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(54) Title: METHOD AND DNA CONSTRUCTS FOR INCREASING THE PRODUCTION LEVEL OF CARBOHYDRATE DEGRADING ENZYMES IN FILAMENTOUS FUNGI

(57) Abstract: The invention is related to a method and DNA constructs for obtaining in a filamentous fungus host a higher production level of a carbohydrate degrading (CD) enzyme, having in its original state a catalytic module (CAT) and a carbohydrate binding module (CBM) separated by a linker region. The DNA construct comprising a truncated actinomycetes, preferably *Nonomuraea flexuosa* (Nf) derived DNA sequence encoding a truncated form of the CD enzyme, for example Nf Xyn1 1A, Nf Xyn10A, and is introduced into a filamentous fungal host. Said CD enzyme contains the catalytically active region of CAT but lacks part or all of the CBM, or all of the CBM and part or all of the linker region and is expressed and secreted under the control of regulatory sequences comprising at least a signal sequence, but also promoters, terminators and DNA sequences encoding a secretable carrier protein or domains thereof, preferably originating from filamentous fungi are included. The production level obtained with DNA sequence having the shortened DNA sequence encoding the truncated form of the CD enzyme is higher than the production level obtained with DNA construct encoding the corresponding full length CD enzyme.



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Method and DNA Constructs for Increasing the Production Level of Carbohydrate Degrading Enzymes in Filamentous Fungi

Technical Field of the Invention

5 The present invention is related to molecular biology and particularly to methods and DNA constructs for increasing the production level in a filamentous fungal host when producing carbohydrate degrading (CD) enzymes, which in their native, unmodified state have a catalytic module (CAT) and a carbohydrate binding module (CBM) separated by a linker region. Carbohydrate degrading enzymes with the structure defined above are found among
10 filamentous fungi and bacteria, such as strains of actinomycete, including *Nonomuraea flexuosa* Xyn11A or Xyn10A. For high yield production, a shortened DNA sequence, which encodes a truncated form of the desired carbohydrate degrading enzymes, is used.

Background of the Invention

15 Plant cell walls consist mainly of a complex mixture of polysaccharides, primarily cellulose, lignin and hemicellulose. In most plant material, xylan is the major hemicellulose component, consisting of a main chain of 1,4-linked beta-D-xylopyranosyl residues that often carry acetyl, arabinosyl and glucuronosyl substituents. Carbohydrate degrading enzymes are useful as feed additives, because of their beneficial effects on the adsorption of feed components and in
20 prebleaching of kraft pulp, wherein they are used as simple and cost-effective alternatives to toxic chlorine-containing chemicals. The main enzyme needed to enhance the delignification of kraft pulp is endo- β -1,4-xylanase (EC 3.2.1.8), but the presence of other enzymes such as mannanase, lipase, and α -galactosidase have been shown to improve the effect of enzymatic treatment. In enzyme-aided bleaching, pretreatment with xylanase removes xylan while
25 preserving the cellulose content. Thereby, the need of bleaching chemicals is decreased and/or the brightness of the paper is increased. In feed applications, the beneficial effects obtained after enzyme addition, including increased growth rate and feed efficiency, result from the reduction of intestinal viscosity and release of nutrients from grain endosperm and aleurone layers.

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The use of enzymatic treatments in both feed and pulp and paper industry has dramatically increased. As a corollary, the demand of carbohydrate degrading enzymes has also increased. This puts a pressure on the development of new efficient and cost-effective methods for the production of sufficient amounts of carbohydrate degrading enzymes having properties suitable

for use in said industries. Xylanases that are active and stable at high temperature and alkaline pH are especially desirable in many industrial processes, due to the high temperatures and the alkaline conditions used in bleaching as well as the high temperatures used in downstream processing, e.g. pelleting.

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Actinomycetes strains are known to produce thermostable enzymes with alkaline optima. Especially, thermophilic actinomycetes strains are a useful source of xylanases for industrial processes. Their activities and stability at high temperature are adequate for bleaching processes and other applications in which it is beneficial to perform the enzyme treatment at high temperatures. Useful genes have been cloned from e.g. *Thermomonospora fusca*,
10 *Nonomuraea flexuosa* DSM43186, previously named as *Actinomadura flexuosa* or *Microtetraspora flexuosa* as well as from some *Streptomyces* species. The cloning of two *Nonomuraea flexuosa* xylanases has been described in US 5,935,836, US 6,300,114, US 6,506,593 and US 6,667,170.

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The desired high temperature resistant carbohydrate degrading enzymes with extreme pH optima originate from relatively unstudied bacteria. Typically, they have low production levels and are unsuitable for industrial production in large scale. Little or practically no experience exists about fermentation of said bacteria. Accordingly, transfer of a gene originating from said
20 microbes, and encoding the desired enzyme, into a heterologous host organism is a feasible alternative for producing the desired enzyme.

Bacterial enzymes have been produced in bacterial hosts and yeasts, as disclosed, for example, in US 5,306,633 and US 5,712,142. US 5,712,142 describes a method for increasing the
25 thermostability of a bacterial cellulase from *Acidothermus cellulyticus* either by proteolytic cleavage or by expressing a shortened or truncated form of the gene encoding the full size cellulase in the yeast host, *Pichia pastoris*. WO 0196382 A2 describes a method for increasing the thermostability of *Rhodothermus marinus* cellulase by expressing a truncated form of the gene in *Escherichia coli*. Accordingly, several bacterial carbohydrate degrading enzymes have
30 been described and it is known how to improve their thermostability.

Truncation of a multidomain xylanase from the anaerobic bacterium *Neocallimastix patriciarum* was shown to improve the expression in *E. coli*, as disclosed in WO 9325693 A1.

Filamentous fungi, including *Aspergillus*, *Trichoderma*, and *Penicillium*, are known as effective producers of homologous and heterologous proteins. They are by far the most preferred host organisms for large scale production of industrial enzymes, including bulk production of amylases, glucoamylases, cellulases, xylanases, etc. The first attempts to produce bacterial enzymes in filamentous fungi were discouraging. The yields of the bacterial enzymes were low, not exceeding a few tens of milligrams per liter. In many of the reports, the enzymes were detected only intracellularly (Jeenes, *et al.*, Biotechnol. Genet. Eng. Rev., **9**, 327-367, 1991; van den Hondel, *et al.*, In J.W. Bennet and L.L. Lasure (ed.) More genetic manipulations in fungi: Academic Press, San Diego, Calif.).

Genetic fusion strategies have been developed and used in order to improve yields of heterologous proteins in filamentous fungi as disclosed in US 5,364,770 and WO 94/21785 and reviewed by Gouka *et al.*, Appl. Microbiol. Biotechnol., **47**, 1-11, 1997. Production of bacterial carbohydrate degrading enzymes from filamentous fungi, using gene fusions comprising a DNA sequence encoding a complete or a partial filamentous fungus secretable protein (polypeptide) as a carrier protein has been described in WO 97/27306 and US 2003/0148453. Using expression cassettes disclosed in said patent applications and comprising DNA sequences encoding a bacterial carbohydrate degrading enzyme fused in frame with a complete or partial filamentous fungus secretable protein, Paloheimo, *et al.*, (Appl. Environ. Microbiol., **69**, 7073-7082, 2003) have demonstrated that the production levels are remarkably increased when the gene encoding the bacterial enzyme is fused in frame with a filamentous fungal secretable polypeptide having an intact domain structure.

The objective of the present invention is to further improve the production levels of carbohydrate degrading enzymes, particularly, bacterial enzymes produced by recombinant DNA techniques from filamentous fungal hosts.

The Summary of the Invention

The invention is related to a method and DNA constructs for improving the production levels of carbohydrate degrading enzymes using recombinant DNA techniques and filamentous fungi as hosts.

The carbohydrate degrading enzymes (CD) of the present invention are active on carbohydrate substrates and are such enzymes, wherein the complete enzyme in its original (native or unmodified) state has three characteristic domains or regions, which are a catalytic module (CAT) containing the active site and a carbohydrate binding module (CBM), which modules are separated by a linker region. Some carbohydrate binding modules, such as the carbohydrate binding module of AM50 (Nf Xyn10A) may have more than one sub-domain.

The method for obtaining the increased production level comprises a first step, wherein a DNA construct is designed. The DNA construct, which is introduced into a filamentous fungus host comprises regulatory sequences and a shortened DNA sequence encoding a truncated form of the desired carbohydrate degrading enzyme, which comprises the catalytically active region of CAT and lacks the CBM module or part of it, or all of the CBM and part or all of the linker region. The regulatory sequences are preferably derived from filamentous fungi and comprise at least one signal sequence.

The DNA sequence encoding the truncated form of the carbohydrate degrading enzyme originates from a bacterial strain, which may be an actinomycetes strain, as exemplified by *Nonomuraea*. Xylanases from *Nonomuraea flexuosa*, particularly, the thermostable xylanases Nf Xyn11A and Nf Xyn10A, are examples of carbohydrate degrading enzymes, the yields of which may be increased by applying recombinant DNA techniques and shortened DNA sequences encoding the truncated target carbohydrate degrading enzyme, which lacks part or all of the CBM, or all of the CBM and part or all of the linker region.

Such DNA sequences may be natural sequences isolated from bacterial genomes. They may be synthetic sequences prepared by known methods, such as PCR. Synthetic sequences include the codon optimized DNA sequences, in which the nucleotide codons of natural bacterial DNA sequences are modified to resemble the codon usage in the filamentous fungus host. The truncated forms of the carbohydrate degrading enzyme encoded by such codon optimized sequences have the catalytic activity corresponding to the enzyme encoded by a natural DNA sequence.

The truncated form of said carbohydrate degrading enzyme is expressed under the control of regulatory sequences originating from the same or different filamentous fungi. The regulatory sequences comprise at least a signal sequence derived from a filamentous fungus. The yield

can be even more improved when the carbohydrate degrading enzyme described above is expressed under the control of regulatory sequences consisting of promoter sequences, terminator sequences, and particularly, DNA sequences encoding a carrier protein (polypeptide) including a signal sequence. The regulatory sequences may be obtained from the same or
5 different filamentous fungi and combined in arbitrary ways. This means that the origin of the regulatory sequences and their order may vary.

The DNA sequences encoding the carrier protein may either include the whole coding region of the mature secretable carbohydrate degrading protein or a selected region or domain thereof
10 which in its native secretable form consists of the N-terminal catalytic module (CAT) and a C-terminal carbohydrate binding domain (CBD) separated by a linker (hinge) region. The carrier protein may include the catalytic module (CAT), or the catalytic module (CAT) and part or all of the linker (hinge) region, or the catalytic module (CAT) and all of the linker (hinge) region and part or all of the carbohydrate binding domain (CBD). *T. reesei* Man5A core region or
15 Man5A core/hinge region (Stålbrand *et al.*, Appl. Environ. Microbiol., **61**: 1090-1097, 1995) are examples of such carrier proteins.

The secretable carbohydrate degrading protein may include only the catalytic domain (CAT) which can be used as a carrier polypeptide. *T. reesei*, XYNI and XYNII are examples of such
20 proteins (Törrönen *et al.*, Biotechnol., **10**: 1461-1465). Some secretable proteins have their CBD in the N-terminal end of the enzyme. Example of such an enzyme is *T. reesei* Cel6A (CBHII) (Teeri *et al.*, Gene, **51**, 43-52, 1987). A Cel6A CBD consists of an A, an A+B or an A+B+B' regions, wherein A is a carbohydrate binding domain, B is a hinge (linker) region, and B' is a duplicated hinge (linker) region. These regions can be used alone or in different
25 combinations as possible carrier polypeptides.

Signal sequences may be selected from various secretable filamentous fungal polypeptides, especially *Trichoderma* and *Aspergillus* signal sequences, including the Man5A signal sequence (Stålbrand *et al.*, Appl. Environ. Microbiol., **61**, 1090-1097, 1995) and the Cel6A
30 signal sequence (Teeri *et al.*, Gene, **51**, 43-52, 1987).

Promoter sequences may be selected from various filamentous fungal promoters, especially from *Trichoderma* and *Aspergillus* promoters, including a strong *Trichoderma cel7A (cbh1)*

promoter. Terminator sequences are also obtainable from filamentous fungi, especially preferred is a *Trichoderma cel7A* terminator sequence.

When the shortened DNA sequence is a shortened *Nf xyn11A* (*am24* (SEQ ID NO: 3) or *am24** (SEQ ID NO: 5)) expressed and secreted solely under the control of a promoter and signal sequence originating from a filamentous fungi without any carrier protein or domains thereof, the production level of the truncated Nf Xyn11A is at least 2 times higher in shake flasks and often more than 8-10 times higher in a fermentation cultivation than the production level obtained when the corresponding full length *Nf xyn11A* is expressed and secreted under the control of the same promoter and signal sequence without a carrier protein or parts thereof.

When the shortened DNA sequence is a shortened *Nf xyn11A* (*am24* (SEQ ID NO: 3) or *am24** (SEQ ID NO: 5)) expressed and secreted under the control of a promoter, signal sequence and a DNA sequence encoding a carrier protein (polypeptide) or domains thereof, all regulatory sequences originating from filamentous fungi, the production level of the truncated Nf Xyn11A is at least two times higher (in shake flasks) than with a DNA sequence encoding the corresponding full length Nf Xyn11A expressed and secreted under the control of the same promoter, signal and carrier protein sequence or domains thereof. Even higher production levels are obtained in fermentation cultivations. It is to be noted that a further improvement was still achieved when a DNA sequence encoding a truncated bacterial enzyme was used, even if an improved production level was already known to be achieved, when DNA sequences encoding a full length bacterial enzyme, were expressed and secreted under the control of intact domains of filamentous fungal secretable carrier protein (Paloheimo, *et al.*, Appl. Environ. Microbiol., **69**, 7073-7082, 2003).

The feasibility of the present invention is demonstrated using the sequences listed below.

SEQ ID NO:1	<i>Nf xyn11A</i> nucleotide sequence (AJ508952), the coding region is from nt 303 to nt 1337. The GenBank sequence AJ508952 will show any revisions made to the sequence.
SEQ ID NO:2	<i>am35</i> nucleotide sequence, <i>Nf xyn11A</i> coding region for the mature Nf Xyn11A (AM35) protein
SEQ ID NO:3	<i>am24</i> nucleotide sequence, shortened form of <i>am35</i> , includes a STOP codon

	SEQ ID NO:4	<i>am35*</i> nucleotide sequence, like <i>am35</i> but 9 codons are changed in the sequence (Example 10) (the changes do not alter the encoded amino acid sequence)
5	SEQ ID NO:5	<i>am24*</i> nucleotide sequence, like <i>am24</i> but 9 codons are changed in the sequence like in <i>am35*</i> (Example 10)
	SEQ ID NO:6	<i>Nf xyn10A</i> nucleotide sequence (AJ508953), coding region is from nt 194 to nt 1672. The GenBank sequence AJ508953 will show any revisions made to the sequence.
10	SEQ ID NO:7	<i>am50</i> nucleotide sequence, <i>Nf xyn10A</i> coding region for the mature Nf Xyn10A (AM50) protein
	SEQ ID NO:8	The nucleotide sequence encoding the AM50 core and linker regions, includes a STOP codon
	SEQ ID NO:9	The nucleotide sequence encoding the AM50 core region, includes a STOP codon
15	SEQ ID NO:10	Nf Xyn11A amino acid sequence (AJ508952) encoded by the <i>Nf xyn11A</i> gene. The GenBank sequence AJ508952 will show any revisions made to the sequence.
	SEQ ID NO:11	r33.4 kDa = AM35 = amino acid sequence for the full length mature Nf Xyn11A protein encoded by <i>am35</i> and <i>am35*</i> genes
20	SEQ ID NO:12	AM24, amino acid sequence for the truncated form from AM35 encoded by <i>am24</i> and <i>am24*</i> genes
	SEQ ID NO:13	r23.8 kDa, amino acid sequence for the truncated form from AM35
	SEQ ID NO:14	r22.0 kDa, amino acid sequence for the truncated form from AM35
25	SEQ ID NO:15	Nf Xyn10A, the amino acid sequence (AJ508953) encoded by the <i>Nf xyn10A</i> gene. The GenBank sequence AJ508953 will show any revisions made to the sequence.
	SEQ ID NO:16	AM50, amino acid sequence for the full length mature Nf Xyn10A
	SEQ ID NO:17	AM50 core + linker, amino acid sequence for the truncated form from AM50
30	SEQ ID NO:18	AM50 core, amino acid sequence for the truncated form from AM50
	SEQ ID NO:19	AM50 core + linker + α/β domains of the tail, amino acid sequence for the truncated form from AM50
	SEQ ID NO:20	AM50 core + linker + α domain of the tail, amino acid sequence for the truncated form from AM50

SEQ ID NO:21	Amino acid sequence for the synthetic linker sequence coding for a Kex2-like protease cleavage signal Lys-Arg included in all the constructs to ensure cleavage of the fusion protein
5 SEQ ID NO:22	An additional amino acid sequence preceding the Kex2 site in the expression cassette pALK1264, pALK1131 and pALK1134
SEQ ID NO:23	The N-terminal amino acid sequence of mature Nf Xyn11A and recombinant Xyn11A polypeptides, named as r33.4 kDa, r23.8 kDa and r22.0 kDa
10 SEQ ID NO:24	A nucleotide sequence for the <i>Nru</i> I recognition site introduced into a Kex2 linker
SEQ ID NO:25	A nucleotide sequence for the Kex2 linker which facilitates the construction of fusions

Short Description of the Drawings

15 **Figure 1** shows the nucleotide sequence of the *Nonomuraea flexuosa xyn11A* gene and the deduced amino acid sequence. The stop codon is shown by an asterisk below the sequence. The mature N-terminal amino acid sequence, determined from the purified Xyn11A protein, is underlined. The active site glutamic acids are marked with an open box. The location of the linker and carbohydrate-binding module is indicated by a dotted line below the amino acid
20 sequence. The cleavage sites of the r23.8 kDa and r22.0 kDa polypeptides are marked by a triangle. The sites for putative N-glycosylation in eukaryotic hosts are bolded.

