBD region is indicated by superscript numbers.

Fig. 14A. The expression cassette used in transformation of *Trichoderma reesei* protoplasts for production of the 50KB+CBD fusion protein. The 50KB+CBD gene is under control of *T. reesei cbh*I promoter (cbhI prom) and transcription termination is ensured with the addition of the *cbh*I terminator (term). The *amdS* gene (amdS) and the *cbh*I 3' flanking region (cbhI 3') are included. Fig. 14B. Amino acid sequence of the junction point of the *M. albomyces* 50KB linked to the *T. reesei* CBHI linker+CBD. The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid in the CBD region is indicated by superscript numbers.

Fig. 15A. The expression cassette used in the transformation of *Trichoderma reesei* protoplasts for producing the recombinant *Thermoascus aurantiacus* CBHI+CBD fusion proteins. The CBHI+CBD gene was under the control of the *cbh1 (cel7A)* promoter (*cbh1* prom) and termination of transcription was ensured by using the *cbh1* terminator sequence (term). The *amdS* gene was included as a transformation marker. Fig. 15B. Amino acid sequence of a junction point at which *Thermoascus aurantiacus* CBHI protein is fused to linker peptide of *Trichoderma reesei* CBHI followed by the cellulose-binding domain (CBD). The amino acids contained in the linker region are underlined, and the amino acid sequence of the CBD region is marked by italics. The first amino acid in the CBD region is indicated by superscript numbers.

Fig. 16. The performance of strains RF5977 and RF6090 expressing fusion proteins of the invention compared to a commercial 20K preparation in denim treatment. Increase of lightness as a function of enzyme dosage at washing conditions described in Examples 8 and 9.

Fig. 17. Effect of the 20K+CBD fusion proteins and corresponding commercial enzyme preparations on the strength of the denim fabric. Fig. 17A. Tear strength (N), warp. Fig. 17B. Tear strength (N), weft.

Fig. 18 shows the depilling effect of the 20K+CBD fusion protein.

Fig. 19 illustrates the performance of the 20K+CBD fusion protein in detergent application. The figure shows the colour matching difference between the anti-greying article 224 washed with or without the enzyme, and the original (unwashed) article; A. at 40°C and B. at 60°C.

Fig. 20 illustrates the performance of 20K+CBD fusion protein in detergent application. The figure shows the colour matching difference between

the anti-greying article 224 washed with enzyme, and without enzyme; A. at 40°C and B. at 60°C.

Detailed description of the invention

The present invention is based on efforts to further improve neutral cellulases, in particular those described in WO97/14804, aiming at reducing the loss of the strength of the fabric in the enzyme treatment. In some applications the 20K cellulase has shown undesirable properties in relation to fiber strength, possibly due to the small size. The simple hypothesis was that an increase in the size of the enzyme would decrease the ability of the enzyme to penetrate into the fibers, thereby weakening the fibers to a smaller extent, i.e. the enzyme would be less aggressive. To do this the fusion protein approach suggested in WO97/14804 was used, and fusions constructs containing a neutral cellulase core of a *Melanocarpus* species and a tail consisting of a linker/CBD of an acid cellobiohydrolase I of *T. reesei* were designed. Surprisingly, however, contrary to the suggestions of the prior art, fully stable fusion protein constructs could not be obtained, but the fusion partners separated from each other in the culture conditions. This was presumably due to the presence of protease(s).

To produce stable fusion proteins, one approach was to design novel junction constructs having no adjacent hydrophobic amino acids (e.g., V, I, L, F, and W) in order to prevent cleavage by aspartylproteases. However, although the constructs produced fusion proteins, some degradation was occasionally observed.

Based on the alignment of neutral cellulases naturally containing a linker/CBD tail, further constructs were produced and finally these constructs proved to be most stable and most useful for further testing. In addition, fusion constructs were designed which carried mutations in the CBD resulting in reduced or minimal affinity or adsorption to cellulose (Linder et al. 1995).

The novel constructs produced improved strength properties, as was the aim. Surprisingly, the stable cellulase fusion proteins additionally showed unexpected improvement in washing performance, and were as high as even six times as efficient as their "parent" cellulases. However, the production yields maintained at about the same level. This means that only one sixth of the amount of the cellulase activity presently needed is enough for achieving the same washing performance of the prior art cellulase. This produces considerable savings in the production step, and also in the logistics and storage,

thereby decreasing the environmental burden. Also the undesired effects of the cellulase preparations are reduced, thereby bringing further savings for the final users of the enzyme product. Considering that about 2 billion pairs of denim jeans are produced annually, and most of them are finished with cellulase, the advantage is highly significant.

Accordingly, the present invention provides a novel cellulase fusion protein comprising

A. an optionally modified first amino acid sequence of a cellulase core derived from one species, and

B. an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species,

wherein a junction region has been introduced between said first amino acid sequence and said second amino acid sequence, whereby a stable fusion protein is obtained.

In a preferred embodiment of the invention the junction region has the following general formula:

$$^{1}A - ^{2}B - ^{3}C - ^{4}D - ^{5}E - ^{6}F$$

wherein

^{1}A is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{1}A is Gly or Val, most preferably Gly;

^{2}B is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, Phe, Val, Glu, Asp, Gln, and Asn; preferably ^{2}B is Pro, Gln, or Glu;

^{3}C is selected from a group consisting of Gly, Ala, Lys, Leu, Pro, Ile, Val, Ser, and Thr; preferably ^{3}C is Ile;

^{4}D is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{4}D is Gly or Pro;

^{5}E is selected from a group consisting of Ser, Pro and Thr; preferably ^{5}E is Ser; and

^{6}F is selected from a group consisting of Ser, Thr or is absent, preferably ^{6}F is Ser or is absent; wherein ^{1}A is attached at the C-terminal amino acid sequence of the cellulase core and ^{6}F is attached at the N-terminal amino acid sequence of the linker and/or domain (CBD).

In a specially preferred embodiment of the invention the junction region has the following general formula:

^{1}Gly – ^{2}B – ^{3}Ile – ^{4}D – ^{5}Ser – ^{6}F

wherein

^{2}B is Pro, Gln, or Glu;

^{4}D is Gly or Pro;

^{5}E is Ser; and

^{6}F is ^{6}F is Ser or is absent.

In another specially preferred embodiment of the invention the junction region has the following general formula:

^{1}Val – ^{2}Gln – ^{3}Ile – ^{4}Pro – ^{5}Ser – ^{6}Ser.

In another specially preferred embodiment of the invention the junction region has the following general formula:

^{1}Gly – ^{2}Glu – ^{3}Ile – ^{4}Gly – ^{5}Ser.

In another specially preferred embodiment of the invention the junction region has the following general formula:

^{1}Gly – ^{2}Pro – ^{3}Ile – ^{4}Gly – ^{5}Ser.

In a preferred embodiment of the invention the first amino acid sequence is from a neutral cellulase and the second amino acid sequence is from an acid cellulase.

In another preferred embodiment of the invention the first amino acid sequence is from a cellulase of family 45 (Cel 45) and the second amino acid sequence is from cellulase of family 7 (Cel 7).

As used in the present context the expression "cellulase core" or "core" means the catalytic domain/core (CD) of an enzyme expressing cellulase activity. Such a catalytic domain may be in its naturally occurring form (i.e. intact) or, preferably, is modified as defined below. The expressions "derivative" and functional variant denote polypeptides expressing the same cellulase activity but including modifications as defined below.

In the present context conventional one-letter amino acid codes and three-letter amino acid codes are used. Thus, A and Ala denote alanine, R and

Arg denote arginine, N and Asn denote asparagine, D and Asp denote aspartic acid, Cys and C denote cysteine, E and Glu denote glutamic acid, Q and Gln denote glutamine, G and Gly denote glycine, H and His denote histidine, I and Ile denote isoleucine, L and Leu denote leucine, K and Lys denote lysine, M and Met denote methionine, F and Phe denote phenylalanine, P and Pro denote proline, S and Ser denote serine, T and Thr denote threonine, W and Trp denote tryptophan, Y and Tyr denote tyrosine, and V and Val denote valine. In addition to naturally occurring L-amino acids, D-amino acids could be used.

In the cellulase fusion proteins of the invention, the neutral cellulase is preferably of fungal origin. The neutral cellulase can be derived from genera of *Melanocarpus*, *Humicola*, *Thielavia*, *Myceliophthora*, *Fusarium*, *Acremonium, Chrysosporium, Thermoascus, Scopulariopsis, Myriococcum, Talaromyces,* or *Chaetomium.* Specifically preferred are *Melanocarpus sp,* with *Melanocarpus albomyces* being especially preferred. The acid cellulase used in the cellulase fusion proteins of the invention originate from *Trichoderma sp.* or *Hypocrea*, especially from *Trichoderma reesei.*

In a specifically preferred embodiment of the invention the first amino acid sequence is 20 K cellulase of *Melanocarpus albomyces* of SEQ ID. NO: 2 or a derivative thereof, and the second amino acid sequence is the linker and/or CBD of *Trichoderma reesei* cellobiohydrolase I of SEQ ID. NO: 4 or a derivative thereof.

In one preferred embodiment of the invention the cellulase fusion proteins contain modifications in cellulase core and/or in the linker and/or CBD. As used in the present context the expression "modified" refers to mutations, such as a deletion, insertion, or substitution of one or more amino acids, or other modifications, such as glycosylations. Examples of such mutations include the substitution of conserved tyrosine residues at positions 31 (corresponding tyrosine Y492 of the mature polypeptide) and/or 32 (corresponding tyrosine Y493 of the mature polypeptide) with an aliphatic amino acid, preferably with alanine, and/or with an aromatic amino acid, such as tryptophan, of CBD of *Trichoderma reesei* CBHI as described by Linder *et al.*, 1995. Further examples of such mutations include interlinker mutations of *Trichoderma reesei* CBHI as described by Srisodsuk *et al.*, 1993, such as deletions of amino acids from position 434 to 444 and from position 434 to 460 of the mature *Trichoderma* CBHI sequence. Further examples of such mutations include the deletion of Ala at position 207, the deletion of Val at position 208, the substitu-

tion of Phe209Trp, and insertion of Pro after position 206 in 20 K cellulase sequence of *Melanocarpus albomyces* of SEQ ID. NO: 2.

The cellulase fusion proteins of the invention are stable. In the context of the present invention the expression "stable cellulase fusion protein" means that at least 20%, preferably at least 40%, more preferably at least 70%, most preferably 90%-100%, of the produced cellulase fusion protein contains uncleaved junction region between the amino acid sequences during the fermentation. This means that 20%-100%, preferably 40%-100%, more preferably 70% -100% of the produced cellulase have the first and the second amino acid sequence fused together. The expression "stable cellulase fusion protein" additionally means that the cellulase fusion protein preparation may be stable as such or has been stabilized by, e.g., heat treatment or adjusting pH or by adding stabilizers or agents reducing protease activity or by separating the fusion protein from the culture. The heat treatment in the present context means a treatment at temperature, which allows the fusion protein in the preparation to be maintained adequately stable. The heat treatment can be, e.g., a treatment at pH 6.0 at 65°C for 60 to 70 minutes.

In the present context the expression "intact fusion protein" means that the junction between the first and the second amino acid sequence in the fusion protein of the invention remains unbroken, although there may or may not appear terminal degradation in said sequences.

In one preferred embodiment of the cellulase fusion protein of the invention, the first amino acid sequence is a *Melanocarpus albomyces* 20K sequence having SEQ ID NO: 2 or a functional variant thereof. In another preferred embodiment of the first amino acid sequence is *Melanocarpus albomyces* 50K sequence having SEQ ID NO: 6 or a functional variant thereof. In another preferred embodiment of the first amino acid sequence is *Melanocarpus albomyces* 50KB sequence having SEQ ID NO: 8 or a functional variant thereof. In another preferred embodiment of the first amino acid sequence is *Thermoascus aurantiacus* CBHI sequence having SEQ ID NO: 10 or a functional variant thereof. In yet one preferred embodiment of the cellulase fusion protein of the invention the second amino acid sequence is the linker and cellulase binding domain sequence having SEQ ID NO: 4 of *Trichoderma reesei* cellobiohydrolase I or a functional variant thereof.

Thus in a highly preferred embodiment of the cellulase fusion protein of the invention, the first amino acid sequence of cellulase core is selected

from SEQ ID. NO: 37, 38, 39, 40, 41, 42, and 43, especially SEQ ID. NO: 39, and the second amino acid sequence of a linker and/or CBD sequence is selected from SEQ ID. NO: 44, 45, 46, 47, 48, 49, and 50. In a special embodiment of the invention, the first amino acid sequence of cellulase core is SEQ ID. NO: 39 and the second amino acid sequence of a linker and/or CBD sequence is SEQ ID. NO: 47, 49, or 50.

The present invention further relates to an expression vector comprising a first polynucleotide sequence encoding an optionally modified first amino acid sequence of a cellulase core derived from one species, and a second polynucleotide sequence encoding an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species, and a polynucleotide encoding a specific junction region connecting said first and second polynucleotide sequences, said polynucleotide sequences encoding the respective amino acid sequences as specifically defined above.

The present invention further relates to cellulase preparations containing one or more cellulase fusion proteins of the invention alone or together with additional enzymes and additives according to the special application in question.

The present invention further relates to the uses of and methods for using the cellulase fusion protein preparations of the invention for purposes specifically disclosed below.

The cellulase fusion protein preparations of the invention are especially useful in the textile and detergent industry. These cellulases show highly improved abrasion effect and visible and measurable increase of lightness. They show acceptable backstaining and good as well as focused contrast in biostoning. They are useful in the textile industry for biofinishing of fabrics or garments, e.g., depilling, defuzzing, color clarification, harshness reduction, creation of different finishes (for example, a 'peach skin,' 'worn out,' 'sand washed,' or 'antique look' effect) and for biofinishing of yarn, for example, reduction of hairiness and improvement of smoothness. Having such good depilling properties is very unusual for neutral cellulases, since enzymes used in industrial biofinishing applications are typically acid cellulases. Neutral cellulase having excellent depilling properties like the 20K+CBD fusion protein enables biofinishing treatment simultaneously during dyeing, leading to considerably savings. Also the colour fastness is often better at neutral than acid conditions.

Additional uses include the use in detergent compositions to improve fabric care properties by antipilling, antigraying, color clarification and softening, and to improve textile cleaning effect, for instance soil removal.

As used in the present context the expression "biostoning" of fabric or garment means the use of enzymes in place of, or in addition to, pumice stones for the treatment of fabric or garment, especially denim.

As used in the present context the expression "biofinishing" refers to the use of enzymes in a controlled hydrolysis of cellulosic fibers in order to modify the fabric or yarn surface in a manner that prevents permanently pilling, improves fabric handle like softness and smoothness, clears the surface structure by reducing fuzzing, which results in clarification of colors, improves the drapability of the fabric, improves moisture absorbability, which may improve also the dyeability.

As used in the present context the expression "backstaining" refers to the tendency of released dye to redeposit on the surface of the fabric fibers.

As used in the present context the expression "detergent" refers to a cleansing agent that can contain surface active agents (anionic, non-ionic, cationic and ampholytic surfactants), builders and other optional incredients such as anti-redeposition and soil suspension agents, optical brighteners, bleaching agents, dyes and pigments and hydrolases. Suitable listing of the contents of detergents is given in U.S. Patent No. 5,433,750, a suitable list of surfactants is given in U.S. Patent No. 3,664,961.

By an amino acid sequence that is an "equivalent" or a “derivative” of a specific amino acid sequence is meant an amino acid sequence that is not identical to the specific amino acid sequence, but rather contains at least some amino acid changes (deletions, substitutions, inversions, insertions, etc) that do not essentially affect the biological activity of the protein as compared to a similar activity of the specific amino acid sequence, when used for a given application.

The biological activity of a cellulase is its catalytic activity, and/or its ability to bind to cellulosic material.

An expression vector is a cloning plasmid or vector capable of expressing DNA encoding the cellulase fusion proteins of the invention after transformation into a desired host. When a fungal host is used, the gene of interest is preferably provided to a fungal host as part of a cloning or expression vehicle that integrates into the fungal chromosome, or allows the gene of inter-

est to integrate into the host chromosome, or as an autonomously replicating plasmid. Sequences that are part of the cloning vehicle or expression vehicle may also be integrated with said DNA during the integration process. In addition, in fungi the expression vector or parts thereof can be targeted into predetermined loci.

The DNA encoding the fusion proteins of the invention is also preferably placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences provided by the vector (which integrate with the gene of interest). Alternatively, the control sequences can be those at the insertion site.

The expression control sequences of an expression vector will vary depending on whether the vector is designed to express a certain gene in a prokaryotic or in a eukaryotic host (for example, a shuttle vector may provide a gene for selection in bacterial hosts). Expression control sequences can contain transcriptional regulatory elements such as promoters, enhancer elements, and transcriptional termination sequences, and/or translational regulatory elements, such as translational initiation and termination sites.

A polynucleotide molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the nucleotide sequence which encodes the polypeptide.

An operable linkage is a linkage in which a sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the sequence under the influence or control of the regulatory sequence. Two DNA sequences (such as a promoter region sequence linked to the 5' end of the protein encoding sequence) are said to be operably linked if function of promoter results in the transcription.

The vectors of the invention may further comprise other operably linked regulatory elements such as enhancer sequences.

In a preferred embodiment, genetically stable transformants are constructed whereby the DNA encoding the cellulase fusion proteins of the invention is integrated into the host chromosome by transformation with a vector, which harbors sequences promoting integration of said vector into the chromosome.

Cells that have stably integrated DNA encoding the cellulase fusion proteins of the invention into their chromosomes are selected by also introduc-

ing one or more markers, homologous or heterologous, which allow for selection of host cells which contain the expression vector in the chromosome, for example the marker may provide biocide resistance, e.g., resistance to antibiotics, or heavy metals, such as copper, or markers complementing an auxotrophic mutation in the host chromosome, and the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transformation.

Once the vector or DNA sequence of the invention containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable means, including transformation as known in the art. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of transformed cells.

Suitable expression and production host systems are for example the production system developed for the fungus host *Trichoderma* (EP 244 234), or *Aspergillus* production system, such as *A. oryzae* or *A. niger* (WO 9708325 and WO 9533386, US 5,843,745, US 5,770,418), or the production system developed for *Fusarium*, such as *F. oxysporum* (Malardier et al., 1989). Suitable production systems developed for bacteria are a production system developed for *Bacillus*, for example *B. subtilis* or for *E. coli,* or for actinomycete *Streptomyces*. Suitable production systems developed for yeasts are systems developed for *Saccharomyces, Shizosaccharomyces* or *Pichia pastoris*. Production systems in some other microbes or in mammalian cells or in plants are also possible.

Expression of the cloned gene sequence(s) results in the production of the desired protein, or in the production of a fragment of this protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner.

Fragments are understood to be parts of nucleic acid molecules long enough to code for the described protein or a biologically active fragment thereof. The term "derivative" means in this context that the nucleotide sequences of these molecules differ from the sequences of the above-described nucleic acid molecules in one or more positions and are highly homologous to said sequence. Homology is understood to refer to a sequence identity of at least 40%, particularly an identity of at least 60%, preferably more than 80% and still more preferably more than 90%. The deviations from the nucleic acid

molecules described above can be the result of deletion, substitution, insertion, addition or combination. Homology furthermore means that the respective nucleotide sequences or encoded proteins are functionally and/or structurally equivalent.

As used in the present context the expressions "enzyme preparation" and "cellulase preparation" refers to any enzyme product, which contains at least one cellulase fusion protein. Thus, such an enzyme preparation may be a spent culture medium or filtrate containing one or more cellulase fusion proteins or one or more cellulase fusion proteins and other enzymes, an isolated cellulase fusion protein or a mixture of one or more cellulase fusion proteins or a mixture of one or more cellulase fusion proteins and one or more other enzymes. In addition to the cellulase fusion protein activity, such a preparation may contain additives, such as stabilizers, buffers, preservatives, surfactants and/or culture medium components. Preferred additives are such, which are commonly used in enzyme preparations intended for the application, where the enzyme preparation is used. The enzyme preparation may be in the form of liquid, powder or granulate.

By “spent culture medium” is here meant the culture medium of the host comprising the produced enzymes. Preferably the host cells are separated from the said medium after the production.

The enzyme preparation may comprise one or more cellulase fusion proteins of the present invention or other cellulase enzymes together with one or more cellulase fusion proteins of the present invention. For example, cellulase fusion proteins having different properties may be combined to make the enzyme preparation more useful for different conditions.

To obtain the enzyme preparations of the invention, the hosts having the desired properties (that is, hosts capable of expressing economically feasible quantities of the cellulase fusion proteins of the invention) are cultivated under suitable conditions, the desired enzymes are secreted from the hosts into the culture medium, and the enzyme preparation is recovered from said culture medium by methods known in the art.

The enzyme preparation may comprise in addition to cellulase fusion protein, one or more other enzymes, which may be for example amylases, laccases and/or peroxidases. Alternatively, before, during or after the treatment with the cellulase fusion protein of the present invention, another enzyme treatment may be carried out. The enzyme treatment may comprise, for exam-

ple, one or more amylase treatments, one or more cellulase treatments and/or one or more peroxidase and/or laccase treatments. Which other enzymes are included to the enzyme preparation or are used in the enzyme treatment, depends on the application.

The enzyme preparation can be the culture medium with or without the native or transformed host cells, or is recovered from the same by the application of methods well known in the art. However, because the cellulase fusion proteins of the invention are secreted into the culture media and display activity in the ambient conditions of the cellulolytic liquor, it is an advantage of the invention that the enzyme preparations of the invention may be utilized directly from the culture medium with no further purification. If desired, such preparations may be lyophilized or the enzymatic activity otherwise concentrated and/or stabilized for storage. The enzyme preparations of the invention are very economical to provide and use because (1) the enzymes may be used in a crude form; isolation of a specific enzyme from the culture medium is unnecessary and (2) because the enzymes are secreted into the culture medium, only the culture medium need be recovered to obtain the desired enzyme preparation; there is no need to extract an enzyme from the hosts. Preferably the host for such production is *Trichoderma*, and especially *T. reesei*.

