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(54) Title: MODIFIED XYLANASES EXHIBITING IMPROVED EXPRESSION

(57) Abstract: A modified Family (11) xylanase enzyme comprising a sequence that introduces a functional consensus glycosylation site is provided. Non-limiting examples of introduced glycosylation sites include mutation of the amino acid at position (34, 131, 180, 182), or a combination thereof, to an asparagine. The indicated amino acid position in the Family (11) xylanase is determined from sequence alignment of the xylanase of interest with that of a *Trichoderma reesei* xylanase II amino acid sequence. The introduced consensus glycosylation site facilitates increased expression efficiency of the modified xylanase when compared to the expression efficiency of a corresponding xylanase from which the modified xylanase was derived, using similar host strains and growth conditions.

MODIFIED XYLANASES EXHIBITING IMPROVED EXPRESSION

[0001] The present invention relates to xylanases with improved expression, more particularly to the improved expression and secretion of xylanases from a host.

BACKGROUND OF THE INVENTION

[0002] Xylanases, produced by many species of filamentous fungi and bacteria, are a group of enzymes with wide commercial utility. A major application of xylanases is for biobleaching pulp in the production of paper. In addition, xylanases have been used as clarifying agents in juices and wines, as enzymatic agents in the washing of precision devices and semiconductors and they are also used for improving digestibility of poultry and swine feed.

[0003] Most xylanases exploited for industrial applications are members of Family 11, showing diversity in their biochemical and biophysical properties. For example, thermostable xylanases have been isolated from bacteria (US 6,667,170), fungi (US 6,635,464), or other extreme thermophiles (Lüthi et al. 1990; Winterhalter et al. 1995; Simpson et al. 1991). Alternatively, xylanase performance has been optimised for various industrial applications via protein engineering (e.g. U.S. 5,759,840; U.S. 5,866,408; U.S. 5,405,769; and Turunen et al., 2001).

[0004] Successful implementation of xylanase enzymes in industrial applications requires economical production from a host microbe, which secretes the xylanase into the culture broth during submerged fermentation. This is particularly necessary for the large-scale production of xylanases from thermophiles or extreme thermophiles that are difficult to culture or do not secrete sufficiently high levels of protein. Typically, the host microbe for the production of industrial enzymes is a filamentous fungus such as *Trichoderma, Aspergillus* or *Fusarium*, an actinomycete such as *Streptomyces* or a species of *Bacillus* bacteria. This means that the genes encoding a target xylanase, whether isolated from a different organism or from protein engineering of a xylanase gene from the host organism, must be cloned into the production host in such a way that the gene is operably linked to the DNA sequences that will facilitate its expression and secretion from the host.

[0005] Expression and secretion of exogenous proteins by genetic modification of industrial strains of *T. reesei* has remained a significant challenge for many years (Conesa et al., 2001). Expression of heterologous proteins in *T. reesei* elicits an Unfolded Protein Response (UPR; Saloheimo et al., 1999), which results from an accumulation of unfolded or misfolded nascent polypeptides in the lumen of the endoplasmic reticulum (ER). Because of the limited information currently available on the mechanisms regulating folding and secretion of the Family 11 xylanases from *T. reesei*, several strategies have been implemented to facilitate high-level expression of related exogenous xylanases in *T. reesei* host strains. These include the use of highly inducible promoters, such as those of the *T. reesei* cellulase genes, and replacement of the native cellulase genes with xylanase expression constructs containing highly inducible promoters.

[0006] Expression of bacterial xylanases from *T. reesei* may require fusion of the xylanase to a carrier *T. reesei* polypeptide with an intact domain structure, such as the catalytic core or binding domains of the *T. reesei* mannanase I or CBH II proteins (Paloheimo, et al., 2003). This strategy, alone or in combination with deletion of one or more cellulase gene(s) from the host *T. reesei* strain, was disclosed in US 6,635,464 and US 6,667,170 to direct the expression thermophilic Family 11 xylanases from both bacterial (*A. flexuosa*) and fungal (*C. thermophilus*) sources. Although the carrier polypeptide certainly increased the production and secretion of the heterologous xylanases disclosed in US 6,635,464 and US 6,667,170 from *T. reesei* host strains, it is not always desirable to have a carrier polypeptide attached to the xylanase enzyme for industrial applications. In these cases, the carrier polypeptide would need to be removed by proteolysis subsequent to secretion of the fusion protein into the culture broth and prior to its use in the application. However proteolysis adds both time and cost to the overall production of the target xylanase due to cost and incubation time required for the proteolysis step itself as well as potential yield losses of the target xylanase during the proteolytic removal of the carrier polypeptide.

[0007] This strategy of using a fusion of a target protein to a carrier protein native to the host cell has also been employed successfully to increase the production and secretion of mammalian chymosin from *Aspergillus* (Van den Brink *et al.*, WO 02/36752 and WO 03/106484). WO 03/106484 discloses further improvements in the production and secretion of glucoamylase-chymosin fusion proteins from *Aspergillus* by the introduction of an N-glycosylation motif within the artificial linker polypeptide between the chymosin and

glucoamylase fusion partners or within the chymosin peptide sequence. However, there was no demonstration of the benefits of chymosin production from *Aspergillus* via the introduction of a glycosylation motif in a construct not containing a fusion partner.

[0008] Sagt *et al.* (2000) report improvements in secretion of a target protein from a heterologous eukaryotic host via introduction of an N-glycosylation motif within the target protein. In this report, introduction of an N-glycosylation site into the sequence of a hydrophobic mutant of either a fungal cutinase or of native llama antibody fragments resulted in increased secretion of the target protein from *Saccharomyces* of *Pichia* yeast host. However, introduction of the glycosylation site into the native fungal cutinase did not result in any increase in expression from the heterologous yeast hosts.

[0009] WO 02/02597 reports the production of the FSH-alpha subunit and glucocerebrosidase polypeptides containing a glycosylation site. The goal of these studies was to improve the stability and expression of these polypeptides. However, the applicability of the method was only demonstrated using the addition of short nucleotide sequences encoding the N-glycosylation motif rather than via direct modification of the primary peptide sequence.

[0010] It is an object of the present invention to provide modified xylanases exhibiting improved expression.

SUMMARY OF THE INVENTION

[0011] The present invention relates to xylanases with improved expression, more particularly to the improved expression and secretion of xylanases from a host.

[0012] The present invention provides a modified Family 11 xylanase comprising, a sequence that introduces a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived. The modified Family 11 xylanase may comprise a substitution of an amino acid at a position selected from the group consisting of position 34 (X34N), position 131 (X131N), position 180 (X180N), position 182 (X182N), and a combination thereof, to an asparagine, the position determined from sequence alignment of the Family 11 xylanase with the amino acid

sequence of *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1. The present invention also pertains to a modified Family 11 xylanase as described above, and further comprising, a substitution of an amino acid at a position selected from the group consisting of position 36 (X34N-S36T), position 182 (X180N-S182T), position 184 (X182N-S184T), and a combination thereof, to a threonine. Preferably, the modified Family 11 xylanase comprises a X131N mutation. Also provided is a modified Family 11 xylanase selected from the group consisting of: ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5', Xln1-131N, and *S. lividans* xlnC-131N.

[0013] The present invention is directed to a modified Family 11 xylanase as described above, wherein the modified xylanase when expressed in a *Trichoderma* host strain exhibits an increase in expression efficiency of at least 40% when compared to the expression efficiency of a Family 11 xylanase from which the modified xylanase is derived.

[0014] The present invention also provides a modified Family 11 xylanase genetic construct comprising a promoter operatively linked to a secretion signal that is operatively linked to a coding region, the coding region comprising a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived, the modified xylanase genetic construct resulting in an increase in expression efficiency of an encoded modified xylanase when compared to the expression efficiency of an encoded Family 11 xylanase from which the encoded modified xylanase was derived.

[0015] Furthermore, there is provided a genetically modified microbe comprising the modified Family 11 xylanase genetic construct as just described. Preferably, the genetically modified microbe comprises a member of the genus of *Trichoderma* or *Hypocrea*. Furthermore, the genetically modified microbe comprises a secretion signal that is a *Trichoderma* secretion signal, for example a *Trichoderma* xylanase secretion signal.

[0016] The present invention also pertains to a genetically modified microbe comprising a coding region that encodes a modified xylanase selected from the group consisting of: ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5',Xln1-131N, and *S. lividans* xlnC-131N.

[0017] The present invention provides a method of processing food or feed comprising, treating the food or feed with an additive comprising the modified Family 11 xylanase

comprising, a sequence that introduces a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived. For example the food or feed additive may be selected from the group consisting of a poultry feed additive, a swine feed additive, a food additive used in baking, or a food additive used in brewing.

[0018] The present invention also pertains to a method of paper pulp manufacturing comprising treating the pulp with a modified family 11 xylanase comprising, a sequence that introduces a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived.

[0019] The present invention pertains to a use of a modified xylanase comprising, a sequence that introduces a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived, in an industrial or food or feed process. The industrial process may be paper pulp manufacturing.

[0020] The present invention provides for modified Family 11 xylanases with improved expression and secretion from a *Trichoderma* host without any apparent change in the biochemical properties of the enzyme. The resulting increase in the specific xylanase production and overall protein productivity of the strain facilitates the economical manufacturing of Family 11 xylanase products for industrial applications. Furthermore, in embodiments of the invention, N-glycosylation sites may be introduced into regions of conserved sequence homology at the beginning, middle or end of the Family 11 peptide sequence. This is achieved without any adverse effects on the function of the xylanase.

[0021] This summary of the invention does not necessarily describe all features of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

[0023] **FIGURE 1** shows the alignment of the amino acid sequences of Family 11 xylanases with numbering from N-terminus, where Bp - *Bacillus pumilus* (SEQ ID NO:31); Ca - *Clostridium acetobutylicum* P262 xynB (SEQ ID NO:33); Cs - *Clostridium stercorarium*

xynA (SEQ ID NO:34); Rf - *Ruminococcus flavefaciens* (SEQ ID NO:35); Tr2 - *Trichoderma reesei* xyn2 (SEQ ID NO:1); Tv - *Trichoderma viride* (SEQ ID NO:42); Th - *Trichoderma harzianum* (SEQ ID NO:41); Sc - *Schizophyllum commune* xynA (SEQ ID NO:36); An - *Aspergillus niger*, var. awamori (SEQ ID NO:42); Ak - *Aspergillus kawachii XynC* (SEQ ID NO:43); At - *Aspergillus tubigensis* (SEQ ID NO:29); Tr1 - *Trichoderma reesei* xyn1 (SEQ ID NO:2); Aa - *Aspergillus awamorovar.kawachi Xyn B* (SEQ ID NO:28); Fs - *Fibrobacter succinogenes Xyn II* (SEQ ID NO:44); Ss - *Streptomyces* sp. 36a (SEQ ID NO:37); SlB - *Streptomyces lividans* xynB (SEQ ID NO:38); SlC - *Streptomyces lividans* xynC (SEQ ID NO:39); Tl – *Thermomyces lanuginosus Xyn* (SEQ ID NO:45); Tf - *Thermomonospora fusca* TfxA (SEQ ID NO:40); Bc - *Bacillus circulans* (SEQ ID NO:30); Bs - *Bacillus subtilis* (SEQ ID NO:32);

[0024] **FIGURE 2** shows a map of the vector pC/XHML-TV used to direct the expression of a modified xylanase in *T. reesei.*

[0025] **FIGURE 3** shows a map of the general mutagenesis vector pALT-H(I)TXn, where "n" is a descriptor, for example "13" or "18", and the vector may comprise a 131 N mutation (i.e., pALT-ITXn), or the vector may not comprise a 131N mutation (i.e., pALT-HTXn). For example when "n" is 18, and the vector does not comprise the 131N mutation, the vector is pALT-HTX18.