Figure 2A shows the nucleotide sequence of the *N. flexuosa xyn10A* gene and the deduced amino acid sequence. The stop codon is shown by an asterisk below the sequence. The tryptic
25 peptide sequences, obtained from the purified Xyn10A protein, are shown by underlining below the amino acid sequence. The active site glutamic acids are marked with an open box. The putative location of the linker and carbohydrate-binding module, consisting of the α , β and γ subdomains (Fujimoto, *et al.*, J. Mol. Biol., **300**, 575-585, 2000) is indicated by a dotted line below the amino acid sequence. The QxW repeats present in the "ricin superfamily" CBMs
30 (Fujimoto, *et al.*, J. Mol. Biol., **300**, 575-585, 2000; Hirabayashi, *et al.*, J. Biol. Chem., **273**, 14450-14460, 1998) are bolded. The nucleotide sequence continues in **Figure 2B**.

Figure 2B shows the continuation of the nucleotide sequence depicted in **Figure 2A**.

Figure 3 shows the structure of the expression cassette pALK945, constructed to produce the recombinant mature *N. flexuosa* Xyn11A protein using *T. reesei* Man5A core/hinge as a carrier polypeptide. The gene fusion was expressed from *cel7A* promoter and termination of transcription was ensured by using *cel7A* terminator sequence. The *man5A* sequence included
5 encoded the amino acids M₁ - G₄₀₆ (Stålbrand, *et al.*, J. Biotechnol., **29**, 229-242, 1993). The *amdS* gene (Kelly and Hynes, EMBO J., 4: 475-479) was included as a transformation marker and the *cel7A* 3'-flanking region was included, together with the *cel7A* promoter, to target the expression cassette into the *cel7A* locus by homologous recombination. Synthetic linker sequence coding for an additional Arg was included to ensure cleavage of the fusion protein.
10 The amino acids coded by the *man5A* sequence are in regular font, those of *xyn11A* in italics and the synthetic amino acid for proteolytic cleavage is in bold.

Figure 4A shows a sample of *T. reesei* cultivation medium from which the recombinant xylanase polypeptides (Example 7) were purified run on SDS-polyacrylamide gel after
15 Coomassie Blue staining (lane 2) and after Western blot analysis (lane 3). The location of the r33.4 kDa and r23.8 kDa polypeptides and the fusion protein are shown by arrows. The r22.0 kDa form was not visible in the gel/Western blot shown, due to its low amount in the culture supernatant. Sizes of the molecular weight markers (on lane 1) were, from the top to the bottom: 104, 80, 46.9, 33.5, 28.3, 19.8 kDa. A polyclonal antibody made against the purified
20 Nf Xyn11A was used for detection of the heterologous xylanase forms from the Western blot filter.

Figure 4B shows the samples of purified native Nf Xyn11A (lane 2), and the recombinant xylanase forms r33.4 kDa polypeptide (lane 3), r23.8 kDa polypeptide (lane 4) and r22.0 kDa
25 polypeptide (lane 5) run on SDS-polyacrylamide gel and stained with Coomassie Blue. The molecular weight markers (lane 1) are the same as in **Figure 4A**.

Figure 5A shows temperature profiles of the purified Xyn11A xylanases at pH 5. Effect of temperature was determined by incubation of the reaction mixture at pH 5 and different
30 temperatures for 60 min. The relative (%) activity is expressed as percentage of maximum activity at the optimum temperature. The relative activities at pH 6 (not shown) were similar to those obtained at pH 5.

Figure 5B shows temperature profiles of the purified Xyn11A xylanases at pH 7. Effect of temperature was determined by incubation of the reaction mixture at pH 7 and different temperatures for 60 min. The relative (%) activity is expressed as percentage of maximum activity at the optimum temperature.

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Figure 5C shows temperature profiles of the purified Xyn11A xylanases at pH 8. Effect of temperature was determined by incubation of the reaction mixture at pH 8 and different temperatures for 60 min. The relative (%) activity is expressed as percentage of maximum activity at the optimum temperature.

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Figure 6A shows residual activity of the purified enzymes after incubation at 80°C at pH 5. Thermostability was determined by incubating the Xyn11A polypeptides at pH 5 for a period of 0, 15, 30, 45, 60, 75, 90, 150 and 245 min in the absence of substrate, after which the residual activities were measured at pH 7 and 70°C for 5 min.

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Figure 6B shows residual activity of the purified enzymes after incubation at 80°C at pH 7. Thermostability was determined by incubating the Xyn11A polypeptides at pH 7 for a period of 0, 15, 30, 45, 60, 75, 90, 150 and 245 min in the absence of substrate, after which the residual activities were measured at pH 7 and 70°C for 5 min.

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Figure 7 shows the overall structure of the expression cassettes constructed to study the effect of the truncation of the *am35* gene on xylanase production levels in *Trichoderma reesei*. The gene fusions were expressed from *cel7A* promoter and termination of transcription was ensured by using *cel7A* terminator sequence. The carrier polypeptides used were encoded either by the *man5A* core/hinge sequence (M₁-G₄₁₀ in pALK1022, pALK1692, pALK1309 and pALK1131), the *cel6A* CBD sequence (M₁-S₈₆) in pALK1285 and pALK1502 or the *man5A* sequence encoding for a fragment of the Man5A core (M₁-V₂₂₇ in pALK1264, pALK1151 and pALK1154). The Cel6A CBD block A codes for the tail of the protein and B for the hinge region. In the plasmids pALK1118 and pALK1276 the xylanase gene was fused to the *man5A* signal sequence. A synthetic linker sequence coding for a Kex2-like protease cleavage signal Lys-Arg (included as RDKR (SEQ ID NO:18)) was included in all the constructs to ensure cleavage of the fusion protein. The expression cassette pALK1264, pALK1131 and pALK1134 also included an additional sequence coding for GQCGG (SEQ ID NO:19) preceding the Kex2 site. The amino acids coded by *am35* (SEQ ID NO: 2) and *am35** (SEQ ID NO: 4) are D₄₄—

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N₃₄₄ (the full length mature protein, Fig. 1; SEQ ID NO: 11) and by the truncated *am35/am35** sequences (*am24* (SEQ ID NO: 3) and *am24**; SEQ ID NO: 5) are D₄₄-L₂₆₃ (Fig. 1; SEQ ID NO: 12). In *am35** (SEQ ID NO: 4) and *am24** (SEQ ID NO: 5) nine codons have been changed as described in Example 10. The changes do not alter the encoded amino acid sequences. The *amdS* marker gene and the *cel7A* 3'-flanking region (to target the expression cassette into the *cel7A* locus by homologous recombination) were included in all the constructs after the *cel7A* terminator sequence (identically to the pALK945 expression cassette shown in Fig. 3).

Figure 8 shows a Coomassie Blue stained SDS-polyacrylamide gel, in which a sample of the culture supernatant of a *T. reesei* transformant producing the truncated form of Nf Xyn11A xylanase (AM24) was run. The Cel6A CBD was used as a carrier polypeptide. The purified r23.8 and r22.0 kDa forms were run in parallel on the gel. Lanes: 1. A molecular weight marker (the molecular mass of the relevant marker proteins is shown), 2. and 3. Culture supernatant from a *T. reesei* transformant producing the truncated Xyn11A, 3. Purified r22.0 kDa xylanase, 5. Purified r23.8 kDa xylanase.

Figure 9A shows the expression cassette for producing a truncated form of Nf Xyn10A, the Nf Xyn10A core, in *T. reesei*. The core includes the amino acids A₄₅-N₃₄₅ (Fig. 2; SEQ ID NO: 18). The *cel7A* promoter, *cel6A* signal peptide and *cel7A* terminator sequences are used (as in the construct pALK1285 and pALK1502, Fig. 7) for Nf Xyn10A production. The Cel6 CBD (A+B) is used as a carrier polypeptide (as in the constructs pALK1285 and pALK1502, Fig. 7). A sequence encoding a Kex2 site (RDKR (SEQ ID NO:18)) is included between the carrier and the xylanase sequences. The *amdS* marker and the *cbh1* 3'-fragment are included as in the constructs for producing Nf Xyn11A.

Figure 9B shows the expression cassette for producing a truncated form of Nf Xyn10A, the Nf Xyn10A core/linker in *T. reesei*. The core/linker includes the amino acids A₄₅-S₃₆₇ (Fig. 2; SEQ ID NO: 17). The other sequences (*cel7A* promoter, *cel6A* signal peptide, *cel6A* CBD encoding sequence, *cel7A* terminator, the sequence encoding the Kex2 site, the *amdS* marker and the *cbh1* 3'-fragment) are as in the constructs in Fig. 9A.

Figure 9C shows the expression cassette for producing the full-length Nf Xyn10A in *T. reesei*. The full-length xylanase includes the Nf Xyn10A amino acids A₄₅-A₄₉₂ (the full-length protein

from the N-terminal end, Fig. 2; SEQ ID NO: 16). The other sequences (*cel7A* promoter, *cel6A* signal peptide, *cel6A* CBD encoding sequence, *cel7A* terminator, the sequence encoding the Kex2 site, the *amdS* marker and the *cbh1* 3'-fragment) are as in the constructs in Fig. 9A.

5 List of nucleotide sequence accession numbers

AJ508952 *xyn11A* nucleotide sequence of *N.flexuosa* DSM43186

AJ508953 *xyn10A* nucleotide sequence of *N.flexuosa* DSM43186

Donor organisms

10 *Nonomuraea flexuosa* DSM43186 (ATCC35864)

General Description of the Invention

Abbreviations and Nomenclature

- 15 Nf *Nonomuraea flexuosa*
xyn10A gene for family 10 xylanase
 Xyn10A protein for family 10 xylanase
xyn11A gene for family 11 xylanase
 Xyn11A protein for family 11 xylanase
- 20 CBM, CBD carbohydrate binding module/domain,
 Partial structure of carbohydrate degrading enzyme
 CBM carbohydrate binding module
 herein the term CBM is used for truncated carbohydrate degrading enzymes
 CBD carbohydrate binding domain
 25 herein the term CBD is used for domains of carrier protein
- cel7A (cbh1)* cellobiohydrolase 1 gene in *Trichoderma reesei*
cel7B (egl1) endoglucanase 1 gene of *Trichoderma reesei*
cel6A (cbh2) cellobiohydrolase 2 gene of *Trichoderma reesei*
cel5A (egl2) endoglucanase 2 gene of *Trichoderma reesei*
- 30 *man5A* mannanase gene of *Trichoderma reesei*
Nfxyn11A *N. flexuosa* xylanase gene from the family 11
Nfxyn10A *N. flexuosa* xylanase gene from the family 10
am35 *Nfxyn11A* gene encoding the full-length form of the Nf Xyn11A mature
 protein (amino acids D₄₄ – N₃₄₄)

- am24* shortened form of *Nf xyn11A* encoding the truncated form of the NfXyn11A mature protein (amino acids D₄₄ – L₂₆₃)
- am35** *am35* but including the following changes in codons:
 Gly₅₃ GGG to GGC, Ala₆₆ GCG to GCC, Gly₆₈ GGG to GGC, Arg₈₅ CGG to CGC, Gly₈₈ GGG to GGC, Gly₁₀₀ GGA to GGC, Arg₁₀₁ CGG to CGC, Arg₁₀₂ CGG to CGC and Val₁₀₄ GTG to GTC
- am24** *am24* but including the same changes in codons as *am35**
- am50* *Nf xyn10A* gene encoding the full-length form of the Nf Xyn10A mature protein (amino acids A₄₅ – A₄₉₂)
- 10 AM35 same as r33.4 kDa, see below, encoded by *am35* and *am35**
- AM24 truncated form from AM35, encoded by *am24* and *am24** (amino acids D₄₄ - L₂₆₃)
- r33.4 kDa full-length recombinant Nf Xyn11A mature protein (amino acids D₄₄ – N₃₄₄)
- r23.8 kDa cleavage form of the full-length recombinant Nf Xyn11A mature protein (amino acids D₄₄ – V₂₆₀)
- 15 r22.0 kDa cleavage form of the full-length recombinant Nf Xyn11A mature protein (amino acids D₄₄ – N₂₃₆)
- AM50 full-length recombinant Nf Xyn10A mature protein (amino acids A₄₅ – A₄₉₂), encoded by *am50*.

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Classification of Carbohydrate-Active Enzymes

Information about the nomenclature of carbohydrated degrading enzymes including cellulases, xylanases, mannanases, and others can be found at Coutinho, P.M.& Henrissat, B. (1999) Carbohydrate-Active Enzymes server at URL:

25 <http://afmb.cnrs-mrs.fr/CAZY/>.

General Description of the Invention

Terminology

30 In the present invention most definitions have the same meaning they generally have in the corresponding scientific fields. Some terms are used in somewhat different way. Therefore, they are explained in more detail below.

The term “**carbohydrate degrading (CD) enzyme**” means an enzyme active on carbohydrate substrate and having a catalytic module (CAT) and a carbohydrate binding module (CBM) separated by a linker region. The terms “domain” or “region” are also used for the term “module”. Bacterial carbohydrate degrading enzymes are obtainable from several different bacterial strains, including actinomycete strains of *Nonomuraea*, *Thermomonospora*, particularly *Nonomuraea flexuosa* or *Thermomonospora fusca*. The method seems to be applicable to enzymes obtainable from some other microorganisms, for example, xylanases obtainable from *Chaetomium*, particularly *C. thermophilum*, which is a filamentous fungus. Carbohydrate degrading enzymes include cellulose and hemicellulose degrading enzymes, particularly some specific mannan degrading enzymes. Particularly useful are thermostable xylanases including Nf Xyn10A or Nf Xyn11A. Information about carbohydrate degrading enzymes and their structures are found for example in Gilkes, *et al.*, Eur. J. Biochem., **202**, 367-377, 1991, Teeri, *et al.*, J. Biotechnol., **24**, 169-176, 1992, Stålbrand, *et al.*, Appl. Environ. Microbiol., **61**, 1090-1097, 1995, Tomme, *et al.*, (1995) Enzymatic Degradation of Insoluble Polysaccharides (Saddler & Penner, Eds.) Cellulose-binding domains: classification and properties. pp 142-163, American Chemical Society, Washington). In most carbohydrate degrading enzymes the CBM is situated in the C-terminal end of the molecule, but some enzymes, for example *T. reesei* Cel6 (CBHII) and Cel5A (EGII) have their CBM in the N-terminal end of the enzyme. The invention, it is the provision of higher production levels by removal of the CBM or part of it or the CBM and the linker or part of it of the bacterial carbohydrate degrading enzyme is believed to work even if the CBM is not situated in the C-terminal end of carbohydrate degrading enzyme used to exemplify the invention.