The enzyme preparations of the invention may be provided as a liquid or as a solid, for example, in a dried powder or granular or liquid form, especially non-dusting granules, or a stabilized liquid, or the enzyme preparation may be otherwise concentrated or stabilized for storage or use. It is envisioned that enzyme preparations containing one or more of the neutral cellulases of the invention can be further enriched or made partially or completely deficient in specific enzymatic activities, so as to satisfy the requirements of a specific utility in various applications e.g. in the textile industry. A mixture of enzyme activities secreted by a host and especially a fungus, can be chosen to be advantageous in a particular industrial application, for example biostoning.

The enzyme preparations of the invention can be adjusted to satisfy the requirements of specific needs in various applications in the textile, detergent or the pulp and paper industry.

Blends may be prepared with other macromolecules that are not necessarily all produced from the same host (for example, other enzymes such as endoglucanases, proteases, lipases, peroxidases, oxidases or amylases) or chemicals that may enhance the performance, stability, or buffering of the de-

sired enzyme preparation. Non-dusting granules may be coated. Liquid enzyme preparations can be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid, or sodium chloride, according to established methods.

Protected forms of the enzymes of the invention may be prepared as described in EP 238,216.

The enzyme preparations of the invention can contain a surfactant which can be anionic, non-ionic, cationic, amphoteric or a mixture of these types, especially when used as a detergent composition, Useful detergent compositions are described e.g. in WO 94/07998, U.S. Patent No. 5,443,750 and U.S. Patent No. 3,664,961.

If required, a desired enzyme may be further purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

The enzyme preparations of this invention are especially useful in textile industry preferably in biostoning and in biofinishing or in detergent industry. Other useful areas are in pulp and paper industry.

Stone washing has three steps: desizing, abrasion and after-treatment. The first step, desizing process is normally the first wet treatment of jeans and means the removal of starch or other sizing agents applied usually to the warp yarns to prevent damage during the weaving process. Alpha-amylases are used to remove starch-based size for improved and uniform wet processing. After desizing the jeans are normally rinsed with water or continued directly with the abrasion step.

The second step, abrasion, can be performed with enzymes or pumice stones or both. In all cases mechanical action is needed to remove the dye, and the treatment is usually carried out in washing machines, like drum washers. The term “abraded” means herein the appearance of denim fabric when it has been treated by cellulase enzymes or stones, or both. As a result of uneven dye removal there are contrasts between dyed areas and areas from which dye has been removed. Synonymous expressions are “stone washed look” or “worn look”. In enzymatic stone washing, or biostoning, abrasion with pumice stones is completely or partially eliminated and cellulase is added to facilitate the abrasion of Indigo dye from the fiber surface. The cellulase treatment may be done using neutral or acid cellulases or both. If a fabric is not cellulase treated or stone washed, the appearance of the fabric is said to be

"dull", since the fashionable contrasts would be missing. When more faded effect is desired, bleaching using chemical agents and/or enzymatic methods such as laccase treatment can be carried out.

Abrasion is generally followed by the third step, after-treatment, that includes washing and rinsing steps during which detergents, optical brighteners or softeners may be used. After the enzymatic treatment the reaction must be stopped in order to prevent damage of the treated materials, for example by temperature and/or pH inactivation, the latter comprising a thorough rinsing and/or detergent wash-off. This ensures that the mechanical strength of the fiber is not further compromised by the continued presence of the enzyme.

By "denim" is meant, in connection of this invention, denim fabric, usually denim garments, particularly jeans. Advantageously the denim is Indigo dyed denim. Denim can also be treated with Indigo, with derivatives of Indigo or denim dyed with Indigo together with some other dye, for example Indigo-dyed denim with sulphur bottom.

Treatment with a cellulase(s) can completely replace treatment with pumice stones (for example, 1 kg commercial enzyme vs. 100 kg stones). However, cellulase treatment can be combined with pumice stone treatment when it is desired to produce a heavily abraded finish. A peach skin effect in which a fine protruding hair-like covering is created is also achieved by a wash combining a neutral cellulase with pumice stones. The cellulases of this invention are especially useful to provide abraded look and to minimize backstaining in biostoning.

Biostoning is preferably performed from about pH 4.5-9.5, and most preferably between pH 6.0-8.0. The temperature of the reaction can range from about 40-80°C, preferably between 50-70°C, and more preferably between 55-65°C, and most preferably at 60°C. The liquor ratio (the ratio of the volume of liquid per weight of fabric) may range from about 2:1 - 30:1 preferably 4:1- 15:1, and most preferably 5:1-10:1. The enzyme dosage can range from about 5-8000 NCU/g fabric, preferably 20-3000 NCU/g fabric and most preferably 30-1500 NCU/g fabric. The treatment time can range between 15 min – 4 h, more preferably 20 min – 90 min and most preferably 30 min – 60 min. It should be emphasized that the enzyme dosage depends greatly on the type of the fabrics, machinery, process conditions (pH, temperature, liquor ratio, treatment time, denim load, process scale) and type of enzyme preparation and like. If desired, pumice stones can be used in combination with the fusion

cellulase proteins. The enzyme dosage required will then be significantly lower. A person skilled in art is capable in defining suitable dosages and conditions.

The cellulase fusion proteins of the invention are useful in the textile industry for biofinishing of fabrics or garments e.g. depilling, defuzzing, color clarification, harshness reduction, the creation of different finishes (for example, a 'peach skin,' 'wornout,' 'sand washed,' or 'antique look' effect) and biofinishing of yarn (for example reduction of hairiness, improvement of smoothness). The cellulase fusion proteins of this invention can be used in biofinishing in acid and in neutral conditions using basically the same conditions as in biostoning.

The cellulase fusion proteins of this invention are useful in detergent compositions to improve fabric care properties by antipilling, antigraying, color clarification and softening, and to improve textile cleaning effect, for instance soil removal.

The textile material that is treated with the enzyme preparations of the invention may be manufactured of natural cellulose containing fibers or manmade cellulose containing fibers or mixtures thereof. Examples of natural cellulosics are cotton, linen, hemp, jute and ramie. Examples of manmade cellulosics are viscose, cellulose acetate, cellulose triacetate, rayon, cupro and lyocell. The above-mentioned cellulosics can also be employed as blends of synthetic fibers such as polyester, polyamide or acrylic fibers. The textile material may be yarn or knitted or woven or formed by any other means.

The cellulases of the invention, besides being especially useful for the treatment of fabric, are useful in general in any area requiring cellulase activity.

In the pulp and paper industry, neutral cellulases can be used, for example, in deinking of different recycled papers and paperboards having neutral or alkaline pH, in improving the fiber quality, or increasing the drainage in paper manufacture. Other examples include the removal of printing paste thickener and excess dye after textile printing, and as a treatment for animal feed. For example, if the intended application is improvement of the strength of the mechanical pulp, then the enzyme preparations of the invention may provide one or more of these proteins so as to enhance or facilitate the ability of cellulose fibers to bind together. In a similar manner, in the application of pulp refining, the cellulase fusion protein preparations of the invention may provide one or more of these proteins at a level that enhance or facilitate such swell-

ing. Of the fusion proteins of the invention especially suitable for pulp applications are those with a *Melanocarpus albomyces* 50KB or *Thermoascus aurantiacus* CBHI core.

The cellulase fusion proteins of the present invention provide unexpected advantages when used in textile industry and especially in biostoning. The cellulase fusion proteins of the invention are considerably more efficient than the cellulases of prior art. In biostoning at least two-fold, usually at least three-fold and even six-fold lower dosages in terms of neutral cellulase activity units dosed on the weight of the fabric could be used, without impairing the strength of the fabric. In other words, up to six times higher performance is achieved by using the cellulase fusion proteins of the present invention. Since the production-yield of the cellulase fusion proteins of the invention corresponds to that of the known 20K cellulase, the overall production efficiency is significantly improved. This can be directly proportioned to great savings in the amounts of the enzyme needed: the possibility to use reduced amounts of the enzyme offers a considerable economical value in terms of both the manufacture and use, including the logistics.

The invention is described in more detail in the following examples, which are not be interpreted to narrow the scope of the invention but only to clarify the use of the invention.

EXAMPLE 1. Construction of the expression vectors for 20K+CBD fusion proteins

Standard molecular biology methods were used in the isolation, purification and enzyme treatments of DNA (plasmids, DNA fragments), in polymerase chain reactions (PCR), in *E. coli* transformations, etc. The basic methods used are described in the standard molecular biology handbooks, e.g., Sambrook *et al.* (1989) and Sambrook and Russell (2001).

Plasmids constructs were designed to join the *Melanocarpus albomyces* 20K (Cel45A, AC# AJ515703; SEQ ID. NO: 1) coding sequence with the coding sequence of the linker and CBD of the *Trichoderma reesei* CBHI (AC# AR088330; Srisodsuk *et al.* 1993; SEQ ID. NO: 3). Altogether six different junctions were designed as described in Table 1.

For constructs #1 and #2 set forth in Table 1, a unique *Nru*I site was introduced at the end of the 20K coding sequence. This site enables direct fusion after the codon for the serine #213 of the mature 20K with any DNA fragment with a blunt end. A PCR reaction was run with the primers 20K_Nco

(SEQ ID NO: 11) and 20K_NruXho (SEQ ID NO: 14) with the plasmid pALK1480 (Fig. 1) as the template using the program A (Table 3). pALK1480 has the genomic copy of the *M. albomyces cel45A* (encoding the Cel45A or 20K) inserted under the *T. reesei cbh1* promoter as an exact fusion and having the *cbh1* terminator downstream the gene in the pUC19 vector (New England Biolabs, Inc., USA). The PCR reaction mixture contained 1x DyNAzyme™ EXT reaction buffer (Finnzymes, Finland), 8 mM Mg^{2+} (the final concentration adjusted with added $MgCl_2$), 0.2 mM dNTPs, 0.5 μM of each primer, 1.0 units of DyNAzyme™ EXT DNA polymerase (Finnzymes, Finland), and approximately 50ng/100μl of the template. The PCR product was digested with *Nco*I and *Xho*I restriction enzymes and the fragment was isolated from the agarose gel after electrophoresis. The similarly cut and isolated 6.1 kb fragment of pALK1480 was ligated with the PCR fragment, and transformed into *E. coli* XL1-Blue (Stratagene, USA). The plasmid DNA was isolated from the transformants, and one suitable candidate was verified by sequencing. The resulting plasmid was designated as pALK1429.

PCR reactions were performed separately as above with primer pairs 1_BamMly (SEQ ID NO: 16) + XhoAge (SEQ ID NO: 15) and 2_BamMly (SEQ ID NO: 17) +XhoAge (SEQ ID NO: 15) with pALK492 as the template (Fig. 2), and the resulting PCR products, containing the linker and CBD, were digested with *Mly*I (producing a blunt end just before the desired first codon of the coding sequence of the linker and CBD) and *Age*I. pALK492 carries about 6.9 kb *Pst*I fragment of *T. reesei* QM6a chromosomal DNA harboring the *cbh1/cel7A* gene subcloned into the *Pst*I site of pUC19. pALK1429 obtained above was digested with *Nru*I and *Age*I, and the vector part was isolated and ligated separately with the two digested PCR products obtained above, and transformed into *E. coli* XL1-Blue. Plasmid DNAs were isolated, verified by sequencing and the resulting plasmids were designated as pALK1430 (carrying the 1_BamMly + XhoAge PCR product as an insert) and as pALK1431 (carrying the 2_BamMly + XhoAge PCR product as an insert).

Table 1. Different junctions constructed between the *Melanocarpus albomyces* 20K and the *Trichoderma reesei* CBHI linker+CBD

Construct #	Core-linker junctions	20K-core template pALK1480		linker+CBD template pALK492		Plasmid constructions
		5' primer	3' primer	5' primer	3' primer	
1	...hddggfavfkaps.-gstgn...	20K_NcoI	20K-NruXho_GPI	1_BamMly_oligo	XhoAge_oligo	**pALK1434 <-pALK1430 <- pALK1429**
2	...hddggfavfkaps.-ggnppg...	20K_NcoI	20K-NruXho_GPI	2_BamMly_oligo	XhoAge_oligo	**pALK1435 <- pALK1431 <- pALK1429**
3	...hddggfa.fGPIgs-tgn...	20K_NcoI_2	20K-NruXho_GPI	3_BamMly_oligo	XhoAge_oligo	**pALK1768 <- pALK1764 <- pALK1758**
4	...hddggfWGEIgs-tgn...	20K_NcoI_3	20K-NruXho_WGEI	3_BamMly_oligo	XhoAge_oligo	**pALK1769 <- pALK1765 <- pALK1759**
5	...hddggfPavQIPSs-tgn...	20K_NcoI_2	20K-NruXho_PavQIPS	3_BamMly_oligo	XhoAge_oligo	**pALK1770 <- pALK1766 <- pALK1760**
6	...hddggfaWGEIgs-tgn...	20K_NcoI_3	20K-NruXho_WGEI-2	3_BamMly_oligo	XhoAge_oligo	**pALK1775 <- pALK1774 <-pALK1773**

In second column, the leftmost part is the *Melanocarpus* derived sequence and the rightmost is the *Trichoderma* derived sequence. Lower cases indicate original sequences, upper cases indicate the modified sequence, period (.) indicate a deleted amino acid and a hyphen indicates the junction point joined by ligating the relevant plasmids. First amino acid of the *Melanocarpus* sequence is histidine #201 of the mature sequence and in constructs #1, #3, #4 and #6 the first amino acid of the *Trichoderma* sequence is glycine #427, in construct #2 glycine #434 and in construct #5 serine #428 of the mature sequence.

Table 2. Primers used

Primer	Length nts	Sequence	Sequence ID NO:
20K_Nco	27	5′- TACGCCATGGTCGTCCAGTCGACCAGC	11
20K_Nco_2	35	5'-TACGCCATGGTCGTCCAGTCGACCAGCACGGGCGG	12
20K_Nco_3	46	5'-TACGCCATGGTCGTCCAGTCGACCAGCACGGGCGGCGACCTCGGCA	13
20K_NruXho	40	5'-CGTACTCGAGTCATCGCGAGGGGGCCTTGAAGACGGCGAA	14
XhoAge	30	5'-TGACTCGAGACCGGTGCGTCAGGCTTTCGC	15
1_BamMly	34	5'-TAGGATCCGAGTCCCATTGGCAGCACCGGCAACC	16
2_BamMly	36	5'-TAGGATCCGAGTCCTAGCGGCGGCAACCCTCCCGGC	17
3_BamMly	34	5'-TAGGATCCGAGTCCCATTACCGGCAACCCTAGCG	18
20K-NruXho_GPI	55	5'-CGTACTCGAGTCATCGCGAGCCGATGGGGCCGAAGGCGAAGCCGCCGTCGTCGTG	19
20K-NruXho_WGEI	52	5'-CGTACTCGAGTCATCGCGAGCCGATCTCGCCCCAGAAGCCGCCGTCGTCGTG	20
20K-NruXho_PavQIPS	58	5'CGTACTCGAGTCATCGCGACGAGGGGATCTGGACGGCGGGGAAGCCGCCGTCGTCGTG	21
20K-NruXho_WGEI-2	52	5'-CGTACTCGAGTCATCGCGAGCCGATCTCGCCCCAGGCGAAGCCGCCGTCGTC	22
50KB_NruIXhoI	37	5′-TCGTCTCGAGTCGCGATGGGGCCGAAGCGGATGTTGG	23
50KB_SphI	31	5′-GGAGGGCATGCCCAACAGCAGCGAGATCACC	24
2_50K_NruISpeI	38	5′-CGGCACTAGTTCGCGACCCGATCTCGCCCCAGCGCAGG	25
50K_XhoI	26	5′ CGCCGAGGGCCGGCTCGAGAGCATCC	26

Table 3. PCR reaction programs used

	Program			
Step	A	B	C	D
1	95°C 5 min	95°C 5 min	98°C 1 min	98°C 1 min
2	95°C 1 min	95°C 1 min	98°C 30 s	98°C 30 s
3	55°C 1 min	60°C 1 min	72°C 1 min	65°C 30 s
4	72°C 1 min	72°C 1 min	GOTO 2 29x	72°C 1 min
5	GOTO 2 24x	GOTO 2 24x	72°C 10 min	GOTO 2 29x
6	4 °C HOLD	72°C 1 min	4°C HOLD	72°C 10 min
7		4°C HOLD		4°C HOLD

The *amdS* marker and *T. reesei cbh1* 3' flanking region were inserted into vectors pALK1430 and pALK1431 as follows: pALK424 (US patent 5,837,51; Fig. 3) was cut with *EcoR*I and *Spe*I, the resulting 4.8 kb fragment was made blunt by the Klenow fill-in reaction, and ligated separately with plasmids pALK1430 and pALK1431 cut with *Stu*I, respectively, and transformed into *E. coli* XL1-Blue. The plasmid DNAs were isolated and the desired orientation of the inserts was checked by digestion with appropriate restriction enzymes. The verified plasmids were designated as pALK1434 (insert from pALK1430) and pALK1435 (insert from pALK1431), respectively (Table 1).

For constructs #3, #4, #5 and #6 set forth in Table 1 a different approach was taken. The coding sequence of the 20K and the different modified junction points (Table 1) were designed to end at the serine endocing codon, which forms a part of the added *Nru*I site. For all these constructs the same insert was used to provide the coding sequence of the major part of the linker and CBD. The latter was constructed as follows. A PCR reaction was performed with the reaction mixture described above (except without added Mg^{2+}) and using the primer pair 3_BamMly (SEQ ID NO: 18) and XhoAge (SEQ ID NO: 15) and pALK492 DNA as the template. The program B in Table 3 was used. The resulting PCR product was digested with *BamH*I and *Xho*I, isolated and ligated with the similarly cut vector part of pBluescript II KS+ (Stratagene, USA), and transformed into *E. coli* XL1 Blue. The plasmid DNA was isolated, checked by digestion with appropriate restriction enzymes and verified by sequencing. One plasmid candidate with the desired sequence was chosen and designated as pALK1767.

For construct #3 in Table 1 a PCR reaction was performed using the primer pair 20K_Nco_2 (SEQ ID NO: 12) and 20K-NruXho_GPI (SEQ ID NO: 19) and pALK1480 DNA as the template. Two reaction mixtures were used: one with the composition described above for the construction of pALK1767, and the other with added DMSO to 3% (v/v). These two reaction mixtures were split, and run with programs C and D in Table 3. All reactions produced DNA fragments of expected size, and the preparations were combined and digested with *Nco*I and *Xho*I. The DNA fragment were isolated and ligated with a similarly cut and isolated 6.1 kb fragment of pALK1480, and transformed into *E. coli* XL1 Blue. The plasmid DNA was isolated, checked by digestion with appropriate restriction enzymes and verified by sequencing. One plasmid candidate with the desired sequence was chosen and designated as pALK1758.

For construct #4 in Table 1 a PCR reaction was performed using the primer pair 20K_Nco_3 (SEQ ID NO: 13) and 20K-NruXho_WGEI (SEQ ID NO: 20) and pALK1480 DNA as the template. The PCR reaction mixture contained 1x Phusion™ GC reaction buffer (Finnzymes, Finland), 0.2 mM dNTPs, 0.5 μM of each primer, 3% (v/v) DMSO and 1.0 units of Phusion™ DNA polymerase (Finnzymes, Finland) and approximately 70 ng/100 μl of the template. The reaction mixture was split, and run with programs C and D in Table 3. Both reactions produced DNA fragments of expected size, and the preparations were combined and digested with *NcoI* and *XhoI*. The DNA fragment was isolated and ligated with a similarly cut and isolated 6.1 kb fragment of pALK1480, and transformed into *E. coli* XL1 Blue. The plasmid DNA were isolated, checked by digestion with appropriate restriction enzymes and verified by sequencing. One plasmid candidate with the desired sequence was chosen and designated as pALK1759.

For construct #5 in Table 1 a PCR reaction was performed using the primer pair 20K_Nco_2 (SEQ ID. NO: 12) and 20K-NruXho_PavQIPS (SEQ ID. NO: 21) and pALK1480 DNA as the template. Two reaction mixtures were used: one with the composition described above for the construction of pALK1759, and the other without the DMSO. These two reaction mixtures were split, and run with programs C and D in Table 3. All reactions produced DNA fragments of expected size, and the preparations were combined and digested with *NcoI* and *XhoI*. The DNA fragment was isolated and ligated with the similarly cut and isolated 6.1 kb fragment of pALK1480, and transformed into *E. coli* XL1 Blue. The plasmid DNAs were isolated, checked by digestion

with appropriate restriction enzymes and verified by sequencing. One plasmid candidate was chosen and designated as pALK1760; it had acquired a mutation in the unique *XhoI* site, but this posed no problem for further subcloning.

For construct #6 in Table 1 a PCR reaction was performed using the primer pair 20K_Nco_3 (SEQ ID. NO: 13) and 20K-NruXho_WGEI-2 (SEQ ID. NO: 22) and pALK1480 DNA as the template (70ng/100µl). The same reaction mixture composition was used as for the construction of plasmid pALK1767, and it was run with the program C in Table 3. The preparation was digested with *Nco*I and *Xho*I. The DNA fragment was isolated and ligated with a similarly cut and isolated 6.1 kb fragment of pALK1480, and transformed into *E. coli* XL1 Blue. The plasmid DNAs were isolated, checked by digestion with appropriate restriction enzymes and verified by sequencing. One plasmid candidate was chosen and designated as pALK1773.

Plasmids pALK1758, pALK1759, pALK1760 and pALK1773 were separately cut with *Nru*I and *Age*I, and the vector parts were isolated. Each preparation was ligated with a 235 bp fragment isolated from pALK1767 after *Mly*I and *Age*I digestion, and each ligation mixture was transformed separately into *E. coli* XL10-Gold. The plasmid DNAs were isolated, checked by digestion with appropriate restriction enzymes and verified by sequencing. The verified plasmids were designated as pALK1764, pALK1765, pALK1766, and pALK1774, respectively (Table 1).