[0026] **FIGURE 4** shows a map of the general vector pc/xH(I)TXn-TV used to direct the expression of a modified xylanases in *T. reesei*, where "n" is the descriptor, for example "13", "18", "18(R135Y)", or "1(N131Q) and the vector may comprise a 131 N mutation (i.e., pc/xITXn-TV), or the vector may not comprise a 131N mutation (i.e., pc/xHTXn-TV). For example when "n" is 18, and the vector does comprise the 131N mutation, the vector is pc/XITXn-TV.

FIGURE 5 shows maps of the vectors used to direct the expression of native (and modified) *T. reesei* xylanase I and *S. lividans* xylanase C, respectively, in *T. reesei.* Figure 5A: pc/xXYN1-(T118N)-TV; Figure 5B: pc/xXYLC-(T128N)-TV

[0027] **FIGURE 6** shows the pH activity profiles for the modified xylanase ITX1, and its native counterpart HTX18.

[0028] **FIGURE 7** shows the temperature activity profile for the modified xylanase ITX1 and its native counterpart HTX18.

DESCRIPTION OF PREFERRED EMBODIMENT

[0029] The present invention relates to xylanases with improved expression, more particularly to the improved expression and secretion of xylanases from a host.

[0030] The following description is of a preferred embodiment. Xylanases and modified xylanases, as outlined herein, may be used for the purposes of bleaching paper pulp or other applications requiring activities typically at temperatures and pH above that of the wild-type enzyme. For the biobleaching of pulp, the preferred xylanase is derived from a xylanase classified in Family 11 (see Table 1).

[0031] Xylanases are produced by many species of filamentous fungi and bacteria, and can be classified into two families, Family 10 or 11, based primarily on structural and mechanistic similarities (Henrissat, 1991). Family 11 xylanase enzymes are a group of small enzymes of relatively low molecular mass (approximately 20 kDa, and about 200 amino acid residues).

[0032] The Family 11 xylanases secreted by *T. reesei* are not glycosylated, which is consistent with the absence of a consensus N-glycosylation motif in the amino acid sequence of xylanase I (Törrönen et al., 1992) However, xylanase II is also not glycosylated, despite the presence of two N-glycosylation consensus motifs in its sequence. In contrast, *T. reesei* cellulases are N-glycosylated at asparagine residues within the consensus motif Asn-Xaa-Ser/Thr, where Xaa is any amino acid other than proline (or N-X-S/T, where X is any amino acid other than proline). However, not all of the potential sites within the various cellulase enzymes are glycosylated (Hui et al., 2001 and 2002). This suggests that the *Trichoderma* organism does not recognize some consensus motifs in native amino acid sequences.

[0033] The present invention provides a modified Family 11 xylanase comprising a glycosylation sequence or motif, for example, but not limited to Asn-Xaa-Ser/Thr, Asn-Xaa-Thr, or Asn-Xaa-Ser, that is otherwise not present in the corresponding xylanase from which the modified xylanase is prepared or derived.

[0034] Furthermore, the present invention provides a modified Family 11 xylanase having one or more than one amino acid selected from positions 34, 131, 180 and 182 substituted to an asparagine (Asn or N) wherein the position is determined from sequence alignment of the Family 11 xylanase with *Trichoderma reesei* xylanase II amino acid

sequence defined in SEQ ID NO:1. Such a substitution may be described as: X34N, X131N, X180N or X182N, where the amino acid "X" is substituted by asparagine or "N" at the indicated position. For example in X131N, indicates that the amino acid "X" at position 131 (as determined from sequence alignment of the Family 11 xylanase with *Trichoderma reesei* xylanase II (TrX II) amino acid sequence defined in SEQ ID NO:1) is substituted by asparagine or "N". Preferably, the mutation is at position 131, producing X131N, or its corresponding position in another Family 11 xylanase as determined by sequence alignment with TrX II (SEQ ID NO:1). It has been observed that the modified xylanase comprising one or more than one of these mutations, for example, the X131N substitution, exhibits an improved expression efficiency compared to the Family 11 xylanase from which the modified xylanase was produced or derived. Examples of constructs comprising the X34N, X131N, X180N or X182N mutations include ITX5 and ITX5', ITX1 and ITX2, ITX3 and ITX3', and ITX4 and ITX4', respectively.

[0035] Additional mutations for example, X34N-S36T, X180N-S182T, and X182N-S184T may also be introduced into the Family 11 xylanase to produce the consensus sequence Asn-Xaa-Thr, thereby ensuring that a Thr is positioned upstream from the Asn within the xylanase. Examples of constructs comprising the X34N,S36T; X180N,S182T; or X182N,S184T mutations include ITX5', ITX3', and ITX4', respectively.

[0036] The modified xylanase of the present invention may be derived from any Family 11 xylanase, for example a xylanase that is native to *Trichoderma*, including but not limited to *T. reesei* xylanase II, *T. reesei* xylanase I, *Trichoderma viride* xylanase, or a xylanase from *Aspergillus, Fusarium*, an actinomycete such as *Streptomyces* for example, but not limited to, *Streptomyces lividans* xylanase B and *Streptomyces lividans* xylanase C, or a xylanase from *Bacillus, Thermobifida, Actinamadura, Chaetomium, or Thermatoga* .

[0037] Modification of *T. ressei* xylanase I (TrX I) to introduce an equivalent mutation at position 131, as determined by comparison with the sequence of *T. ressei* xylanase II (TrX-II; SEQ ID NO:1), requires a mutation at position 118 of TrX-I (i.e. the mutation T118N). In this case *T. ressei* xylanase I, with a substitution at T118N, comprises an equivalent mutation to that of X131N as found in TrX II (see Figure 1 for alignments of "Tr1" and "Tr2"). Similarly, modification of *S. lividans* xylanase C, to introduce an equivalent mutation to that at position 131, as determined by comparison with the sequence of TrX-II, requires a mutation at position 128 of *S. lividans* xylanase C (i.e. the mutation

T128N). In this case xylanase T128N in *S. lividans* xylanase C comprises an equivalent mutation to that of X131N in TrX-II (see Figure 1 for alignments of "SlC" and "Tr2").

[0038] By the term "xylanase", it is meant an enzyme that hydrolyzes xylan to xylose. Xylanases may possess varying properties, including structure (molecular weight, three-dimensional orientation, amino acid composition, and active site) and catalytic activity (rate and kinetics of xylan hydrolysis, and ability to act on other substrates) as is known to one of skill in the art.

[0039] The modified xylanase of the present invention may be derived from a native, or wild-type xylanase, or it may be derived from an already altered xylanase that has been mutagenized and selected or genetically engineered using standard protocols as would be known to one of skill in the art, for example site directed mutagenesis, chemical mutagenesis, or equivalent methods, to alter its pH profile, temperature profile, substrate specificity, or a combination thereof. Examples of such altered xylanases include those disclosed herein, for example but not limited to HTX18 and HTX18-R135Y. Additional examples of altered, or genetically engineered, xylanases that may also be further modified as described herein, include those that are known to one of skill in the art, for example but not limited to those disclosed in WO 00/29587, WO 01/92487 and WO 03/046169 (which are incorporated herein by reference), and include, but are not limited to, TrX-DS1; TrX-162H-DS1; TrX-162H-DS2; TrX-162H-DS4; TrX-162H-DS8; TrX-75A; TrX-HML-105H; TrX-HML-75A-105H; TrX-HML-75C-105R; TrX-HML-75G-105R; TrX-HML-75G-105R-125A-129E; TrX-HML-75G-105H-125A-129E; TrX-HML-75A-105H-125A-129E; TrX-HML-75A-105R-125A-129E; TrX-157D-161R-162H-165H; TrX-HML-AHAE; TrX-HML-AHAE-R; TrX-HML-AHAE-RR; TrX-HML-AHAE-RRR; TrX-HML-AHA-RR-DRHH; TrX-HML-AHAE-RR-DRHH; TrX-HML-AHAE-RRR-DRHH; TrX-116G; TrX-118C; TrX- HML-AHCAE-R; TrX-H-11D-ML-AHGAE-RR; TrX-HML-AHGAE-R; TrX-H-11D-ML-AHGCAE-RR; TrX-H-11D-ML-AHCAE-RR.

[0040] A native xylanase or wild-type xylanase is a xylanase that has not been modified or altered outside of the regular course of nature. A native xylanase may comprise mutations that occur naturally.

[0041] By "*Trichoderma reesei* xylanase II sequence alignment" or "TrX numbering" it is meant the numbering associated with the position of amino acids based on

the amino acid sequence of *Trichoderma reesei* xylanase II (also referred to as TrX II; see Table 1, Tr2; Figure 1; and SEQ ID NO:1). TrX II is a member of the Family 11 xylanases. Family 11 xylanases exhibit a substantial degree of sequence similarity (see Figure 1), therefore, by aligning the amino acids to optimize the sequence similarity between xylanase enzymes and by using the amino acid numbering of TrX II (*Trichoderma reesei* xylanase II) as the basis for numbering, the positions of amino acids within other xylanase enzymes can be determined relative to TrX II.

[0042] Structural studies indicate that Family 11 xylanases from bacterial and fungal origins share the same general molecular structure (e.g. U.S. 5,405,769), exhibiting three types of secondary structure: beta-sheets, turns and a single alpha helix. A xylanase can be classified as a "Family 11 xylanase" if it comprises similarity to other Family 11 xylanases, in particular two glutamic acid residues at positions 86 and 177 (based on *Trichoderma reesei* xylanase II (TrX II) amino acid numbering) that may serve as catalytic residues. Family 11 xylanases may include those listed in Table 1. Preferably, the xylanase is a *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase I, *Trichoderma viride* xylanase, *Streptomyces lividans* xylanase B, *Streptomyces lividans* xylanase C, or a xylanase from *Aspergillus, Fusarium,* or *Bacillus.*

TABLE 1. Representative Family 11 xylanase enzymes from bacteria and fungi.

Microbe	Xylanase	SEQ ID NO:	Swiss Prot No
Aspergillus niger	Xyn A	SEQ ID NO:43	-
Aspargillus awamori var.kawachi	Xyn B	SEQ ID NO:28	P48824
Aspergillus kawachii	Xyn C	SEQ ID NO:43	-
Aspergillus tubigensis	Xyn A	SEQ ID NO:29	-
Bacillus circulans	Xyn A	SEQ ID NO:30	P09850
Bacillus pumilus	Xyn A	SEQ ID NO:31	P00694
Bacillus subtilis	Xyn A	SEQ ID NO:32	P18429
Cellulomonas fimi	Xyn D	-	P54865
Chainia spp	Xyn	-	-
Clostridium acetobutylicum	Xyn B	SEQ ID NO:33	-
Clostridium stercorarium	Xyn A	SEQ ID NO:34	P33558
Fibrobacter succinognes	Xyn II	SEQ ID NO:44	-
Neocallimasterix patriciarum	Xyn A	-	P29127
Nocardiopsis dassonvillei	Xyn II	-	-
Ruminococcus flavefaciens	Xyn A	SEQ ID NO:35	P29126
Schizophyllum commune	Xyn A	SEQ ID NO:36	P35809
Streptomyces sp. No. 36a	Xyn	SEQ ID NO:37	
Streptomyces lividans	Xyn B	SEQ ID NO:38	P26515
Streptomyces lividans	Xyn C	SEQ ID NO:39	P26220
Streptomyces thermoviolaceus	Xyn II	-	-
Thermomonospora fusca	Xyn A	SEQ ID NO:40	-
Thermomyces lanuginosus	Xyn A	SEQ ID NO:45	O43907
Trichoderma harzianum	Xyn	SEQ ID NO:41	P48793
Trichoderma reesei	Xyn I	SEQ ID NO:2	P36218
Trichoderma reesei	Xyn II	SEQ ID NO:1	P36217
Trichoderma viride	Xyn	SEQ ID NO:42	-

[0043] A modified xylanase of the present invention is any xylanase that is engineered to introduce or comprise a changed glycosylation site when compared to the xylanase from which the modified xylanase was prepared or derived.