The term “**truncated form of the carbohydrate degrading enzyme**” means an enzyme, particularly a bacterial enzyme, for example, from an actinomycetes strain, which lacks part of the amino acid sequence. Typically, this means that a part or all of the CBM, or alternatively all of the CBM and a part or all linker region is missing from the truncated form. Truncated form of the enzyme contains an intact catalytic domain or at least a region of the catalytic domain which is essential in catalytic activity of the carbohydrate degrading enzyme (CD). The truncated enzyme is expressed and secreted under the control of suitable regulatory sequences, including promoters and signal sequences.

The term “**catalytically active region of CAT**” means that the region carries at least the enzyme active site, which provides the specificity for its particular substrate and contributes the amino

acid residues that directly participate in the making and breaking of chemical bonds. The active site amino acid residues are conserved within different CD families. The active sites of family 10 and family 11 endo -1,4-xylanases, for example, contain two conserved glutamic acids, which are essential for catalytic activity. The catalytically active region of CAT has similar catalytic activity than the corresponding intact CAT or the intact carbohydrate degrading enzyme and can be measured, for example, with enzyme activity assays.

The term **“DNA construct”** means an expression or transformation construct. The DNA construct comprises at least a shortened DNA sequence, which originates from a bacterium and encodes a shortened form of said bacterial carbohydrate degrading enzyme preferably in combination with appropriate regulatory sequences, which include a promoter, a signal sequence with or without a carrier sequence, and a terminator sequence originating from a filamentous fungus. The DNA construct includes at least the DNA sequences, which are essential for competent expression and secretion of the shortened form of the desired carbohydrate degrading enzyme. The DNA constructs can be provided in two different forms, as an expression cassette or an expression plasmid.

In an expression plasmid the DNA construct may further contain plasmid elements and reporter gene sequences for replication and selection in *E. coli*. An expression cassette favourably consists of the DNA sequences, which are essential for the expression and secretion of the bacterial enzymes in filamentous fungi. The expression cassette does not include plasmid elements and reporter sequences. The selection marker can be included in either the expression plasmid/cassette or it can be separately transformed to the filamentous fungal host by using co-transformation method. In other words, plasmid elements and reporter sequences are removed from the expression plasmids in order to obtain the expression cassettes for transformation. However, both forms may include and preferably include sequences, which enable locus targeted transformation in the filamentous fungal host. The DNA construct may thereby be targeted to a selected locus in the genome of the host.

The term **“regulatory sequences”** means DNA sequences controlling the expression and secretion of the carbohydrate degrading enzyme having the structure defined above. The regulatory sequences used in the present invention preferably originate from filamentous fungi, such as *Aspergillus* and *Trichoderma*, particularly *Trichoderma reesei*.

The regulatory sequences comprise at least one signal sequence derived from a filamentous fungus, and also at least one promoter from a filamentous fungus, including the strong *Trichoderma cel7A (cbh1)* promoter. In a preferred embodiment of the invention the shortened DNA sequence encoding the truncated form of the carbohydrate degrading enzyme, is fused in frame with a DNA sequence encoding a carrier protein (polypeptide). The carrier protein is preferably encoded by DNA sequences, which may be obtained from and put together of domains derived from the same or different filamentous fungi and encode one or more, preferably intact domains of a filamentous fungal secretable enzyme. The structure of an intact domain resembles the corresponding region in the full length enzyme due to correct folding of the primary amino acid sequence. Therefore, secretion pathways of carrier polypeptides containing one or more intact domains of the filamentous fungal secretable enzyme are expected to be similar to the native full length enzyme. Applicable, secretable enzymes may comprise a core domain containing the active or catalytic site of the enzyme and a substrate binding domain or carbohydrate binding domain (CBD) linked by a hinge region, but the carrier protein may also be a secretable enzyme naturally consisting solely of the core region, as exemplified by certain filamentous fungal xylanases, e.g. *T. reesei* XYN I and XYN II. An intact CBD or CBD and linker may also be used in order to achieve a high production level. Different domains or regions of filamentous secretable enzymes derived from different filamentous fungi may be put together in varying and arbitrary order. The DNA sequences encoding the regulatory sequences including the carrier protein may be constructed by combining DNA sequences derived from different filamentous fungal sources.

The most preferred combinations include constructs, wherein the carrier protein is a *T. reesei* Man5A core or Man5A core/hinge region, or a *T. reesei* Cel6A CBD (A, A+B or A+B+B'), wherein A means the substrate binding domain or tail; B means the linker (hinge) region; and B' means a duplicated linker(hinge) region. Said DNA sequences encoding the carrier polypeptide are preferably combined with a *cel7A (cbh1)* promoter. It is possible to construct a multitude of other combinations. The invention includes but is not limited to the following domains: *Trichoderma* CBDs from Cel6A (CBHII), and Cel5A (EGII) carrying a CBD domain in their N-terminal end, xylanase *Trichoderma* (XYN I, XYN II) and *Trichoderma* CBDs from Cel7A (CBHI), Cel7B (EGI) Cel61A (EGIV), Cel45 (EGV) and Cel74 (EGVI) carrying a CBD in their C-terminal end. *Aspergillus* proteins/polypeptides encoded by *glaA* (glucoamylase) and *Penicillium* proteins/polypeptides encoded by *xynA* (Alcocer, *et al.*, Appl. Microbiol. Biotechnol., **60**, 726-732, 2003) and the corresponding structures from other organisms.

Some other filamentous fungus secretable proteins having applicable domains are found among the carrier polypeptides described in Gouka, *et al.*, Appl. Microbiol. Biotechnol., 47, 1-11, 1997.

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The most preferred combinations include the Man5A core or core/hinge region as a carrier polypeptide, the Kex-2 linker (SEQ ID NO: 21) with or without the additional sequence (SEQ ID NO: 22) preceeding the Kex-2 linker and the truncated forms of AM35 polypeptide, i.e. AM24 (SEQ ID NO: 12), r23.8 kDa polypeptide (SEQ ID NO: 13) or r22.0 kDa polypeptide (SEQ ID NO: 14) or the Man5A core or core/hinge region as carrier polypeptide, the Kex-2 linker (SEQ ID NO: 21) with or without the additional sequence (SEQ ID NO: 22) preceeding the Kex-2 linker and the truncated forms of AM50 polypeptide (SEQ ID NO: 17; SEQ ID NO: 18; SEQ ID NO: 19; SEQ ID NO: 20).

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15 The most preferred combinations further include the Cel6A CBD (A, A+B or A+B+B') region as a carrier polypeptide, the Kex-2 linker (SEQ ID NO: 21) with or without the additional sequence (SEQ ID NO: 22) preceeding the Kex-2 linker and the truncated forms of AM35 polypeptide, i.e. AM24 (SEQ ID NO: 12), r23.8 kDa polypeptide (SEQ ID NO: 13) and r22.0 kDa polypeptide (SEQ ID NO: 14) or the Cel6A CBD (A, A+B or A+B+B') region as a carrier polypeptide, the Kex-2 linker (SEQ ID NO: 21) with or without the additional sequence (SEQ ID NO: 22) preceeding the Kex-2 linker and the truncated forms of AM50 polypeptide (SEQ ID NO: 17; SEQ ID NO: 18; SEQ ID NO: 19; SEQ ID NO: 20).

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In the present invention the term "**filamentous fungus**" is used in several connections including host organism and donor organism for regulatory sequences. The preferred filamentous fungi, include any transformable filamentous fungi in which expression can be achieved, most preferably they include but are not limited to *Trichoderma*, *Aspergillus*, and *Penicillium* strains. Particularly preferred hosts and donor organisms are *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Trichoderma viridae*, *Trichoderma koningii*, *Aspergillus niger*, *Aspergillus sp.*, *Penicillium sp.*, *Humicola sp.*, including *Humicola insolens*, *Chrysosporium sp.*, *Fusarium sp.*, *Hypocrea sp.* and *Emericella sp.*, etc.

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The term "**an increased production level**" means that the production level of the truncated form of the bacterial carbohydrate degrading enzyme is higher than the production level of the

full length enzyme in a similar construct which is otherwise identical but differs in the length of the DNA sequence encoding the bacterial enzyme. The increase in production level is in this case measured as an increase of activity (Table 4), because the increase in activity in this case is independent of the specific activity, as demonstrated by the results shown in Table 1. It is to be noted that the increase in efficacy or in the production level is measured as xylanase activity from the culture media of isogenic single-copy transformants or host cells containing only one copy of the DNA construct in question. Furthermore, the isogenic single copy is located in the same locus, for example in the *cbh1* locus. Therefore, it is unambiguously excluded that the increase in production level would be caused by introduction of multiple copies of the DNA construct or of differences in the site of integration. The transformants analysed differ only in that the DNA constructs of the present invention comprise a shortened DNA sequence encoding a truncated form of the full length carbohydrate degrading enzyme, whereas the prior art DNA construct comprises a DNA sequence encoding the corresponding full length enzyme. The production levels are accordingly based upon unambiguous comparisons.

The Detailed Description of the Invention

In the present invention it is demonstrated that production levels of carbohydrate degrading (CD) enzymes, particularly bacterial enzymes, produced in a *Trichoderma reesei* host may be increased not only by fusion strategies as described by Paloheimo, *et al.*, Appl. Environ. Microb., **69**, 7073-7082, 2003, but also by using truncated DNA sequences originating from actinomycetes strains including *Nonomuraea flexuosa*. By expressing said shortened DNA sequences yields of the desired truncated carbohydrate degrading enzyme increase. The functions, including specific activities of the truncated enzymes are the same or similar to those obtained with the corresponding full-length enzyme. The preparations obtained with the method and constructs of the present invention are useful in industrial processes requiring high temperatures and pHs.

In the following examples the invention is described in more detail.

Example 1

Methods used in the analysis and characterisation of proteins (polypeptides)

a. Protein and enzyme assays

Protein concentrations in the culture media and purified enzyme samples were assayed after TCA precipitation by the method of Lowry, *et al.* (J. Biol. Chem., **193**, 265-275, 1951) using bovine serum albumin as a standard protein. During enzyme purifications, proteins were monitored at 280 nm. Xylanase activity was assayed by using 1% (w/v) birch xylan (Roth no. 7500, Roth, Karlsruhe, Germany) as a substrate in 50 mM McIlvaine citrate-phosphate buffer according to the method of Bailey, *et al.*, (Enzyme Microb. Technol., **3**, 53-157, 1992). During enzyme purification and determination of the specific activity of the pure proteins the assay was performed at pH 7, 60°C for 5 min, otherwise as stated in the figure legends. For characterization of the purified Xyn11A polypeptides the buffer was supplemented with 0.1% BSA, except for the determination of thermostability which was performed without BSA.

b. Protein electrophoresis

The samples were run on 12% polyacrylamide slab gels containing 0.1% SDS on a Mini Protean II electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, Calif.) and stained with Coomassie Brilliant Blue R250. Detection of the xylanase and xylanase polypeptides on the Western blot filters was carried out with a polyclonal rabbit antibody raised against the purified Nf Xyn11A xylanase prepared at Diabor Ltd. (Oulu, Finland) and the Protoblot AP System (Promega Corp., Madison, Wisc.).

c. Preparation and sequencing of peptides

Purified fractions containing xylanase activity were subjected to tryptic digestion as described in Fagerström and Kalkkinen (Biotechnol. Appl. Biochem., **21**, 223-231, 1995). Sequencing was performed by Edman degradation in a gas-pulsed-liquid-phase sequencer (Kalkkinen and Tilgmann, J. Protein Chem., **7**, 242-243, 1988) and the phenylthiohydantoin amino acids were analyzed on-line by using narrow bore reverse phase HPLC. For N-terminal sequencing, SDS-PAGE gels were blotted on a PVDF filter and the protein spots were directly subjected to Edman degradation as above. The sequencing was performed at the Institute of Biotechnology (Helsinki, Finland). The C-termini of the purified recombinant polypeptides were determined at the Protein Analysis Center at the Karolinska Institutet (Stockholm, Sweden).

d. Estimation of pI and determination of the molecular mass

The pI of the purified xylanases was estimated by chromatofocusing on a 4 ml prepacked Mono P HR 5/20 column (Amersham Biosciences) equilibrated with 75 mM Tris-CH₃COOH (pH 9.5) and eluted with 10% Polybuffer 96 (Amersham Biosciences) in 75 mM Tris-CH₃COOH

(pH 6.3). The pH was measured from the fraction containing xylanase activity. Molecular masses of the purified xylanases were determined by Bruker Biflex Reflector MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

5 **Example 2**

DNA techniques used in constructing plasmids and strains

Standard DNA methods (Sambrook, *et al.*, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989) were used in constructing plasmids, transforming *E. coli* and performing Southern blots. Each enzyme and kit was used according
10 to the instructions from the supplier. The enzymes for DNA modifications were purchased from Roche Diagnostics GmbH (Mannheim, Germany), New England Biolabs (Beverly, Mass.) and Finnzymes (Espoo, Finland). Qiagen columns (Qiagen GmbH, Hilden, Germany) or Magic Miniprep kits (Promega, Madison, Wisc.) were used in the plasmid isolations. The oligonucleotides were either synthesized using ABI 381A DNA synthesizer or ordered from
15 Sigma-Genosys. The sequencing reactions were analysed either by using ABI 373A or ABI Prism™ 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Polymerase chain reactions (PCR) were performed using PTC-100 Programmable Thermal Controller (MJ Research Inc, Watertown, Mass.). DNA fragments for subcloning and transformations were isolated from low melting point agarose gels (BioWhittaker Molecular Applications Inc.,
20 Rockland, Maine) by the freeze-thaw-phenol method (Benson, BioTechniques, 2, 66-68, 1984), by using β -agarase (New England Biolabs) or by using the Qiaex II Gel Extraction Kit (Qiagen GmbH).

The genomic DNAs were isolated as described in (Raeder and Broda, Lett. Appl. Microbiol., 1,
25 17-20, 1985). Digoxigenin labeled (Roche Diagnostics GmbH) expression cassettes were used as probes in the Southern blot hybridizations.

Example 3

Microbial strains used as hosts and in constructing plasmids, growth media and growth 30 conditions

Plasmids were propagated in *Escherichia coli* XL1-Blue or XL10-Gold (Stratagene, La Jolla, Calif.). The vector backbones used in the plasmid constructions were pUC18 (EMBL Database Accession No L09136), pUC19 (L09137), pBluescript SK- and pBluescript II KS + or KS-

(Stratagene). All *E. coli* cultivations were performed over night at 37 °C in Luria-Bertani medium into which ampicillin had been added (50 µg/ml).