The *amdS* marker and *T. reesei cbh1* 3' flanking region were inserted into vectors pALK1764, pALK1765, pALK1766, and pALK1774 as follows: pALK424 was cut with *EcoR*1 and *Spe*1, the resulting 4.8 kb fragment was made blunt by the Klenow fill-in reaction, and ligated separately with plasmids pALK1764, pALK1765, pALK1766, and pALK1774 cut with *Stu*I, respectively, and transformed into *E. coli* XL10-Gold. Plasmid DNAs were isolated and the desired orientation of the inserts was checked by digestion of appropriate restriction enzymes. The verified plasmids were designated as pALK1768, pALK1769, pALK1770, and pALK1775, respectively (Table 1) (Fig. 10).

EXAMPLE 2. Production of the fusion 20K+CBD proteins in *T. reesei*

8.7 kb linear expression cassettes from the plasmids pALK1434 and pALK1435 were isolated from the vector backbone after *EcoRI* digestion and transformed to *T. reesei* A47 protoplasts. The transformations were performed as described in Penttilä *et al.* (1987) with the modifications described in Kar-

hunen *et al*. (1993) selecting with acetamide as the sole nitrogen source. The transformants were purified on selection plates through single conidia prior to sporulating them on PD (Potato Dextrose Agar).

The 20K+CBD production of the transformants was analysed from the culture supernatants of the shake flask cultivations (50 ml). The transformants were grown for 7 days in a complex cellulase-inducing medium (Joutsjoki *et al*. 1993) buffered with 5% KH_2PO_4 at pH 5.5. The enzyme activity of the fusion protein was measured as the release of reducing sugars from carboxymethylcellulose (3% CMC) at 50°C in 50 mM Hepes buffer pH 7.0 in 10 min (NCU activity, nkat; Bailey and Nevalainen, 1981; Haakana *et al*., 2004). NCU activities of the best producing transformants are presented in Table 4. The genotypes of the chosen transformants were confirmed by using Southern blots, in which several genomic digests were included and the respective expression cassette was used as a probe. The 20K+CBD protein was detected from the culture supernatants using the polyclonal antibodies raised against the purified *Melanocarpus albomyces* 20K neutral cellulase (Haakana *et al*. 2004) and the ProtoBlot Western blot AP system (Promega). The Western blot analyses showed that the fusion 20K+CBD enzymes were produced mainly as stable fusion proteins in *T. reesei*.

Table 4. NCU activities of the selected 20K+CBD transformants from shake flask cultivations

Transformant	Construction No.	RF Number	Neutral cellulase activity, NCU/ml	Endogenous cellulase phenotype
A47/pALK1434/#20	#1	RF5580	3278	CBHI-
A47/pALK1434/#23	#1	RF5581	2091	(CBHI+)
A47/pALK1434/#37	#1	RF5582	2330	CBHI-
A47/pALK1435/#3	#2	RF5583	3624	CBHI-
A47/pALK1435/#7	#2	RF5584	3211	CBHI-
A47/pALK1435/#11	#2	RF5585	1172	(CBHI+)
A47/pALK1435/#14	#2	RF5586	3152	CBHI-

In Table 4, the construction number refers to Table 1; RF number refers to that the transformants were named as RF strains.

The possible targeting of the expression cassette to the *cbh1* (*cel7A*) locus was screened as a CBHI-negative phenotype by Western blot. The detection of the CBHI protein was performed using the monoclonal antibodies CI-258 or CI-261 (Aho *et al.*, 1991) and the ProtoBlot Western blot AP system (Promega, USA). The genotypes of the chosen transformants were confirmed by using Southern blots, in which several genomic digests were included and the respective expression cassette was used as a probe.

8.7 kb linear expression cassettes from the plasmids pALK1768, pALK1769, pALK1770, and pALK1775 prepared in Example 1 were isolated from the vector backbone after *EcoRI* digestion and transformed to *T. reesei* RF5796 and RF5798 protoplasts (both strains originating from the strain QM6a (Bailey and Nevalainen, 1981) and having the phenotype CBHI- CBHII- EGI- EGII- for the endogenous *T. reesei* cellulases) selecting with acetamide as the sole nitrogen source. The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

The 20K+CBD production of the transformants was analyzed from the culture supernatants of the shake flask cultivations (50 ml). The transformants were grown for 7 days in a complex cellulase-inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH_2PO_4 at pH 5.5. The NCU activity of the produced 20K+CBD fusion proteins was then assayed as described above. NCU activities of the selected transformants are presented in Table 5. The genotypes of the chosen transformants were confirmed by using Southern blots in which several genomic digests were included and the respective expression cassette was used as a probe. The 20K+CBD protein was detected from the culture supernatants using the polyclonal antibodies raised against the purified *M. albomyces* 20K neutral cellulase (Haakana *et al.* 2004) and the ProtoBlot Western blot AP system (Promega, USA). The Western blot analyses showed that the fusion 20K+CBD enzyme was produced by the transformants. Some cultures showed also a band reacting with the anti-20K antiserum, and having the mobility of the wild type 20K protein, indicating that possibly some cleavage of the linker+CBD had taken place during the cultivation. The 20K+CBD fusion protein produced by the pALK1770 transformants was chosen further studies due to its stability.

Table 5. NCU activities of the selected 20K+CBD transformants from shake flask cultivations

Transformant	Construction No.	RF number	Neutral cellulase activity, NCU/ml
RF5796/pALK1768/#6	#3	RF5966	3622
RF5796/pALK1768/#7	#3	RF5967	1316
RF5796/pALK1768/#9	#3	RF6035	6605
RF5798/pALK1768/#11	#3	RF5970	1525
RF5798/pALK1768/#17	#3	RF5971	2885
RF5798/pALK1768/#20	#3	RF5972	2598
RF5796/pALK1769/#7	#4	RF5968	4344
RF5796/pALK1769/#10	#4	RF5969	4858
RF5796/pALK1769/#11	#4	RF6036	6145
RF5798/pALK1769/#4	#4	RF5973	4505
RF5798/pALK1769/#8	#4	RF5974	4895
RF5796/pALK1770/#13	#5	RF5975	3073
RF5796/pALK1770/#17	#5	RF5976	2256
RF5796/pALK1770/#22	#5	RF5977	2107
RF5798/pALK1770/#10	#5	RF5978	1907
RF5798/pALK1770/#14	#5	RF5979	3661
RF5796/pALK1775/#8	#6	RF6078	2431
RF5796/pALK1775/#13	#6	RF6079	3505
RF5796/pALK1775/#21	#6	RF6080	2541
RF5798/pALK1775/#22	#6	RF6081	1697
RF5798/pALK1775/#29	#6	RF6082	3096

In Table 5, the construction number refers to Table 1; RF number refers to that the transformants were named as RF strains.

T. reesei strains RF5582, RF5583, RF6036, RF5977, and RF5978 were grown in a bioreactor for applications tests. Some preparations were heat treated (pH 6.0, 65 ºC, 60 – 70 min) in order to inactivate any remaining *T.*

reesei endogenous enzyme activity. The 20K+CBD is relatively heat stable (Miettinen-Oinonen *et al.* 2004), and does not denature during the treatment.

EXAMPLE 3. Production of the fusion 20K+CBD affinity mutant proteins in *T. reesei*

Melanocarpus albomyces 20K (*cel45A*, AC# AJ515703) enzyme was fused to the cellulose-binding domain (CBD) of *Trichoderma reesei* CBHI, in which the conserved tyrosine residues at positions 31 (corresponding to Y492 of the mature polypeptide) and/or 32 (corresponding to Y493 of the mature polypeptide) were mutated to alanine as described by Linder *et al.*, 1995. In addition, the tyrosine residue at position 31 was replaced by tryptophan, an amino acid naturally found in the CBD region of e.g. *Humicola grisea* CBHI (Azevedo *et al.*, 1990) and *T. reesei* EGV (Cel45A, Saloheimo *et al.*, 1994). The mutated CBDs were constructed by PCR, and the amino acid substitutions of Y31A, Y32A, Y31W and Y31A_Y32A were included in the cellulose-binding domain of *T. reesei* CBHI (numbering according to amino acid sequence of CBD). In all constructs, the forward primer 3_BamMly: 5´-TAGGATCCG-AGTCCCATTACCGGCAACCCTAGCG-3´ (SEQ ID. NO: 18) was used. The reverse primers used for the amplification of different CBD_{mut} products are described in Table 6. The PCR reaction mixtures contained 1x PfuUltra™ HF reaction buffer (Stratagene, USA) providing 2 mM Mg^{2+} concentration, 0.2 mM dNTPs, 2 μM of each primer and 1.5 units of PfuUltra™ HF DNA polymerase (Stratagene, USA) and approximately 45 ng of pALK492 plasmid as a template. The pALK492 (Fig. 2) plasmid contains the *T. reesei cbh1* gene. The conditions for the PCR reactions were the following: 2 min initial denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min annealing at 65°C (±5 °C gradient), 2 min extension at 72°C and a final extension at 72°C for 10 min.

Table 6. Reverse PCR primers designed for amplifying mutated CBD products

Primer	Length (nts)	Sequence, reverse	Amino acid substitution	Sequence Id. No
XhoAge_Y31A	69	5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCTTTACAGGCACTGAGAGTAGGCAGGGTTCAGG	Y31A	SEQ ID. NO: 27
XhoAge_Y32A	69	5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCTTTACAGGCACTGAGAGGCGTAAGGGTTCAGG	Y32A	SEQ ID. NO: 28
XhoAge_Y31W	69	5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCTTTACAGGCACTGAGAGTACCAAGGGTTCAGG	Y31W	SEQ ID. NO: 29
XhoAge_Y31A-Y32A	69	5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCTTTACAGGCACTGAGAGGCGGCAGGGTTCAGG	Y31A_Y32A	SEQ ID. NO: 30

All primer combinations produced the specific DNA fragment in PCR reactions at annealing temperature of 60°C. The PCR products were isolated from these reactions, digested with *Xho*I and *Bam*HI restriction enzymes and then cloned to pBluescript II KS+ (Stratagene, USA). The plasmids obtained were named as pALK1884 (Y31A mutation), pALK1885 (Y32A mutation), pALK1886 (Y31W mutation) and pALK1887 (Y31A_Y32A mutations). The PCR fragments in the plasmids were confirmed by sequencing. The *Mly*I and *Age*I digested inserts of the plasmids pALK1884 to pALK1887 were further ligated to a *Nru*I and *Age*I digested pALK1760 vector fragment containing the full-length *Melanocarpus albomyces* 20K gene fused to the *T. reesei cbh1 (cel7A)* promoter and terminator. The C-terminal part of the 20K gene in pALK1760 was modified so that ligation of the CBD fragment produced a junction point of PAVQIPSS (construct #5), which was shown to result in a stable fusion product in *T. reesei* as described in Examples 1 and 2. At the final step, the *amdS* marker was added as a blunt-ended *Spe*I-*EcoR*I fragment (4.5 kb) of p3SR2 plasmid (Fig. 6) to obtain expression plasmids of pALK1877 (Y31A mutation), pALK1878 (Y32A mutation), pALK1879 (Y31W mutation), and pALK1880 (Y31A_Y32A mutation) for production of fusion 20K+CBD_{mut} enzymes in *T. reesei*. The amino acid sequences of the 20K protein fusion to linker peptide followed by the mutated CBD region are presented in Fig. 11B. The expression plasmids were confirmed by sequencing, and the 8.4 kb linear expression cassettes (Fig. 11A) were isolated from the vector backbone after *Not*I digestion and were transformed to *T. reesei* RF5796 protoplasts. The transformations were performed in Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993), selecting with acetamide as a sole nitrogen source. The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

The 20K+CBD_{mut} production of the transformants was analysed from the culture supernatants of the shake flask cultivations (50 ml). The transformants were grown for 7 days in a complex cellulase-inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH_2PO_4 at pH 5.5. The enzyme activity of the fusion protein was measured as the release of reducing sugars from carboxymethylcellulose (3% CMC) at 50°C in 50 mM Hepes buffer pH 7.0 in 10 min (NCU activity, nkat; Bailey and Nevalainen, 1981; Haakana *et al.*, 2004). NCU activities of the best producing transformants are presented in Table 7. The genotypes of the chosen transformants were confirmed by using

Southern blots in which several genomic digests were included and the respective expression cassette was used as a probe. The 20K+CBD_{mut} protein was detected from the culture supernatants using polyclonal antibodies raised against the purified *Melanocarpus albomyces* 20K neutral cellulase (Haakana *et al*. 2004) and the ProtoBlot Western blot AP system (Promega). The Western blot analyses showed that the fusion 20K+CBD_{mut} enzymes were produced as stable fusion proteins in *T. reesei*.

Table 7. NCU activities of the selected 20K+CBD_{mut} transformants from shake flask cultivation

Transformant	Amino acid substitution	RF number	Neutral cellulase activity, NCU/ml
pALK1877/#26	Y31A	RF6084	3658
pALK1877/#34	Y31A	RF6085	2447
pALK1878/#02	Y32A	RF6086	3434
pALK1878/#13	Y32A	RF6088	2915
pALK1879/#13	Y31W	RF6090	2545
pALK1879/#24	Y31W	RF6091	3452
pALK1880/#06	Y31A_Y32A	RF6092	3415
pALK1880/#25	Y31A_Y32A	RF6094	2727

RF number refers to that the transformants were named as RF strains.

The strains RF6084 to RF6086, RF6088, RF6090 to RF6092 and RF6094 were fermented to obtain material for the application tests (see EXAMPLES 9 - 11).

EXAMPLE 4. Production of the fusion 20K+CBD linker deletion proteins in *T. reesei*

Melanocarpus albomyces 20K enzyme was fused to the cellulose-binding domain (=CBD) of *Trichoderma reesei* CBHI, which was further modified by introducing deletions to the interdomain linker peptide. Linker deletions were designed according to Srisodsuk *et al*., 1993. Deletion of amino acids from position 434 to 444 (Mutant ΔG-444) of the mature polypeptide removes approximately one-third of the linker including the glycine- and proline-rich repeated sequence but leaving all the putative *O*-glycosylation sites intact. Dele-

tion of residues from position 434 to 460 (Mutant ΔG-460) removes practically all of the linker (Fig. 12A). Additional 20K+CBD linker deletions having an affinity double mutation of Y31A_Y32A in the CBD region were also constructed.

PCR reactions were performed to introduce the deletions to linker peptide as well as the amino acid substitution to the CBD region. The PCR amplifications were done as described in Example 3, except that the annealing temperature of 60°C (±5 °C gradient) was used. The forward primers

5´-TAGGATCCGAGTCCCATTACCGGCAACCCTAGCACCACC-ACCACCCGCCGCCCAGCC -3´ (SEQ ID. NO: 31)

and

5´-TAGGATCCGAGTCCCATTACCGGCAACCCTAGCCC-TACCCAGTCTCACTACGGCCAGTGC-3´ (SEQ ID. NO: 32)

were used for synthesizing the linker deletions of ΔG-444 and ΔG-460, respectively. Correspondingly, the reverse primer

5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCT-TTACAGG-3 (SEQ ID. NO: 33)

was used to amplify the intact CBD region of the *T. reesei* CBHI. The Y31A_Y32A mutation to the CBD region was generated with the reverse primer

5´-TGACTCGAGACCGGTGCGTCAGGCTTTCGCACGGAGCTT-TACAGGCACTGAGAGGCGGCAGGGTTCAGG-3´ (SEQ ID. NO: 30)

All primer combinations produced the specific DNA fragment in PCR reactions from 55.2°C to 65.0°C range of annealing temperatures. Expression plasmids pALK1893 (ΔG-444 deletion), pALK1896 (ΔG-460 deletion), pALK1899 (ΔG-444 deletion, Y31A_Y32A mutation), and pALK1952 (ΔG-460 deletion, Y31A_Y32A mutation) were constructed as described in Example 3. The amino acid sequences of the 20K protein fusion to truncated linker peptide followed by the intact or mutated CBD are presented in Fig. 12B. The 8.3 kb linear expression cassettes were isolated from the vector backbone after *EcoR*I digestion and were transformed to *T. reesei* RF5796 protoplasts. Transformation, transformant purification, shake flask cultivations, activity measurements, Southern blot hybridizations, and Western blot analyses were performed as described in Example 3.

Table 8. NCU activities of the selected 20K+CBD linker deletion transformants from shake flask cultivation

Transformant	Linker Deletion / Amino acid substitution	RF number	Neutral cellulase activity, NCU/ml
pALK1893/#08	ΔG-444	RF6107	1182
pALK1893/#10	ΔG-444	RF6108	2058
pALK1896/#05	ΔG-460	RF6110	2576
pALK1896/#07	ΔG-460	RF6111	2628
pALK1899/#07	ΔG-444, Y31A_Y32A	RF6112	1947
pALK1899/#20	ΔG-444, Y31A_Y32A	RF6114	2462
pALK1952/#01	ΔG-460, Y31A_Y32A	RF6115	2428
pALK1952/#17	ΔG-460, Y31A_Y32A	RF6116	1738

RF number refers to that the transformants were named as RF strains.

The selected strains RF6107, RF6108, RF6110 to RF6112, and RF6114 to RF6116 were fermented to obtain material for the application tests (Examples 9 to 11). The Western blot analyses showed that the fusion 20K+CBD linker deletion enzymes were produced as stable fusion proteins in *T. reesei*.

EXAMPLE 5. Production of the recombinant *Melanocarpus albomyces* 50K+CBD fusion protein in *T. reesei*

Plasmid constructs were designed to join the *Melanocarpus albomyces* 50K (*cel7A*, AC# AJ515704) coding sequence with the linker region and cellulose binding domain (CBD) of the *T. reesei* CBHI (*cel7A*, AC# AR088330; Srisodsuk *et al*. 1993). Plasmid pALK1237 (Fig. 4), which is a basis for the new constructs, contains the *cel7A* gene under control of *T. reesei cbh1* promoter as an exact fusion.

First, a unique *Nru*I restriction site was introduced near the C-terminus of the 50K coding sequence. This enables direct fusion of any blunt-ended DNA after amino acid S393 of the mature 50K polypeptide (Fig. 13B). A

PCR reaction was performed with primers 2_50K_NruISpeI (5' CGGCAC-TAGTTCGCGACCCGATCTCGCCCCAGCGCAGG 3'; SEQ ID. NO: 25) and 50K_XhoI (5' CGCCGAGGGCCGGCTCGAGAGCATCC 3'; SEQ ID. NO: 26) using pALK1237 as a template. The PCR reaction contained 1x DyNAzyme™ EXT reaction buffer (Finnzymes, Finland), 0.25 mM dNTPs, 0.5 µM of each primer, 2.0 units of DyNAzyme™ EXT DNA polymerase (Finnzymes, Finland) and approximately 50ng/100µl of pALK1237 template DNA. The conditions for PCR amplification was as follows: 5 min initial denaturation at 96°C, followed by 25 cycles of 15 s at 96°C, 60 s annealing at 56°C or 61°C, 60 s extension at 72°C, and a final extension at 72°C for 10 min. The PCR product was digested with *Xho*I and *Spe*I restriction enzymes and purified from the agarose gel. The purified PCR fragment was ligated into the 6.9 kb *Xho*I-*Spe*I restriction fragment of plasmid pALK1237 and transformed into *E. coli* XL1-Blue (Stratagene, USA). Plasmid DNA was isolated from the transformants and three candidates were verified by sequencing. The selected clone was designated as pALK1703.

The *T. reesei* CBHI linker+CBD was amplified by PCR with primers 3_BamMly_50 (5' TTGGATCCGAGTCGCAGCACCGGCAACCCTAGCG 3'; SEQ ID. NO: 36) and XhoAge (5' TGACTCGAGACCGGTGCGTCAGGCTTTCGC 3'; SEQ ID. NO: 15) using pALK492 as a template. The PCR reaction conditions were as described above, except that the extension time in the amplification reaction was 90 s. The PCR product was digested with *Mly*I and *Age*I enzymes and purified from the agarose gel. The linker+CBD containing PCR fragment was ligated into the 6.8 kb *Age*I-*Nru*I restriction fragment of pALK1703 and transformed into *E. coli* XL1-Blue (Stratagene, USA). Transformants were analyzed as described above and a suitable clone was designated as pALK1704.

To enable selection of *T. reesei* transformants the *amd*S marker gene and the *T. reesei cbh*I 3' flanking region was inserted into the vector plasmid pALK1704. A 4.8 kb *Eco*RI-*Spe*I restriction fragment of pALK424 (US 5,837,515) was isolated and the fragment ends were filled-in with Klenow enzyme. The blunt-ended *amd*S marker fragment was ligated into the *Stu*I digested pALK1704 and transformed into *E. coli* XL1-Blue (Stratagene, USA). Plasmid DNA was isolated from transformants and the desired orientation of the insert was verified by restriction enzyme digestion. The selected transformant was designated as pALK1708.

A 9.2 kb linear expression cassette (Fig. 13A) from pALK1708 backbone was isolated by *Eco*RI digestion, transformed into *T. reesei* RF5636 pro-

toplasts (derived from the strain QM6a; Bailey and Nevalainen, 1981), and transformants selected with acetamide as sole nitrogen source. The host strain lacks three major endogenous cellulases: CBHII (Cel6A), EGI (Cel7B) and EGII (Cel5A). Transformation was performed according to Penttilä *et al.* (1987) with modifications described by Karhunen *et al.* (1993). Transformants were purified on selection plates through single conidia prior to sporulating them on PD.