[0044] Non-limiting examples of such modifications include X34N, X34N-S36T, X131N, X180N, X180N-S182T, X182N, or X182N-S184T (TrX numbering), or a combination thererof. Preferably, the modified xylanase is a Family 11 xylanase. The modified xylanase of the present invention may comprise *Trichoderma reesei* xylanase I or II enzymes, or the *Streptomyces lividans* xylanase B or C enzymes. It is generally recognized that the amino acid sequence of a natural xylanase may be tailored to alter its biochemical or biophysical properties. An example of a modified xylanase of the present invention comprises the X131N mutation, or its equivalent as determined by comparing the sequence alignment of the xylanase of interest with that of TrX II (SEQ ID NO:1) and other modifications, substitutions or deletions relative to the corresponding native xylanase. Several examples of modified xylanases that are not to be considered limiting are shown in Table 2.

[0045] The substitution at position 131 to asparagine, in conjunction with a Thr/Ser at position 133, which is highly conserved in Family 11 xylanases, results in the creation of a N-glycosylation motif: Asn-Xaa-Thr/Ser. It has been observed that xylanases comprising the 131N mutation result in an increased production of xylanase. Without wishing to be bound by theory, the introduction of the N-glycosylation motif may result in increased expression efficiency, decreased degradation, increased secretion, or a combination thereof, of the modified xylanase when compared to the native xylanase enzyme lacking the 131N modification. The modified xylanase may exhibit improved expression from a *Trichoderma* host strain and exhibit similar biochemical and biophysical properties, in comparison to the corresponding native Family 11 xylanase. Similar mutations may also be prepared at other sites adjacent to conserved Thr/Ser in xylanase, for xample but not limited to X34N, X180N and X182N (ITX 2, ITX 3 and ITX 4, respectively; see Table 2), to produce the Asn-Xaa-Thr/Ser sequence. In each of these locations the amino acid Ser is conserved, and 3-D modelling of the folded protein indicates that these sites would be positioned on the outer surface of the protein. Additional modifications that may be made include X34N-S36T, X180N-S182T or X182N-S184T (ITX5', ITX3' and ITX4', respectively) to produce the glycosylation motif Asn-Xaa-Thr.

[0046] Those skilled in the art are aware that amino acid substitutions can be made by a number of methods, for example site-directed or random mutagenesis to alter the primary peptide sequence of the xylanase to produce a consensus N-glycosylation motif. Any suitable

method may be used to introduce the X34N, X131N, X180N, X182N, X34N-S36T, X180N-S182T or X182N-S184T mutation into the Family xylanase gene. For example, the N-glycosylation motifs may be introduced by direct substitution of one or more codons within the primary xylanase sequence rather than addition of extra nucleotides encoding the N-glycosylation motif so as to increase the production of the xylanase from the host without changing the biophysical and biochemical properties of the enzyme.

[0047] By direct substitution it is meant that the glycosylation site is introduced by introducing specific nucleotide changes within the xylanase coding region that alter the primary peptide sequence without changing its length through the addition or deletion of one or more amino acids.

[0048] As shown in Example 11, and with reference to Figures 5 and 6, the ITX1 xylanase, comprising the T131N mutation and otherwise having similar mutations as HTX18 (see Table 2; ITX1 lacks the Y135R mutation), has a similar pH and temperature activity profile as the HTX18 xylanase. Thus the addition of the T131N mutation does not alter the biophysical and biochemical properties of the xylanase. Furthermore, as shown in Tables 3 and 4 of Examples 9 and 10, respectively, the ITX1 enzyme is produced with an increase in expression efficiency of about 73% -100% when compared to HTX18 or HTX18(R135Y).

TABLE 2: Examples of Modified xylanases

Xylanase	Description
TrX-HML	TrX with N10H, Y27M, and N29L (see U.S. 5,759,840)
HTX13	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E
HTX18	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, Y135R, H144R, N157D, Q161R, Q162H and Q165R
ITX1	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, **T131N**, H144R, N157D, Q161R, Q162H and Q165R
ITX2	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, **T131N**, Y135R, H144R, N157D, Q161R, Q162H and Q165R
ITX3	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, Q165R and **F180N**
ITX3'	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, Q165R, **F180N and S182T**
ITX4	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, Q165R and **S182N**
ITX4'	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, Q165R, **S182N and S184T**
ITX5	TrX with N10H, Y27M, N29L, **Q34N**, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and Q165R
ITX5'	TrX with N10H, Y27M, N29L, **Q34N, S36T**, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and Q165R
HTX18-R135Y	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and Q165R
Xln1(131N)	*T. reesei* xylanase I with mutation **T118N**
S. lividans xlnC(131N)	*S. lividans* xylanase C with mutation **T128N**

[0049] "Expression efficiency" is the amount of active enzyme, or enzymatic activity, produced by a production host. The expression efficiency may be calculated as the quantity of active enzyme or enzymatic activity generated per unit volume of the fermentation culture when all fermentation conditions remain constant. A first xylanase will be considered as having higher expression efficiency compared to a second xylanase if the first xylanase is produced in levels that are higher than a second xylanase by the same host at the same fermentation conditions. For example if the first xylanase is produced at an amount that is greater than about 40% to about 2500%, or an amount there between, than the second xylanase by the same host at the same fermentation conditions, then the expression efficiency of the first xylanase is greater than that the second xylanase. For example, the first xylanase may be produced at an amount that is greater than 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1250, 1500, 1750, 2000, 2250, or 2500%, or an amount there between, than the second xylanase by the same host at the same fermentation conditions. Preferably, the

first xylanase is produced at an amount that is at least 50% more than the second xylanase by the same host at the same fermentation conditions (see Examples 9 and 10).

[0050] By "operatively linked" it is meant that the particular sequences interact either directly or indirectly to carry out an intended function, such as mediation or modulation of gene expression. The interaction of operatively linked sequences may, for example, be mediated by proteins that interact with the operatively linked sequences.

[0051] By "xylanase gene" it is meant a region of DNA that includes the sequence that encodes the xylanase enzyme. The xylanase gene may encode a native or a modified xylanase. A xylanase gene may further comprise a promoter, secretion signal, coding region and transcriptional terminator.

[0052] A "xylanase genetic construct" refers to nucleic acid sequence comprising the elements necessary to produce and secrete a native xylanase, or a modified xylanase. Preferably, the xylanase genetic construct is optimized to permit expression from a suitable production host, for example but not limited to, production from a *Trichoderma* host. These elements include:

A xylanase coding region.

[0053] A xylanase coding region comprises the DNA sequence necessary to encode a functional xylanase as isolated from extracellular culture filtrates. The xylanase coding region may be comprised of a sequence encoding a native xylanase, a sequence encoding an altered xylanase that has previously been engineered, a modified xylanase as described herein, and a combination thereof. The modified xylanase coding region may include the X34N, X131N, X180N, X182N, X34N-S36T, X180N-S182T or X182N-S184T mutation (TrX II numbering), but does not include a secretion signal at the amino terminal end. The xylanase coding region may be from a Family 11 xylanase gene that has previously been altered (see non-limiting examples provided above, for example but not limited to those disclosed in WO 00/29587, WO 01/92487 and WO 03/046169; which are incorporated herein by reference), or it may be from a natural Family 11 xylanase, for example from a *Trichoderma* or *Streptomyces* gene. For example, but not to be considered limiting, the modified xylanase coding region of the present invention may be derived from a natural or an engineered coding region of *T. reesei xln1, T. reesei xln2, or S. lividans xlnC.*

[0054] As understood by one of skill in the art, a natural coding region can be altered or engineered by replacement, substitution, addition, or elimination of one or more nucleic acids without changing its function (i.e. xylanase activity). The practice of this invention is not constrained by such alterations to the xylanase coding region.

A secretion signal.

[0055] A "secretion signal" is a peptide sequence present within the secreted protein, typically at the amino terminus of a secreted protein, which directs entry of the protein into the endoplasmic reticulum (ER); the secretion signal may subsequently be cleaved from the mature secreted protein by a signal peptidase.

[0056] The coding region of a modified xylanase gene of the present invention may be operably linked to a DNA sequence encoding any secretion signal (i.e., linked in such a manner that the transcribed sequence may be directed to the ER) that is functional in a desired production host, for example, but not limited to, *Trichoderma*. The xylanase secretion signal may, for example, be from any secreted *Trichoderma* protein, for example from a *Trichoderma* xylanase, or from another fungal or bacterial protein. Without wishing to be limiting, the secretion signal may be from the *Trichoderma reesi* xylanase I (*xln1*) gene or xylanase II (*xln2*) gene.

[0057] Those skilled in the art are aware that a natural secretion signal can be modified by replacement, substitution, addition, or elimination of one or more nucleic acids without changing its function as a secretion signal. The practice of the invention is not constrained by such alterations to the secretion signal.

A promoter.

[0058] The practice of this invention is not constrained by the choice of promoter in the genetic construct. It is preferred that the promoter is functional in the production host. The promoter is operably linked to the coding region of the modified xylanase gene, or it is operatively linked to the secretion signal which is operatively linked to the coding region of the modified xylanase gene, so that the promoter controls the expression of the coding region, or the secretion signal and coding region, respectively. Without wishing to be limiting in any manner, preferred promoters that may be used in the practice of the present invention include the *Trichoderma cbh1, cbh2, eg1, eg2, eg3, eg5, xln1* and *xln2* promoters, or a combination of two or more than two of these promoters.

[0059] Those skilled in the art are aware that a natural promoter can be modified by replacement, substitution, addition, or elimination of one or more nucleic acids without changing its function as a promoter. The practice of the invention is not constrained by such alterations to the promoter.

Additional sequences between the secretion signal and the mature xylanase coding region.

[0060] The xylanase genetic construct may contain additional sequences that encode the additional amino acids between the secretion signal and the xylanase coding region, or the modified xylanase coding region as described herein. These sequences, which may be natural or synthetic, may encode one or more of the amino acids of the mature protein corresponding to the secretion signal encoded by the construct or may result from the addition of restriction enzyme sites needed to join the sequences encoding the secretion signal and modified xylanase coding region. The practice of the invention is not constrained by the presence of additional DNA sequences between those encoding the secretion signal and the mature xylanase coding region.

Other elements.

[0061] The xylanase genetic construct may contain a transcriptional terminator that is functional in the production host, as would be known to one of skill in the art. The transcriptional terminator may be positioned immediately downstream of the xylanase coding region. The practice of the invention is not constrained by the choice of transcriptional terminator that is sufficient to direct the termination of transcription by an RNA polymerase in the production host. An example of a transcriptional terminator which is not to be considered limiting in any manner, comprises 1.9 kb of DNA 3' to the stop codon of the *Trichoderma cbh2* gene, as described in Examples 5.1-5.4.

[0062] The xylanase genetic construct may contain a selectable marker for determining transformation of the production host. The selectable marker may be present on the same plasmid vector, upstream or downstream of the genetic construct (i.e., at the 5' or 3' end of the construct), or the selectable marker may be co-transformed with the construct on a separate plasmid vector.