The *Trichoderma reesei* strains ALKO3620 and ALKO4468 were used as parents for the transformations. *T. reesei* ALKO3620 is an endoglucanase II-negative strain. It was constructed from the low protease mutant strain ALKO2221, derived from the strain VTT-D-79125 (Bailey and Nevalainen, Enzyme Microb. Technol., **3**, 153-157, 1981) by UV-mutagenesis (Mäntylä, *et al.*, Abstr. 2nd Eur. Conf. Fungal Genetics, abstr. B52, 1994) as follows. The endoglucanase 2 gene (*cel5A* or *egl2*, originally named as *egl3*; (Saloheimo, *et al.*, Gene **63**, 11-21, 1988)) was replaced by the phleomycin resistance-encoding marker gene from *Streptoalloteichus hindustanus*, Sh *ble* (Drocourt, *et al.*, Nucleic Acids Res., **18**, 4009, 1990). The 3.3 kb *Bgl*III-*Xba*I fragment from the plasmid pAN8-1 (Matheucci, *et al.*, Gene, **8**, 103-106, 1995), containing the *ble* gene flanked by the *Aspergillus nidulans* *gpd* promoter and *trpC* terminator was used. The *cel5A* flanking sequences in the replacement cassette (the 5'-region being the 1.4 kb *Xho*I – *Sac*I fragment about 2.2 kb upstream from the *cel5A* gene and the 3'-region the 1.6 kb *Avr*II – *Sma*I fragment about 0.2 kb from the end of the *cel5A* gene) were isolated from the *egl3* λ clone (Saloheimo, *et al.*, Gene, **63**, 11-21, 1988). The strategy for the replacement was as described in (Suominen, *et al.*, Mol. Gen. Genet., **241**, 523-530, 1993). *T. reesei* ALKO4468 is an endoglucanase I and II-negative strain. It was constructed from the strain ALKO3620 by further replacing the endoglucanase 1 gene, *cel7B* (*egl1*, Penttilä, *et al.*, Gene, **45**, 253-263, 1986), by the *E. coli* hygromycin B phosphotransferase gene, *hph* (Gritz, *et al.*, Gene, **45**, 179-188, 1983), conferring resistance to hygromycin B. The 1.7 kb *Not*I-*Nsi*I fragment from the plasmid pRLM_{EX}30 (Mach, *et al.*, Curr. Genet., **25**, 567-570, 1994) was used in which of the *hph* gene is expressed from the *T. reesei* pyruvate kinase (*pki*) promoter and the transcription is terminated using the *cel6A* terminator sequences. The plasmid pRLM_{EX}30 was kindly provided by Prof. Christian P. Kubicek (Institut für Biochemische Technologie, TU Wien, Austria). The *cel7B* flanking regions were as described in (Suominen, *et al.*, Mol. Gen. Genet., **241**, 523-530, 1993). The single-copy replacements of the *cel5A* and *cel7B* genes by the marker genes in *T. reesei* ALKO3620 and ALKO4468 were verified by Southern blot analysis, as described in (Suominen, *et al.*, Mol. Gen. Genet., **241**, 523-530, 1993).

T. reesei strains were sporulated on PD agar slants (Potato Dextrose Broth, Difco, Detroit, Mis.). The transformants were selected on *Trichoderma* minimal medium containing acetamide as a nitrogen source (Penttilä, *et al.*, Gene, **61**, 155-164, 1987). The fungal mycelia for DNA

isolations were obtained after growing the strains for two days on *Trichoderma* minimal medium containing 2% proteose peptone (Difco). Complex lactose-based cellulase-inducing media (Joutsjoki, *et al.*, Curr. Genet., **24**, 223-228, 1993) were used for enzyme production in shake flasks and fermentations. The transformants were screened using 50 ml cultivations and the mycelium for the RNA isolations was collected from 200 ml cultivations. The shake flask cultivations were grown for 7 days at 30°C, 250 rpm. The laboratory scale fermentor cultivations were performed for 5 days in 1 l Braun Biostat M fermentors (B. Braun).

Example 4

10 Cultivation of *Nonomuraea flexuosa* for enzyme production

Nonomuraea flexuosa DSM43186 (ATCC35864) was cultivated on rolled oats mineral medium plates (medium no. 84, Deutsche Sammlung von Microorganismen und Cellkulturen GmbH Catalogue of Strains, 1983) at 50°C. A sporulating colony was inoculated in XPYB medium, the GPYB medium (Greiner-Mei, *et al.*, Syst. Appl. Microbiol., **9**, 97-109, 1987) supplemented with 0.5% oat spelt xylan (Sigma X-0627, Sigma-Aldrich Corp., St. Louis, Mo.) instead of glucose as described in Holtz *et al.* (Antonie Leeuwenhoek, **59**, 1-7, 1991) and was incubated in shake flask for 2 or 3 days (250 rpm, 50-55°C) after which the shake flask culture was used as a seed culture for the fermentation. The laboratory scale fermentor cultivations were performed for 3 days at 50°C in 1 liter Braun Biostat M fermentors (B. Braun, Melsungen AG, Melsungen, Germany) in the medium described above.

Example 5

Heterologous production of the thermostable *Nonomuraea flexuosa* AM35 xylanase in *Trichoderma*

25 The gene coding for the Nf Xyn11A (AM35) xylanase (*am35* or *Nf xyn11A*; EMBL accession no AJ508952) was isolated from a lambda ZAP Express® library prepared from partially digested (*Sau*3A) and size-fractionated *Actinomadura* (*Nonomuraea*) *flexuosa* DSM43186 (ATCC35864) chromosomal DNA as described in US 6,300,113. The nucleotide sequence of the gene and the deduced amino acid sequence are shown in Fig. 1.

30 The *T. reesei* transformant strain ALKO4396 producing recombinant Nf Xyn11A was constructed by transforming the expression cassette pALK945 (Fig. 3) to *T. reesei* ALKO3620. The *am35* gene is expressed from the *cbh1* promoter, as a fusion to a carrier polypeptide encoded by *man5A* core/hinge sequence. The construction of the plasmid pALK945 and the

strain were performed as described in Paloheimo, *et al.*, Appl. Environ. Microbiol., **69**, 7073-7082, 2003).

T. reesei transformant strain ALKO4396 was sporulated on Potato Dextrose Broth (PD) agar slants (Difco, Detroit, Mis.) at 30°C. For enzyme production in laboratory scale fermentor, the complex lactose based cellulose inducing medium (Joutsjoki *et al.*, Curr. Genet., **24**, 223-228, 1993) was used. Fermentations were performed for 5 days in 1 l Braun Biostat M fermentors.

In addition to the full-length protein, the recombinant *T. reesei* ALKO4396 culture medium was observed, in Western blot, to contain shorter forms of Xyn11A and low amounts of unprocessed Man5A-Xyn11A fusion protein (Fig. 4A).

Example 6

Purification of *N. flexuosa* Xyn11A and the recombinant Xyn11A polypeptides.

Purification of the native Nf Xyn11A xylanase from the culture media of *N. flexuosa* DSM43186 and the recombinant Xyn11A xylanases from *T. reesei* ALKO4396 was performed by combining ion exchange chromatography, hydrophobic interaction chromatography (HIC) and gel filtration in the following way. The growth medium of 1 l fermentation was centrifuged at 8,000 x g for 30 min at 4°C. The supernatant was adjusted to pH 9.1 with 1 M NaOH and diluted with distilled water (until conductivity 4 mS/cm). This sample was applied to a DEAE Sepharose Fast Flow (Amersham Biosciences AB, Uppsala, Sweden) ion -exchanger (SR column, 5x15,5 cm diameter, 300 ml) equilibrated with 20 mM Na₂HPO₄ (pH 9.1) using a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences) at 4°C. Flow rate was 20 ml/min. Column was washed with 400 ml of 20 mM Na₂HPO₄ (pH 9.1).

Flow-through proteins were collected into 100 ml fractions. Elution of the bound proteins from the DEAE-column was accomplished by a linear gradient from 20 mM Na₂HPO₄ (pH 9.1) to 20 mM Na₂HPO₄ (pH 9.1) containing 0.5 M NaCl at 20 ml/min for 30 min, 5 ml fractions were collected. Finally column was washed with 300 ml of 20 mM Na₂HPO₄ (pH 9.1) containing 0.5 M NaCl. Fractions were analysed for xylanase activity and purity of proteins was assessed by SDS-PAGE and Western blots.

The flow-through fractions containing xylanase activity were pooled (up to 500-600 ml) and adjusted to contain 2 M NaCl and applied to a Phenyl Sepharose 6 Fast Flow (Amersham

Biosciences) column (XR50/30 column, 5x11 cm, 215 ml) equilibrated with 40 mM Na₂HPO₄ (pH 9.1) containing 2 M NaCl. After washing with 400 ml of 40 mM Na₂HPO₄ (pH 9.1) containing 2 M NaCl elution was performed at 20 ml/min with a two-step gradient. First the proteins were separated using linear gradient (400 ml) of decreasing NaCl concentration (2-0 M) in 40 mM Na₂HPO₄ (pH 9.1). After washing with 200 ml of 40 mM Na₂HPO₄ (pH 9.1) the proteins were eluted with an increasing gradient (600 ml) of 0-60% ethylene glycol in 40 mM Na₂HPO₄ (pH 9.1). Finally the column was washed with 200 ml of 60% ethylene glycol in 40 mM Na₂HPO₄ (pH 9.1). 10 ml fractions were collected and analysed for xylanase activity and purity.

Pooled HIC-fractions containing xylanases of different molecular masses were concentrated on an Amicon ultrafiltration unit (Millipore Corp., Billerica, Mass.) with Diaflo® (PM10 62mm 10 PK) membrane, cut-off 10 kDa. Concentrated sample (10 ml) was subjected to gel exclusion chromatography on a HiLoad 26/60 Superdex 75 prep grade column (2,6x61 cm, 320 ml) equilibrated with 40 mM Na₂HPO₄ (pH 9.1) containing 0.1 M NaCl. Elution was performed with 400 ml of 40 mM Na₂HPO₄ (pH 9.1) containing 0.1 M NaCl. 6 ml fractions were analysed for xylanase activity purity.

With *N. flexuosa* culture medium roughly half of the xylanase activity loaded on the DEAE Sepharose FF column was found in the flow-through and half was bound to the DEAE column. With *T. reesei* culture medium the xylanase activity was in the flow-through. Most of the native *T. reesei* proteins, e.g. cellulases and the mannanase core/hinge region used as a fusion partner were bound to the DEAE column.

The flow-through fractions containing xylanase activity were pooled for the HIC run. After the Phenyl Sepharose 6 FF column three different forms of the recombinant Xyn11A xylanase were separated (Fig. 4B). The 37 kDa form (on SDS-PAGE, lane 3) corresponds to the native Nf Xyn11A purified from the *N. flexuosa* culture medium. The shorter forms had molecular masses of 30 kDa (lane 4) and 27 kDa (lane 5) on SDS-PAGE. These shorter forms eluted from the Phenyl Sepharose 6 FF column with 0 M NaCl, first the 30 kDa form and the 27 kDa form in subsequent fractions. The 37 kDa polypeptide eluted at 15-30% ethylene glycol concentration. The 30 kDa xylanase was further purified using Superdex 75 gel filtration.

Example 7**Characterization of the native Xyn11A and the recombinant Xyn11A polypeptides**

Characteristics of the native Nf Xyn11A and the three recombinant Xyn11A polypeptides are presented in Table 1. The molecular masses estimated from the SDS-PAGE were significantly higher than deduced from the amino acid sequence or determined by analysing the protein with the mass spectrometer. The molecular mass of the native Nf Xyn11A was determined to be 32857 Da, corresponding the calculated molecular weight (32876 Da) of the mature enzyme. It represents the full-length protein consisting of the catalytic domain and the CBM separated by the linker region. The molecular mass of the recombinant full-length Xyn11A was 33429 Da, which is 572 Da higher than that of the native Nf Xyn11A and 553 Da higher than the calculated value. The molecular masses of the 30 kDa and 27 kDa polypeptides were determined to be 23769 Da and 21974 Da, respectively. The recombinant Xyn11A polypeptides were named as r33.4 kDa, r23.8 kDa and r22.0 kDa polypeptides (Table 1) on the basis of their molecular masses on mass spectrometry. The N-terminus of all four polypeptides was DTTITQ (SEQ ID NO:20) which suggests different C-terminal processing in the r23.8 kDa and r22.0 kDa polypeptides. The C-terminal processing sites are shown in Fig. 1.

The specific activities of the Nf Xyn11A and the recombinant Xyn11A polypeptides were similar on birch xylan substrate (15568-17367 nkat/mg) (Table 1). Also, the pIs of the full-length proteins, Nf Xyn11A and the r33.4 kDa polypeptide were very similar (8.5 vs. 8.6), but they both differed from the value calculated from the amino acid sequence (7.9). The pIs of the r23.8 kDa and r22.0 kDa polypeptides lacking the CBMs were lower than those of the full-length enzymes (7.6 and 8.2 vs. 8.5-8.6).

Example 8**Temperature and pH dependences of the purified Xyn11A xylanases**

The pH and temperature optima for xylanase activity were determined by incubating the Xyn11A samples at different pH values (pH 5-8) at temperatures from 60°C to 80°C for 60 min. Both at pH 5 (Fig. 5A) and pH 6 (data not shown) the maximal activity was reached at 80°C, and at pH 7 at 70°C (Fig. 5B). At all pHs the native Nf Xyn11A was the most thermophilic. This was seen especially at pH 8, where the optimum for Nf Xyn11A was at 70°C, and for the recombinant Xyn11A polypeptides at 60°C (Fig. 5C). The thermostability of the enzymes was determined in the absence of BSA. At 70°C no reduction in enzyme activity was found even after several hours of incubation. At 80°C the reduction was dependent of pH and the enzyme

form. At pH 5 the r22.0 kDa and r23.8 kDa polypeptides were more stable (half-lives of 123 min and 157 min) than the full-length Xyn11A polypeptides, r33.4 kDa and Nf Xyn11A (half-lives of 13 min and 32 min) (Fig. 6; Table 1). The enzymes behave similarly also at pH 7, although the half-lives were shorter, 63-95 min for the r22.0 kDa and r23.8 kDa polypeptides, and 17-31 min for the full-length enzymes.

Example 9

Bleaching experiments using the purified Xyn11A forms

The *N. flexuosa* cultivation medium, the purified native Xyn11A and the recombinant Xyn11A polypeptides produced in *T. reesei* were tested in a single-stage peroxide bleaching with Finnish oxygen-delignified softwood kraft pulp (starting brightness 34% ISO, kappa number 20 and dry matter content 29.9%) at 100 nkat/g pulp dry matter *N. flexuosa* supernatant also 50 nkat/ml). The purified enzymes were supplemented with *T. reesei* culture medium to stabilize the enzymes. The amount of culture medium corresponded to the ratio of thermoxylanase activity and total protein content in the original recombinant *T. reesei* culture media used for enzyme purifications. After these additions the mixtures resembled the enzyme preparations to be used in actual industrial applications. Pulp treatments were carried out at 3% consistency at 80°C and pH 8 for one hour. Reference pulp was treated similarly without enzyme addition. After enzyme treatments the pulp was washed with distilled water. Chelation was performed by adding EDTA to 0.2% of dry matter and carried out at 3.0% consistency at pH 5 for one hour. Pulp was bleached at 10% pulp consistency at 80°C for three hours (H₂O₂ 3%, NaOH 3%, diethylenetriamine pentaacetic acid 0.2%, MgSO₄ 0.5% by volume), after which pulp was acidified with H₂SO₄, washed with distilled water and made into paper handsheets.

Reducing sugars in the chelated pulp were analyzed by the dinitrosalicylic acid method. The quality of the bleached and washed pulps was analyzed by determining the kappa number according to the TAPPI Test Method T 236 and viscosity according to the SCAN28 Scandinavian method. Brightness of the handsheets was analysed according to ISO 2470. Peroxide consumption was determined by titration.

In the single-step peroxide bleaching experiment performed with the *N. flexuosa* cultivation medium lignin removal (0.7-1.1 Kappa units depending on enzyme dosage) and brightness increase (1.0 –1.1 ISO units) was obtained with no reduction in the pulp strength determined by viscosity (Table 2). The *T. reesei* culture medium increased the brightness by 0.9 ISO units. A

further increase of 1.1-1.6 ISO units was obtained with the purified recombinant Xyn11A polypeptides, r33.4 kDa, r23.8 kDa and r22.0 kDa - the r22.0 kDa polypeptide being the least efficient (Table 2).

5 Example 10

Construction of the expression cassettes for heterologous production of Nf Xyn11A (AM35) and the truncated Nf Xyn11A (AM24) in *T. reesei*

For production of Nf Xyn11A (AM35) and the truncated Nf Xyn11A (AM24) in *T. reesei* several expression cassettes were constructed that either include a fungal signal sequence or the
 10 fungal signal sequence and a variable carrier polypeptide for these two proteins. The *am35* /*am35** (see below) and *am24/am24** (see below) genes were expressed from the *T. reesei cel7A* promoter. The expression cassettes constructed are listed in Table 3 and their general structure is shown in Fig. 6. The Table 3 also includes the other relevant information on the constructs. The promoter, transcription terminator and 3'-flanking sequences were as described
 15 in (Karhunen, *et al.*, Mol. Gen. Genet., **241**, 515-522, 1993). The gene coding for acetamidase (*amdS*) was used as a marker in the transformations. The *amdS* gene was isolated from p3SR2 (Kelly and Hynes, EMBO J., **4**, 75-479, 1985). A 3.1 kb *Spe*I-*Xba*I fragment was ligated between the *cel7A* terminator and 3'-flanking region. In addition to these two genes, *am35* and *am24*, with the native codon usage, the following 9 changes were made to codons in some of
 20 the constructs (see Table 3), to make the codons more favorable to *T. reesei*: Gly₅₃ GGG to GGC, Ala₆₆ GCG to GCC, Gly₆₈ GGG to GGC, Arg₈₅ CGG to CGC, Gly₈₈ GGG to GGC, Gly₁₀₀ GGA to GGC, Arg₁₀₁ CGG to CGC, Arg₁₀₂ CGG to CGC and Val₁₀₄ GTG to GTC. The genes including the changes described above were designated as *am35** and *am24**. The changes made did not change the amino acid sequence encoded by the genes, compared to
 25 *am35* and *am24*.