The production of 50K+CBD fusion protein of the transformants was analyzed from the culture supernatants of shake flask cultivations (50 ml). Transformants were grown for 7 days in a complex cellulose-inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH_2PO_4 at pH 5.5. The enzyme activity of the fusion protein was measured as the release of reducing sugars from carboxymethylcellulose (3% CMC) at 50°C in 50 mM Hepes buffer pH 7.0 (NCU activity; Bailey and Nevalainen 1981; Haakana et al. 2004). The activity of the transformants varied from 2035 to 3633 NCU/ml. The 50K+CBD protein was detected from the culture supernatants by ProtoBlot Western blot AP system (Promega) using polyclonal antibodies raised against the purified *Melanocarpus albomyces* 50K neutral cellulase (Haakana *et al.* 2004). The Western blot analysis showed that the 50K+CBD fusion protein produced from *T. reesei* is stable. The genotypes of the chosen transformants were analysed by Southern blotting using the expression cassette as a probe. The possible targeting of the expression cassette to the *cbh*I locus was also verified by Western blotting using monoclonal CBHI antibodies (CI-261, Aho *et al.* 1991) to detect CBHI protein.

EXAMPLE 6. Production of the recombinant *Melanocarpus albomyces* 50KB+CBD fusion protein in *T. reesei*

Plasmid constructs were designed to join the *Melanocarpus albomyces* 50KB (*cel7B*, AC# AJ515705) coding sequence with the linker region and cellulose binding domain (CBD) of the *T. reesei* CBHI (*cel7A*, AC# AR088330; Srisodsuk *et al.* 1993). Plasmid pALK1241 (Fig. 5), which is a basis for the new constructs, contains the *cel7B* gene under control of *T. reesei cbh1* promoter as an exact fusion.

First, a unique *Nru*I restriction site was introduced near the C-terminus of the 50KB coding sequence. This enables direct fusion of any blunt-ended DNA after amino acid S426 of the mature 50KB polypeptide (Fig. 14B). A PCR reaction was performed with primers 50KB_NrulXhoI *(5'*

TCGTCTCGAGTCGCGATGGGGCCGAAGCGGATGTTGG 3'; SEQ ID. NO: 23) and 50KB_SphI (5' GGAGGGCATGCCCAACAGCAGCGAGATCACC 3'; SEQ ID. NO: 24) using pALK1241 as a template. The PCR reaction contained 1x DyNAzyme™ EXT reaction buffer (Finnzymes, Finland), 0.25 mM dNTPs, 0.5 µM of each primer, 2.0 units of DyNAzyme™ EXT DNA polymerase (Finnzymes, Finland) and approximately 50 ng/100µl of pALK1241 template DNA. The conditions for PCR amplification was as follows: 5 min initial denaturation at 96°C, followed by 25 cycles of 15 s at 96°C, 60 s annealing at 56°C or 60°C, 60 s extension at 72°C, and a final extension at 72°C for 5 min. The PCR product was digested with *Xho*I and *Sph*I restriction enzymes and purified from the agarose gel. The purified PCR fragment was ligated into the 6.9 kb *Xho*I-*Sph*I restriction fragment of plasmid pALK1241 and transformed into *E. coli* XL 1-Blue (Stratagene, USA). Plasmid DNA was isolated from the transformants and one candidate was verified by sequencing. The selected clone was designated as pALK1705.

The *T. reesei* CBHI linker+CBD was amplified by PCR with primers 3_BamMly_50 (5' TTGGATCCGAGTCGC AGCACCGGCAACCCTAGCG 3'; SEQ ID. NO: 18) and XhoAge (5' TGACTCGAGACCGGTGCGTCAGGCTTTCGC 3'; 15) using pALK492 as a template. The PCR reaction conditions were as described above, except that the extension time in the amplification reaction was 90 s. The PCR product was digested with *Mly*I and *Age*I enzymes and purified from the agarose gel. The linker+CBD containing PCR fragment was ligated into the 7.2 kb *Age*I-*Nru*I restriction fragment of pALK1705 and transformed into *E. coli* XL 1-Blue (Stratagene, USA). Transformants were analyzed as described above and a suitable clone was designated as pALK1706.

To enable selection of *T. reesei* transformants the *amdS* marker gene and the *T. reesei cbhI* 3' flanking region were inserted into the vector plasmid pALK1706. A 4.8 kb *Eco*RI-*Spe*I restriction fragment of pALK424 (US 5,837,515) was isolated and the fragment ends were filled-in with Klenow enzyme. The blunt-ended *amd*S marker fragment was ligated into the *Stu*I digested pALK1706 and transformed into *E. coli* XLI-Blue (Stratagene, USA). Plasmid DNA was isolated from transformants and the desired orientation of the insert was verified by restriction enzyme digestion. The selected transformant was designated as pALK1709.

A 9.6 kb linear expression cassette (Fig. 14A) from pALK1709 backbone was isolated by *Eco*RI digestion, transformed into *T. reesei* RF5636 pro-

toplasts, and transformants selected with acetamide as sole nitrogen source. The host strain lacks three major endogenous cellulases: CBHII (Cel6A), EGI (Cel7B) and EGII (Cel5A). Transformation was performed according to Penttilä *et al.* (1987) with modifications described by Karhunen *et al.* (1993). Transformants were purified on selection plates through single conidia prior to sporulating them on PD.

The production of 50KB+CBD fusion protein of the transformants was analyzed from the culture supernatants of shake flask cultivations (50 ml). Transformants were grown for 7 days in a complex cellulose-inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH_2PO_4 at pH 5.5. The cellobiohydrolase activity of the fusion protein was measured using 4-methylumbelliferyl-β-D-lactoside substrate (MUL activity; van Tilbeurgh *et al.* 1988). The 50KB+CBD protein was detected from the culture supernatants by ProtoBlot Western blot AP system (Promega) using polyclonal antibodies raised against the purified *Melanocarpus albomyces* 50KB cellulase (Haakana *et al.* 2004). In Western blot analysis no wild type 50KB protein was detected showing that the 50KB+CBD fusion protein produced from *T. reesei* is stable. The genotypes of the chosen transformants were analysed by Southern blotting using the expression cassette as a probe. The possible targeting of the expression cassette to the *cbh*I locus was also verified by Western blotting using monoclonal CBHI antibodies (CI-261, Aho *et al.* 1991) to detect CBHI protein.

EXAMPLE 7. Production of the recombinant *Thermoascus aurantiacus* CBHI+CBD fusion proteins in *T. reesei*

Thermoascus aurantiacus CBHI (AC# AF478686, Hong *et al.*, 2003; SEQ ID. NO: 9) was fused to linker and CBD of *Trichoderma reesei* CBHI (AC# AR088330, Srisodsuk *et al.* 1993; SEQ ID. NO: 3). First, the coding sequence of the linker and the CBD of *T. reesei* CBHI was synthesized by PCR using following primers:

5´-TTAAACATATGTTATCTACTCCAACATCAAGGTCGGACCCATTGGCAGCACCGGCAACCCTAGCGGC-3´ (forward sequence, SEQ ID. NO: 34)

and

5´-TATATGCGGCCGCACCGGTGCGTCAGGCTTTCGCACGGAGCTTTACAGGC-3´ (reverse sequence, SEQ ID. NO: 35).

The PCR reaction mixture contained 1x DyNAzyme™ EXT reaction buffer (Finnzymes, Finland), 15 mM Mg^{2+}, 0.2 mM dNTPs, 2 μM of each primer, 0.6 units of DyNAzyme™ EXT DNA polymerase (Finnzymes, Finland),

and approximately 75ng/30µl of the pALK492 template. The pALK492 plasmid contains the *T. reesei cbh*1 *(cel7A)* gene. The conditions for the PCR reaction were the following: 2 min initial denaturation at 98°C, followed by 30 cycles of 30 sec at 98°C, 30 sec annealing at 68°C (±4°C gradient), 30 sec extension at 72°C and a final extension at 72°C for 10 min. The specific DNA fragment in PCR reaction was obtained at annealing temperature range from 64°C to 68.5°C. The synthesized CBD fragment, containing also 3´-terminal nucleotide sequence of *Thermoascus aurantiacus cbh*1 gene, was digested *Nde*I and *Not*I restriction enzymes and the fragment was isolated from the agarose gel after electrophoresis. Thereafter, the isolated PCR fragment was ligated to the *Nde*I and *Not*I digested pALK1649 (Fig. 7) vector fragment containing the full-length *Thermoascus aurantiacus cbh*1 gene. The plasmid obtained was named as pALK1888, and the PCR amplified fragment in the plasmid was confirmed by sequencing. As a result of fusion, the C-terminal part of the *Thermoascus aurantiacus* CBHI in the pALK1888 plasmid contains a junction point of GPIGST (Fig. 15B). The *Sac*II and *Age*I digested insert of the plasmid pALK1888 was ligated to *Sac*II and *Age*I digested pALK1694 (Fig. 8) vector fragment, which results to *Thermoascus aurantiacus* CBHI+CBD fusion to *T. reesei cbh1 (cel7A)* promoter (an exact fusion) and terminator. At the final step, the *amdS* marker fragment was added, as described in Example 3, to obtain expression plasmid of pALK1890 for production of recombinant *Thermoascus aurantiacus* CBHI+CBD fusion enzyme in *T. reesei*. The amino acid sequence of the *Thermoascus aurantiacus* CBHI protein fusion to linker peptide followed by the CBD region of *T. reesei* CBHI is presented in Fig. 15B.

The expression plasmid was confirmed by restriction enzyme digestions, and the 8.9 kb linear expression cassette (Fig. 15A) was isolated from the vector backbone after *Not*I digestion and was transformed to *T. reesei* RF5796 protoplasts. The transformations were performed as in Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

Thermoascus aurantiacus CBHI+CBD production of the transformants was analyzed from the culture supernatants of the shake flask cultivations (50 ml). The transformants were grown for 7 days in a complex cellulase-inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH_2PO_4 at pH 5.5. The cellobiohydrolase activity was assayed using 4-methylumbelliferyl-β-D-

lactoside (MUL) substrate according to van Tilbeurgh *et al.*, 1988. The genotypes of the chosen transformants were confirmed by using Southern blots in which several genomic digests were included and the expression cassette was used as a probe. The SDS-PAGE analyses showed that the recombinant *Thermoascus aurantiacus* CBHI+CBD enzyme was produced as stable fusion protein in *T. reesei*.

EXAMPLE 8. Performance of the fusion 20K+ CBD protein preparations in denim finishing/biostoning

20K + CBD fusion proteins produced using *Trichoderma* as host as described in Example 2 were tested for their ability in biostoning of denim to create abraded look similar to that provided by pumice stones. A commercial 20K preparation efficient in denim finishing was used for comparision.

English jeans made of Indigo dyed denim twill with sulphur bottom were used as test material after desizing with ECOSTONE® A200 alpha-amylase. Warp and weft yarns of the fabric were ring spun. The cellulase treatments were performed with Electrolux's Wascator FOM 71 CLS washer extractor under conditions described in Table 9.

Table 9. Test conditions used in the cellulase treatments

Process parameter	
Denim load	1.3 kg
Water	19 l
Buffer/pH control (pH 6.5)	31.6 g $Na_2HPO_4 \cdot H_2O$ 10.5 g Citric acid
Time	55 min
Temperature	60°C
Cellulase dosage	250 - 3000 NCU/g fabric

Enzymes were dosed as neutral cellulase activity units (NCU) per the weight of the fabric. The cellulase enzyme was inactivated after draining by raising the pH above 11 by adding 5 g of NaOH (10 min, 40°C) and rinsing three times. The jeans were dried in a tumbler. Two pairs of jeans were used in each test.

The biostoning effect/abrasion level was evaluated by measuring the color as reflectance values with Minolta CM 2500 or CM 1000 spectropho-

tometer using L*a*b* color space coordinates (illuminant D65/2°). The color from the face side and the reverse side of denim (data not shown) was measured after desizing (i.e. before the cellulase treatment) and after the cellulase treatment. Each measurement was the average of approximately 40 measurements. Two pairs of jeans were used in each test and the final result is the average of them. Lightness or increase of lightness after enzyme treatment was used for evaluation of abrasion effect (performance or biostoning effect). The results are shown in Tables 10 and 11, where bolding is used to highlight the similar abrasion levels and equivalent dosages. Treatments with 20K or without any enzyme were used for comparison. Some of preparations (Table 11) had been heat treated (pH 6.0, 65°C, 60 to 70 min) in order to inactivate any remaining *T. reesei* endogenous enzyme activity and/or in order to test the effect of heat treatment on the stability of the enzyme.

Table 10. Color measurements of the face side of denim treated with 20K+CBD fusion proteins

Strain No.	Enzyme	NCU/ g fabric	Before cellulase treatment		After cellulase treatment		Increase of L*
			L*	b*	L*	b*	
-	No enzyme	0	16.77	-9.95	18.18	-12.39	1.42
-	20K[1]	3000	16.80	-9.70	24.00	-14.71	7.20
-	20K[1]	**1500**	16.73	-10.05	**22.98**	-14.62	**6.25**
RF6036	20K + CBD	**500**	16.41	-10.14	**22.80**	-14.19	**6.40**
RF5977	20K +CBD	**250**	16.68	-9.91	**22.81**	-14.47	**6.13**
RF5977	20K +CBD	500	16.73	-10.01	24.07	-14.70	7.34
RF5977	20K +CBD	1500	16.71	-9.68	25.63	-14.79	8.93

L* indicates the lightness, -b* is the blue direction, +b* is the yellow direction.

[1]Commercial preparation

Table 11. Color measurements of the face side of denim treated with heat treated 20K+CBD fusion proteins

Strain No.	Enzyme	NCU/g fabric	Before cellulase treatment		After cellulase treatment		Increase of L*
			L*	b*	L*	B*	
-	No enzyme	0	16.77	-9.95	18.18	-12.39	1.42
-	20K[1] (not heat treated)	3000	16.80	-9.70	24.00	-14.71	7.20
-	20K[1] (not heat treated)	**1500**	16.73	-10.05	**22.98**	-14.62	**6.25**
RF5206	20K CBHI-	**3000**	16.80	-10.00	**22.61**	-14.59	**5.81**
RF5582	20K+CBD construct #1, CBHI-	3000	16.83	-10.01	26.39	-15.13	9.56
RF5582	20K+CBD- construct #1, CBHI-	1000	16.61	-9.76	23.98	-14.98	7.37
RF5582	20K+CBD- construct #1, CBHI-	**500**	16.70	-9.93	**22.75**	-14.75	**6.05**
RF5583	20K+CBD- construct #2, CBHI-	3000	16.73	-9.98	24.78	-14.95	8.05
RF5583	20K+CBD- construct #2, CBHI-	**1000**	16.92	-9.92	**22.73**	-14.57	**5.81**
RF5583	20K+CBD- construct #2, CBHI-	500	16.62	-10.03	21.76	-14.37	5.14
RF5977	20K +CBD- construct #5, [2]	**500**	16.56	-9.77	**23.00**	-14.55	**6.45**

L* indicates the lightness. -b* is the blue direction. +b* is the yellow direction.

[1]Commercial preparation, [2] CBHI-, CBHII-, EGI-, EGII-

Results in Table 10 and Fig. 16 show that the washing performance of the 20K+CBD fusion proteins of the invention in denim treatment was greatly improved compared to 20K strains. With stain RF5977 the enzyme dosage as low as 250 NCU/g fabric could be used to obtain similar abrasion level (lightness L*) to that obtained with the 20K dosage of 1500 NCU/g. Thus a 6 times better washing performance was obtained, and the contrast was good. Also the washing performance obtained with strain RF5978 was similar to that obtained with RF5977.

Heat treatment of the fusion protein preparations seemed to somewhat decrease the stone washing effect, for instance with strain RF5977 a dosage of 500 NCU/g fabric was needed to obtain the same abrasion level as with dosage of 250 NCU/g of not heat treated enzyme preparation (Table 10). Nevertheless, a 3-fold improvement in the washing performance was achieved as compared to a prior art preparation.

EXAMPLE 9. Performance of fusion 20K+CBD affinity mutant protein and fusion 20K+ CBD linker deletion protein preparations in denim finishing/biostoning

Fusion 20K + CBD affinity mutant enzymes produced using *Trichoderma* as host described in Example 3 and fusion 20K + CBD linker deletion proteins produced using *Trichoderma* as host as described in Example 4 were tested for their ability in biostoning of denim. A 20K preparation efficient in denim finishing was used for comparison.

The denim and test systems for biostoning were as in Example 8. Also the effect of the cellulase treatment was evaluated as in Example 8. The results of the biostoning test for exemplary fusion 20K+CBD affinity mutant protein and fusion 20K+ CBD linker deletion protein preparations are shown in Table 12.

Strain RF6090 with the Y31W amino acid substitution showed excellent washing performance (ca. 6 times better than 20K) and good contrast. The efficiency of strain RF6090 compared to 20K can clearly be seen also in Fig. 16. The Strain RF6084 with Y31A amino acid substitution was ca. 1.5 times better than 20K. Fusion 20K+CBD_{mut} proteins with a Y32A or Y31A_Y32A amino acid substitution had a lower biostoning effect than 20K. The washing performance of the 20K+CBD linker deletion proteins in denim treatment was greatly improved compared to 20K strain and good contrast was obtained. With strain RF6108 (ΔG-444) the washing performance was at least 6 times better than with 20K and with strain RF6110 (ΔG-460) ca. 3 times better.

Table 12. Color measurements of the face side of denim treated with 20K+CBD affinity mutant and fusion 20K+ CBD linker deletion proteins

Strain No.	Enzyme (amino acid substitution)	Activity/ g fabric	Before cellulase treatment		After cellulose treatment		Increase of L*
			L*	B*	L*	B*	
-	No enzyme	0	16.77	-9.95	18.18	-12.39	1.42
-	20K[1]	3000	16.80	-9.70	24.00	-14.71	7.20
-	20K[1]	**1500**	16.73	-10.05	**22.98**	-14.62	**6.25**
RF6086	20K +CBDmut(Y32A)	1500	16.83	-9.68	22.05	-14.14	5.22
RF6086	20K +CBDmut(Y32A)	**3000**	16.68	-9.76	**23.30**	-14.40	**6.62**
RF6084	20K +CBDmut(Y31A)	**1000**	16.83	-9.42	**23.15**	-14.26	**6.32**
RF6090	20K +CBDmut(Y31W)	**250**	16.79	-9.32	**22.99**	-14.24	**6.21**
RF6090	20K +CBDmut(Y31W)	1000	16.77	-9.22	25.10	-14.74	8.33
RF6094	20K +CBDmut(Y31A_Y32A)	1500	16.78	-9.30	21.66	-14.02	4.89
RF6094	20K +CBDmut(Y31A_Y32A)	**3000**	16.71	-9.36	**22.27**	-14.14	**5.56**
-	20K[1]	**3000**	16.80	-9.70	**24.00**	-14.71	**7.20**
RF6108	20K + CBD(ΔG-444)	**250**	16.65	-9.59	**23.62**	-13.97	**6.97**
RF6110	20K + CBD(ΔG-460)	**1000**	16.81	-9.72	**24.17**	-14.39	**7.37**

L* indicates the lightness. -b* is the blue direction. +b* is the yellow direction. [1]Commercial preparation

EXAMPLE 10. Effect of the 20K+CBD fusion proteins on the strength of the denim

Some of the jeans obtained from washing tests with 20K+CBD fusion proteins (Examples 8 and 9) that had similar abrasion level (L* -value ca. 23 or 24 after cellulase treatment) were selected for the strength measurements. The tear strength after treatment with 20K+CBD fusion proteins and control samples were measured by Elmendof method according to standard SFS-EN ISO 13937-1. The specimens were cut both in the warp and weft direction. The results are shown in Table 13.

The cellulase fusion proteins caused essentially same or lower strength loss as 20K, i.e., with some preparations the strength of the fabric remained even higher. The lowest strength loss both in warp and weft direction was obtained with strain RF6108 with linker deletion ΔG-444. Also affinity mutant RF6090 with Y31W amino acid substitution caused less strength loss than 20K. Strain RF5977 had rather similar effect on the strength of the fabric than 20K.

Some of the jeans washed with heat treated fusion protein preparations (Example 8, Table 11) were also selected for tear strength measurements. It was noticed that the strength of the fabric was improved with some heat treated preparations, but because of the reduced washing performance higher dosages had to be used to obtain the same abrasion level.

Table 13. Tear strength measurements of jeans treated with 20K+CBD fusion proteins of the invention

Strain No.	Enzyme protein	NCU/ g fabric	L*	Warp		Weft	
				Tear strenght (N)	(%)	Tear strenght (N)	(%)
-	No enzyme	0	1.5	62.2	100.0	46.2	100.0
-	20K[1]	1500	22.9	46.3	74.4	31.9	69.0
RF5977	20K +CBD	250	22.9	48.1	77.3	32.1	69.5
RF6086	20K +CBDmut (Y32A)	3000	23.1	46.6	74.9	30	64.9
RF6084	20K +CBDmut (Y31A)	1000	23.1	47.2	75.9	30.6	66.2
RF6090	20K +CBDmut (Y31W)	250	23.2	48.6	78.1	34.4	74.5
RF6094	20K +CBDmut (Y31A_Y32A)	3000	22.3	48.4	77.8	32.9	71.2
-	20K[1]	3000	23.9	47.6	76.5	28.9	62.6
RF6108	20K + CBD (ΔG-444)	250	23.8	54.3	87.3	35.2	76.2
RF6110	20K + CBD (ΔG-460)	1000	24.0	48.4	77.8	29.8	64.5

[1]Commercial preparation

EXAMPLE 11. Comparison of selected 20K+CBD fusion protein preparations with prior art enzyme preparations

Best 20K+CBD fusion proteins from Examples 8 and 9 were tested with other type of denim. 20K preparation (Ecostone® NP8500) efficient in denim finishing and two commercially available prior art preparations, DeniMax® 399S from Novozymes and Mex 500 from Meiji, which is the most concentrated solid enzyme preparation commercially available, were used for comparison.

The test system for biostoning was as in Example 8, except the denim load was 1 kg and the liquor ratio therefore slightly higher. Five pieces of denim ("legs") made of Down Under Denim twill (Bradmill Textiles Pty, Australia) were used for each test after desizing. Warp and weft yarns of the fabric were ring spun. Enzymes were dosed as NCU-activity units, so that similar abrasion levels (measured as lightness of the face side of denim after cellulase treatment) were obtained. The effect of the cellulase treatment was evaluated as in Example 8, except 20 color measurements were measured per leg.