[0063] Choices of selectable markers are well known to those skilled in the art and include genes (synthetic or natural) that confer to the transformed cells the ability to utilize a metabolite that is not normally metabolized by the microbe (e.g., the *A. nidulans amdS* gene

encoding acetamidase and conferring the ability to grow on acetamide as the sole nitrogen source) or antibiotic resistance (e.g., the *Escherichia coli hph* gene encoding hygromycin-β-phosphotransferanse and conferring resistance to hygromycin). If the host strain lacks a functional gene for the marker chosen, then that gene may be used as a marker. Examples of such markers include *trp, pyr4, pyrG, argB, leu,* and the like. The corresponding host strain would therefore have to be lacking a functional gene corresponding to the marker chosen, i.e. lacking in the expression of *trp, pyr, arg, leu* and the like. A non-limiting example of a selectable marker used in the genetic constructs is described in Example 5.1. In this example, the selectable marker is an *E. coli hph* gene expressed using a *Trichoderma* phosphoglycerate kinase (*pgk*) promoter. An alternate selectable marker is described in Example 5.2 and comprises the *Neurospora crassa pyr4* gene expressed from its native promoter.

[0064] The present invention provides genetic constructs and genetically modified production hosts, for example *Trichoderma* strains, expressing modified xylanases that introduce or alter a glycosylation site in xylanase. Non-limiting examples of modified xylanases comprising an introduced glycosylation site include one or more than one of X34N, X131N, X180N, X182N, X34N-S36T, X180N-S182T or X182N-S184T mutation (TrX II numbering).

[0065] The modified xylanase genetic construct of the present invention is not constrained by the method of making the construct which can include, but is not limited to, standard molecular biology techniques such as isolation of plasmid DNA from *E. coli* by alkaline lysis, digestion of plasmid DNA with restriction endonucleases, separation and isolation of DNA fragments by agarose gel electrophoresis, ligation of DNA fragments with T4 DNA ligase, insertion of unique restriction sites at the ends of DNA fragments by polymerase chain reaction or the addition of oligonucleotide linkers, and the blunting of DNA fragments with T4 DNA polymerase or Klenow fragment of *E. coli* DNA polymerase I. Such a procedure is described in Examples 1-5.

[0066] In a further aspect of the present invention, the modified xylanase genetic construct is introduced into and expressed in a desired microbial (production) host. Preferably the expression efficiency for the modified xylanases from the resulting recombinant microbe is increased. For example the expression efficiency may be at least, 40% or more, higher than the expression efficiency for the corresponding Family 11 xylanase produced from the corresponding genetic construct from which the modified xylanase was

derived or produced, in the same microbial host grown under similar fermentation conditions. For example, the first xylanase may be produced at an amount that is greater than 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 425, 450, 474, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1250, 1300, 1500, 1750, 2000, 2250, or 2500%, or any amount there between, than the second xylanase by the same host at the same fermentation conditions. Preferably, the first xylanase is produced at an amount that is at least 50% more than the second xylanase by the same host at the same fermentation conditions (see Examples 9 and 10).

[0067] The practice of the present aspect of the invention is not constrained by the method of introducing the xylanase genetic construct into the microbial host (production host). Methods of introducing the DNA construct into a production host are familiar to those skilled in the art and include, but are not limited to calcium chloride treatment of bacterial cells or fungal protoplasts to weaken the cell membranes, addition of polyethylene glycol to allow for fusion of cell membranes, depolarization of cell membranes by electroporation, or shooting the DNA through the cell wall and membranes via microprojectile bombardment with a particle gun.

[0068] The production host may be a member of the species of *Trichoderma* (which has been classified at various times as *T. viride*, *T. longibrachiatum* and, most recently, as *Hypocrea jecorina*—Simmons, 1977; Bissett, 1984; Cannon 1986; Kuhls et al., 1996). These species are well suited because they produce Family 11 xylanases. In addition, methods have been published for the introduction of DNA constructs into cellulase-producing strains of *Trichoderma* (Lorito et al., 1993; Goldman et al., 1990; Penttila et al., 1987).

[0069] Example 7.1 describes one procedure for introducing a xylanase genetic construct into *Trichoderma* spores using a particle gun. Example 7.2 describes a procedure for introducing a xylanase genetic construct into *Trichoderma* protoplasts treated with polyethylene glycol and calcium chloride.

[0070] An increase in the expression efficiency, for example a 50% enhancement of expression efficiency, over a native Family 11 xylanase, reflects a significant enhancement that is well above the natural variability of the strain and is commercially significant. Results show that the degree of enhancement of xylanase production by this method can be as high as

2-fold and could reach over 10-fold. The measurement of the degree of enhancement of xylanase production is by growth of the culture and measurement of the xylanase activity, as described in Example 8.

[0071] It is understood by those skilled in the art that the specific xylanase activity of an enzyme mixture (in IU/mg protein) may be increased by decreasing the amount of cellulase and other proteins in the enzyme mixture. This can be done as desired, by physical and mechanical separation of the enzyme mixture to remove cellulase and other proteins from the mixture, or by deletion of the cellulase or other genes by recombinant means from the production host so that the expression of cellulase or other proteins is reduced or eliminated. Such methods have little or no effect on the actual production of xylanase by the production host.

[0072] Xylanases and modified xylanases, as outlined herein, may be used for the purposes of bleaching pulp or other applications requiring activities at temperatures and pH above that of the wild-type enzyme. For the bio-bleaching of pulp, a xylanase derived from a xylanase classified in Family 11 (see Table 1) is most commonly used. The modifications as outlined herein may be found in native xylanase proteins, and these native xylanase enzymes, when expressed in alternate (non-native) production hosts, may exhibit the desired features as described herein, and are included within the present invention.

[0073] The practice of the present invention is not constrained by the industrial application of the modified xylanase. Industrial uses of a xylanase produced according to the present invention include, but are not limited to, food processes, for example poultry or swine feed additives, baking or brewing, or industrial processes such as pulp and paper manufacturing.

[0074] The following is a summary of the sequences disclosed in the present invention (SEQ ID NO's: 28 to 45 refer to xylanases from the listed organisms (see Table 2 for more details):

Sequence	Name	Sequence	Name
SEQ ID NO:1	TrxII (Tr2)	SEQ ID NO:25	S182N,S184T
SEQ ID NO:2	TrxI (Tr1)	SEQ ID NO:26	Q34N

SEQ ID NO:3	S75A
SEQ ID NO:4	L105H
SEQ ID NO:5	S125A
SEQ ID NO:6	I129E
SEQ ID NO:7	Y135R
SEQ ID NO:8	H144R
SEQ ID NO:9	N157D
SEQ ID NO:10	Q161R
SEQ ID NO:11	Q162H
SEQ ID NO:12	T165H
SEQ ID NO:13	T131N,R135Y
SEQ ID NO:14	R135Y
SEQ ID NO:15	T128N
SEQ ID NO:16	XynC-5F (Nhe)
SEQ ID NO:17	XynC-3R (Kpn)
SEQ ID NO:18	T118N
SEQ ID NO:19	Xyn1-F
SEQ ID NO:20	Xyn1-R (BamHI)
SEQ ID NO:21	T131N
SEQ ID NO:22	F180N
SEQ ID NO:23	F180N,S182T
SEQ ID NO:24	S182N
SEQ ID NO:27	134N,S36T
SEQ ID NO:28	*Aspargillus awamori var.kawachi*
SEQ ID NO:29	*Aspergillus tubigensis*
SEQ ID NO:30	*Bacillus circulans*
SEQ ID NO:31	*Bacillus pumilus*
SEQ ID NO:32	*Bacillus subtilis*
SEQ ID NO:33	*Clostridium acetobutylicum*
SEQ ID NO:34	*Clostridium stercorarium*
SEQ ID NO:35	*Ruminococcus flavefaciens*
SEQ ID NO:36	*Schizophyllum commune*
SEQ ID NO:37	*Streptomyces sp. No. 36a*
SEQ ID NO:38	*Streptomyces lividans*
SEQ ID NO:39	*Streptomyces lividans*
SEQ ID NO:40	*Thermomonospora fusca*
SEQ ID NO:41	*Trichoderma harzianum*
SEQ ID NO:42	*Trichoderma viride*
SEQ ID NO:43	*Aspergillis niger*
SEQ ID NO:44	*Aspergillis kawachii*
SEQ ID NO:45	*Fibrobacter succinogenes*
SEQ ID NO:46	*Thermomyces lanuginosus*
SEQ ID NO:47	modified *T. reesei* xylanase I
SEQ ID NO:48	modified *S. lividans* xylanase C

[0075] The present invention will be further illustrated in the following examples.

Examples

[0076] Example 1 describes the isolation of genomic DNA from *Trichoderma reesi* strain M2C38 and the genetically modified derivatives of these strains. Examples 2-5 describe the construction of genomic DNA libraries, the cloning of various genes, the modification of xylanase gene sequences and several genetic constructs for expression of modified xylanases from *Trichoderma reesei* strains RutC30 and M2C38. Examples 7-10 describe the transformation and expression of xylanase genetic constructs in *Trichoderma reesei* strains RutC30 and M2C38. Examples 11 and 12 describe the biochemical characterization of modified and native xylanases.

Example 1: Isolation of *Trichoderma reesei* Genomic DNA and Construction of *T. reesei* Genomic Libraries

[0077] *Trichoderma reesei* strain M2C38 is a proprietary strain of Iogen Corporation derived from *Trichoderma reesei* RutC30 (ATCC #56765; Montenecourt and Eveleigh, 1979), which was in turn derived from *Trichoderma reesei* Qm6A (ATCC # 13631; Mandels and Reese, 1957). It is well understood by those skilled in the art that the procedures described herein, the genetic constructs from these strains, and the expression of the genetic constructs in these strains is applicable to all *Trichoderma* strains derived from Qm6A.

[0078] To isolate genomic DNA, 50 ml of Potato Dextrose Broth (Difco) was inoculated with *T. reesei* spores collected from a Potato Dextrose Agar plate with a sterile inoculation loop. The cultures were shaken at 200 rpm for 2-3 days at 28°C. The mycelia was filtered onto a GFA glass microfibre filter (Whatman) and washed with cold, deionized water. The fungal cakes were frozen in liquid nitrogen crushed into a powder with a pre-chilled mortar and pestle; 0.5 g of powdered biomass were resuspended in 5 ml of 100 mM Tris, 50 mM EDTA, pH 7.5 plus 1% sodium dodecyl sulphate (SDS). The lysate was centrifuged (5000g for 20 min, 4°C) to pellet cell debris. The supernatent was extracted with 1 volume buffer (10 mM Tris, 1 mM EDTA, pH 8.0) saturated phenol followed by extraction with 1 volume of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1) in order to remove soluble proteins. DNA was precipitated from the solution by adding 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold 95% ethanol. After incubating for at least 1 h at -20°C, the DNA was pelleted by centrifugation (5000g for 20 min, 4°C), rinsed with 10 ml 70% ethanol, air-dried and resuspended in 1 ml 10 mM Tris, 1 mM EDTA, pH8.0. RNA is digested by the addition of Ribonuclease A (Boehringer Mannheim) added to a final

concentration of 0.1 mg/ml and incubation at 37°C for 1 hour. Sequential extractions with 1 volume of buffer-saturated phenol and 1 volume of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1) are used to remove the ribonuclease from the DNA solution. The DNA is again precipitated with 0.1 volumes of 3M sodium acetate, pH 5.2 and 2.5 volumes of cold 95% ethanol, pelleted by centrifugation, rinsed with 70% ethanol, air-dried and resuspended in 50 µl of 10 mM Tris, 1 mM EDTA, pH 8.0. The concentration of DNA was determined by measuring the absorbance of the solution at 260 nm (p. C1 in Sambrook et al., 1989).