When the full-length *N. flexuosa* Xyn11A was produced, the *am35* and *am35** genes were included in the expression cassettes either as a 1.3 kb fragment ending at the *Mlu*I site about 250 bps after the stop codon or as an exact fusion of the *am35* to the *cbh1* terminator. The
 30 shortened genes, *am24* and *am24** ended to nucleotide 1091 (Fig. 1) and they were exactly fused to *cbh1* terminator including the stop codon. All the genes were fused from the sequence encoding the N-terminal Asp₄₄ (from nucleotide 432, Fig. 1) to the *man5A* signal sequence (pALK1118 and pALK1276, *man5A* nucleotides 1-57) or to a sequence encoding a carrier polypeptide. The carrier polypeptide sequences included the *man5A* core/hinge encoding

sequence (pALK1022, pALK1309, pALK1131 and pALK1692, 1-1359), the sequence encoding a fragment of the *man5A* core (pALK1264, pALK1151 and pALK1154, 1-681) and the *cel6A* CBD/hinge (blocks A and B in pALK1285 and pALK1502, 1-306). For the *man5A* sequence, see Stålbrand, *et al.*, (Appl. Environ. Microbiol., **61**, 1090-1097, 1995). For the
5 *cel6A* sequence, see (Teeri, *et al.*, Gene, **51**, 43-52, 1987). A synthetic sequence coding for the dipeptide Lys - Arg, a target of a Kex2-like protease (Calmels, *et al.*, J. Biotechnol., **17**, 51-66, 1991) was included in the linkers of all the constructs including the carrier protein (not in the signal sequence constructs pALK1118 and pALK1276). In addition, the linkers of pALK1264, pALK1151 and pALK1154 were preceded by a sequence coding for the amino acids Gly – Gln
10 – Cys – Gly – Gly (SEQ ID NO:22). This additional sequence was included to increase the length of the linker between this non-intact carrier and the recombinant xylanase. An identical sequence, naturally occurring in the Man5A polypeptide, is preceding the xylanase sequence in pALK1022.

15 Exact fusions between the *cel7A* promoter and the signal sequences, carrier, linker, *xyn11A* sequences and terminator were synthesized by PCR. An *NruI* recognition site (TCGCGA (SEQ ID NO:24)) was introduced into the Kex2 linker (coded by a sequence CGC GAC AAG CGC (SEQ ID NO:25)) to facilitate the construction of the fusions. The codon CGC was chosen for the arginines in the linker and the third nucleotide of the native codon preceeding the linker was
20 changed to T, when necessary. The modifications made did not change the amino acids encoded by the constructs.

Example 11

Transformation of *Trichoderma* and analysis of the transformants.

25 *T. reesei* protoplasts were transformed with linear expression cassettes isolated from the vector backbones by *EcoRI*. The expression cassettes were transformed to *T. reesei* strain ALKO3620 (Cel5A⁻) and/or to ALKO4468 (Cel5A⁻, Cel7B⁻), see the Table 4. Transformations were performed as in Penttilä, *et al.* (Gene, **61**, 155-164, 1987) with the modifications described in Karhunen, *et al.* (Mol. Gen. Genet., **241**, 515-522, 1993). The transformants were purified on
30 selection plates through single conidia prior to sporulating them on PD. Targeting to the *cel7A* locus was screened as Cel7A-negative phenotype using Minifold I-SRC 96 dot blotter (Schleicher & Schuell, Dassel, Germany). The monoclonal antibody CI-258 or CI-261 (Aho, *et al.*, Eur. J. Biochem., **200**, 643-649, 1991) was used in the detection of Cel7A protein by the ProtoBlot Western blot AP system (Promega). The genotypes of the chosen transformants were

confirmed by using Southern blots in which several genomic digests were included and the respective expression cassettes were used as probes. Strains containing a replacement of the *cel7A* with one copy of the expression cassette were chosen for further studies.

5 Example 12

Production of the AM35 and AM24 xylanases by single-copy *T. reesei* transformants

We have previously shown (Paloheimo, *et al.*, Appl. Environ. Microbiol., **69**, 7073-7082, 2003) that when a carrier polypeptide with an intact domain structure was used, higher production level of AM35 was obtained in *T. reesei* compared to the constructs without a carrier or with a carrier of non-intact domain structure. Also, the recombinant AM35 proteins had the same thermostability as the xylanase activity in the *N. flexuosa* cultivation supernatant. The xylanase activities were stable for at least two hours at 70°C, pH 7. The culture supernatants were also found to increase the brightness of pulp in laboratory scale peroxide of kraft pulp at high temperature and pH in the same way as the culture supernatant from *N. flexuosa*.

Now, the expression cassettes contained either the *am35/am35** gene encoding the full-length Xyn11A or a shortened version from it, *am24/am24**, encoding the truncated Xyn11A protein. Both these proteins were produced using three different carrier polypeptides and also without a carrier polypeptide (only a fungal signal sequence was included). The carriers had either an intact domain structure as Man5A core/hinge of Cel6A CBD/hinge or a non-intact domain structure as Man5A core/hinge fragment.

The expression cassettes shown in Table 3 (Fig. 6) were isolated from the expression plasmids and transformed to *T. reesei* ALKO3620 and/or ALKO4468. The transformants containing single-copy replacement of *cel7A* gene by the expression cassettes were screened for further analysis. Several parallel single-copy strains from each construct were similar in terms of protein and xylanase activity levels analyzed from the culture supernatants of shake flask cultivations. One single-copy representative from each construct was chosen to be cultivated in the fermentor. The culture supernatants from the fermentor cultivations were analysed for the amount of protein and xylanase activities (Table 4).

The results from fermentor cultivations (Table 4) showed that the best xylanase activities were obtained from the *T. reesei* transformants including the constructs in which the shortened genes, *am24* or *am24**, were used. The increase in xylanase activity was observed with all the carriers

tested and also when no carrier protein was included (e.g. RF5024 vs. RF5724, RF5725 vs. ALKO4405, RF5139 vs. RF5013, RF4861 vs. ALKO4823). The changes made to codons did not have an effect on the xylanase production level (ALKO4405 vs. RF5510 and RF4861 vs. RF4878). Also, similar levels of activity were obtained from the transformant with the construct in which the xylanase gene was fused exactly to the terminator sequence compared to the transformant including the construct in which there was a non-exact fusion of the xylanase gene to the terminator (RF5745 vs. ALKO4405). This similar level of activity was obtained even though, for unknown reason, the level of total protein in the culture supernatant of RF5745 including an exact terminator fusion was lower than in the corresponding transformant with a non-exact terminator fusion. Both the *T. reesei* host strains used, transformed with the same expression cassette did produce similar amount of activity (RF5725 vs. ALKO4812).

Because the specific activities of the AM35 and AM24 proteins are very similar to each other (Table 1, r33.4 and r23.8 kDa) it can be concluded that shortening of *Nonomuraea* xylanase gene increases the production level of xylanase in *T. reesei*. When the carriers with an intact domain structure were used (Man5A core/hinge and Cel6A CBD/hinge), the xylanase activity levels measured from the culture supernatants were over three-fold higher for the constructs in which AM24 was produced compared to the corresponding constructs for AM35. For the constructs in which no carrier polypeptide was included or the carrier was a fragment of Man5A (no intact domain structure), the increase in xylanase activity in the culture supernatant was even higher, from over 5- to over 10-fold. Thus, even with no carrier or by using carrier having a non-optimal structure, the yield of the bacterial xylanase could be increased to a surprisingly high level when a shortened xylanase gene was expressed in *T. reesei*.

Increases in the levels of xylanase activity were also observed in the shake flaks cultivations. When no carrier polypeptide was used, the increase in xylanase activity (RF5024 compared to RF5724) was about 2.8-fold and reached 4900 nkat/ml. When Man5A core/hinge was used as a carrier (RF5725 compared to ALKO4405), the increase in activity was about 2.7-fold (RF5725 produced about 21 000 nkat/ml). By using the Cel6A CBD carrier (RF5139 vs. RF5013) the increase in activity was even higher, about 5.6-fold. This high increase was due to the low level of activity in shake flaks from the strains producing AM35 with the Cel6A CBD carrier polypeptide (e.g. RF5013 produced about 3000 nkat/ml, and the activity from RF5139 cultivations was about 16 800 nkat/ml).

The Fig. 8 shows the AM24 xylanase product from a *T. reesei* transformant (Cel6A CBD carrier was used). In addition to the protein having the expected molecular mass, also a xylanase form having a lower molecular mass (corresponding to 22.0 kDa) can be detected from the culture supernatant. This form is due to a proteolytic cleavage of the AM24 protein.

5

Example 13

The use of the *T. reesei* culture supernatants containing the recombinant truncated Nf Xyn11A (AM24) xylanase in bleaching

Culture supernatant from a *T. reesei* transformant producing the recombinant truncated Xyn11A protein (AM24) was tested in bleaching of kraft pulp (Scandinavian birch and pine). The results obtained are shown on Tables 5 and 6. The same brightness could be obtained with lower ClO₂ consumption in both the bleaching experiments when AM24 was used compared to the reference bleaching without a treatment with the xylanase preparation.

15 Example 14

The use of the *T. reesei* culture supernatants containing the recombinant AM24 xylanase in feed application

Two feeding trials with broilers were performed with the AM24 xylanase preparation. The results from the trials demonstrated a good effect of AM24 in poultry. The body-weight in wheat-barley based diet was increased, compared to un-supplemented control birds, by 3.4% and in wheat and soy bean meal by 3.1 – 3.7%. Also, nutrient digestibility was significantly increased as measured by feed conversion rate. The AM24 xylanase preparation was at least as effective as the *T. reesei* XYNII (XYLII) used for years in animal nutrition. The advantage of the AM24 xylanase is the high heat stability compared to e.g. *T. reesei* XYNII (XYLII) which is a great improvement for several feed production procedures.

Example 15

The production of Nf Xyn10A (AM50) and two truncated forms of Nf Xyn10A in *T. reesei*

The gene coding for the Nf Xyn10A (AM50) xylanase (*am50* or *Nfxyn10A*; EMBL accession no AJ508953) was isolated from a lambda ZAP Express® library prepared from partially digested (*Sau3A*) and size-fractionated *Actinomadura* (*Nonomuraea*) *flexuosa* DSM43186 (ATCC35864) chromosomal DNA as described in US 6,300,113. The nucleotide sequence of the gene and the deduced amino acid sequence are shown in Fig. 2. Three expression cassettes (Fig. 9) are constructed for production of the full-length AM50 protein (amino acids A₄₅ – A₄₉₂

from Fig. 2) and two truncated forms from it, the core (A₄₅ – N₃₄₅) and the core/linker missing all the three subdomains from the tail (A₄₅ – S₃₆₇). Also, constructs could be made that encode Xyn10A polypeptides deficient in only the γ -subdomain or the γ - and β -subdomains of the tail. The estimated molecular masses of the different polypeptides would be (from the N-terminal A₄₅, without any added sugar moieties): core 34.0 kDa, core-linker 35.9 kDa, core-linker- α -subdomain of the tail 40.6 kDa, core-linker- α + β -subdomains of the tail 44.8 kDa, core-linker- α + β + γ -subdomains of the tail (full length mature protein) 49.1 kDa. The standard molecular biology methods, PCR reactions and annealing of oligonucleotides are used to make exact fusions between different sequences encoding: the *cbh1* promoter, *cel6A* signal sequence and sequence encoding Cel6A CBD (A+B), Kex2 site (RDKR), sequence encoding *Nf xyn10A* (core or core/hinge or the full-length protein) and *cbh1* terminator. The restriction sites at the end and the beginning of the fragments to be fused are included in the oligonucleotides to enable easy construction of the fusions. Finally, the *amdS* marker gene and the *cbh1* 3'-flanking region will be included as in the constructs made for expressing the *am35/am24* genes. The expression cassettes are isolated from the vector backbones using *EcoRI* digestion and they are transformed to *T. reesei* host strain. The same methods for the transformation, handling and selection of the transformants will be used as in construction of the strains producing Nf Xyn11A: the transformants are purified through single conidia and they are screened in shake flask cultivations by measuring the xylanase activity from the cultivation supernatants. The single-copy transformants in which the expression cassette is replacing the *cbh1* locus will be chosen basing on the results on xylanase production level and Southern blot analysis of the genomes. The increased xylanase production from the strains producing the truncated forms of the Nf Xyn10A protein, compared to the strains producing the full length Nf Xyn10A is shown by using activity assays, SDS-PAGE and Western blot methods.

The culture supernatant(s) are used in the application tests, both for the bleaching of kraft pulp and feed applications, to show the effect of the xylanase(s).

TABLE 1. Characteristics of the purified Nf Xyn11A and the recombinant Xyn11A polypeptides.

Enzyme	MW SDS-PAGE	MW Mass spect	N-terminus	Sp. act. (nkat/mg)	pI	Half-life 80°C, pH 5 (min)	Half-life 80°C, pH 7 (min)
Nf Xyn11A	37	32.9	DTTITQ	15 568	8.5	32	31
Recombinant enzymes							
r33.4 kDa	37	33.4	DTTITQ	16 005	8.6	13	17
r23.8 kDa	30	23.8	DTTITQ	17 367	7.6	157	95
r22.0 kDa	27	22.0	DTTITQ	16 950	8.2	123	63

TABLE 2. Peroxide bleaching with the *N. flexuosa* cultivation medium and the purified recombinant Xyn11A polypeptides.

Xylanase	Brightness/ Brightness increase (ISO)	Kappa	Viscosity (dm ³ /kg)	Reducing sugars (% of dry matter)	Peroxide consumption (%)
<i>N. flexuosa</i> medium ^a	72.9	8.3	890	ND	ND
<i>N. flexuosa</i> medium ^b	73.0	7.9	890	ND	ND
Reference	71.9	9.0	870	ND	ND
r33.4 kDa	64.5/+2.4	8.8	ND	0.30	2.3
r23.8 kDa	64.6/+2.5	8.1	ND	0.31	2.3
r22.0 kDa	64.1/+2.0	8.7	ND	0.30	2.3
<i>T. reesei</i> medium	63.0/+0.9	9.2	ND	0.25	2.3
Reference	62.1	8.7	ND	0.25	2.2

^aEnzyme dosage 50 nkat/g pulp dry matter.

^bEnzyme dosage 100 nkat/g pulp dry matter.

TABLE 3. The constructs used to express the *N. flexuosa am35/am35** gene and the shortened *am35/am35** gene (*am24/am24**).