Two legs with similar abrasion level (L* -value ca. 26 after cellulase treatment) from each washing test were selected for the strength measurements. The tear strength after treatment with 20K+CBD fusion proteins and control samples were measured as in Example 10. The results are shown in Table 14 and Figures 17A and 17B.

The tear strength of weft, which is typically weaker yarn than warp, was higher with fusion proteins of strains RF5977, RF6090, RF6108 and RF6110 than with 20K. With all of the fusion protein strains considerable higher strength both in warp and weft direction was obtained compared to DeniMax 399S and Mex 500. Also 20K strain was less harmful to the strength of the fabric than the other prior art preparations.

Table 14. Tear strength measurements of denim treated with 20K+CBD fusion proteins of the invention, 20K and prior art preparations

Enzyme	Form	L*	Warp	Weft
			Tear strength (N)	Tear strength (N)
Ecostone NP8500	powder	25.9	58.0	40.3
RF5977, 20K+CBD	granula	26.0	56.1	44.4
Mex 500, Meiji	powder	26.1	48.4	31.1
DeniMax 399S, Novozymes	granula	25.5	50.6	38.1
RF6090, 20K+CBD_{mut} (Y31W)	liquid	26.0	57.9	43.4
RF6108, 20K+CBD (ΔG-444)	liquid	25.9	55.6	43.9
RF6110, 20K+CBD (ΔG-460)	liquid	26.2	59.8	45.4

L* indicates the lightness of the face side of denim after cellulase treatment

Example 12. Performance of 20K+CBD preparation in biofinishing (depilling)

The performance of the concentrated 20K+CBD fusion protein preparation in biofinishing was tested. A commercial EGII enriched, acid cellulase preparation (US. 5,874,293) used in biofinishing formulations, and treatment without enzyme were used for comparision. The depilling treatments were performed with Electrolux's Wascator FOM 71 CLS washer extractor under conditions described in Table 15.

Pieces of two kinds of unused pullovers made of 100 % cotton: jersey-based fabric and rib with fuzzy surface were used as test material with filling material. Samples were first pre-washed for 10 min at 60°C with 1 ml/l surfactants/wetting agents (Sandoclean PCJ from Sandos and Imacol CN from Clariant) and rinsed 3 times. After this the cotton knits were treated with cellulase at 60°C for 60 minutes in the presence of the same textile auxiliaries as used in pre-wash. After draining the enzyme was inactivated (for 10 min at 60°C) by raising the pH above 11 with sodium hydroxide. The pieces of knitwear were then rinsed three times and dried in a tumbler.

Table 15. The test conditions/process parameters used in biofinishing treatments

Process parameter	
Fabric load	1.0 kg
Water	15 liter
Sandoclean PCJ and Imacol CN	1 ml/l
pH control pH 5-5.3/pH 6-6.3	Adjustment with acetic acid (80 %)
Time	60 min
Temperature	60°C
Cellulase dosage	0.25 % to 0.63% of the weight of the fabric

The effect of the cellulase treatment was evaluated visually with the naked eye and with a loupe. The results are shown in Table 16 and digital camera photos taken with a macroobjective are shown in Fig.18.

The 20K+CBD fusion protein preparation had excellent biofinishing properties leading to extensive reduction in fuzzing and prevention of the pill formation that can clearly be seen from photos (taken from the rib samples) in Fig.18. Control samples, especially the rib pullover, treated without enzyme contained dense surface fuzzing and severe pilling. With the 20K+CBD preparation a very clean surface of knit wear was obtained already with the smallest dosage (0.25 % of the weight of the fabric, o.w.f.), corresponding to a neutral cellulase activity of 125 NCU/g fabric. This dosage resulted in almost as good effect as a two-fold dosage. Using a dosage of 0.5 % 20K+CBD preparation of the weight of fabric a similar depilling effect was obtained compared to using a dosage of 0.63 % o.w.f. of EG II enriched preparation, that was used at dosage typical for this enzyme concentrate in the biofinishing application. Also the culture medium of 20K+CBD produced by the recombinant host is volumetrically at least two-fold as effective in biofinishing as that of EG II produced by a recombinant host.

Table 16. The results of biofinishing treatments with 20K+CBD fusion protein compared to EGII enriched and control without enzyme

Sample	Dosage g	Dosage, % o.w.f[a)]	Depilling effect[b)]
20K+CBD conc.	5	0.50	+ + + + +
20K+CBD conc.	2.5	0.25	+ + + +
EGII enriched conc.	6.3	0.63	+ + + + +
Control, without enzyme	-	-	-

[a)] of the weight of the fabric

[b)] + + + + + Excellent depilling effect, visually very clean surface.

+ + + + Very good depilling effect, visually clean surface.

- Dense surface fuzzing and/or severe pilling

Example 13. Performance of 20K+CBD preparation in detergent application

The efficiency of a granulated 20K+CBD fusion protein preparation was tested with repeated washing cycles in a household machine using enzyme from 0 to 0.5 % of the weight of detergent formulation (standard detergent ECE 98), which did not contain any enzymes. The test conditions are described in Table 17. Load, detergent dosage and main test conditions were according to the standard EN 60456:2003 but with additional soilings. Fabric samples were taken after 5, 10 and 15 washes.

Table 17. Test design for testing performance of 20K+CBD preparation

Item	Specification
Concentration of enzyme in use	0, 0.1, 0.25, 0.5 %
Washing machine	Siemens 1632 IQ
Washing program	Standard cotton program without fuzzy logic
Detergent	ECE 98, 50 g per wash
pH of detergent in washing liquor	11
Washing temperature	40/60°C
Hardness of water	16°fH
Load	IEC60456:2003 load 4 kg
Added artificial soil Total 38 pieces (37 x 37cm) of each type	Art. No. 101*, cotton soiled with carbon black/olive oil Art. No. 163*, cotton soiled with porridge Art. No. 112*, cotton soiled with cocoa
Test fabric	Anti-greying on Art. No. 224* Tensile strength on Art. No. 224*

* from EMPA Test materialen AG, Switzerland

The test results on anti-greying, carried out on article 224 (ISO 2267) and measured as colour matching differences, are shown in Tables 18 and 19 and Figures 19 and 20. The colour difference was measured by the tristimulus method using Spectraflash 500 colourmeter (illuminant D65/10°).

The 20K+CBD fusion protein preparation showed a positive effect on the tristimulus colour value Y and therefore on anti-greying. The effect of different enzyme concentrations was about the same. Already a very low enzyme concentration (0.10%) was sufficient for anti-greying. Rather similar effects (data not shown) were obtained with 20K+CBD fusion protein compared to commercial detergent cellulase BIOTOUCH®DCC containing 1.8 times higher neutral cellulase activity (NCU).

Article 224 was also used for tensile strength measurements (data not shown) performed according to ISO 13934-1:1999 after 15 washes. None of the enzyme concentrations had a harmful effect on the tensile strength. All results

were within the deviation of each other (variation ±2.5 %). The same applied to the elongation to breaking load.

Table 18. Performance of 20K+CBD fusion protein in detergent application. Colour matching difference between enzyme washed anti-greying article 224 and the original (unwashed) article.

Washing temperature, Enzyme conc. (%)	**Difference (deltaY)**		
	After 5 washes	**After 10 washes**	**After 15 washes**
40°C, 0%	-7.46	-10.39	-12.30
40°C, 0.10%	-5.78	-6.60	-8.01
40°C, 0.25%	-5.52	-6.08	-8.18
40°C, 0.50%	-4.85	-5.24	-7.56
60°C, 0%	-8.65	-11.43	-13.95
60°C, 0.10%	-5.77	-6.77	-8.41
60°C, 0.250%	-4.72	-5.70	-7.46
60°C, 0.50%	-4.33	-6.29	-7.44

Table 19. Performance of 20K+CBD fusion protein in detergent application. Colour matching difference between enzyme washed anti-greying article 224 and wash without enzyme.

Washing temperature, Enzyme conc. (%)	**Diffference (deltaY)**		
	After 5 washes	**After 10 washes**	**After 15 washes**
40°C, 0%	0	0	0
40°C, 0.10%	1.68	3.79	4.29
40°C, 0.25%	1.94	4.31	4.12
40°C, 0.50%	2.61	5.15	4.74
60°C, 0%	0	0	0
60°C, 0.10%	2.88	4.66	5.54
60°C, 0.250%	3.93	5.73	6.49
60°C, 0.50%	4.32	5.14	6.51

REFERENCES

Aho S, V Olkkonen, T Jalava, M Paloheimo, R Bühler, M-L Niku-Paavola, EH Bamford and M Korhola. 1991. Monoclonal antibodies against core and cellulose-binding domains of *Trichoderma reesei* cellobiohydrolases I and II and endoglucanase I. Eur. J. Biochem. 200:643-649.

Azevedo Mde O, Felipe MS, Astolfi-Filho S, Radford A. 1990. Cloning, sequencing and homologies of the cbh-1 (exoglucanase) gene of *Humicola grisea* var. *thermoidea*. J Gen Microbiol. 136: 2569-2576.

Bailey MJ and Nevalainen KMH. 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enz Microbiol Technol. 3: 153-157.

Haakana H, Miettinen-Oinonen A, Joutsjoki V, Mäntylä A, Suominen P, and Vehmaanperä J. 2004. Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei*. Enz Microbiol Technol. 34: 159-167.

Henrissat B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280: 309-316.

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

Hong J, Tamaki H, Yamamoto K and Kumagai H. 2003. Cloning of a gene encoding thermostable cellobiohydrolase from *Thermoascus aurantiacus* and its expression in yeast. Appl Microbiol Biotechnol 63: 42-50.

Joutsjoki VV, Torkkeli TK, and Nevalainen KMH. 1993. Transformation of *Trichoderma reesei* with the *Hormoconis resinae* glucoamylase P *(gamP)* gene: production of a heterologous glucoamylase by *Trichoderma reesei*. Curr. Genet. 24:223-228.

Karhunen T, A Mäntylä, KMH Nevalainen, and PL Suominen. 1993. High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. Mol. Gen. Genet. 241:515-522.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680 – 685.

Linder M, Mattinen ML, Kontteli M, Lindeberg G, Ståhlberg J, Drakenberg T, Reinikainen T, Pettersson G, Annila A. 1995. Identification of functionally important amino acids in the cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I. Protein Science 4: 1056-1064.

Lowry OH, NJ Roseborough, AL Farr and RJ Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol Chem 193: 265-275.

Malardier L, Daboussi MJ , Julien J, Roussel F, Scazzocchio C and Brygoo Y. 1989. Cloning of the nitrate reductase gene (niaD) of Aspergillus nidulans and its use for transformation of Fusarium oxysporum. Gene 15:147-156.

Miettinen-Oinonen A, Londesborough J, Joutsjoki V, Lantto R and Vehmaanperä, J. 2004. Three cellulases from *Melanoarpus albomyces* with applications in the textile industry. Enz Microbiol Technol. 34: 332-341.

Penttilä M, H Nevalainen, M Rättö, E Salminen, and J Knowles. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61:155-164.

Saloheimo A, Henrissat B, Hoffren AM, Teleman O, Penttilä M. 1994. A novel, small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast. Mol Microbiol 13: 219-228.

Sambrook J, EF Fritsch, and T Maniatis. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York, US.

Sambrook J and DW Russell. 2001. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York, US.

Srisodsuk M, Reinikainen T, Penttilä M, Teeri TT. 1993. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. J. Biol. Chem. 268: 20756-20761.

Van Tilbeurgh H, Loonties F, de Bruyne C, Clayssens M 1988. Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. Meth. Enzymol. 160: 45-59.

Ward M, Shan W, Dauberman J, Weiss G, Larenas E, Bower B, Rey M, Clarkson K and Bott R. (1993) Cloning, sequence and preliminary structural analysis of a small, high pI endoglucanase (EGIII) from Trichoderma reesei. Proceedings of the second TRICEL symposium on *TRICHODERMA REESEI* CELLULASES AND OTHER HYDROLASES, Espoo, Finland, 1993, ed. by P. Suominen and T. Reinikainen. Foundation for Biotechnical and Industrial Fermentation Research 8 (1993): 153-158.

Claims

1. A cellulase fusion protein comprising

A. an optionally modified first amino acid sequence of a cellulase core derived from one species, and

B. an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species,

wherein a junction region has been introduced between said first amino acid sequence and said second amino acid sequence, whereby a stable fusion protein is obtained.

2. A cellulase fusion protein of claim 1, wherein the junction region has the following general formula:

$^{1}A - ^{2}B - ^{3}C - ^{4}D - ^{5}E - ^{6}F$

wherein

^{1}A is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{1}A is Gly or Val, most preferably Gly;

^{2}B is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, Phe, Val, Glu, Asp, Gln, and Asn; preferably ^{2}B is Pro, Gln, or Glu;

^{3}C is selected from a group consisting of Gly, Ala, Lys, Leu, Pro, Ile, Val, Ser, and Thr; preferably ^{3}C is Ile;

^{4}D is selected from a group consisting of Gly, Ala, Leu, Pro, Ile, and Val; preferably ^{4}D is Gly or Pro;

^{5}E is selected from a group consisting of Ser, Pro and Thr; preferably ^{5}E is Ser; and

^{6}F is selected from a group consisting of Ser, Thr or is absent, preferably ^{6}F is Ser or is absent; wherein ^{1}A is attached at the C-terminal amino acid sequence of the cellulase core and ^{6}F is attached at the N-terminal amino acid sequence of the linker and/or domain (CBD).

3. A cellulase fusion protein of claim 2, wherein the junction region has the following general formula:

$^{1}Gly - ^{2}B - ^{3}Ile - ^{4}D - ^{5}Ser - ^{6}F$

wherein

^{2}B is Pro, Gln, or Glu;

^{4}D is Gly or Pro;

^{5}E is Ser; and

^{6}F is Ser or is absent.

4. A cellulase fusion protein of claim 2, wherein the junction region has the following formula:

^{1}Val – ^{2}Gln – ^{3}Ile – ^{4}Pro – ^{5}Ser – ^{6}Ser.

5. A cellulase fusion protein of claim 2, wherein the junction region has the formula:

^{1}Gly – ^{2}Glu – ^{3}Ile – ^{4}Gly – ^{5}Ser or ^{1}Gly – ^{2}Pro – ^{3}Ile – ^{4}Gly – ^{5}Ser.

6. A cellulase fusion protein of any one of claims 1 to 5, wherein said first amino acid sequence is from a neutral cellulase and said second amino acid sequence is from an acid cellulase.

7. A cellulase fusion protein of claim 6, wherein said first amino acid sequence is from cellulase of family 45 and said second amino acid sequence is from cellulase of family 7.

8. A cellulase fusion protein of claim 6 wherein said neutral cellulase is of fungal origin.

9. A cellulase fusion protein of claim 8, wherein said neutral cellulase is derived from genera of *Melanocarpus*, *Humicola*, *Thielavia*, *Myceliophthora*, *Fusarium*, *Acremonium, Chrysosporium, Thermoascus, Scopulariopsis, Myriococcum, Talaromyces or Chaetomium,* preferably from a *Melanocarpus sp,* most preferably from *Melanocarpus albomyces.*

10. A cellulase fusion protein of claim 9, wherein said cellulase is *Melanocarpus albomyces* 20 K cellulase, 50K cellulase, 50KB cellulase, or a derivative thereof.

11. A cellulase fusion protein of claim 9, wherein said cellulase is *Thermoascus aurantiacus* CBHI or a derivative thereof.

12. A cellulase fusion protein of claim 6, wherein said acid cellulase is derived from from *Trichoderma sp.* or *Hypocrea*, preferably from *Trichoderma reesei.*

13. A cellulase fusion protein of claim 6, wherein said neutral cellulase is the 20 K cellulase of *Melanocarpus albomyces* of SEQ ID. NO: 2 or a derivative thereof, and the second amino acid sequence is the linker and/or CBD of *Trichoderma reesei* cellobiohydrolase I of SEQ ID. NO: 4 or a derivative thereof.

14. A cellulase fusion protein of any one of claims 1 to 13, wherein said first amino acid sequence and/or said second amino acid sequence is modified.

15. A cellulase fusion protein of claim 14, wherein in said second amino acid sequence amino acid tyrosine at position 492 (position 31 of the CBD) and/or 493 (position 32 of the CBD) of the mature *Trichoderma reesei* CBHI is substituted with an aliphatic amino acid, preferably with alanine, and/or with an aromatic amino acid, preferably with tryptophan.

16. A cellulase fusion protein of claim 15, wherein said second amino acid sequence is selected from a group of SEQ ID. NO: 45, 46, 47, and 48.

17. A cellulase fusion protein of claim 14, wherein in said second amino acid sequence amino acids 434 to 444 or 434 to 460 of the mature *Trichoderma reesei* CBHI sequence are deleted.

18. A cellulase fusion protein of claim 17, wherein said second amino acid sequence is selected from a group of SEQ ID. NO: 49 and 50.

19. A cellulase fusion protein of claim 14, wherein in said first amino acid sequence amino acid valine at position 208 of 20K cellulase sequence of *Melanocarpus albomyces* of SEQ ID. NO: 2 has been deleted.

20. A cellulase fusion protein of claim 14, wherein in said first amino acid sequence amino acid alanine at position 207 of 20K cellulase sequence of *Melanocarpus albomyces* of SEQ ID. NO: 2 has been deleted.

21. A cellulase fusion protein of claim 14, wherein in said first amino acid sequence amino acid phenylalanine at position 209 of 20 K cellulase sequence of *Melanocarpus albomyces* of SEQ ID. NO: 2 has been substituted with Trp.

22. A cellulase fusion protein of claim 14, wherein in said first amino acid sequence amino acid contains an inserted proline after position 206 in 20 K cellulase sequence of *Melanocarpus albomyces* of SEQ ID. NO: 2.

23. A cellulase fusion protein of claim 14, wherein in said first amino acid sequence amino acid is selected from a group of SEQ ID. NO: 37, 38, 39, and 40.

24. A cellulase fusion protein of any one of claims 1 to 23, wherein said fusion protein is stable.

25. A cellulase fusion protein of any one of claims 1 to 23, wherein a preparation containing said fusion protein has further been stabilized by heat treatment.

26. A cellulase fusion protein of claim 1, wherein said first cellulase naturally lacks a CBD.

27. A cellulase fusion protein of claim 1, wherein a CBD has been deleted from said first cellulase.

28. An expression vector comprising a first polynucleotide sequence encoding an optionally modified first amino acid sequence of a cellulase core derived from one species, and a second polynucleotide sequence encoding an optionally modified second amino acid sequence of a linker and/or cellulose binding domain (CBD) derived from another species, and a polynucleotide encoding a junction region connecting said first and second polynucleotide sequences, said polynucleotide sequences encoding the respective amino acid sequences as specifically defined above.

29. An expression vector of claim 28, wherein the first polynucleotide sequence encodes a neutral cellulase core and said second polynucleotide sequence encodes a linker and/or cellulose binding domain (CBD) of an acid cellulase.

30. An expression vector of claim 29, wherein said neutral cellulase is derived from genera of *Melanocarpus*, *Humicola*, *Thielavia*, *Myceliophthora*, *Fusarium*, *Acremonium, Chrysosporium, Thermoascus, Scopulariopsis, Myriococcum, Talaromyces or Chaetomium,* preferably from a *Melanocarpus sp,* most preferably from *Melanocarpus albomyces.*

31. An expression vector of claim 30, wherein said cellulase is *Melanocarpus albomyces* 20 K cellulase, 50K cellulase, 50KB cellulase, or a derivative thereof.

32. An expression vector of claim 30, wherein said cellulase is *Thermoascus aurantiacus* CBHI.

33. An expression vector of claim 28, wherein said acid cellulase is derived from from *Trichoderma sp.* or *Hypocrea*, preferably from *Trichoderma reesei.*

34. An expression vector of claim 28, wherein said neutral cellulase is the 20 K cellulase of *Melanocarpus albomyces* of SEQ ID. NO: 1 or a derivative thereof, and the second amino acid sequence is the linker and/or CBD of

Trichoderma reesei cellobiohydrolase I of SEQ ID. NO: 3 or a derivative thereof.

35. An expression vector of any one of claims 28 to 34, wherein said first amino acid sequence and/or said second amino acid sequence is modified.

36. An expression vector of claim 35, wherein said second polynucleotide sequence encodes an amino acid sequence selected from a group of SEQ ID. NO: 45, 46, 47, and 48.

37. An expression vector of 35, wherein said second polynucleotide sequence encodes an amino acid sequence selected from a group of SEQ ID. NO: 49 and 50.

38. An expression vector of claim 35, wherein said first polynucleotide encodes an amino acid sequence selected from a group of SEQ ID. NO: 37, 38, 39, and 40.

39. An expression vector of claim 28, wherein said first polynucleotide encodes a cellulase, which naturally lacks a CBD.

40. A host cell comprising an expression vector as defined in any one of claims 28 to 39.

41. A host cell of claim 40, which is of fungal origin.

42. A host cell of claim 41, which belongs to filamentous fungi.

43. A host cell of claim 41 or 42 which belongs to the genus *Trichoderma or Aspergillus.*

44. The host cell of claim 43, which is *Trichoderma reesei.*

45. A process for the production of a cellulase fusion protein as defined in any one of claims 1 to 27 comprising the steps of culturing the host cell of any one of claims 41 to 44 and recovering the protein from the culture medium.

46. An enzyme preparation comprising a cellulase fusion protein as defined in any one of claims 1 to 27.

47. A process for biostoning which comprises the step of adding a cellulase fusion protein as defined in any one of claims 1 to 27 or the preparation of claim 46 to cotton containing fabric or garments.

48. The process of claim 47, wherein the fabric or garments is denim.

49. A process for biofinishing, which comprises the step of adding a cellulase fusion protein as defined in any one of claims 1 to 27 or the preparation of claim 46 to textile materials like fabrics or garments or yarn.