[0079] Two plasmid libraries and one phage library were constructed using genomic DNA isolated from *T. reesei* strain M2C38. The plasmid libraries were constructed in the vector pUC119 (Viera and Messing, 1987) as follows: 10 µg genomic DNA was digested for 20 hrs at 37°C in a 100 µl volume with 2 units/µg of *Hindlll*, *BamHI* or *EcoRI* restriction enzymes. The digested DNA was fractionated on a 0.75% agarose gel run in 0.04M Tris-acetate, 1 mM EDTA and stained with ethidium bromide. Gel slices corresponding to the sizes of the genes of interest (based on published information and Southern blots) were excised and subjected to electro-elution to recover the DNA fragments (Sambrook et al., pp. 6.28-6.29). These enriched fractions of DNA were ligated into pUC119 in order to create gene libraries in ligation reactions containing 20-50 µg/ml DNA in a 2:1 molar ratio of vector:insert DNA, 1 mM ATP and 5 units T4 DNA ligase in a total volume of 10-15 µl at 4°C for 16 h. *Escherichia coli* strain HB101 was electroporated with the ligation reactions using the Cell Porator System (Gibco/BRL) following the manufacturer's protocol and transformants selected on LB agar containing 70µg/ml amplicillin.

[0080] The phage library was constructed in the lambda vector λDASH (Stratagene, Inc.) as follows: genomic DNA (3 µg) was digested with 2, 1, 0.5 and 0.5 units/µg *Bam HI* for 1 hour at 37°C to generate fragments 9-23 kB in size. The DNA from each digest was purified by extraction with 1 volume Tris-staturated phenol:choroform:isoamyl alcohol (25:24:1) followed by precipitation with 10 µl 3M sodium acetate, pH 5.2 and 250 µl 95% ethanol (-20°C). The digested DNA was pelleted by microcentrifugation, rinsed with 0.5 ml cold 70% ethanol, air-dried and resuspended in 10 µl sterile, deionized water. Enrichment of DNA fragments 9-23 kB in size was confirmed by agarose gel electrophoresis (0.8% agarose in 0.04 M Tris-acetate, 1 mM EDTA). Digested DNA (0.4 µg) was ligated to 1 µg λDASH arms predigested with *BamHI* (Stratagene) in a reaction containing 2 units T4 DNA ligase

and 1 mM ATP in a total volume of 5 µl at 4°C overnight. The ligation mix was packaged into phage particles using the GigaPack® II Gold packaging extracts (Stratagene) following the manufacturer's protocol. The library was titred using the *E. coli* host strain XL1-Blue MRA (P2) and found to contain 3 x 10^5 independent clones.

Example 2: Isolation of genomics clones from *T. reesei* M2C38 libraries

2.1 Cloning the cellobiohydrolase I (cbh1) and cellobiohydrolase II (cbh2) genes from pUC119 libraries

[0081] *E. coli* HB101 transformants harboring *cbh1 or cbh2* clones from recombinant pUC119-*BamH1*or –*EcoRI* libraries were identified by colony lift hybridization: 1-3 x 10^4 colonies were transferred onto HyBond™ nylon membranes (Amersham); membranes were placed colony-side up onto blotting paper (VWR 238) saturated with 0.5 M NaOH, 1 M NaCl for 5 min to lyse the bacterial cells and denature the DNA; the membranes were then neutralized by placing them colony-side up onto blotting paper (VWR 238) saturated with 1.5 M Tris, pH 7.5 plus 1 M NaCl for 5 min; the membranes were allowed to air-dry for 30 min and the DNA was then fixed to the membranes by baking at 80°C for 2 h.

[0082] ^{32}P-labelled probes were prepared by PCR amplification of short (0.7-1.5 kB) fragments of the *cbh1* and *cbh2* coding regions from the enriched pool of *BamH1 or EcoR1*fragments, respectively, in a labelling reaction containing 10-50 ng target DNA, 0.2 mM each d(GCT)TP, 0.5 µM dATP, 20-40 µCi α-^{32}P-dATP, 10 pmole oligonucleotide primers and 0.5 units Taq polymerase in a total volume of 20 µl. The reaction was subjected to 6-7 cycles of amplification (95°C, 2 min; 56°C, 1.5 min; 70°C, 5 min). The amplified, ^{32}P-labelled DNA was precipitated by the addition of 0.5 ml 10% (w/v) trichloroacetic acid and 0.5 mg yeast tRNA. The DNA was pelleted by microcentrifugation, washed twice with 1 ml 70% ethanol, air-dried and resuspended in 1M Tris pH7.5, 1 mM EDTA.

[0083] Nylon membranes onto which the recombinant pUC119 plasmids had been fixed were prehybridized in heat-sealed bags for 1 h at 60-65°C in 1 M NaCl, 1% SDS, 50 mM Tris, 1 mM EDTA pH 7.5 with 100 µg/ml denatured sheared salmon sperm DNA. Hybridizations were performed in heat-sealed bags in the same buffer with only 50 µg/ml denatured sheared salmon sperm DNA and 5 x 10^6 - 5 x 10^7 cpm of denatured *bgl1, cbh1 or cbh2* probe for 16-20 h at 60-65°C. Membranes were washed once for 15 min with 1 M NaCl, 0.5% SDS at 60°C, twice for 15 min each with 0.3M NaCl, 0.5% SDS at 60°C and

once for 15 min with 0.03M NaCl, 0.5% SDS at 55°C. Membranes were again placed in heat-sealed bags and exposed to Kodak RP X-ray film for 16-48 h at -70°C. The X-ray film was developed following the manufacturer's protocols. Colonies giving strong or weak signals were picked and cultured in 2xYT media supplemented with 70 µg/ml ampicillin. Plasmid DNA was isolated from these cultures using the alkaline lysis method (Sambrook, et al., pp. 1.25-1.28) and analyzed by restriction digest, Southern hybridization (Sambrook, et al., pp. 9.38-9.44) and PCR analysis (Sambrook, et al., pp. 14.18-14,19).

[0084] Clones carrying the *cbh1* gene were identified by colony lift hybridization of the pUC119-*BamH1* library with a 0.7 kb *cbh1* probe prepared using oligonucleotide primers designed to amplify bp 597-1361 of the published *cbh1* sequence (Shoemaker et al., 1983). A *cbh1* clone, pCOR132 was isolated containing a 5.7 kb *BamH1* fragment corresponding to the promoter (4.7 kb) and 1 kb of the *cbh1* structural gene (2.3 kb). From this, a 2.5 kb *EcoR1* fragment containing the *cbh1* promoter (2.1 kb) and 5' end of the *cbh1* coding region (0.4 kb) was subcloned into pUC119 to generate pCB152. Clones carrying the *cbh2* gene were identified by colony lift hybridization of the pUC119-*EcoR1* library with a 1.5 kb *cbh2* probe prepared using oligonucleotide primers designed to amplify bp 580-2114 of the published *cbh2* sequence (Chen et al. 1987). A *cbh2* clone, pZUK600 was isolated containing a 4.8 kb *EcoR1* fragment corresponding to the promoter (600 bp), structural gene (2.3 kb) and terminator (1.9 kb).

2.1 Cloning cbh1 terminator, xylanase II (*xln2*) gene, and phosphoglycerate kinase promoter (*pgk p*). *from* λDASH *libraries*

[0085] Digoxigen-11-dUTP labelled probes were prepared from PCR amplified coding regions of the *cbh1, xln2* and *pgk* genes by random prime labelling using the DIG Labelling and Detection kit (Boehringer Mannheim) and following the manufacturer's protocols. Genomic clones containing the *cbh1, xln2* and *pgk* genes were identified by plaque-lift hybridization of the λDASH library. For each gene of interest, 1 x 10^4 clones were transferred to Nytran® (Schleicher and Schull) nylon membranes. The phage particles were lysed and the phage DNA denatured by placing the membranes plaque-side up on blotting paper (VWR238) saturated with 0.5 M NaOH, 1 M NaCl for 5 min; the membranes were then neutralized by placing them plaque-side up onto blotting paper saturated with 1.5 M Tris, pH 7.5 plus 1 M NaCl for 5 min; the membranes were allowed to air-dry for 30 min and the DNA was then fixed to the membranes by baking at 80°C for 2 h. The membranes

were prehybridized in heat-sealed bags in a solution of 6X SSPE, 5X Denhardt's, 1% SDS plus 100 μg/ml denatured, sheared salmon sperm DNA at 65°C for 2 h. The membranes were then hybridized in heat-sealed bags in the same solution containing 50 μg/ml denatured, sheared salmon sperm DNA and 0.5 μg of digoxigen-dUTP labelled probes at 65°C overnight. The membranes were washed twice for 15 min in 2X SSPE, 0.1% SDS at RT, twice for 15 min in 0.2XSSPE, 0.1% SDS at 65°C and once for 5 min in 2X SSPE. Positively hybridizing clones were identified by reaction with an anti-digoxigenin/alkaline phosphatase antibody conjugate, 5-bromo-4-chloro-3-indoyl phosphate and 4-nitro blue tetrazolium chloride (Boehringer Mannheim) following the manufacturer's protocol. Positively hybridizing clones were further purified by a second round of screening with the digoxigen-dUTP labelled probes.

[0086] Individual clones were isolated and the phage DNA purified as described in Sambrook et al. (1989) pp. 2.118-2.121 with the exception that the CsCl gradient step was replaced by extraction with 1 volume of phenol:choroform:isoamyl alcohol (25:24:1) and 1 volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 0.1 volumes of 3M sodium acetate, pH 5.2 and 2.5 volumes cold 95% ethanol. The precipitated phage DNA was washed with 0.5 ml cold 70% ethanol, air-dried and resuspended in 50 μl 10 mM Tris, 1 mM EDTA pH8.0. Restriction fragments containing the genes of interest were identified by restriction digests of the purified phage DNA and Southern blot hybridization (Sambrook, et al., pp. 9.38-9.44) using the same digoxigen-dUTP labelled probes used to screen the λDASH library. The membranes were hybridized and positively hybridizing fragments visualized by the same methods used for the plaque lifts. Once the desired restriction fragments from each λDASH clone were identified, the restriction digests were repeated, the fragments were resolved on a 0.8% agarose gel in TAE and the desired bands excised. The DNA was eluted from the gel slices using the Sephaglas BandPrep Kit (Pharmacia) following the manufacturer's protocol.

[0087] Clones carrying the *cbh1* gene were identified by colony lift hybridization of the λDASH library (Example 2) with a *cbh1* probe comprising bp 45-2220 of the published *cbh1* sequence (Shoemaker et al.). A 1.8 kb *BamHI* fragment containing the 3' end of the *cbh1* coding region (0.5 kb) and the *cbh1* terminator (1.3 kb) was isolated by restriction digestion of phage DNA purified from a λDASH *cbh1* clone. This fragment was subcloned into the *BamH1* site of the *E.coli* plasmid vector pUC119 to generate the plasmid pCB1Ta.

Clones carrying the *xln2* gene were identified by colony lift hybridization of the λDASH library (Example 2) with a *xln2* probe comprising bp 100-783 of the published *xln2* sequence (Saarelainen et al., 1993). A 5.7 kb *Kpn1* fragment containing the promoter (2.3 kb), coding region (0.8 kb) and terminator (2.6 kb) the *xln2* gene was isolated by restriction digestion of phage DNA purified from a λDASH *xln2* clone. This fragment was subcloned into the *Kpn1* site of pUC119 to generate the plasmid pXYN2K-2. Clones carrying the *pgk* gene were identified by colony lift hybridization of the λDASH library (Example 2) with a *pgk1* probe comprising bp 4-1586 the published *pgk* sequence (Vanhanen et al., 1989). A 5.0 kb *EcoR1* fragment containing the promoter (2.9 kb), coding region (1.6 kb) and terminator (0.5 kb) the *pgk* gene was isolated by restriction digestion of phage DNA purified from a λDASH *pgk* clone. This fragment was subcloned into the *EcoR1* site of pUC119 to generate the plasmid pGK5.0.