Expr. cassette	Carrier protein	Xylanase gene	Term. fusion	Reference
pALK1118	No carrier (<i>man5A</i> ss)	<i>am35</i>	Non-exact	Paloheimo <i>et al.</i> (2003)
pALK1276	No carrier (<i>man5A</i> ss)	<i>am24</i>	Exact	This application
pALK1022	Man5A core/hinge	<i>am35</i>	Non-exact	Paloheimo <i>et al.</i> (2003)
pALK1692	Man5A core/hinge	<i>am35</i>	Exact	This application
pALK1309	Man5A core/hinge	<i>am35*</i>	Non-exact	This application
pALK1131	Man5A core/hinge	<i>am24*</i>	Exact	This application
pALK1285	Cel6A CBD/hinge	<i>am35</i>	Non-exact	Paloheimo <i>et al.</i> (2003)
pALK1502	Cel6A CBD/hinge	<i>am24*</i>	Exact	This application
pALK1264	Man5A fragment	<i>am35</i>	Non-exact	Paloheimo <i>et al.</i> (2003)
pALK1151	Man5A fragment	<i>am24</i>	Exact	This application
pALK1154	Man5A fragment	<i>am24*</i>	Exact	This application

5 **Table 4. Xylanase production from single-copy *Trichoderma reesei* transformants in laboratory scale fermentations.**

Strain	Expression cassette	<i>T. reesei</i> host	Protein (mg/ml)	Xylanase (nkat/ml)
1. <i>T. reesei</i> host strains				
ALKO3620	No cassette		14.4	1 490
ALKO4468	No cassette		9.3	430
2. No carrier (Man5a signal sequence):				
ALKO4766	pALK1118	ALKO4468	8.8	2 460
RF5724	pALK1118	ALKO3620	11.3	3 870
RF5024	pALK1276	ALKO3620	7.7	31 830
3. Man5A core/hinge as a carrier				
ALKO4405	pALK1022	ALKO3620	12.3	14 400
RF5745	pALK1692	ALKO3620	4.3	13 800
RF5510	pALK1309	ALKO3620	11.9	14 600
RF5725	pALK1131	ALKO3620	14.1	45 940
ALKO4812	pALK1131	ALKO4468	12.3	42 980
4. Cel6A CBD (A+B) as a carrier				
RF5013	pALK1285	ALKO3620	7.5	10 750
RF5139	pALK1502	ALKO3620	8.8	37 140
5. Fragment of Man5A as a carrier				
ALKO4823	pALK1264	ALKO4468	8.8	4 940
RF4861	pALK1151	ALKO3620	7.8	26 400
RF4878	pALK1154	ALKO3620	9.8	29 600

Table 5. Thermoxyylanase in bleaching of birch mill kraft pulp

The sequence used was X/D-EO-D, the kappa number of the original birch kraft pulp used (from handsheet) was 14.4 and brightness 44.1%. The activity of the enzyme preparation used was 187 000 nkat/ml (measured at pH 7, 70°C using 5 min reaction time). ND = not determined. All the pH values were measured from pulp slurry at the reaction temperature. The pulp samples were acidified to pH 4.5 with SO₂-water before the sheet preparation to eliminate the residual chemicals. The pulp properties were tested according to ISO standard methods: brightness ISO 2470 (split pulp sheet), kappa number ISO 302. tp = ton of pulp. aCl = active chlorine.

Bleaching no		1717 Ref.	1718	1719	1720	1721
X-stage	Enzyme					
Consist. 8%	Enzyme dosage, l/tp	no	0.3	0.15	0.3	0.15
Temp. 82°C	H ₂ SO ₄ , kg/tp (for pH adjustment)	6.9	7.3	7.3	9.9	9.9
Time 20 min	pH start / final	7.0 / 7.6	7.0 / 7.9	7.0 / 7.9	6.0 / 6.3	6.0 / 6.3
no washing after enzyme treatment						
D0-stage						
Consist. 9%	ClO ₂ dosage, aCl kg/tp	47.0	41.0	41.0	41.0	41.0
Temp. 70°C	ClO ₂ consumpt., aCl kg/tp	47.0	41.0	41.0	41.0	41.0
Time 30 min	final pH	2.8	2.6	3.0	2.4	2.5
(EO)-stage	NaOH dosage, kg/t	21.0	21.0	21.0	21.0	21.0
Consist. 10%	final pH	10.3	10.2	10.3	10.3	10.3
Temp. 85°C	Brightness, %	79.3	79.1	78.9	80.3	79.6
Time 60 min	Kappa number	2.5	2.5	2.9	2.4	2.6
4 bar O ₂	XD(EO) yield, %	93.4	93.2	93.9	91.3	92.5
D2-stage	ClO ₂ dosage, aCl kg/tp	17.0	15.0	15.0	14.0	14.5
Consist 9%	ClO ₂ consumpt., aCl kg/tp	15.6	14.4	14.4	13.2	13.2
Temp 75°C	NaOH kg/tp (for pH adjustment)	2.5	2.2	2.2	2.1	2.1
Time 110 min	final pH	4.3	4.6	4.6	4.3	4.3
	Brightness, %	90.1	90.2	90.1	90.7	90.3
	Kappa number	0.6	0.6	ND	ND	ND
	D2-yield, %	98.7	98.6	97.8	99.0	98.5
	Total bleaching yield, %	92.2	91.9	91.8	90.4	91.1
	Tot. ClO ₂ dosage, aCl kg/tp	64.0	56.0	56.0	55.0	55.5
	Tot. ClO ₂ consumpt, aCl kg/tp	62.6	55.4	55.4	54.2	54.2
	Tot. NaOH kg/tp	23.5	23.2	23.2	23.1	23.1
	Tot. H ₂ SO ₄ , kg/tp	6.9	7.3	7.3	9.9	9.9

Table 6. Thermoxylanase in bleaching of softwood kraft pulp. Sequence X/D-E-D-E-D was used. The Kappa number of the original pulp was 28.9 (from handsheet), brightness 28.2% and viscosity 1180 ml/g. The activity of the enzyme preparation used was 187 000 nkat/ml (measured at pH 7, 70°C, 5 min reaction time). ND = not done. All the pH values were measured from pulp slurry at the reaction temperature. The pulp samples were acidified to pH 4.5 with SO₂-water before the sheet preparation to eliminate the residual chemicals. The pulp properties were tested according to ISO standard methods: brightness ISO 2470 (split pulp sheet), kappa number ISO 302, viscosity ISO 5351/1. tp = ton of pulp. aCl = active chlorine.

Bleaching no		1627 Ref.	1692	1698
X-stage	Enzyme			
Consist. 8%	Enzyme dosage, l/tp	no	0.3	0.3
Temp. 70°C	H ₂ SO ₄ , kg/tp (for pH adjustment)	2.7	2.7	2.7
Time 60 min	pH start / final	7.0 / 7.4	7.0 / 7.3	7.0 / 7.3
D0-stage				
Consist. 9%	ClO ₂ dosage, aCl kg/tp	60.0	54.0	54.0
Temp. 50°C	ClO ₂ consumpt., aCl kg/tp	60.0	54.0	54.0
Time 45 min	final pH	1.7	1.9	1.9
E1-stage	NaOH dosage, kg/t	27.0	27.0	27.0
Consist. 10%	final pH	10.9	11.1	11.1
Temp. 60°C	Brightness, %	49.5	49.6	49.4
Time 60 min	Kappa number	6.1	6.2	6.5
D1-stage	ClO ₂ dosage, aCl kg/tp	24.5	20.0	20.0
Consist 10%	ClO ₂ consumpt., aCl kg/tp	24.5	19.8	20.0
Temp 70°C	NaOH kg/tp (for pH adjustment)	3.4	3.0	2.7
Time 240 min	final pH	3.6	4.2	4.1
	Brightness, %	78.9	ND	77.2
	Kappa number	1.8	ND	ND
E2-stage	NaOH dosage, kg/t	8.0	8.0	8.0
Consist. 10%	final pH	10.6	10.6	10.6
Temp. 70°C	Brightness, %	ND	77.5	ND
Time 60 min	Kappa number	ND	1.5	ND

D2-stage	ClO ₂ dosage, aCl kg/tp	9.0	8.0	9.5
Consist. 10%	ClO ₂ consumpt., aCl kg/tp	7.9	7.6	9.3
Temp. 70°C	NaOH kg/tp (for pH adjustment)	1.1	0.9	1.1
Time 240 min	final pH	4.6	4.4	4.2
	Brightness, %	90.0	89.4	89.9
	Kappa number	0.7	0.7	0.6
	Viscosity ml/g	1030.0	ND	1050.0
	Tot. ClO ₂ , aCl kg/tp	93.5	82.0	83.5
	Tot. NaOH kg/tp	39.5	38.9	38.8
	Tot. H ₂ SO ₄ , kg/tp	2.7	2.7	2.7

CLAIMS

1. A recombinant DNA method for obtaining in a filamentous fungus host a higher production level of a bacterial carbohydrate degrading enzyme, which in its original full length state has a structure consisting of a catalytic module (CAT) and a carbohydrate binding module (CBM) separated by a linker region, **characterized** in that a DNA construct, which comprises regulatory sequences derived from filamentous fungi and a shortened DNA sequence encoding a truncated form of said bacterial carbohydrate degrading enzyme, which contains the catalytically active region of CAT, but lacks part or all of the CBM, or all of the CBM and part or all of the linker region is introduced into filamentous fungal host and allowed to express and secrete said truncated bacterial carbohydrate degrading enzyme, under the control of said filamentous fungal derived regulatory sequences, which include a promoter sequence, a signal sequence with or without a carrier sequence, and a terminator sequence derived from a filamentous fungus; and wherein the higher production level obtained with said DNA construct encoding the truncated form of said bacterial carbohydrate degrading enzyme measured as an increase of activity from single copy transformants is higher than the production level obtained with another corresponding DNA construct, which is identical with said DNA construct except that it comprises a DNA sequence encoding the corresponding full length bacterial carbohydrate degrading enzyme.

2. The method according to claim 1, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme originates from an actinomycete.

3. The method according to claim 1, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme is derived from *Nonomuraea*.

4. The method according to claim 1, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme is derived from *Nonomuraea flexuosa*.

5. The method according to claim 1, wherein the bacterial carbohydrate degrading enzyme is a truncated form of a xylan degrading enzyme.

6. The method according to claim 1, wherein the bacterial carbohydrate degrading enzyme is a truncated form of a thermostable xylanase.

7. The method according to claim 1, wherein the bacterial carbohydrate degrading enzyme is a truncated form of Nf Xyn11A.

8. The method according to claim 1, wherein the bacterial carbohydrate degrading enzyme is a truncated form of Nf Xyn10A.

9. The method according to claim 1, wherein the truncated form of the bacterial carbohydrate degrading enzyme is the AM24 (SEQ ID NO:12), the r23.8 kDa polypeptide (SEQ ID NO:13), or the r22.0 kDa polypeptide (SEQ ID NO:14).

10. The method according to claim 1, wherein the truncated form of the bacterial carbohydrate degrading enzyme is the AM50 core and linker region (SEQ ID NO:17), the AM50 core region (SEQ ID NO:18), the AM50 core and linker and the α/β region of the tail (SEQ ID NO:19), or the AM50 core and linker and α region of the tail (SEQ ID NO:20).

11. The method according to claim 1, wherein the DNA sequences encoding the carrier polypeptide are derived from filamentous fungi and encode selected regions or domains of a secretable carbohydrate degrading enzyme.

12. The method according to claim 11, wherein the DNA sequence encodes the carrier polypeptide Man5A core or core/hinge region, or Cel6A CBD consisting of a A, A+B or A+B+B', wherein A is carbohydrate binding domain, B is a hinge region, and B' is a duplicated hinge region.

13. The method according to claim 1, wherein the promoter sequences comprise *Trichoderma* or *Aspergillus* promoters.

14. The method according to claim 13, wherein the *Trichoderma* promoter is *cel7A (cbh1)* promoter.

15. The method according to claim 1, wherein the higher production level achieved with the DNA construct encoding the truncated form of the bacterial carbohydrate degrading enzyme is at least 2 times higher than the production level achieved with another corresponding DNA construct, which is identical with said DNA construct except that it comprises a DNA sequence encoding the corresponding full length carbohydrate degrading enzyme.

16. A DNA construct for obtaining in a filamentous fungus host a higher production level of a bacterial carbohydrate degrading enzyme having in its original full length state a catalytic module (CAT) and a carbohydrate binding module (CBM) separated by a linker region, **characterized** in that said DNA construct comprises regulatory sequences derived from filamentous fungi including a promoter, a signal sequence with or without a carrier sequence, and a terminator sequence and a shortened DNA sequence encoding a truncated form of said bacterial carbohydrate degrading enzyme, which contains a catalytically active region of CAT but which lacks part or all of the CBM, or all of the CBM part or all of the linker region, wherein the production level of said DNA construct encoding the truncated bacterial carbohydrate degrading enzyme measured as an increase of activity from single copy transformants is higher than the production level obtained with another corresponding DNA construct which is identical with said DNA construct, except that it comprises a DNA sequence encoding the corresponding full length bacterial carbohydrate degrading enzyme.

17. The DNA construct according to claim 16, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme originates from an actinomycete.

18. The DNA construct according to claim 16, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme originates from *Nonomuraea*.

19. The DNA construct according to claim 16, wherein the DNA sequence encoding the truncated form of the bacterial carbohydrate degrading enzyme originates from *Nonomuraea flexuosa*.

20. The DNA construct according to claim 16, wherein the DNA sequence encoding the bacterial carbohydrate degrading enzyme is a DNA sequence encoding a truncated form of a xylan degrading enzyme.

5 21. The DNA construct according to claim 16, wherein the DNA sequence encoding the bacterial carbohydrate degrading enzyme is a DNA sequence encoding a truncated form of a thermostable xylanase.

10 22. The DNA construct according to claim 16, wherein the DNA sequence encoding the bacterial carbohydrate degrading enzyme is a DNA sequence encoding a truncated form of Nf Xyn11A.

15 23. The A DNA construct according to claim 16, wherein the DNA sequence encoding the bacterial carbohydrate degrading enzyme is a DNA sequence encoding a truncated form of Nf Xyn10A.

20 24. The DNA construct according to claim 16, wherein the shortened DNA sequence encodes the AM24 (SEQ ID NO:12), the r23.8 kDa polypeptide (SEQ ID NO:13), or the r22.0 kDa polypeptide (SEQ ID NO:14).

25 25. The DNA construct according to claim 16, wherein the shortened DNA sequence encodes the AM50 core + linker (SEQ ID NO:17), the AM50 core (SEQ ID NO:18), the AM50 core + linker + α/β domain of the tail (SEQ ID NO:19), or the AM50 core + linker + α domain of the tail (SEQ ID NO:20).

30 26. The DNA construct according to claim 16, wherein the DNA sequences encoding the carrier polypeptide are DNA sequences derived from the same or different filamentous fungi and encode selected regions or domains of a secretable carbohydrate degrading enzyme.

27. The DNA construct according to claim 26, wherein the DNA sequence encodes the carrier polypeptide Man5A core or core/hinge region, or a Cel6A CBD consisting of a A, A+B or A +B + B' region, wherein A is a carbohydrate binding domain, B is hinge domain and B' is a duplicated hinge domain.

28. The DNA construct according to claim 16, wherein the promoter sequences comprise *Trichoderma* or *Aspergillus* promoters.

5 29. The DNA construct according to claim 28, wherein the *Trichoderma* promoter is cel7A (*cbh1*) promoter.

10 30. The DNA construct according to claim 16, wherein the higher production level achieved with said DNA construct encoding the truncated form of the bacterial carbohydrate degrading enzyme is at least two times higher than the production level achieved with another corresponding DNA construct, which is identical with said DNA construct, except that comprises a DNA sequence encoding the full length carbohydrate degrading enzyme.

15 31. The use of the method according to any of claims 1-15 for producing an enzyme preparation for use in industrial processes requiring high temperatures.

32. The use of the DNA construct according to any of claims 16-30 for producing an enzyme preparation for use in industrial processes requiring high temperatures.