50. The process of claim 49, wherein the textile materials are manufactured of natural cellulose containing fibers or manmade cellulose containing fibers or are mixtures thereof.

51. The process of claim 49, wherein the textile materials are blends of synthetic fibers and cellulose containing fibers.

52. A detergent composition comprising a cellulase fusion protein as defined in any one of claims 1 to 27 or the enzyme preparation of claim 46 and auxiliaries, such as surface active agents, surfactants, bleaching agents or builders.

53. A method of treating cellulosic fiber containing textile material, wherein said method comprises contacting said textile material with the detergent composition of claim 52.

54. A method for treating wood-derived pulp or fiber, which comprises the step of adding a cellulase fusion protein as defined in any one of claims 1 to 27 or the preparation of claim 46 to wood-derived mechanical or chemical pulp or secondary fiber.

55. A method for improving the quality of animal feed, which comprises treating plant material with a cellulase fusion protein as defined in any one of claims 1 to 27 or the preparation of claim 46.

Fig. 1

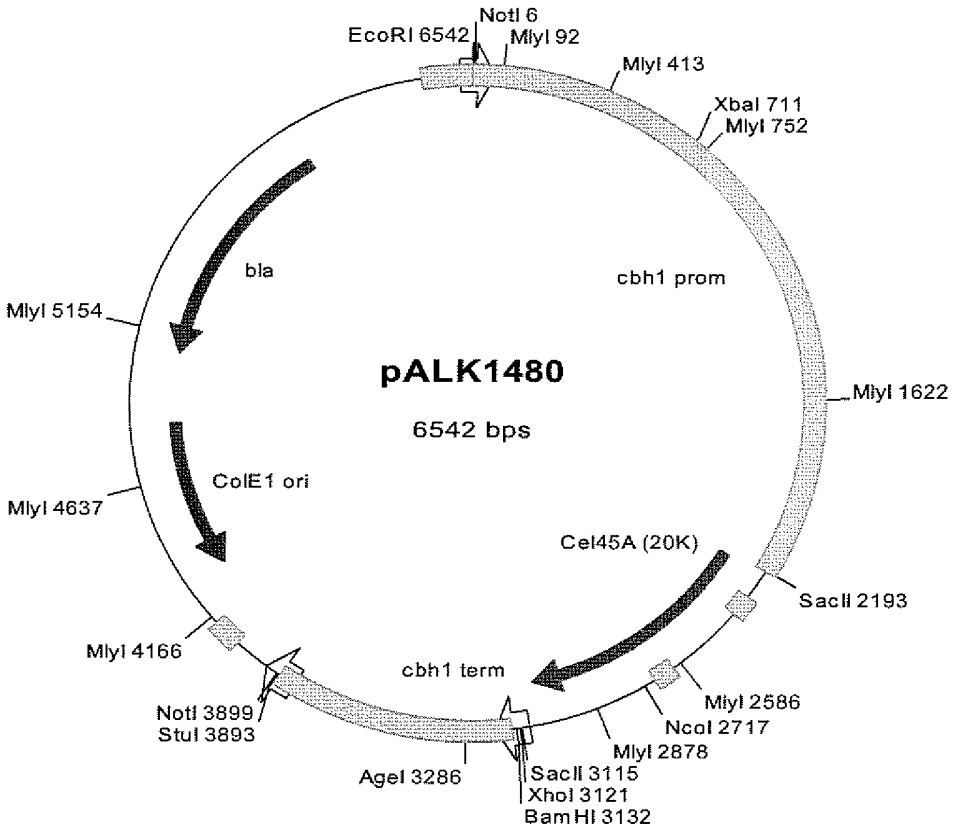

NotI 6
EcoRI 6542
MlyI 92
MlyI 413
XbaI 711
MlyI 752
bla
cbh1 prom
MlyI 5154
pALK1480
6542 bps
MlyI 1622
ColE1 ori
MlyI 4637
Cel45A (20K)
SacII 2193
MlyI 4166
cbh1 term
MlyI 2586
NcoI 2717
NotI 3899
StuI 3893
MlyI 2878
AgeI 3286
SacII 3115
XhoI 3121
BamHI 3132

Fig. 2

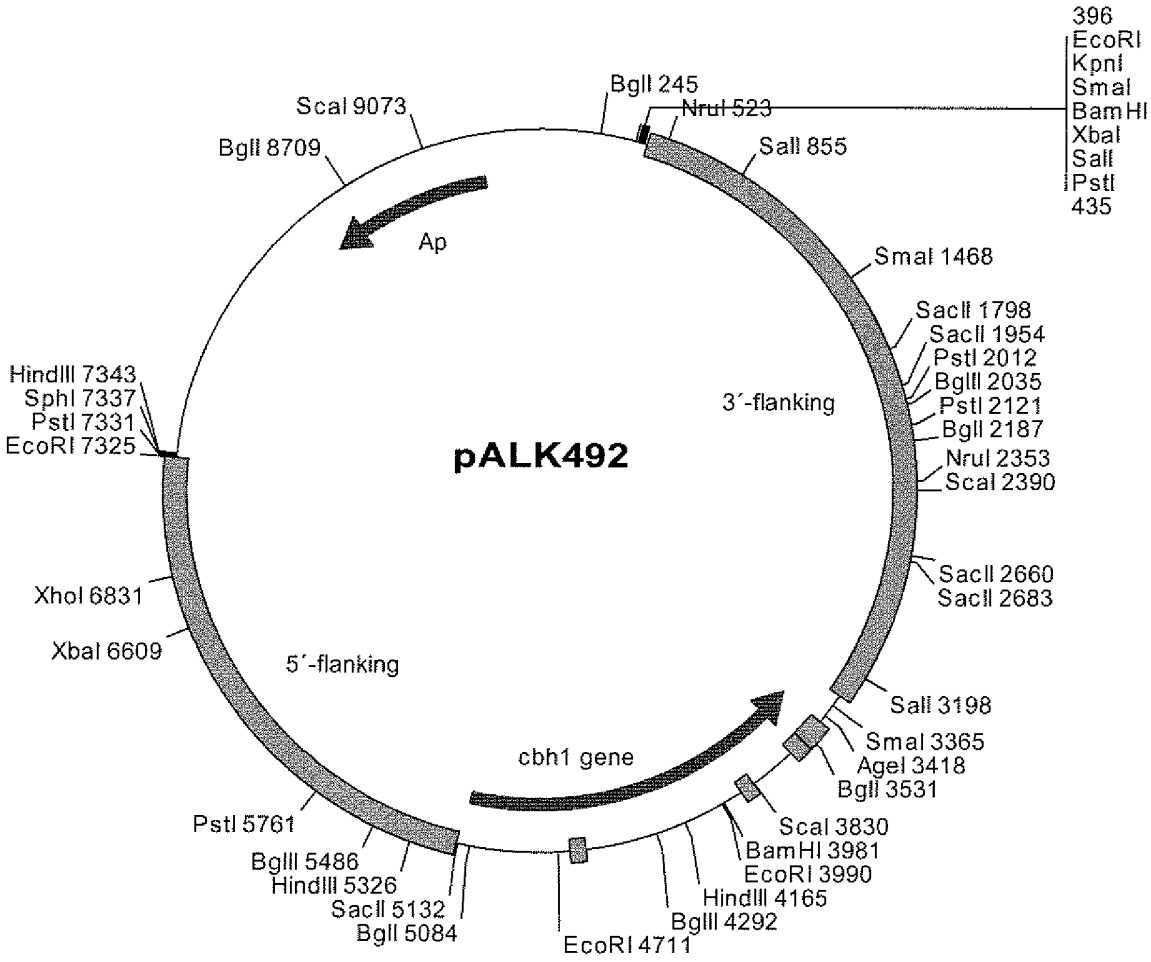

pALK492
396
EcoRI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
435
BglII 245
NruI 523
SalI 855
SmaI 1468
SacII 1798
SacII 1954
PstI 2012
BglII 2035
PstI 2121
BglII 2187
NruI 2353
ScaI 2390
SacII 2660
SacII 2683
SalI 3198
SmaI 3365
AgeI 3418
BglII 3531
ScaI 3830
BamHI 3981
EcoRI 3990
HindIII 4165
BglII 4292
EcoRI 4711
PstI 5761
BglII 5486
HindIII 5326
SacII 5132
BglII 5084
XhoI 6831
XbaI 6609
HindIII 7343
SphI 7337
PstI 7331
EcoRI 7325
ScaI 9073
BglII 8709
Ap
3´-flanking
5´-flanking
cbh1 gene

Fig. 3

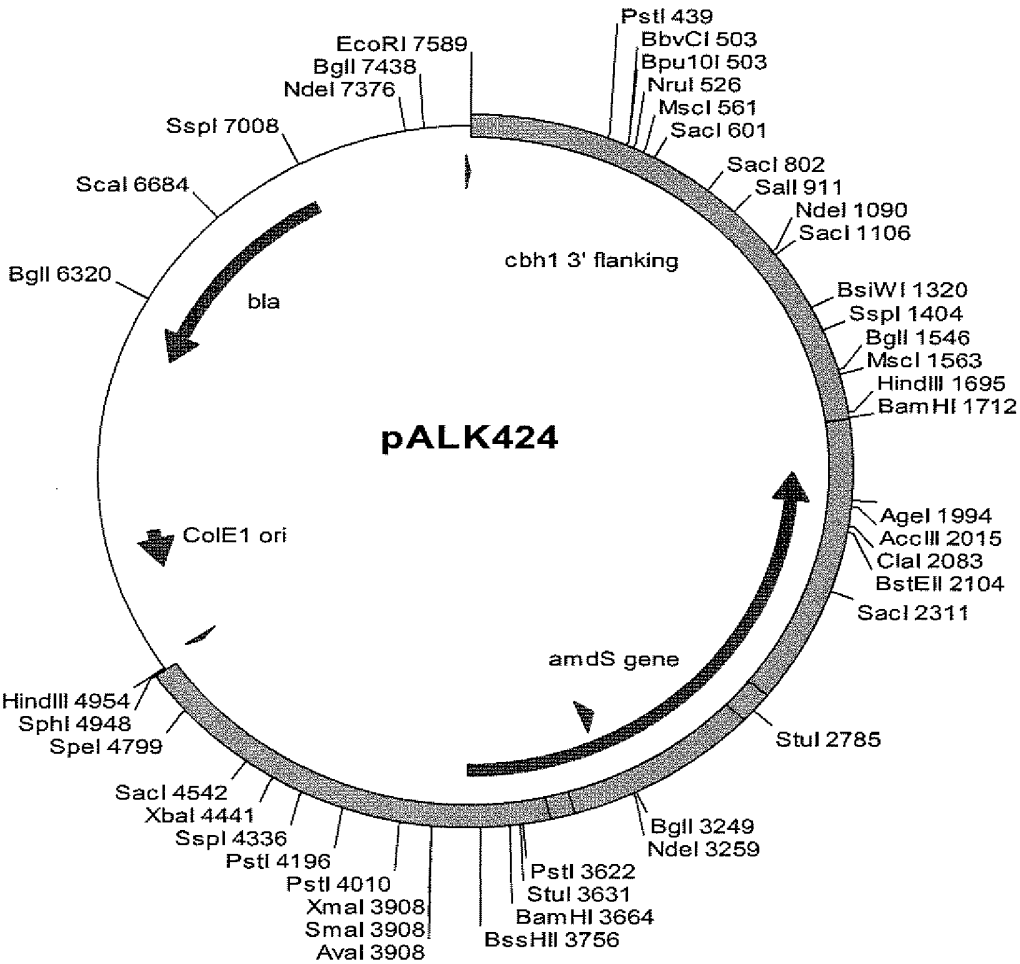

pALK424
cbh1 3' flanking
bla
ColE1 ori
amdS gene
PstI 439
BbvCI 503
Bpu10I 503
NruI 526
MscI 561
SacI 601
SacI 802
SalI 911
NdeI 1090
SacI 1106
BsiWI 1320
SspI 1404
BglI 1546
MscI 1563
HindIII 1695
BamHI 1712
AgeI 1994
AccIII 2015
ClaI 2083
BstEII 2104
SacI 2311
StuI 2785
BglI 3249
NdeI 3259
PstI 3622
StuI 3631
BamHI 3664
BssHII 3756
AvaI 3908
SmaI 3908
XmaI 3908
PstI 4010
PstI 4196
SspI 4336
XbaI 4441
SacI 4542
SpeI 4799
SphI 4948
HindIII 4954
BglI 6320
ScaI 6684
SspI 7008
NdeI 7376
BglI 7438
EcoRI 7589

Fig. 4

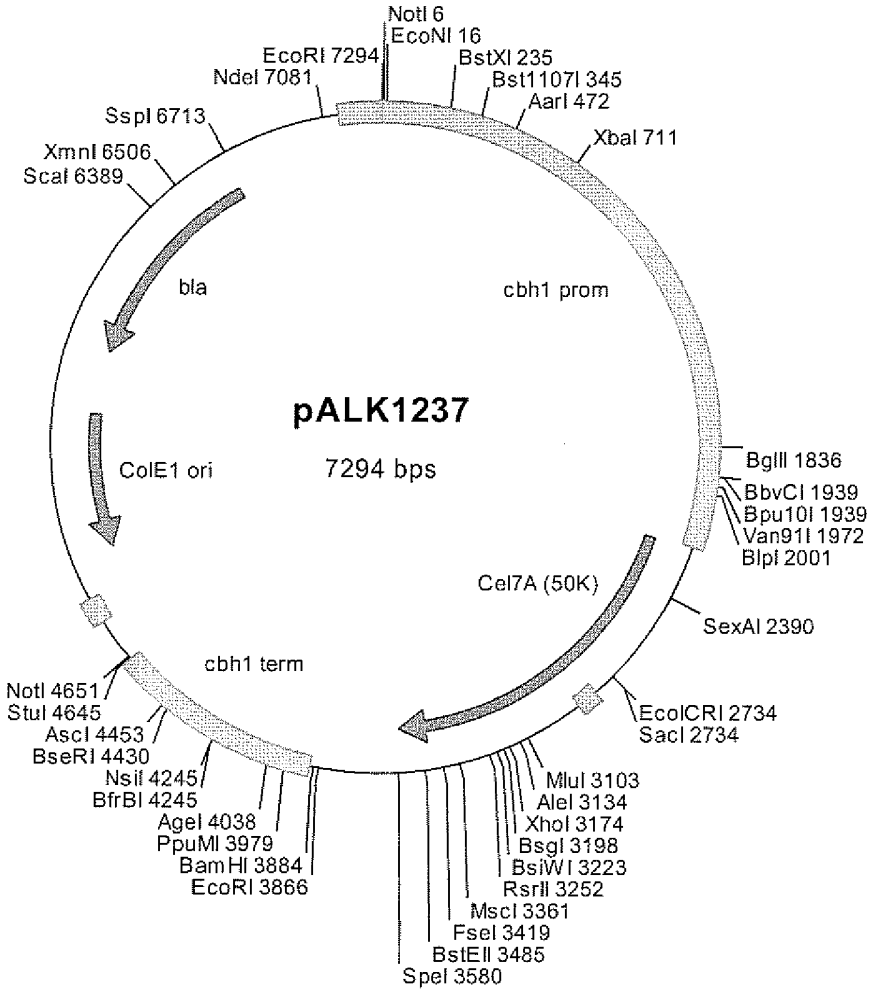

pALK1237
7294 bps
NotI 6
EcoNI 16
BstXI 235
Bst1107I 345
AarI 472
XbaI 711
BglII 1836
BbvCI 1939
Bpu10I 1939
Van91I 1972
BlpI 2001
SexAI 2390
EcoICRI 2734
SacI 2734
MluI 3103
AleI 3134
XhoI 3174
BsgI 3198
BsiWI 3223
RsrII 3252
MscI 3361
FseI 3419
BstEII 3485
SpeI 3580
NotI 4651
StuI 4645
AscI 4453
BseRI 4430
NsiI 4245
BfrBI 4245
AgeI 4038
PpuMI 3979
BamHI 3884
EcoRI 3866
EcoRI 7294
NdeI 7081
SspI 6713
XmnI 6506
ScaI 6389
bla
ColE1 ori
cbh1 prom
cbh1 term
Cel7A (50K)

Fig. 5

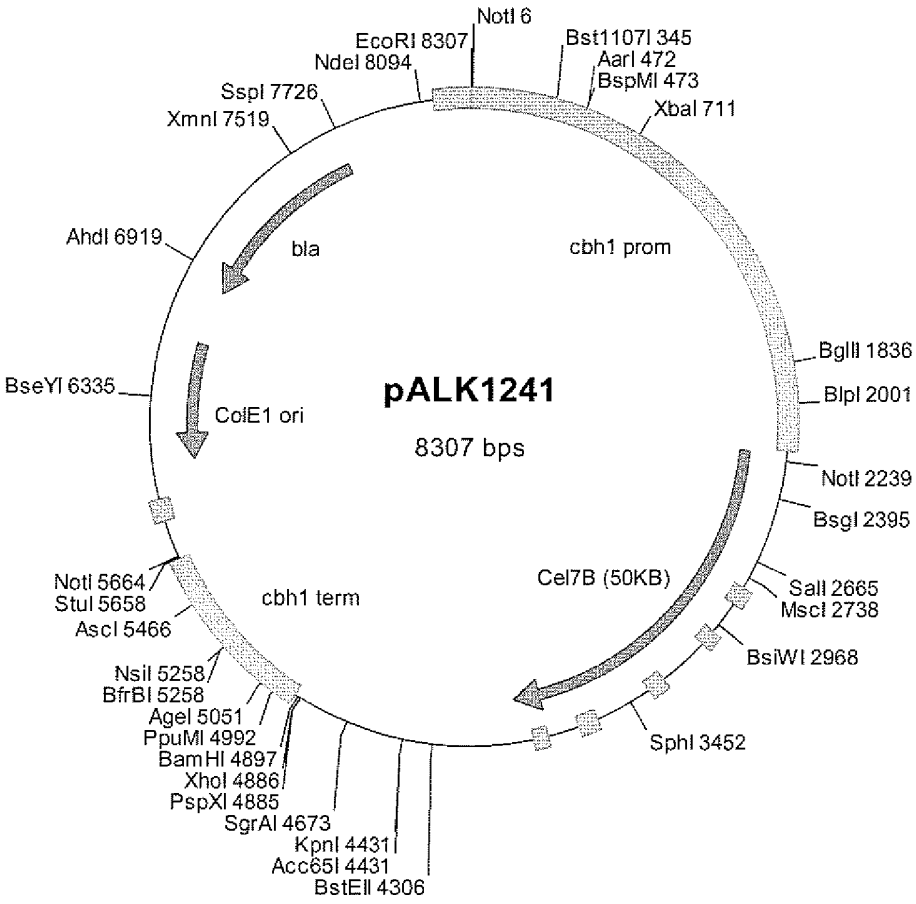

pALK1241
8307 bps
NotI 6
EcoRI 8307
NdeI 8094
SspI 7726
XmnI 7519
AhdI 6919
BseYI 6335
NotI 5664
StuI 5658
AscI 5466
NsiI 5258
BfrBI 5258
AgeI 5051
PpuMI 4992
BamHI 4897
XhoI 4886
PspXI 4885
SgrAI 4673
KpnI 4431
Acc65I 4431
BstEII 4306
Bst1107I 345
AarI 472
BspMI 473
XbaI 711
BglII 1836
BlpI 2001
NotI 2239
BsgI 2395
SalI 2665
MscI 2738
BsiWI 2968
SphI 3452
bla
cbh1 prom
ColE1 ori
cbh1 term
Cel7B (50KB)

Fig. 6

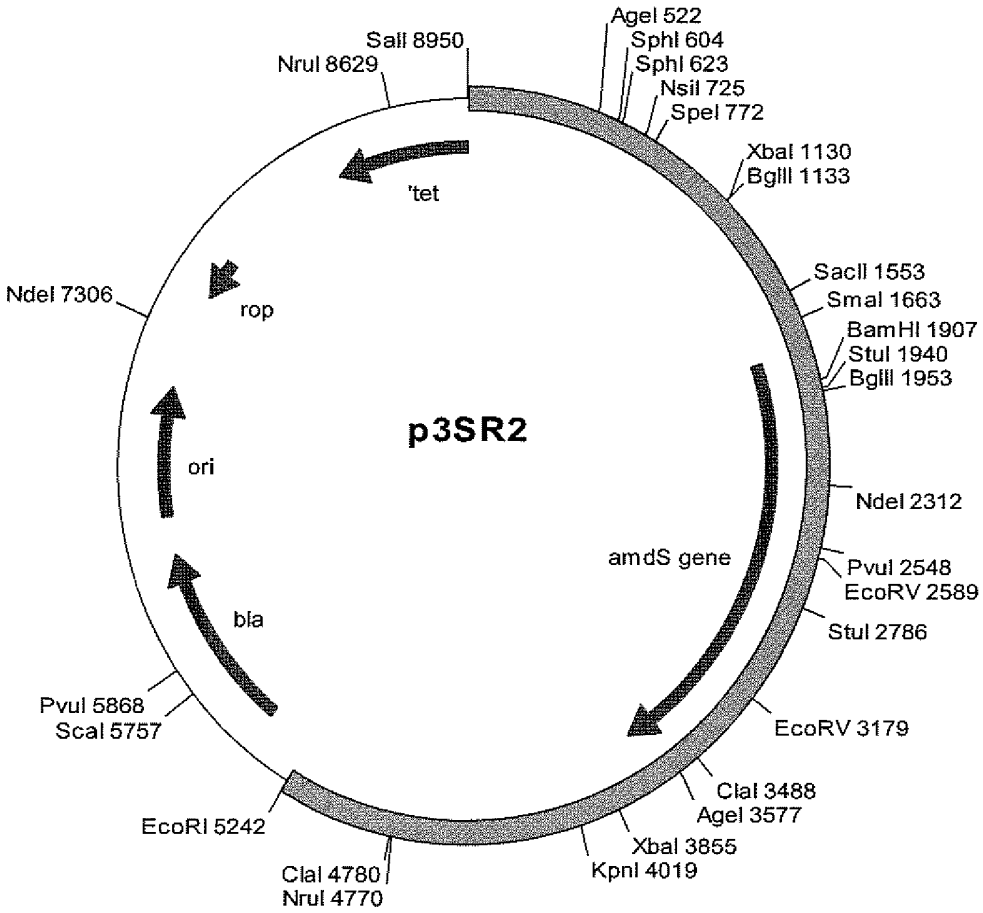

AgeI 522
SphI 604
SphI 623
NsiI 725
SpeI 772
XbaI 1130
BglII 1133
SacII 1553
SmaI 1663
BamHI 1907
StuI 1940
BglII 1953
NdeI 2312
PvuI 2548
EcoRV 2589
StuI 2786
EcoRV 3179
ClaI 3488
AgeI 3577
XbaI 3855
KpnI 4019
ClaI 4780
NruI 4770
EcoRI 5242
PvuI 5868
ScaI 5757
NdeI 7306
SalI 8950
NruI 8629
'tet
rop
ori
bla
amdS gene
p3SR2