Example 3: Cloning and modification of the *T. reeesei* xylanase I and *S. lividans* xylanase C genes

[0088] Xylanase C (*xyl*C; SEQ ID NO:39) was amplified from genomic DNA isolated from *Streptomyces lividans* using primers that introduced a *Nhe*I site upstream and a *Kpn*I site downstream of the coding sequence. Megaprimer PCR was used to introduce the T128N mutation into *xyl*C. The mutagenic primer was used in conjunction with the reverse primer to introduce a *Kpn*I site downstream. The resulting PCR product was isolated and used as a reverse primer in conjunction with the forward primer to introduce a *Nhe*I site upstream. The sequence of the modified *S. lividans* xylanase C is shown in SEQ ID NO: 48). Primer sequences are listed below:

T128N:	CCCTCCGTGG AAGGCAACAA GACCTTCCAG (SEQ ID NO: 15)
XynC-5F(Nhe):	GCCCACGCCG CTAGCACCAT CACC (SEQ ID NO: 16)
XynC-3R(Kpn):	CGTCCACCGG TACCAGGTCA ACC (SEQ ID NO: 17)

[0089] The gene encoding xylanase I (*xyn*I; SEQ ID NO:2) was amplified from genomic DNA isolated from *T. ressei* strain M2C38 using primers that introduced a *Nhe*I site upstream and a *BamH*I site downstream of the coding sequence. Megaprimer PCR was used to introduce the T118N mutation into *xyn*I. The mutagenic primer was used in conjunction with the reverse primer to introduce a *BamH*I site downstream. The resulting PCR product

was isolated and used as a reverse primer in conjunction with the forward primer to introduce a *Nhe*I site upstream. The sequence of the modified *T. ressei* xylanase C is shown in SEQ ID NO: 47).Primer sequences are listed below:

T118N:	CCATCCAGGG CAACGCGACC TTC (SEQ ID NO: 18)
XynI-F:	CGTCGTGCTA GCATCAACTA CGAC (SEQ ID NO: 19)
XynI-R (BamHI):	GGATCCTAGT TGCTGACAC (SEQ ID NO: 20)

[0090] The amino acid sequences for the native, unmodified *T. reesei* xylanase I and *S. lividans* xylanase C encoded by the genetic constructs described in Examples 5.4 and 5.5 are provided as SEQ ID NO: 2 and SEQ ID NO: 39, respectively.

Example 4: Mutagenesis of *T. reesei* xylanase II to generate the variants HTX18, ITX1-5, ITX3'-5', HTX18(R135Y)

4.1 Introduction of mutations N10H, 27M, Y29L:

[0091] Genetic engineering of the *xln2* gene from strain M2C38 was performed by cassette mutagenesis of a synthetic *xln2* gene (Sung et al., 1995; also see WO 01/92487 and WO 03/046169; which are incorporated herein by reference). Specifically, a double-stranded ApaI/PinAI fragment comprising codons 8-33, in which codons 10, 27 and 29 were altered as indicated in SEQ ID: 2, was synthesized *in vitro*. This fragment was then used to replace the native *xln2* sequence in the plasmid pUC/Xln (Sung et al., 1993). The synthetic DNA comprising codons 32-190 in pUC/XLN was replaced by the corresponding genomic fragment of *T. reesei xln2*, containing a 108 bp intron at codon 58, which was amplified using genomic *T. reesei* DNA as a template and introducing a unique *PinAI* site at codons 31 and 32 and a unique *BamHI* directly downstream of the TAG stop codon. This generates pUC/HTX4.

4.2 Introduction of mutations 75A, 105H, 125A, 129E:

[0092] A 3.2 kb *Sst*I fragment containing the promoter regions, the *xln2* gene, and part of the *cbh2* terminator was isolated from pC/XHML-TV (see example 5.1, below) and cloned into the *Sst*I site in the polylinker of the mutagenesis vector, pALTER®-1 (Promega). Four sequential rounds of mutagenesis were performed to alter specific amino acids using primers specifically designed to incorporate the desired mutations:

S75A: AGCTACCTCG CCGTGTACGG (SEQ ID NO:3),

L105H: CCACCAAGCA CGGCGAGGT (SEQ ID NO:4),

S125A: ACGCAGCGCG TCAACGCCCC GTCCATCATC GGC (SEQ ID NO:5), and

I129E: AACGCCCCGT CCATCGAGGG CACCGCCACC TTT (SEQ ID NO:6)

(see WO 01/92487 and WO 03/046169; which are incorporated herein by reference, for associated methods); this generated the plasmid pALT-HTX13 (the plasmid is shown in its generic form, "pALT-H(I)TXn" in Figure 3. The incorporation of all mutations was verified by DNA sequence analysis.

4.3 Introduction of mutations Y135R, H144R, N157D, Q161R, Q162H and T165H

[0093] Four sequential rounds of mutagenesis were performed on the plasmid pALT-HTX13 using the Promega Altered Sites® II *in vitro* Mutagenesis System to introduce the six targeted amino acid substitutions and generate pALT-HTX18, as follows:

- addition of the 135R and 144R mutations to pALT-HTX13 using primer sequences:

Y135R: GGCACCGCCA CCTTTCGCCA GTACTGGTCC (SEQ ID NO: 7) and

H144R: GTCCGCCGCA ACCGCCGCTC GAGCGGCTC (SEQ ID NO:8)

to make pALT-HTX13+135R/144R;

- addition of the 157D and 162H mutations to pALT-HTX13+135R/144R using primer sequences:

N157D: AACCACTTCG ACGCGTGG (SEQ ID NO: 9) and

Q162H: GGCTCAGCAC GGCCTGACG (SEQ ID NO:11),

to make pALT-HTX13+135R/144R/157D/162H;

- addition of the 161R mutation to pALT-HTX13+135R/144R/157D/162H using the primer sequence

Q161R: TTCGACGCGT GGGCTCGCCA CGGCCTGACG CTC (SEQ ID NO: 10),

to make pALT-HTX13+135R/144R/157D/161R/162H;

-addition of the 165H mutation to pALT-HTX13+135R144R157D/161R/162H using the primer sequence

T165H: GCTCGCCACG GCCTGCACCT CGGGACGATG GAT (SEQ ID NO: 12),

to make pALT-HTX18. The plasmid is shown in its generic form, "pALT-H(I)TXn" in Figure 3. The incorporation of all mutations was verified by DNA sequence analysis.

4.4 Reversion of mutation Y135R and introduction of T131N into HTX18

[0094] One round of mutagenesis was performed on the plasmid pALT-HTX18 using the Promega Altered Sites ® II *in vitro* Mutagenesis System and the primer sequence:

T131N, R135Y: GGCAACGCCA CCTTTTACCA GTACTGGTCC (SEQ ID NO:13)

to introduce the T131N and R135Y mutations and generate pALT-ITX1. The plasmid is shown in its generic form, "pALT-H(I)TXn" in Figure 3. The incorporation of all mutations was verified by DNA sequence analysis

4.5 Reversion of the Y135R mutation in HTX18

[0095] One round of mutagenesis was performed on the plasmid pALT-HTX18 using the Promega Altered Sites® II *in vitro* Mutagenesis System and the primer sequence:

R135Y: GGCACCGCCA CCTTTTACCA GTACTGGTCC (SEQ ID NO14),

to introduce the R135Y mutations and generate pALT-HTX18R135Y. The plasmid is shown in its generic form, "pALT-H(I)TXn" in Figure 3. The incorporation of all mutations was verified by DNA sequence analysis

4.6 Introduction of glycosylation sites at positions 34, 131, 180 and 182 within HTX18:

[0096] One round of mutagenesis was performed on the plasmid pALT-HTX18 using the Promega Altered Sites ® II *in vitro* Mutagenesis System and the primer sequences:

T131N: CCGTCCATCG AGGGCAACGC CACCTTTCGC (SEQ ID NO: 21)

F180N: GTGGAGGGTT ACAACAGCTC TGGCTCTGCT (SEQ ID NO: 22)

F180N, S182T: GTGGAGGGTT ACAACAGCAC CGGCTCTGCT (SEQ ID NO: 23)

S182N: GGTTACTTTA GCAACGGCTC TGCTTCCATC (SEQ ID NO: 24)

S182N, S184T: GGTTACTTTA GCAACGGCAC CGCTTCCATC (SEQ ID NO: 25)

Q34N: GGTCCCGGCG GGAACTTCTC CGTCAACTGG (SEQ ID NO: 26)

Q34N, S36T: GGTCCCGGCG GGAACTTCAC CGTCAACTGG (SEQ ID NO: 27)

to generate the plasmids pALT-ITXn and pALT-ITXn' (where n equals 2 through 5). A generic map of these plasmids, "pALT-H(I)TXn", is shown in Figure 3. The incorporation of all mutations was verified by DNA sequence analysis

Example 5: Construction of vectors directing the expression of native and modified Family 11 xylanases in *Trichoderma reesei*.

5.1 Construction of pC/XHML-TV:

[0097] A 2.4 kb fragment containing the promoter and secretion signal of the *xln2* gene (bp -2150 to +195 where +1 indicates the ATG start codon and +193-195 represent codon 32) was amplified with *Pwo* polymerase from the genomic *xln2* subclone pXYN2K-2 using a *xln2*-specific primer containing a *PinA1* at bp 190-195 or codons 31 and 32 and the pUC reverse primer (Cat. No. 18432-013, Gibco/BRL) which anneals downstream of the *Kpn1* site at the 5' end of the *xln2* gene. This *xln2* PCR product was inserted as a blunt-ended fragment into the SmaI site of the pUC119 polylinker in such an orientation that the *BamHI* site of the polylinker is 3' to the *PinAI* site; this generated the plasmid pUC/XynPSS(Pin). The same *xln2* PCR product was reisolated from pUC/XynPSS(Pin) by digestion with *EcoR1* (which was amplified as part of the pUC119 polylinker from pXYN2K-2) and *BamHI* and inserted into the plasmid pBR322L (a derivative of pBR322 containing an *Sph1-Not1-Sal1* adaptor between the original Sph1 and Sal1 sites at bp 565 and 650), also digested with *EcoRI* and *BamHI*, to generate the plasmid pBR322LXP. To facilitate high level expression of the HTX4 xylanase, a 1.3 kb *HindIII* fragment comprising bp -1400 to -121 of the *xln2* promoter in pBR322LXP was replaced with a 1.2 kb *HindIII* fragment comprising bp -1399 to -204 of the *cbh1* promoter which was isolated by *HindIII* digestion of pCOR132; this generated the plasmid pBR322LXC. Finally, the EcoR1 site of pBR322LXC was then

blunted with Klenow and *Spe1* linkers (Cat. No. 1086, New England Biolabs) were added to generate pBR322SpXC.

[0098] A fragment containing codons 1-190 of the xylanase gene containing the mutations N10H, Y27M, N29L was isolated from the plasmid pUC/HTX4 (described in example 4.1, above) by digestion with *NheI* and *BamHI* inserted into pCB219N-N digested with *NheI* and *BamHI* to generate pHTX4/C2ter. To make pCB219N-N, a *cbh2* terminator fragment was amplified from the pZUK600 (described in Example 2, above) template using a primer homologous to bp 2226-2242 of the published 3' untranslated region of the *cbh2* gene (Chen et al., 1987) containing a short polylinker comprising *XbaI-NheI-BamHI-SmaI-KpnI* sites at the 5' end and the pUC forward primer (Cat. No. 1224, New England Biolabs) which anneals upstream of the *EcoR1* site at the 3' end of *cbh2* in pZUK600. This fragment was digested at the engineered *XbaI* and *EcoR1* sites and inserted into the corresponding sites of pUC119 to generate pCB219. An *EcoR1-Not1* adaptor (Cat. No. 35310-010, Gibco/BRL) was inserted into the unique *EcoR1* site of pCB219 to generate pCB219N. A 2.7 kb fragment comprising codons 9-190 of the HTX4 gene and the *cbh2* terminator was isolated from pHTX4/C2ter by digestion with *PinAI* and *NotI* and inserted into pBR322SpXC digested with *PinAI* and *NotI* to generate the expression cassette pXHML-EC.