[illegible]

FIG. 1

1 ttcggcagcctattgacaaaatttctgtgaatgtttccacacttctctgcagacggccccgcgcgatcatgggtgcaccgggtcggcgggaccggtgctcoga
101 cgccattcgggggtgtgcgcctgcggcgcggtgatcccggggactcccgcggttccctttccgtgtccctctaataatggaggtcaggaATGGGCG
1 m g v

201 TGAACGCCTTCCCCAGACCCGGAGCTCGGCGGTTCACCGCGGGCTGTACCGGGCCCTGGCCCGGCCACGGTGAGCGTGGTTCGGCGTGCACGGCCCT
4 n a f p p r p g a r r f t g g l y r a l a a a t v s v v g v v t a l

301 GACGGTGACCCAGCCCGCCAGCGCGCGGCGAGCAGCTCGCCCGAGGTGCCCGCAGACACACCGGTACTTCGGCGTGGCCATCGCCCGCGAACAGGCTC
37 t v t q p a s a A A S T L A E G A A Q H N R Y F G V A I A A N R L
#1696 #1697

401 ACCGACTCGGTCTACACCAACATCGCGAACCGCGAGTTCAACTCGGTGACGGCGGAGAACGAGATGAAGATCGACGCCACCGAGCCGCGAGCAGGGCGGT
70 T D S V Y T N I A N R E F N S V T A E N E M K I D A T E P Q Q G R F
#1698 #1704

501 TCGACTTACCCAGGCGCCGGGATCTACAACTGGGGCGCGCCAGAACGGCAAGCAGGTCCGCGGCCACACCCCTGGCCTGGCACTCGCAGCAGCCCGCAGTG
104 D F T Q A D R I Y N W A R Q N G K Q V R G H T L A W H S Q Q P Q W

601 GATGCAGAACTCAGCGGCCAGGCGCTGCGCCAGGCGATGATCAACCACATCCAGSGGTTCATGTCTACTACCGGGGCAAGATCCCGATCTGGGACGTG
137 M Q N L S G Q A L R Q A M I N H I Q G V M S Y Y R G K I P I W D V

701 GTGAACGAGGCGTTTCGAGGACGGAACTCCGGCGCGCGGTGCGACTCCAACCTCCAGCGCACCGGTAAAGATTGGATCGAGGTCCGCTTCGGCACCGCCC
170 V N E A F E D G N S G R R C D S N L Q R T G N D W I E V A F R T A R

801 GCCAGGGGACCCCTCGGCCAAGCTCTGCTACAACGACTACAACATCGAGAATCGAAACGCGGCCAAGACCCAGGCGGTCTACAACATGGTTCGGGGACTT
204 Q G D P S A K L C Y N D Y N I E N W N A A K T Q A V Y N M V R D F
#1703

901 CAAGTCCCGCGGCGTGCCTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTCAGTCGCGTTC
237 K S R G V P I D C V G F Q S H F N S G N P Y N P N F R T T L Q Q F

1001 GCGGCCCCGCGGTGACGTCAGGTCACCGAGCTGGACATCGAGAACGCCCCCGCCAGACCTACGCCAGCGTGTATCCGGGACTGCCCTGGCCCGTGGACC
270 A A L G V D V E V T E L D I E N A P A Q T Y A S V I R D C L A V D R

FIG. 2A

1101 GCTGCACCGGCATCACCGTCTGGGGTGTCCGGACAGCGACTCCTGGCGCTCGTACAGAACCCGGTGTGTTTCGACAACAACGGCAACAAGACGAGGC
304 C T G I T V W G V #1699 R D S D S W R S Y Q N P L L F D N N G N K Q A

1201 CTACTACGGGTGCTCGACGCCCTGAACGAGGGTCCGACGAGTGGGCGCCCGTCCAACCCCGGTCTCGCCGCCCGCGGGTGGCGGTTCGGGGCAG
337 Y Y A V L D A L N E G S D D G G G P S N P P V S P P G G S G Q

1301 ATCCGGGGGTGGCCTCCAACCGGTGCATCGACGTGCCGAACGGCAACCGCCGACGGCACCCAGGTCCAGCTGTACGACTGCCACACAGCGGTTCCAACC
370 I R G V A S N R C I D V P N G N T A D G T Q V Q L Y D C H S G S N **Q**

1401 AGCAGTGGACCTACACCTCGTCCGGTGAGTTCGGCAACAAGTGCCTGGACGGCGGGCGGTCCAGCAACGGTGGTCCAGATCTACAG
404 **Q** W T Y T S S G E F R I F G N K C L D A G G S S N G A V Q I Y S

1501 CTGCTGGGGCGGCCAACAGAAAGTGGGAGCTCCGGGCCGACGGCACCAATCGTGGGCGTGCAGTCCGGGTGTGCCCTCGACGCGGTGGGTGGCGGCACC
437 C W G G A N **Q** K W E L R A D G T I V G V Q S G L C L D A V G G G T

1601 GGCAACGGCACGGGTGCAGCTCTACTCTGCTGGGGCGGCCAACACAGAAAGTGGTCTTACAACGCCCTGATCCCCGGTGTGATCGACCCCTAGTtaggc
470 G N G T R L Q L Y S C W G G N N **Q** K W S Y N A * 492

1701 cgtctccggtaacggcaccgtcggaccggaggcgtcccttgttcgtccaggacggaagaccgggtctgagcagggcgcgatcggaacacccatggtggga
1801 ggcacgaaagcggagggtcgattccgagactccgggaagtggaggtgttcctccacctga 1864

FIG. 2B

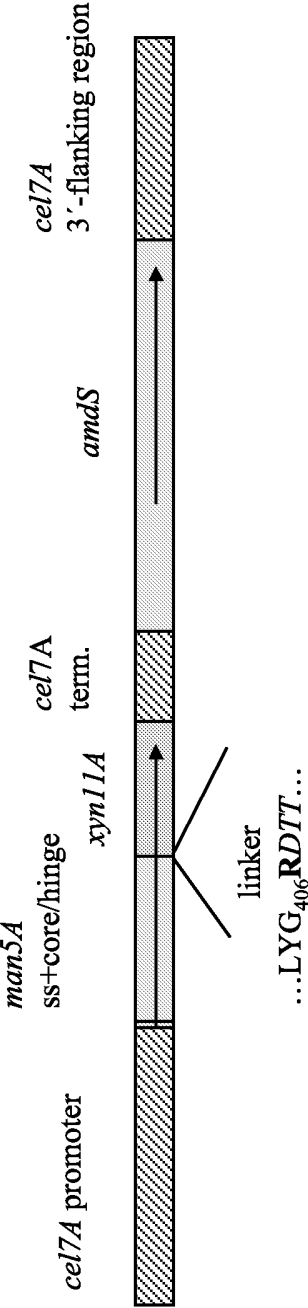


FIG. 3

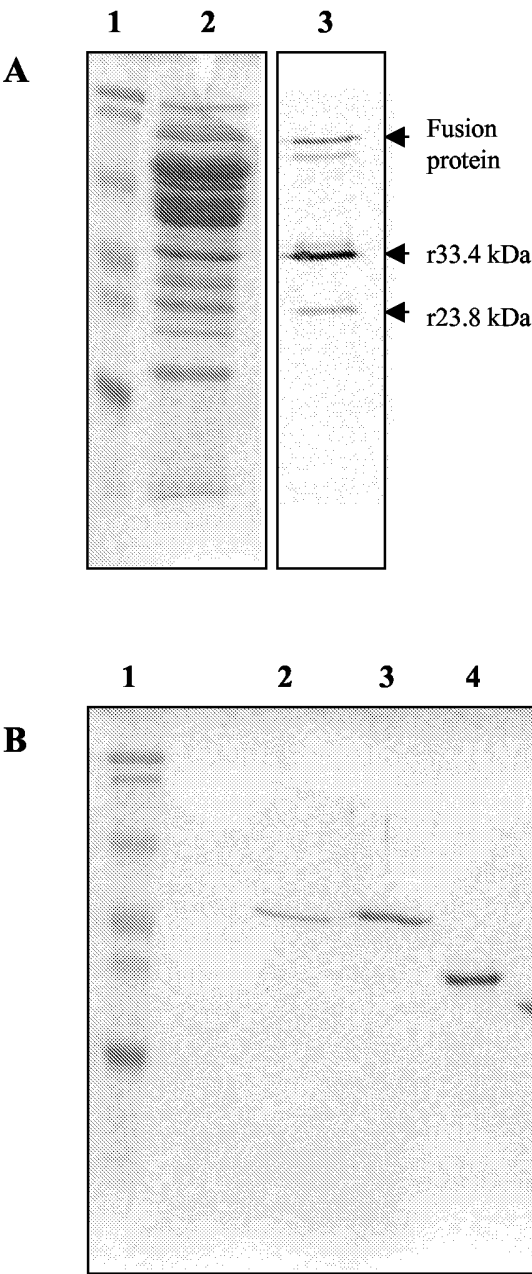
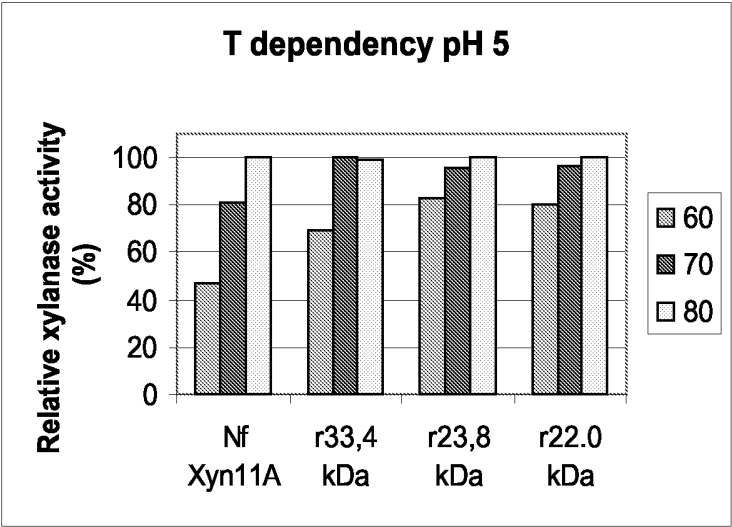


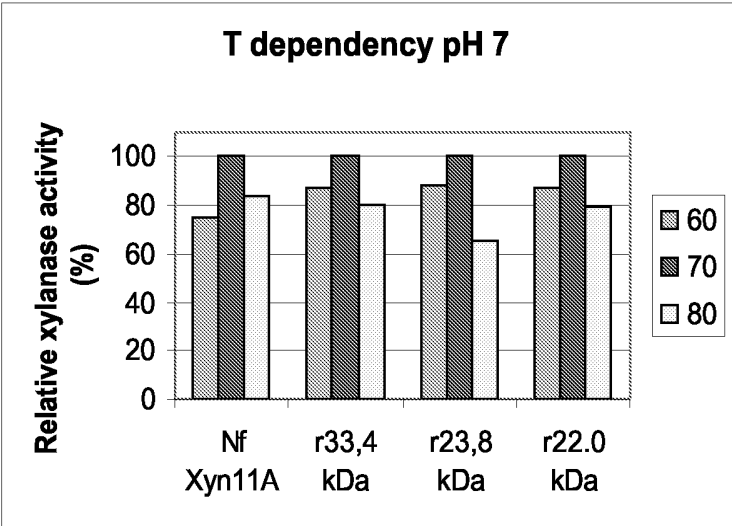
FIG. 4

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A.



B.



C.

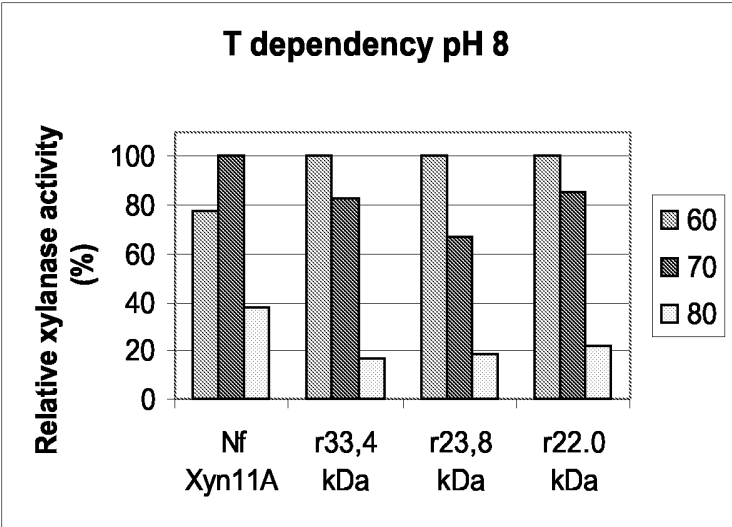


FIG. 5

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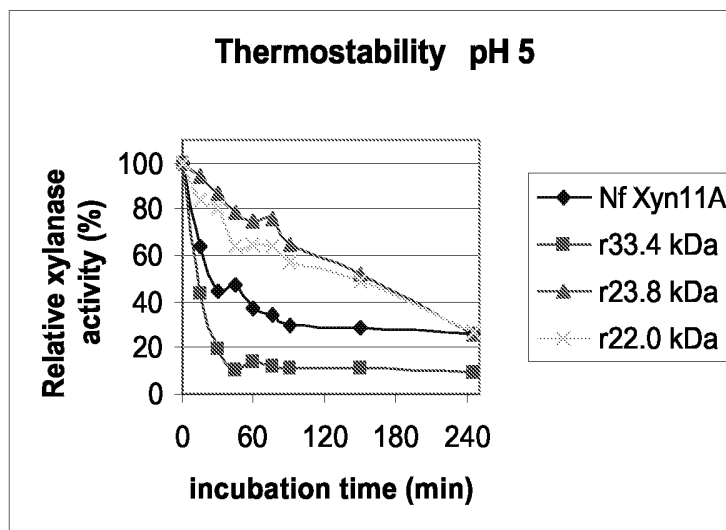
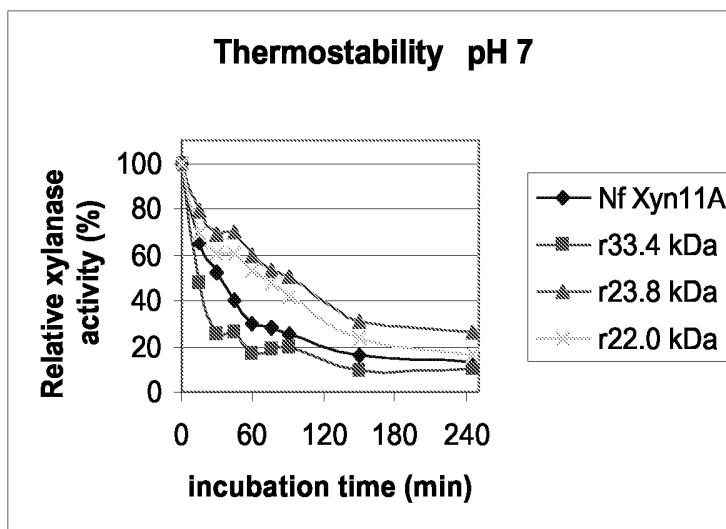
A.**B.**

FIG. 6

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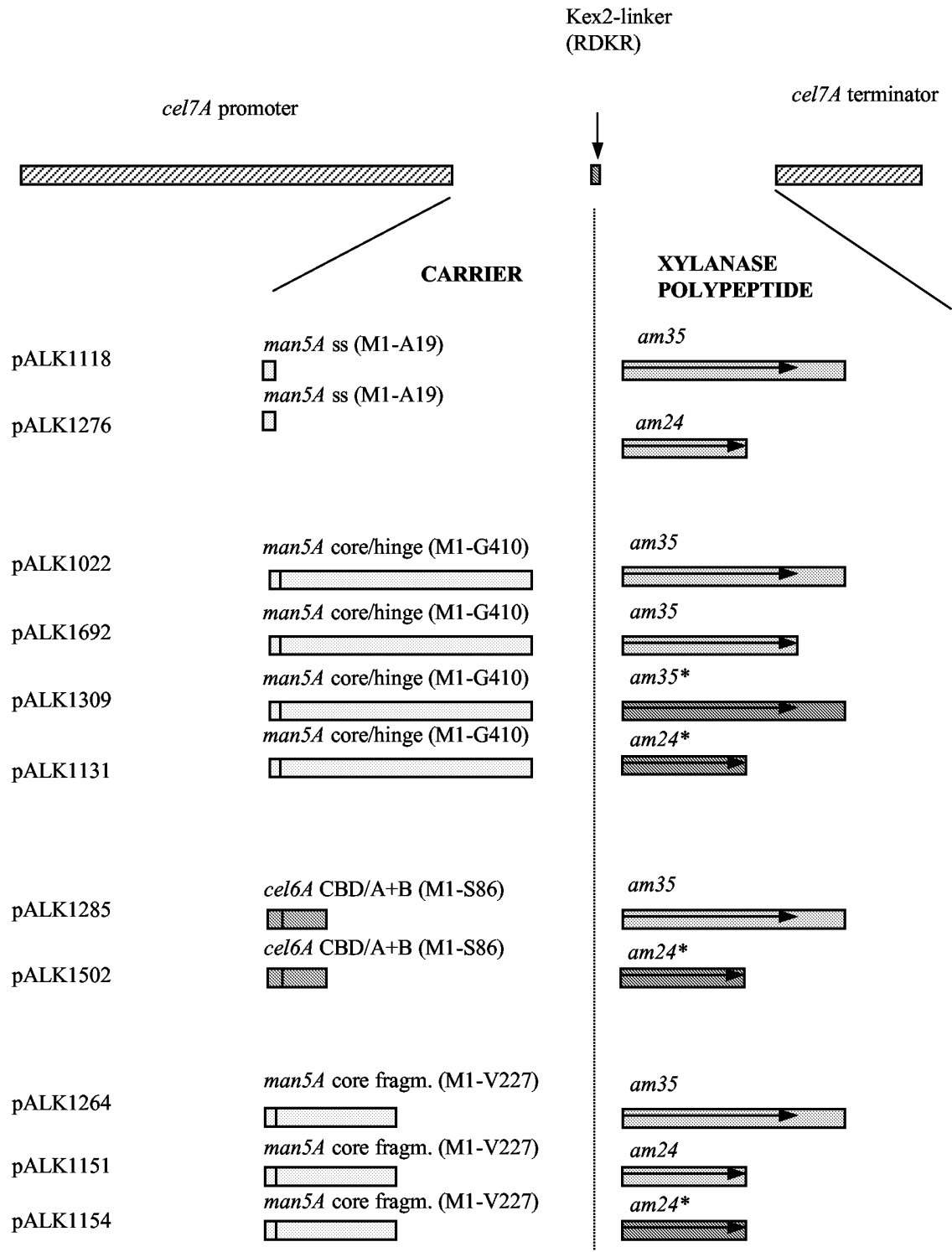