Fig. 7

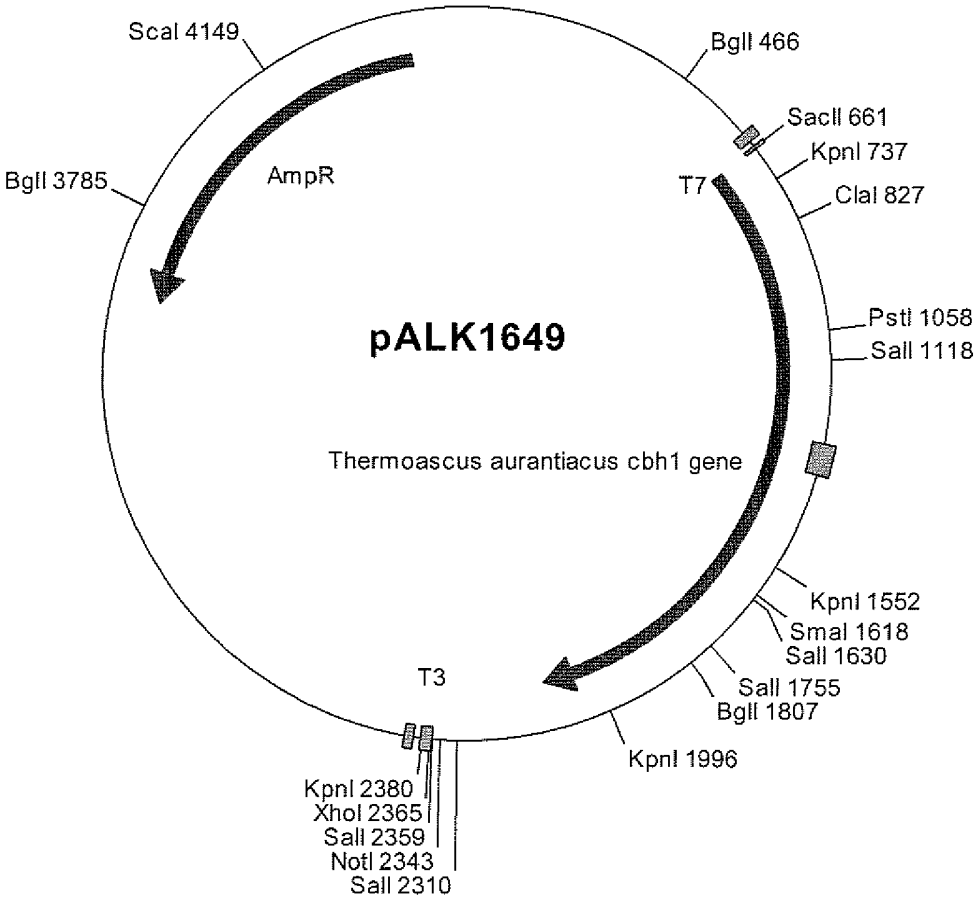

ScaI 4149
BglI 466
SacII 661
KpnI 737
ClaI 827
PstI 1058
SalI 1118
KpnI 1552
SmaI 1618
SalI 1630
SalI 1755
BglI 1807
KpnI 1996
KpnI 2380
XhoI 2365
SalI 2359
NotI 2343
SalI 2310
BglI 3785
AmpR
T7
T3
pALK1649
Thermoascus aurantiacus cbh1 gene

Fig. 8

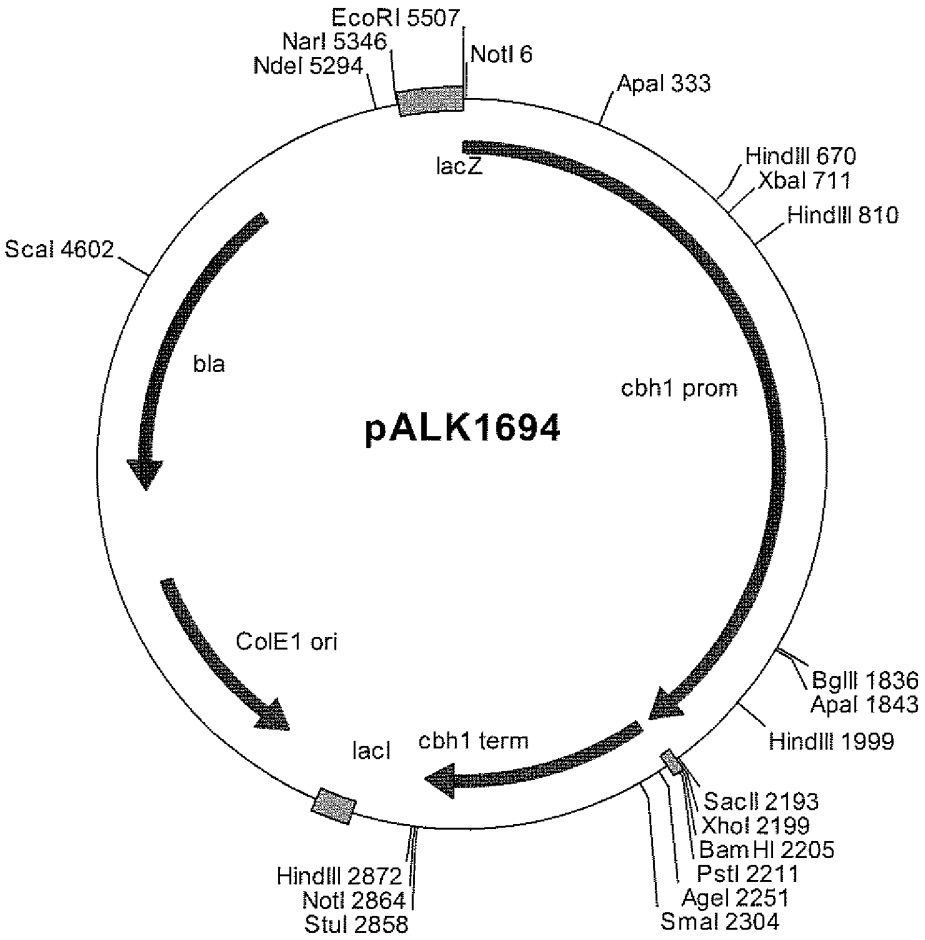
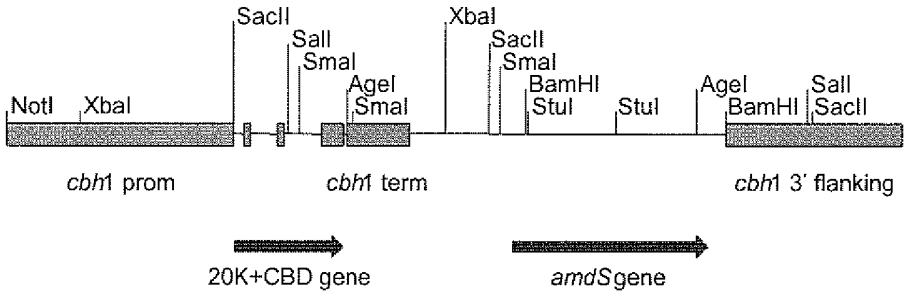

EcoRI 5507
NarI 5346
NdeI 5294
NotI 6
ApaI 333
HindIII 670
XbaI 711
HindIII 810
lacZ
ScaI 4602
bla
cbh1 prom
pALK1694
ColE1 ori
BglII 1836
ApaI 1843
HindIII 1999
lacI
cbh1 term
SacII 2193
XhoI 2199
BamHI 2205
PstI 2211
AgeI 2251
SmaI 2304
HindIII 2872
NotI 2864
StuI 2858

Fig. 9

A)



B)

20K+CBD fusion protein:

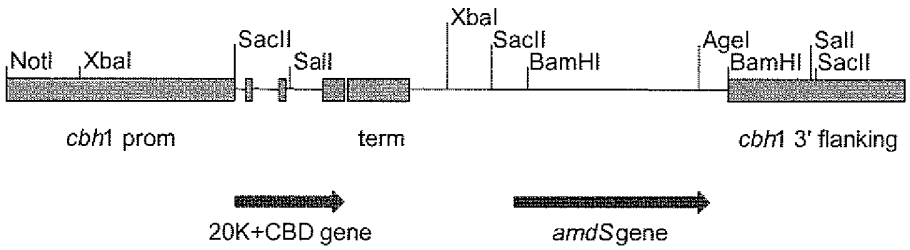
	M. albomyces	*T. reesei* CBHI	
	20K	Linker	*CBD*

pALK1434: D G G F A V F K A P S G S T G N P S...*T*[1] *Q ... Y Y S Q C L*

pALK1435: D G G F A V F K A P S G G N...*T*[1] *Q ... Y Y S Q C L*

Fig. 10

A)



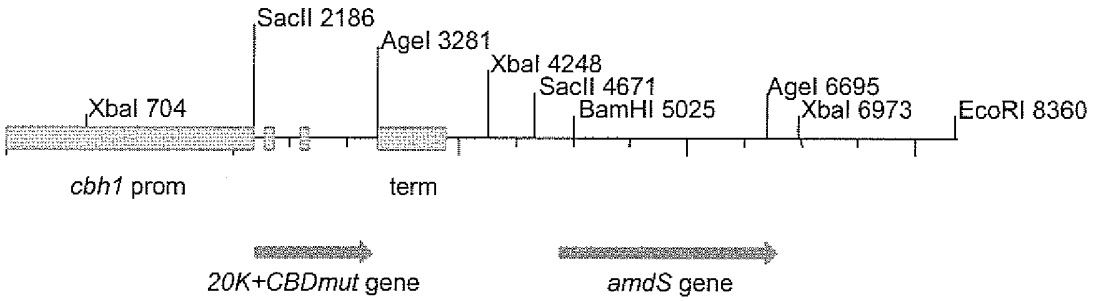
B)

20K+CBD fusion protein:

	M. albomyces		*T. reesei* CBHI		
	20K	Junction	Linker	*CBD*	
pALK1768:	**D G G F A F** G P I G S T G	N P S...	*T*[1]	*Q ... Y Y S Q C L*	
pALK1769:	**D G G F** W G E I G S T G	N P S...	*T*[1]	*Q ... Y Y S Q C L*	
pALK1770:	**D G G F** P A V Q I P S S T G	N P S...	*T*[1]	*Q ... Y Y S Q C L*	
pALK1775:	**D G G F A** W G E I G S T G	N P S...	*T*[1]	*Q ... Y Y S Q C L*	

Fig. 11

A)



B)

20K+CBD$_{mut}$ fusion protein:

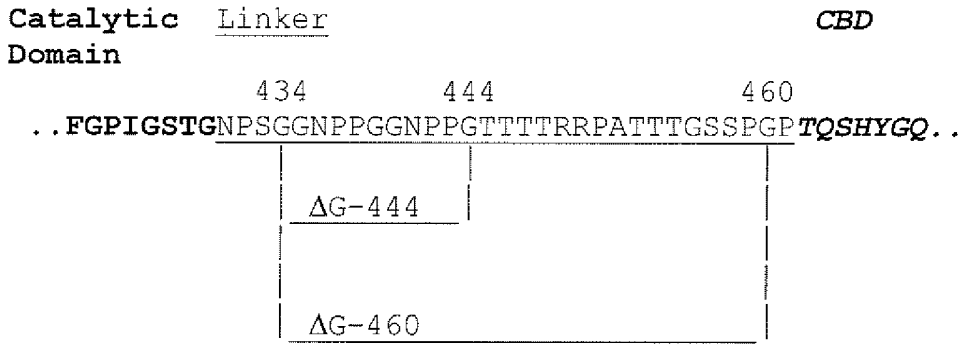
M. albomyces *T. reesei* CBHI
20K Junction Linker *CBD*

D G G F P A V Q I P S S T G N P S...*T*[1] *Q S H Y*...*N P Y*[31] *Y*[32] *S Q C L*

pALK1877: ***N P A***[31] ***Y***[32] ***S Q C L***
pALK1878: ***N P Y***[31] ***A***[32] ***S Q C L***
pALK1879: ***N P W***[31] ***Y***[32] ***S Q C L***
pALK1880: ***N P A***[31] ***A***[32] ***S Q C L***

Fig. 12

A)



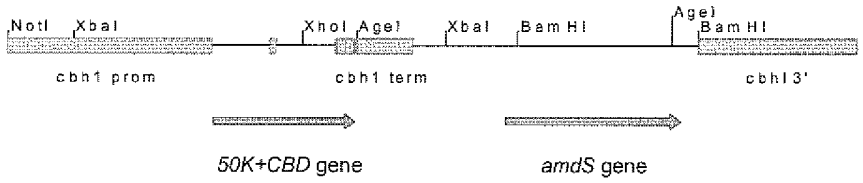
B)

20K+CBD linker deletions:

	20K	Junction	Linker	CBD
	M. albomyces		*T. reesei* CBHI	
pALK1893:DGGF	PAVQIPSS	TG	NPSTTTTRRPATTTGSSPGP	$T^{1}Q...P\ Y^{31}\ Y^{32}\ S\ Q\ C\ L$
pALK1896:DGGF	PAVQIPSS	TG	NPSP	$T^{1}Q...P\ Y^{31}\ Y^{32}\ S\ Q\ C\ L$
pALK1899:DGGF	PAVQIPSS	TG	NPSTTTTRRPATTTGSSPGP	$T^{1}Q...P\ A^{31}\ A^{32}\ S\ Q\ C\ L$
pALK1952:DGGF	PAVQIPSS	TG	NPSP	$T^{1}Q...P\ A^{31}\ A^{32}\ S\ Q\ C\ L$

Fig. 13

A)



B)

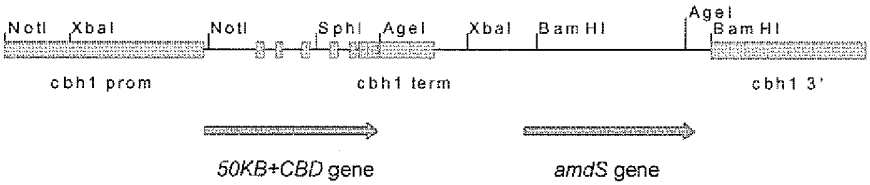
***Melanocarpus albomyces* 50K fusion protein:**

M. albomyces	*T. reesei* CBHI
50K	Linker *CBD*

N L R W G E I G S T G N P S ...*T*[1] *Q S H Y...N P Y Y S Q C L*

Fig. 14

A)



B)

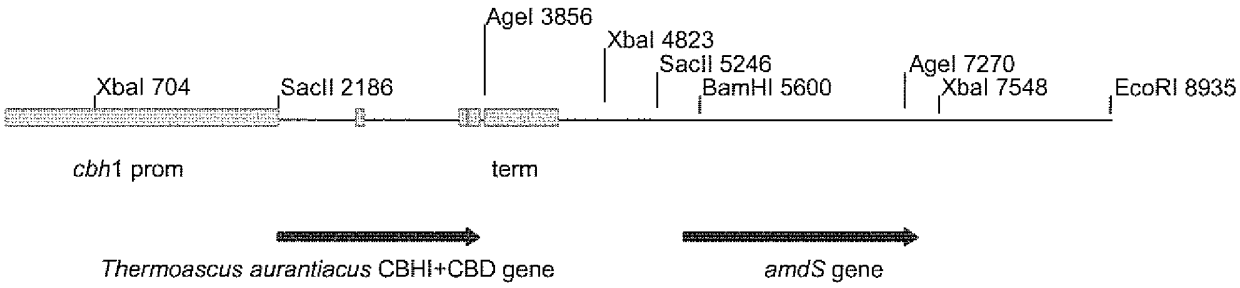
***Melanocarpus albomyces* 50KB fusion protein:**

M. albomyces	*T. reesei* CBHI
50KB	Linker *CBD*

N I R F G P I G S T G N P S ...*T*[1] *Q S H Y...N P Y Y S Q C L*

Fig. 15

A)



B)

***Thermoascus aurantiacus* CBHI+CBD fusion protein:**

T. aurantiacus *T. reesei* CBHI
CBHI Junction Linker *CBD*

N I K V G P I G S T G N P S...*T*[1] *Q S H Y...N P Y Y S Q C L*

Fig. 16

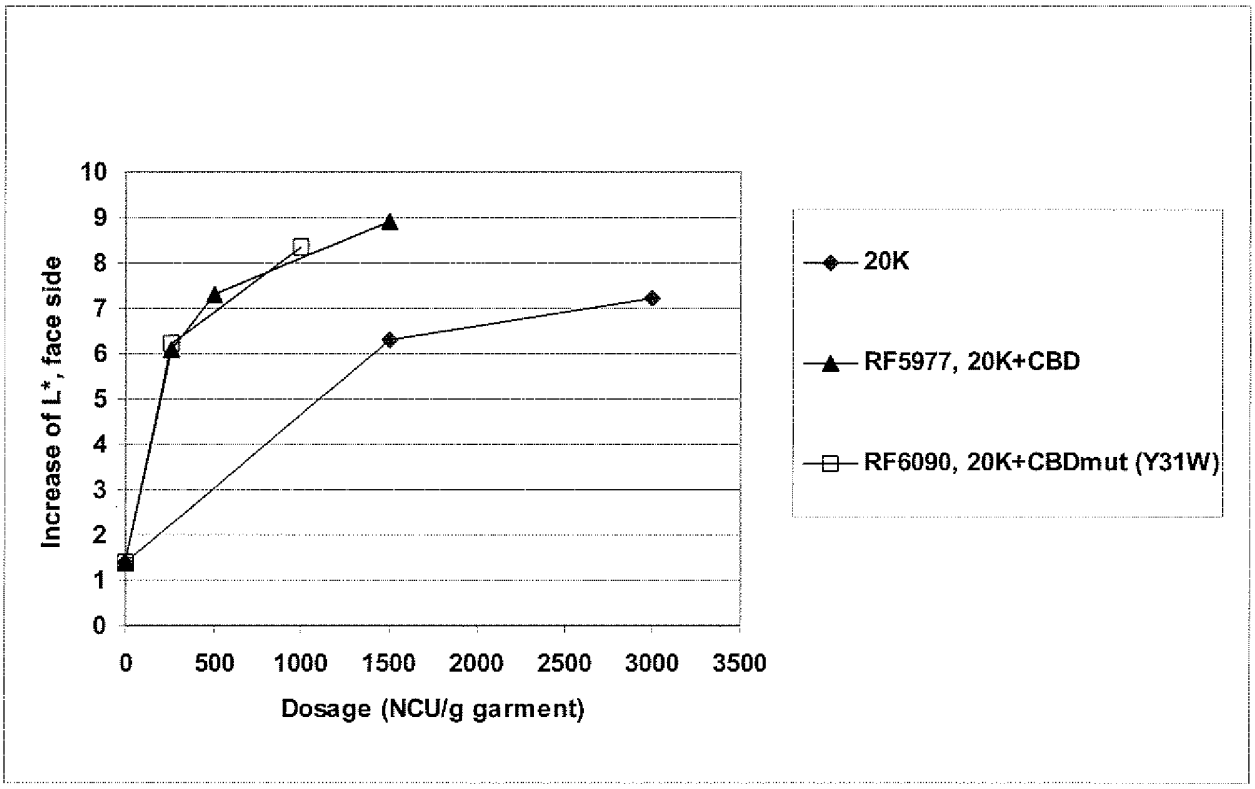
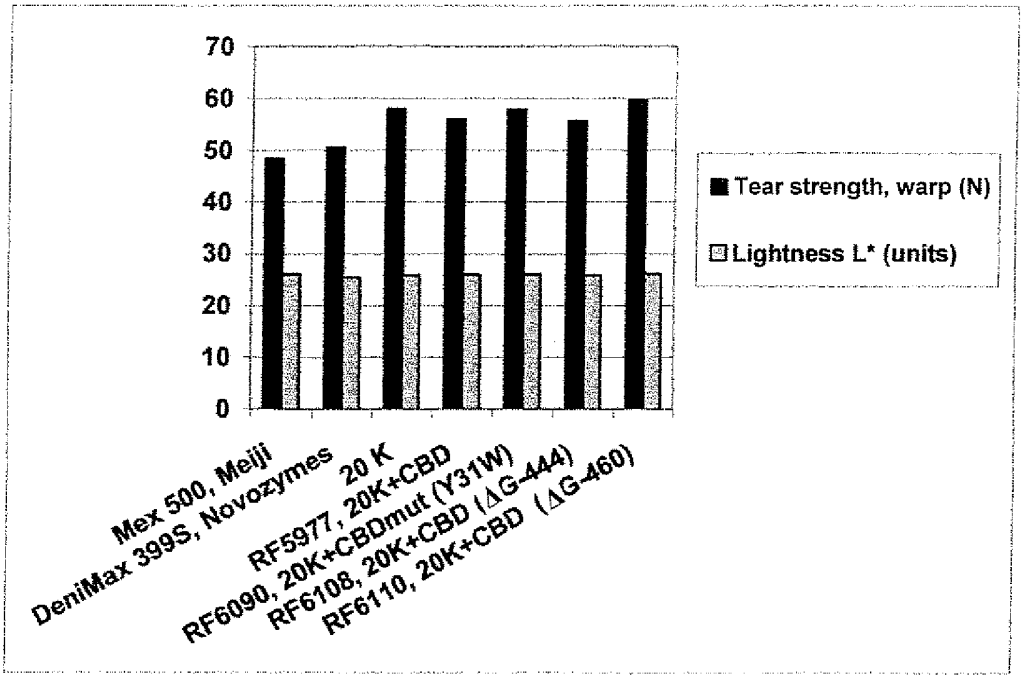