[0099] The *E. coli* hygromycin phosphotransferase gene (*hph*) used as a selectable marker for *T. reesei* was amplified with *Pwo* polymerase from the plasmid pVU1005 (Van den Elzen et al., 1989). The primers were designed to introduce *Sph1* and *Kpn1* sites at the 5' and 3' ends of the *hph* coding region (bp 211-1236 of the published *hph* sequence, Gritz and Davies, 1983), respectively. The PCR product was digested with *Sph1* and *Kpn1* and inserted into the corresponding sites in the polylinker region of pUC119. The resulting plasmid, pHPT100, was used as the starting plasmid for the construction of the selection cassette.

[00100] Two new linker regions were introduced into plasmid pHPT100 to facilitate the cloning of the promoter and terminator fragments. A *HindIII-XbaI-XhoI-SphI* linker was inserted between the *HindIII* and *SphI* sites as well as a *KpnI-NotI-SacI* linker which was inserted between the *KpnI* and *SacI* sites of pUC119 polylinker remaining in pHPT100. This construct was designated as pHPT102. The primers used to amplify the *pgk* promoter (Vanhanen et al., 1991) were designed to introduce an *XhoI* site and a *SphI* site at positions -970 and +1 of the promoter respectively. These sites were subsequently used to insert the *pgk* promoter into the *XhoI* and *SphI* sites of pHPT102 to generate pHPT115. A 1.3 kb *cbh1*

terminator fragment was amplified with *Pwo* polymerase from pCB1Ta using a primer annealing to the 3' untranslated region of *cbh1* (bp 1864-1899 of the published *cbh1* sequence) containing a *Kpn1* site at bp 1877-1882 and the pUC reverse primer (Cat. No., 18432-013, Gibco/BRL) which anneals downstream of the *EcoR1* site at the 3' end of the *cbh1* terminator in pCB1Ta. The *cbh1* terminator PCR product was digested with *Kpn1* and inserted into the unique *Kpn1* site of pHPT115 to generate the selection cassette plasmid pHPT136. The *cbh1* terminator in the selection cassette plasmid pHPT136 was replaced with a 2.6 kb *Kpn1* fragment containing the *xln2* transcriptional terminator. The *xln2* terminator was amplified with *Pwo* polymerase from the genomic subclone pXYN2K-2 using a primer to introduce a *KpnI* site directly downstream of bp 780 of the published *xln2* sequence (Saarelainen et al. 1993) and the pUC forward primer (Cat. No. 18431-015, Gibco/BRL) which anneals downstream of the 3' end of the *xln2* gene in pXYN2K-2. The *xln2* terminator PCR product was digested with *Kpn1* and ligated to a 5.1 kb *Kpn1* fragment from pHPT136 containing the *pgk* promoted-*hph* gene in pUC119 to generate the selection cassette plasmid pHPT136X, thus maintaining the unique *Not1* site at the 3' end of the selection cassette.

[00101] To make the transformation vector, the expression cassette from pC/XHML-EC was isolated by *NotI* digestion, blunting the *NotI* site with Klenow DNA polymerase, and *SpeI* digestion. At the same time, the selection cassette plasmid was prepared to accept this fragment by digestion with *XhoI*, blunting of the *XhoI* site with Klenow DNA polymerase and subsequent digestion with *XbaI*. The *SpeI*-expression cassette-*NotI*° fragment was inserted between the *Xba1* and *Xho1*° sites upstream of the selection cassette of pHPT136X. The final transformation vector, pC/XHML-TV (Figure 2), was linearized by digestion with *NotI* prior to introduction into *T. reesei* M2C38 via microprojectile bombardment as described in Example 7.

5.2 Construction of pC/XH(I)TXn-TV:

[00102] Each 3640 bp *Sac* I fragment containing the promoter regions, the modified *xln2* genes and part of the *cbh2* terminator from pALT-H(I)TXn (described in Example 4) was cloned into the *Sac* I site of a plasmid containing the remaining *cbh2* terminator sequence in pSP72. This generates the expression cassette containing plasmids, pc/xH(I)TXnPSP. The selection cassette containing plasmid, pNCBglNSNB(r), was derived from a *N. crassa pyr4* containing plasmid, pFB6 (Radford et al, 1985). A 3.2 kb *Bgl* II fragment from pFB6 containing the *N. crassa pyr4* gene (GenBank accession M13448) as

well as its promoter, terminator and some 5' UTR sequences was cloned into the *Bam* HI site of pUC119 modified to contain *Not* I, *Sma* I, *Nhe* I and *Bgl* II sites in the polylinker (between *Eco* RI and *Sac* I) to generate pNCBgl-NSNB(r). A 2238 bp *Kpn* I fragment containing the entire *N. crassa pyr4* coding region, promoter and terminator sequences was isolated from pNCBgl-NSNB(r) and cloned into the unique *Kpn I* site of pc/xHTX18PSP to generate pc/xHTX18-TV (the plasmid is shown in its generic form "pc/xH(I)TXn-TV ", in Figure 4).

5.3 Construction of pc/xHTX18(R135Y)-TV

[00103] The 3640 bp *Sac* I fragment containing the promoter regions, the modified *xln2* gene and part of the *cbh2* terminator from pALT-HTX18(R135Y) was cloned into the *Sac* I site of a plasmid containing the remaining *cbh2* terminator sequence in pSP72. This step generates the expression cassette containing plasmid pc/xHYX18(R135Y)PSP. The 2238bp *Kpn* I fragment containing the entire *N. crassa pyr4* coding region, promoter and terminator sequences was isolated from pNCBgl-NSNB(r) (described in Example 5.2, above) and cloned into the unique *Kpn I* site of the expression cassette-containing plasmids to generate pc/xHTX18(R135Y)-TV (the plasmid is shown in its generic form "pc/xH(I)TXn-TV ", where "n" is "18(R135Y)" in Figure 4).

5.4 Construction of pc/xxyn1-TV and pc/xxyn1-T118N-TV

[00104] The 675 bp wild type and modified *xyn*I PCR products (described in example 3, above) were digested with *Nhe*I and *BamH*I and inserted into the corresponding sites in the plasmid pCB219N-N (described in example 5.1, above) to generate the plasmids pX1C2ter and pX1(118N)C2ter. An ~ 1.6 kb fragment comprising bp -1399 to -204 of the cbh1 promoter, bp -121 to -1 of the xln2 promoter and the sequence encoding the *xln2* secretion signal was amplified from the plasmid pBR322LXC (described in example 5.1) using primers to introduce a XbaI site at bp -1399 of the *cbh1* promoter and an *NheI* site directly downstream of the Gln codon comprising the first amino acid of the mature xylanase II protein. This PCR product was digested with *XbaI* and *NheI* and inserted upstream of the native and modified xylanase I coding regions in the corresponding sites of the plasmids pX1C2ter and pX1(118N)C2ter to generate the expression cassette plasmids pc/xxyn1-EC and pc/xxyn1-T118N-EC. The expression cassettes were excised by digestion with *Xba*I, blunting of the *Xba*I site with Klenow DNA polymerase and digestion with *Eco*RI. The *N. crassa pyr4*-containing plasmid pNCBglNSNB(r) (described in Example 5.2, above) was prepared to accept this fragment by digestion with *Not*I, blunting of the *Not*I site with Klenow

DNA polymerase and digestion with *Eco*RI. The *Eco*RI-xynI expression cassette-*Xba*I° fragment was inserted between the *Eco*RI and *Not*I° sites downstream of the selection cassette to produce the transformation vectors pc/xxyn1-TV and pc/xxyn1-T118N-TV (Figure 5). The final transformation vectors were linearized by digestion with *XbaI* prior to introduction into protoplasts of *T. reesei* M2C38aux5 via PEG-mediated transformation of protoplasts as described in Example 7.

5.5 Construction of pc/xxynC-T128N-TV

[00105] The xylanase I expression cassette plasmid, pc/xxln1-EC, was digested with *NheI* and *KpnI* to drop-out the xylanase I coding region and the larger fragment was ligated with the 700 bp modified *xynC* PCR product (described in example 3, above) digested with *NheI* and *KpnI* to generate the expression cassette plasmid pc/xxynC-T128N-EC. The expression cassette was excised by digestion with *NotI*, blunting of *NotI* site with Klenow DNA polymerase and digestion with *XbaI*. At the same time, the *hph*-containing selection cassette plasmid pHPT136 (described in example 5.1, above) was prepared to accept this fragment by digestion with *XhoI*, blunting of the *XhoI* site with Klenow DNA polymerase and subsequent digestion with *XbaI*. The *XbaI-xynC* expression cassette-*NotI*° fragment was inserted between the *Xba1* and *Xho1*° sites upstream of the selection cassette of pHPT136. The final transformation vector, pc/xxynC-T128N-TV (Figure 5), was linearized by digestion with *XbaI* prior to introduction into protoplasts *T. reesei* RutC30 via PEG-mediated transformation as described in Example 7.

Example 6: Isolation of a *pyr4* auxotroph of *Trichoderma reesei* strain M2C38

[00106] In order to use the *N. crassa pyr4* gene as a selectable marker, a spontaneous *pyr4* auxotroph of M2C38 was isolated as follows: 1 x 10^6 spores of M2C38 were plated onto minimal media containing 5 mM uridine and 0.15% (w/v) of the uridine analog 5-fluoroorotic acid (FOA) as previously described for the selection of *pyr4* auxotrophs of *T. reesei* (Berges and Barreau, 1991). The ability to grow on FOA-containing media will allow for selection of mutants disrupted in either the *pyr2* gene encoding orotate phosphoribosyl transferase or the *pyr4* gene encoding orotidine 5'-phosphate decarboxylase. Spontaneous FOA-resistant colonies were subjected to secondary selection of minimal media with and without uridine. Spores of FOA-resistant colonies that could not grow on minimal media were then

transformed with pNCBglNSNB(r) (described in Example 5.2) by microprojectile bombardment and selected for growth on minimal media. Only those strains that were complemented by the *N. crassa pyr4* gene in pNCBglNSNB(r) will grow on minimal media and are true *pyr4* auxotrophs. Using these procedures, auxotroph 5 (M2C38aux5) was selected as a stable *pyr4* auxotroph of M2C38.

Example 7: Transformation of the *Trichoderma reesei* M2C38.

7.1 Transformation via microprojectile bombardment

[00107] The Biolistic PDS-1000/He system (BioRad; E.I. DuPont de Nemours and Company) was used to transform spores of *T. reesei* strain M2C38 and all procedures were performed as recommended by the manufacturer. Gold particles (median diameter of 0.6 um, BioRad Cat. No. 1652262) were used as microcarriers. The following parameters were used in the optimization of the transformation: a rupture pressure of 1100 psi, a helium pressure of 29 mm Hg, a gap distance of 0.95 cm, a macrocarrier travel distance of 16 mm, and a target distance of 9 cm. Plates were prepared with $1x10^6$ spores on miminal media agar (see example 7.2, below). Bombarded plates were incubated at 28°C. Transformants can be observed after 3-6 days growth at which time the colonies are transferred to MM agar in individual petri plates and allowed to grow and sporulate.