FIG. 7

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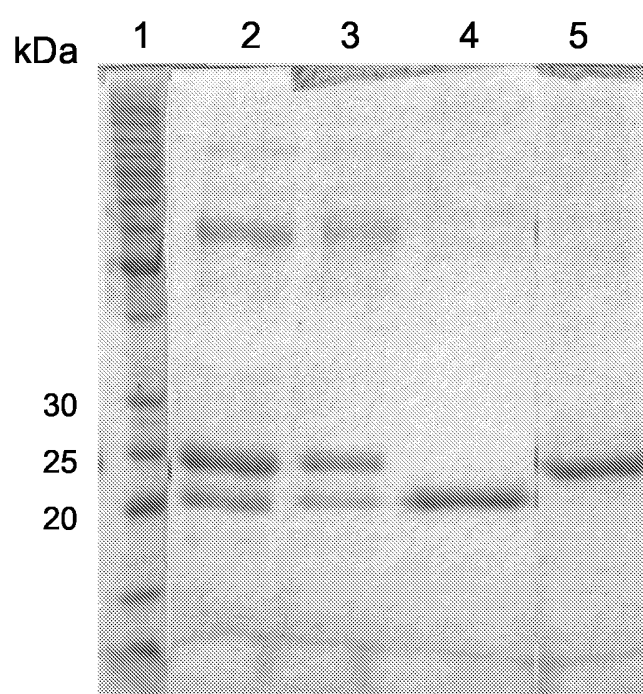
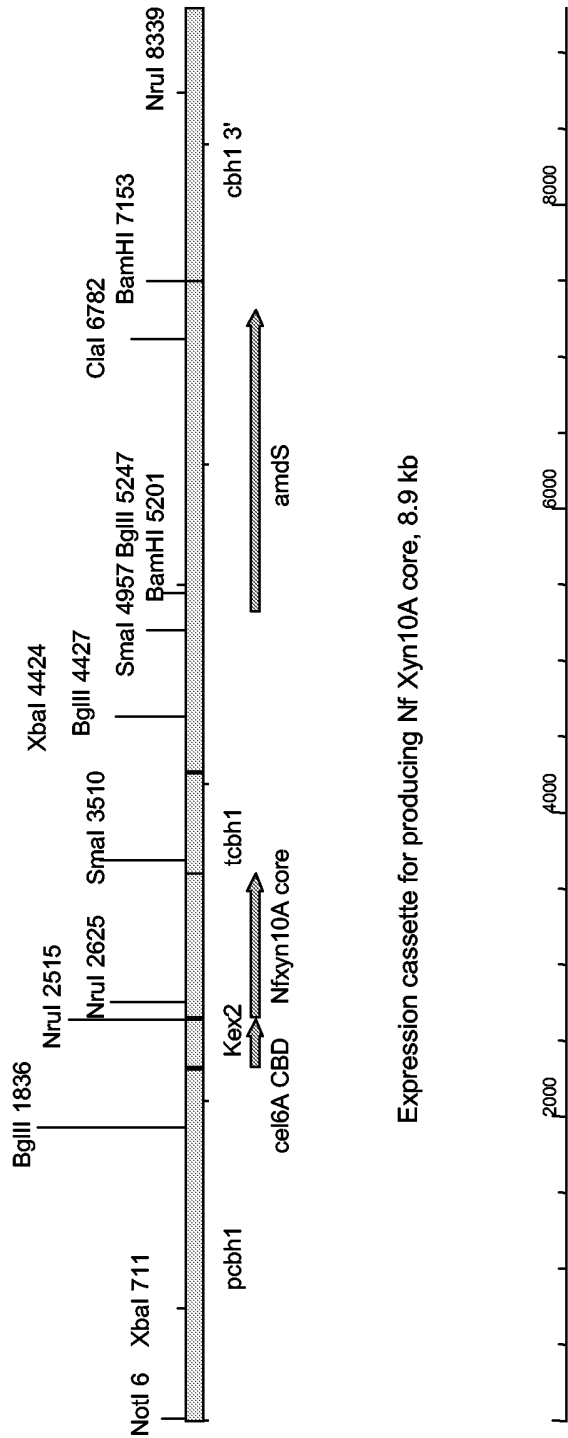


FIG. 8



Expression cassette for producing Nf Xyn10A core, 8.9 kb

FIG. 9A

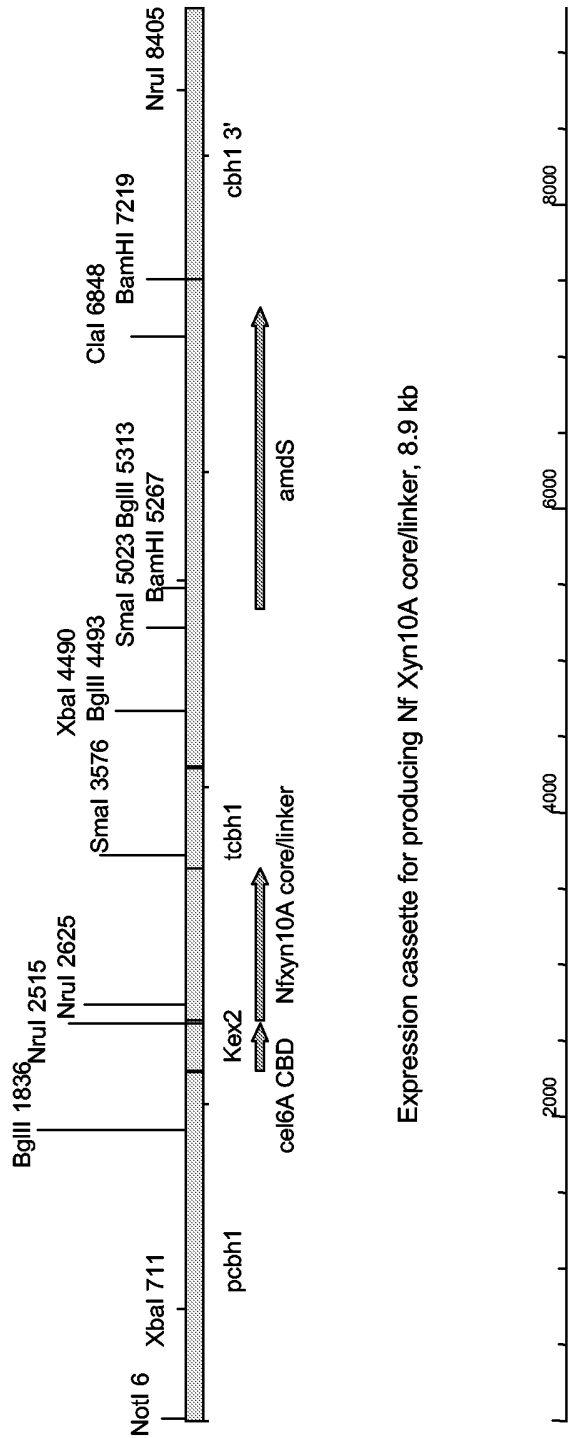
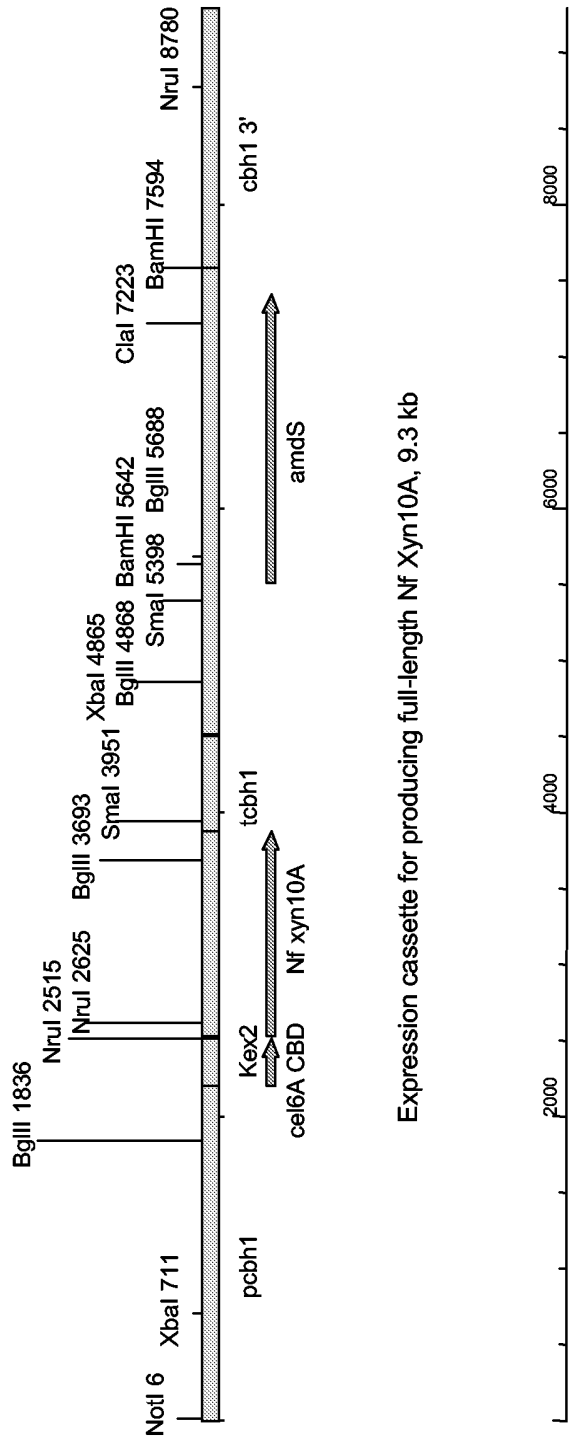


FIG. 9B



Expression cassette for producing full-length Nf Xyn10A, 9.3 kb

FIG. 9C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2005/050123

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/24, 15/56, 15/67, 15/80, D21C 9/10, C12S 3/08

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)

IPC7: C12N, D21C, C12S

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
FI, SE, NO, DK classes as aboveElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-INTERNAL, WPI, PAJ, BIOSIS, CHEM. ABS. DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9727306 A1 (ALKO GROUP LTD) 31 July 1997 (31.07.1997) see pages 43-45 and 54-62	1-32
Y	PALOHEIMO M. et al. High yield production of bacterial xylanase in the filamentous fungus <i>Trichoderma reesei</i> requires a carrier polypeptide with an intact domain structure. Applied and Environmental Microbiology. December 2003, Vol. 69, No. 12, pages 7073-7082 see the whole document	1-32
Y	WO 9325693 A1 (HAZLEWOOD GEOFFREY PETER et al.) 23 December 1993 (23.12.1993) see page 4, lines 12-17, pages 9 and 14	1-32
Y	WO 0196382 A2 (PROKARIA EHF) 20 December 2001 (20.12.2001) see page 4, lines 6-29, page 5, lines 12-26 and page 8, lines 8-21	1-32
Y	WO 9015860 A1 (GENENCOR INC) 27 December 1990 (27.12.1990) see abstract and page 15	1-32

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

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"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

22 June 2005 (22.06.2005)

Date of mailing of the international search report

23 June 2005 (23.06.2005)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2005/050123

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1408108 A2 (GENENCOR INT) 14 April 2004 (14.04.2004) see the whole document	1-32
A	RIXON J.E. et al. Do the non-catalytic polysaccharide-binding domains and linker regions enhance the biobleaching properties of modular xylanases? Applied Microbiology and Biotechnology. 1996, Vol. 46, pages 514-520 see the whole document	1-32
A	US 6083734 A (CHUANG MING-HON et al.) 04 July 2000 (04.07.2000) see the whole document	1-32

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI2005/050123

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
WO 9727306 A1	31/07/1997	US 2003148453 A1	07/08/2003
		US 2001024815 A1	27/09/2001
		US 6667170 B1	23/12/2003
		ID 15848 A	14/08/1997
		EP 0876494 A1	11/11/1998
		CA 2241968 A1	31/07/1997
		AU 1547797 A	20/08/1997
		US 6300114 B1	09/10/2001
.....			
WO 9325693 A1	23/12/1993	FI 945928 A	16/02/1995
		NO 944851 A	14/02/1995
		FI 945889 A	08/02/1995
		NZ 253280 A	22/09/1997
		NZ 252937 A	28/10/1996
		JP 8501444T T	20/02/1996
		JP 7508647T T	28/09/1995
		EP 0652961 A1	17/05/1995
		EP 0746607 A1	11/12/1996
		CA 2138399 A1	23/12/1993
		CA 2138383 A1	23/12/1993
		BR 9306580 A	08/12/1998
		BR 9306576 A	12/01/1999
		WO 9325671 A1	23/12/1993
		AU 4347993 A	04/01/1994
		AU 4298393 A	04/01/1994
		AU 696768 B2	17/09/1998
		AU 696724 B2	17/09/1998
.....			
WO 0196382 A2	20/12/2001	US 2002102699 A1	01/08/2002
		EP 1297004 A2	02/04/2003
		AU 6421201 A	24/12/2001
.....			

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI2005/050123

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
WO 9015860 A1	27/12/1990	US 2003224489 A1	04/12/2003
		US 6130063 A	10/10/2000
		US 5679543 A	21/10/1997
		FI 110124B B1	29/11/2002
		JP 3153234B2 B2	03/04/2001
		JP 4500313T T	23/01/1992
		ES 2120947T T3	16/11/1998
		EP 0429628 A1	05/06/1991
		DK 429628T T3	31/05/1999
		DE 69032616T T2	11/03/1999
		DE 69032616D D1	08/10/1998
		CA 2034487 A1	17/12/1990
		AU 5846790 A	08/01/1991
		AU 627334 B2	20/08/1992
		AT 170556T T	15/09/1998
.....			
EP 1408108 A2	14/04/2004	DK 1032686T T3	27/06/2005
		DE 69829308D D1	14/04/2005
		AT 290599T T	15/03/2005
		US 2002081702 A1	27/06/2002
		US 2002076792 A1	20/06/2002
		WO 0009707 A1	24/02/2000
		US 6287839 B1	11/09/2001
		JP 2004500010T T	08/01/2004
		EP 1088080 A1	04/04/2001
		CA 2330245 A1	24/02/2000
		AU 4409499 A	06/03/2000
		AU 752901 B2	03/10/2002
		WO 9925846 A2	27/05/1999
		NZ 504197 A	01/03/2002
		JP 2001523463T T	27/11/2001
		EP 1032686 A2	06/09/2000
		CA 2309764 A1	27/05/1999
		AU 1419099 A	07/06/1999
		AU 749780 B2	04/07/2002
		US 6187577 B1	13/02/2001
.....			
US 6083734 A	04/07/2000	JP 11318474 A	24/11/1999
		CA 2262989 A1	04/09/2000
.....			