Increase of L*, face side
10
9
8
7
6
5
4
3
2
1
0
0
500
1000
1500
2000
2500
3000
3500
Dosage (NCU/g garment)
20K
RF5977, 20K+CBD
RF6090, 20K+CBDmut (Y31W)

Fig 17

A


70
60
50
40
30
20
10
0
Tear strength, warp (N)
Lightness L* (units)
Mex 500, Meiji
DeniMax 399S, Novozymes
20 K
RF5977, 20K+CBD
RF6090, 20K+CBDmut (Y31W)
RF6108, 20K+CBD (ΔG-444)
RF6110, 20K+CBD (ΔG-460)

B

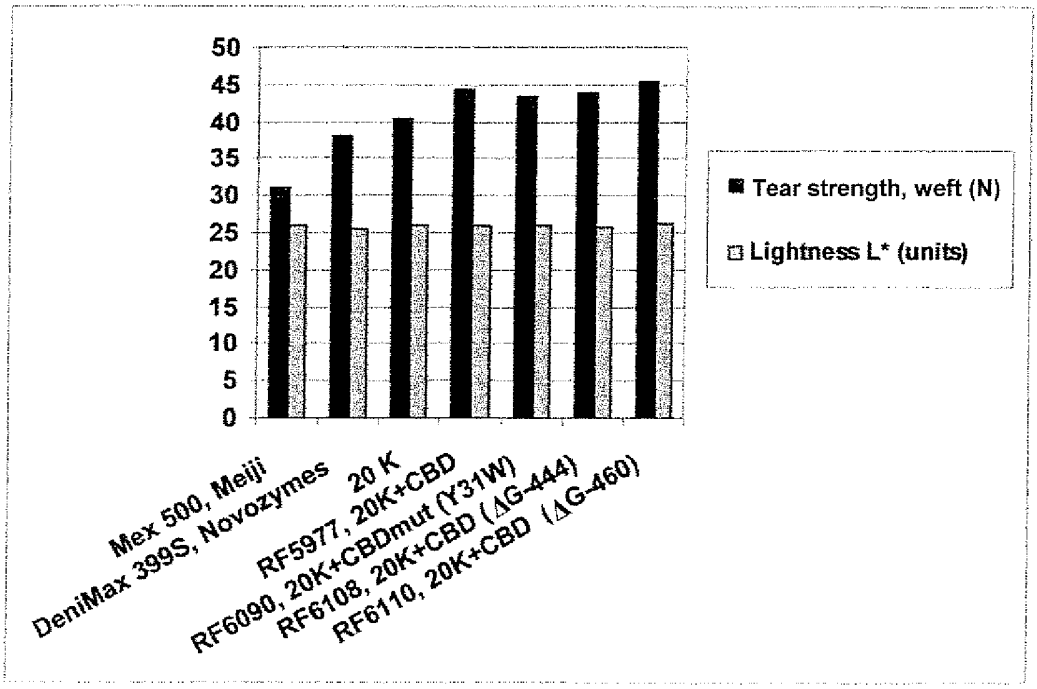
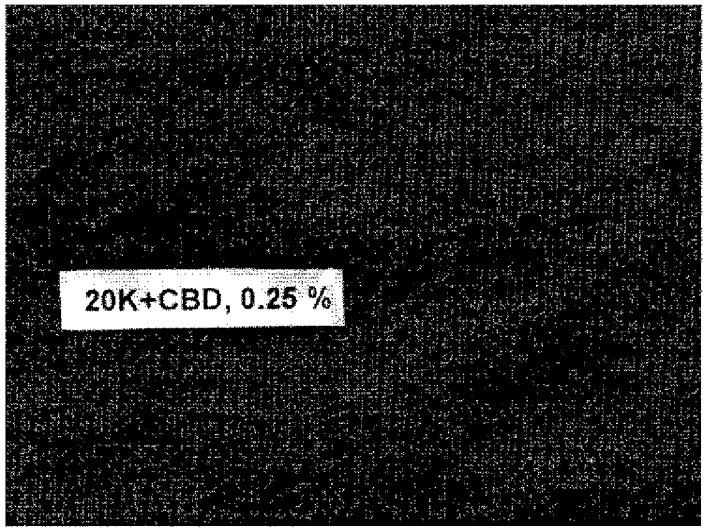

50
45
40
35
30
25
20
15
10
5
0
Tear strength, weft (N)
Lightness L* (units)
Mex 500, Meiji
DeniMax 399S, Novozymes
20 K
RF5977, 20K+CBD
RF6090, 20K+CBDmut (Y31W)
RF6108, 20K+CBD (ΔG-444)
RF6110, 20K+CBD (ΔG-460)

Fig. 18



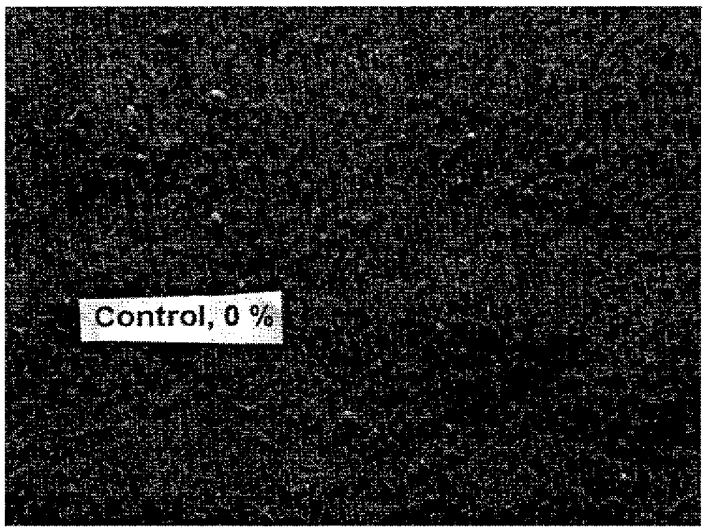


Fig. 19 A

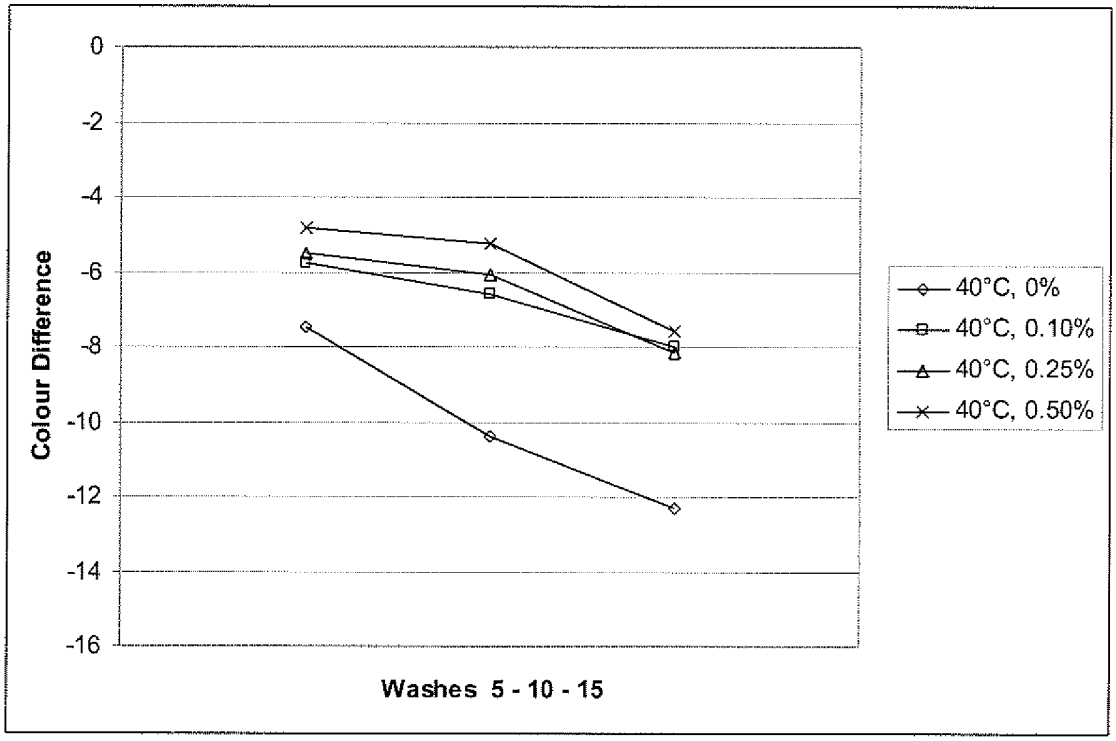


Fig. 19 B

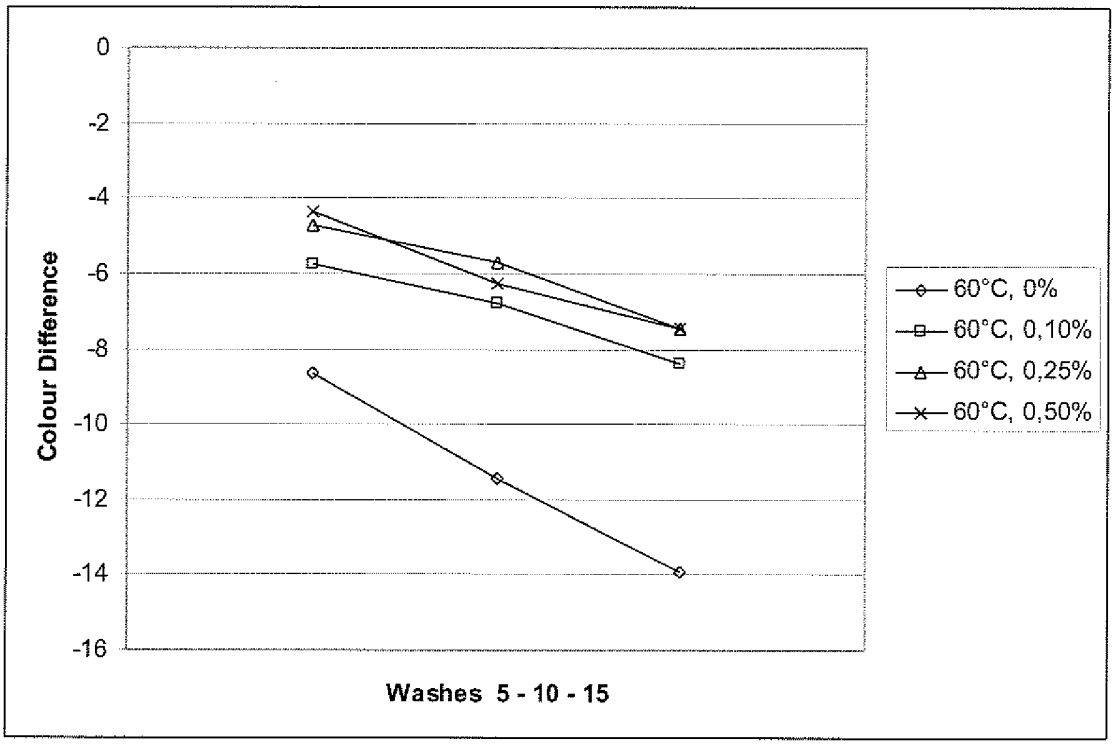


Fig. 20 A

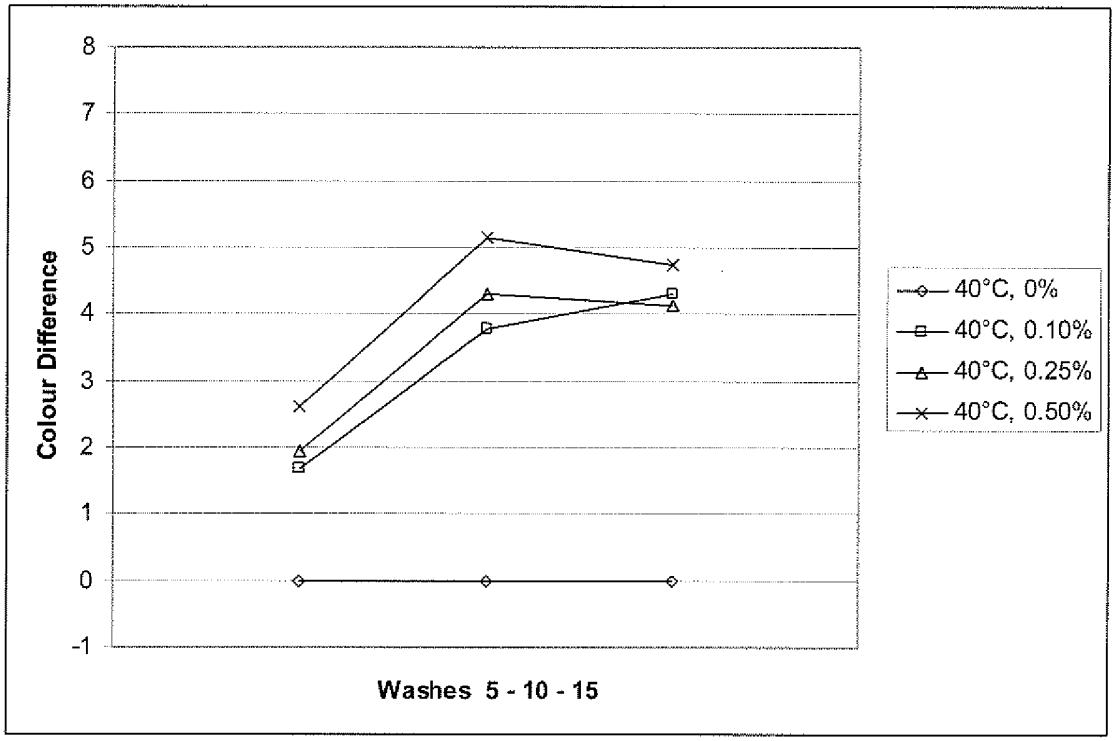
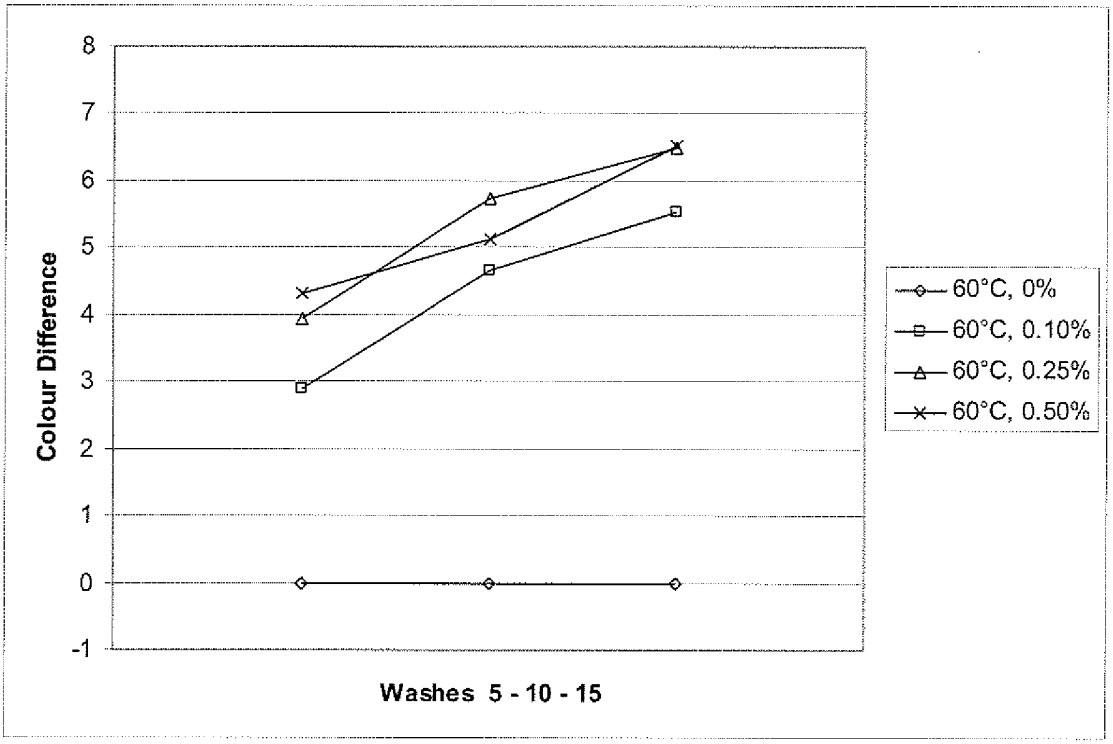


Fig. 20 B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2006/050161

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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)

IPC8: C12N, C11D, D06M, A23K

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

FI, SE, NO, DK

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

EPO-Internal, WPI, PAJ, BIOSIS, EMBASE, CHEM.ABS., SEQUENCE SEARCHES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 9117244 A1 (NOVO NORDISK A/S) 14 November 1991 (14.11.1991)	
X	See Example 6, Figures 10 and 15 A-D	1, 6-55
A		2-5
	WO 9812307 A1 (NOVO NORDISK A/S) 26 March 1998 (26.03.1998)	
X	See chapter #The enzyme (endo-β-1,4-glucanase) variants of the invention# on pages 11-13 and Examples 3 and 4	1, 6-55
A		2-5
	WO 9743409 A2 (NOVO NORDISK A/S) 20 November 1997 (20.11.1997)	
X	See Example 3, SEQ ID NOs. 65-74	1, 6-41, 45-55
A		2-5, 42-44

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2006 (20.06.2006)	28 June 2006 (28.06.2006)

Name and mailing address of the ISA/FI
National Board of Patents and Registration of Finland
P.O. Box 1160, FI-00101 HELSINKI, Finland
Facsimile No. +358 9 6939 5328

Authorized officer
Stiina Kaikkonen
Telephone No. +358 9 6939 500

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2006/050161

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Kim H. et al. Functional Analysis of a hybrid endoglucanase of bacterial origin having a cellulose binding domain from a fungal exoglucanase. Applied Biochemistry and Biotechnology, Nov.-Dec. 1998, Vol. 75, No. 2-3, pages 193-204	
X		1, 6-40, 45-55
A		2-5, 41-44
A	US 6184019 B1 (MIETTINEN-OINONEN ARJA et al.) 06 February 2001 (06.02.2001), See from page 13 line 55 to page 14 line 20, and Example 26	1-55

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2006/050161

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 6-55 (all partly)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Box No. II 2.

The subject matter of independent claims 1 and 28 is unclear (Article 6 PCT) for the following reasons:
The characterizing portion of claim 1 lacks clarity and conciseness. Claim 1 only describes a junction region through the desirable function of it, namely that “a junction region has been introduced..., whereby a stable fusion protein is obtained.” Claim 1 does not give the formula or sequence of the junction region, nor does it state what is meant by “stable”.
Claim 28 lacks referral to any specific claim containing amino acid sequences. The expression "said polynucleotide sequences encoding the respective amino acid sequences as specifically defined above" is not considered sufficient in referring to these sequences.
Based on the above grounds a meaningful search over the whole of the claimed scope is not possible. The search is mainly based on those parts of the claims that are clear, supported, and disclosed, and where the junction region is defined by a formula or a sequence, claims 2-5.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI2006/050161

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
WO 9117244 A1	14/11/1991	FI 20031726 A	25/11/2003
		GR 3023734T T3	30/09/1997
		US 5763254 A	09/06/1998
		US 5686593 A	11/11/1997
		US 5457046 A	10/10/1995
		KR 191564 B1	15/06/1999
		FI 925080 A	09/11/1992
		JP 3228419B2 B2	12/11/2001
		ES 2100229T T3	16/06/1997
		EP 0531315 A1	17/03/1993
		DK 0531315T T3	22/09/1997
		DE 69125371T T2	14/08/1997
		DE 69125371D D1	30/04/1997
		CA 2082259 A1	10/11/1991
		BR 9106434 A	04/05/1993
		AU 7791091 A	27/11/1991
		AU 652153B B2	18/08/1994
		AT 150788T T	15/04/1997
WO 9812307 A1	26/03/1998	DE 69735767D D1	01/06/2006
		AT 324437T T	15/05/2006
		US 2005009166 A1	13/01/2005
		JP 2004065255 A	04/03/2004
		US 2003092097 A1	15/05/2003
		JP 2000514311T T	31/10/2000
		EP 0937138 A1	25/08/1999
		CN 1230987 A	06/10/1999
		CA 2265914 A1	26/03/1998
		BR 9711479 A	24/08/1999
		AU 4200797 A	14/04/1998
WO 9743409 A2	20/11/1997	US 6270968 B1	07/08/2001
		EP 0898618 A2	03/03/1999
		AU 3025597 A	05/12/1997

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI2006/050161

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
US 6184019 B1	06/02/2001	US 2004185498 A1	23/09/2004
		US 2004142444 A1	22/07/2004
		US 2002168751 A1	14/11/2002
		WO 9714804 A1	24/04/1997
		EP 0857216 A1	12/08/1998
		CN 1204368 A	06/01/1999
		CA 2232245 A1	24/04/1997
		BR 9611114 A	13/07/1999
		AU 7299796 A	07/05/1997

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2006/050161

CLASSIFICATION OF SUBJECT MATTER

Int.Cl.
C12N 9/42 (2006.01)
C12N 15/62 (2006.01)
C12N 15/63 (2006.01)
C12N 15/80 (2006.01)
C11D 3/386 (2006.01)
D06M 16/00 (2006.01)
A23K 1/165 (2006.01)