7.2 Protoplast transformations using polyethylene glycol(PEG) and $CaCl_2$.

[00108] 5×10^6 spores of M2C38aux5 are plated onto sterile cellophane on Potato Dextrose agar supplemented with 5 mM uridine and are incubated for 20 hours at 30°C to facilitate spore germination and mycelial growth. Cellophane discs with mycelia are transferred to 10 ml of a protoplasting solution containing 7.5 g/l Driselase and 4 g/l beta-glucanase (InterSpex Products Inc., Cat. Nos. 0465-1 and 0439-2, respectively) in 50 mM potassium phosphate buffer, pH 6.5 containing 0.6 M ammonium sulfate (Buffer P). The mycelial mat is digested for 5 hours at 28°C with shaking at 60 rpm. Protoplasts are recovered by centrifugation at 1000-1500 x g for 10 min at room temperature. Protoplasts are washed with 5 ml of Buffer P and centrifuged again at 1000-1500 x g for 10 min at room temperature. Protoplasts are resuspended in 1 ml of STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCL, pH 7.5) and separated from undigested mycelia by filtration through sterile No. 60 MIRACLOTH™ and collected into a sterile microcentrifuge tube.

[00109] For transformation, 0.1 ml of resuspended protoplasts (approximately 5 x 10^6 protoplasts) are combined with 2 µg vector DNA and 25 µl of PEG solution (25% PEG 4000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). After incubation on ice for 30 min, 1 ml of PEG solution is added and the mixture incubated for 5 min at room temperature. Transformation mix is diluted with 2 ml of 1.2 M sorbitol in PEG solution and 0.75 ml of the mix is added to 25 mL of molten MMSS agar media (see below) cooled to about 47°C and the protoplast suspension poured over MM agar (see below). Plates are incubated at 30°C until colony growth is visible. Transformants are transferred to individual plates containing MM agar and allowed to sporulate. Spores are collected and plated at high dilution on MM agar to isolate homokaryon transformants, which are then plated onto PDA to allow for growth and sufficient sporulation to inoculate the screening cultures described in Example 9, below.

Minimal medium (MM) agar contains:

Reagent	Per L
KH_2PO_4	10 g
$(NH_4)_2SO_4$	6 g
$Na_3Citrate\text{-}2H_2O$	3 g
$FeSO_4\text{-}7H_2O$	5 mg
$MnSO_4\text{-}H_2O$	1.6 mg
$ZnSO_4\text{-}7H_2O$	1.4 mg
$CaCl_2\text{-}2H_2O$	2 mg
Agar	20 g
20% Glucose f.s.	50 ml
1 M MgSO4-$7H_2O$ f.s.	4 mL
	pH to 5.5

[00110] MMSS agar contains the same components as MM agar plus 1.2 M sorbitol, 4 mM $MgSO_4$, 1 g/L YNB (Yeast Nitrogen Base w/o Amino Acids from DIFCO Cat. No.291940) and 0.12 g/l amino acids (-Ura DO Supplement from CLONTECH Cat. No.8601-1).

Example 8: Detection of xylanase activity in *T. reesei* culture filtrates

Detection of thermophilic xylanase activity due to expression of HTX18, HTX18(R135Y), ITX1-5, and ITX3'-5

[00111] The presence of thermophilic xylanase activity in culture filtrates of *T. reesei* transformants is determined by measuring the release of reducing sugars from a soluble arabinoxylan substrate at 65°C. Specifically, 30 µl of an appropriate dilution of culture filtrate is pre-incubated at 65°C for 5 min. Subsequently, 300 µl of a solution of 1.5% wheat arabinoxylan (Megazyme International) redissolved in pH 7.0 phosphate buffer containing 0.04% Tween, also pre-incubated at 65°C for 5 min, is added to the enzyme sample in a

microcentrifuge tube. The tubes are vortexed briefly to facilitate mixing and then the reaction is incubated at 65°C for 20 min. The enzymatic hydrolysis reaction is stopped by the addition of 150 µl of the stopping solution containing 43.64 mM 2-hydroxy-3,5-dinitrobenzoic acid, 0.93M sodium potassium tartrate, 0.4M sodium hydroxide and 0.4 M potassium hydroxide. The resulting solution is then boiled for 10 minutes to facilitate reaction of the 2-hydroxy-3,5-dinitrobenzoic acid with the reducing sugars released from the arabinoxylan substrate by the enzyme. The tubes are cooled in a cold water bath for 5 minutes and then 1.5 ml of deionized water is added. The absorbance of the solution is measured at 530 nm. The amount of reducing sugar released by the thermophilic xylanases during the incubation is calculated from a standard curve of A530 measurements of several dilutions of a pure xylose solution reacted with the same stopping solution.

Detection of xylanase I activity due to overexpression of native or modified T. reesei xylanase I and S. lividans xylanase C-131N

[00112] Detection of xylanase I activity in culture filtrates of *T. reesei* strains overexpressing the native or modified xylanase I was carried out as described in Section 8.1, above, except that the incubations were carried out at 40°C and the 1.5% wheat arabinoxylan substrate was prepared in acetate buffer at pH 4.0 containing 0.04% Tween.

[00113] Detection of xylC-131N activity in culture filtrates of *T. reesei* strains overexpressing the modified S. lividans xylanase C was carried out as described in Section 8.1, above, except that the incubations were carried out at 40°C and the 1.5% wheat arabinoxylan substrate was prepared in acetate buffer at pH 6.0.

Example 9: Production of modified xylanases in liquid cultures

[00114] Individual colonies of *Trichoderma* are transferred to PDA plates for the propagation of each culture. Sporulation is necessary for the uniform inoculation of shake flasks which are used in testing the ability of the culture to produce the thermophilic xylanases and cellulase. The culture media is composed of the following:

Component	g/L
$(NH_4)_2SO_4$	6.35
KH_2PO_4	4.00
$MgSO_4$-$7H_2O$	2.02
$CaCl_2$-$2H_2O$	0.53
CSL	6.25
$CaCO_3$	10.00

Carbon source**	5-200
Trace elements*	1 mL/L

*Trace elements solution contains 5 g/l $FeSO_4*7H_20$; 1.6 g/l $MnSO_4*H_20$; 1.4 g/l $ZnSO_4*7H_20$.
** glucose, Solka floc, lactose, cellobiose, sophorose, corn syrup, or Avicel. The carbon source can be sterilized separately as an aqueous solution at pH 2 to 7 and added to the remaining media initially or through the course of the fermentation.

[00115] Individual transformants are grown in the above media in 150 mL cultures in 1-litre flasks or in 1 mL cultures in 24-well microplates. The initial pH is 5.5 and the media sterilized by steam autoclave for 30 minutes at 121°C prior to inoculation. For both native and transformed cells, spores are isolated from the PDA plates as described in Example 8 and 10^4-10^6 spores per ml are used to inoculate each culture. The cultures are shaken at 200-300 rpm at a temperature of 28°C for a period of 6 days. The biomass is separated from the filtrate containing the secreted protein by filtration through GF/A glass microfibre filters (Whatman) or by centrifugation at 12000 rpm. The protein concentration is determined using the Bio-Rad Protein Assay (Cat. No. 500-0001). Xylanase activity is determined as described in Example 8. Strains expressing the highest xylanase activity from each construct and exhibiting high overall protein production were selected for growth in 14-litre pilot fermentations.

Example 10: Production of xylanases in 14L fed-batch fermentations.

[00116] *T. reesei* strains were grown on Potato Dextrose Agar at 28-30°C until a confluent lawn of spores was obtained. Spores were collected and used to inoculate 750 ml of Berkeley media (10 g/l glucose, 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.31 g/l $MgSO_4*7H_20$, 0.53 g/l $CaCl_2$; 5.1 g/l dry corn steep, 5 mg/l $FeSO_4*7H_2O$; 0.8 mg/l $MnSO_4*H_2O$, 0.7 mg/l $ZnSO_4*7H_2O$) in a 2 L baffled flask. After 3 days of growth at 28°C and 150 rpm, this culture was used to inoculate 10 L of fermentation medium with the following initial composition: 13 g/l glucose, 2.2 g/l $(NH_4)_2SO_4$, 1.39 g/l KH_2PO_4, 0.7 g/l $MgSO_4*7H_20$, 0.185 g/l $CaCl_2$, 6 g/l dry corn steep, 3.75 mg/l $FeSO_4*7H_2O$; 1.2 mg/l $MnSO_4*H_2O$, 1.05 g/l $ZnSO_4*7H_2O$. A fed-batch aerobic fermentation using one or more of the inducing carbohydrate sources listed in Example 9 is run for 6 days at pH 4.5 and 28-30°C in a 14L New Brunswick Microferm fermentor. After 6 days, the culture is filtered over Harborlite and the culture filtrate adjusted to pH 4.5 and preserved with 0.5 % benzoate to prevent microbial growth.

[00117] Expression of the modified xylanases did not significantly alter the growth of the *Trichoderma* host strains, as all fermentations accumulated similar amounts biomass by the end of 6 days of growth (Table 3). Biomass concentration in fermentor samples was determined as follows: 5-10 g of fermentation broth is weighed and recorded. The fermentation broth is then filtered over a pre-weighed glass micro-fiber filter paper (Whatman) and washed with water. The filtered biomass is dried overnight in a 100°C oven. The weight of the dried biomass is determined by subtracting the mass of the filter paper from the mass of the dried biomass plus filter paper. The biomass is calculated as follows:

$$\text{Biomass (g/L)} = \frac{\text{Mass of dry biomass (g)}}{\text{Mass of wet biomass (g)}} \times \text{Density of sample (g/mL)} \times \frac{1000\ \text{mL}}{\text{L}}$$

[00118] Strains producing the modified xylanases comprising any of the X34N, X131N, X180N or X182N mutations (see Table 2 for description of mutations) produced higher levels of total protein than strains producing the corresponding unmodified xylanases (Table 3). The protein concentration in daily fermentor samples was determined using the Bio-Rad Protein Assay (Cat. No. 500-0001).

Table 3: Expression of modified xylanases from transformed *T. reesei* strains (biomass and xylanase activity).

Strain	Enzyme	New N-X-S/T site	Protein (mg/ml)	Biomass (g/l)	Xylanase Activity (XU/ml)	increase in expression fold x (% increase)[b]
RutC30	Native xylanase	--	40.89	30.35	812.5[e]	--
M2C38	Native xylanase	--	23.93	18.11	141[a]	--
P67AB	HTX18	None	18.9	20.1	3302	--
2013B	HTX18-R135Y	None	34.9	24.0	6166	1.86 x (86%)
P210A	ITX1 (T131N, R135Y)	131N	44.7	26.5	15288	4.6 x (360%)[c] 2.5 x (150%)[d]
P284A	ITX2	131N	42.9	21.3	9691	2.9 x (190%)
P304B	ITX3	180N	43.2	20.5	6546	2.0 (100%)x
P321H	ITX3'	180N/182T	40.5	22.8	7192	2.2 x (120%)
P322B	ITX4	182N	39.0	20.2	11083	3.4 x (240%)
P323B	ITX4'	182N/184T	33.0	19.2	12061	3.6 x (260%)
P331B	ITX5	34N	34.5	22.5	5275	1.6 x (60%)
P336B	ITX5'	34N/36T	38.0	22.6	6933	2.1 x (110%
P300A	Xyn 1	No	31.0	23.2	136[a]	-
P279A	Xyn 1-131N	118N	31.75	25.5	2042[a]	15.0 x (1400%)
P348C	xlnC-131N	128N	35.2	23.8	2365[e]	ND

[a] measured at 40°C, pH 4.0

[b] relative to the expression of the corresponding unmodified xylanase comprising the same primary amino acid sequence except for the newly introduced glycosylation motif

[c] xylanase expression efficiency relative to P67AB expressing a modified xylanase comprising Y135R mutation but without 131N

[d] xylanase expression efficiency relative to strain 2013B expressing a modified xylanase comprising neither the T131N nor the Y135R mutation.
[e] measured at 40°C, pH 6.0

[00119] Xylanase activity was determined as described in Example 8.

[00120] Strains P210A and P284A, comprising modified xylanase genetic constructs containing the N-glycosylation motif N-X-T at positions 131-133 of the *T. reesei* xylanase II sequence in addition to the mutations present in HTX18, produce 4.6- and 2.9 fold higher xylanase activity than the HTX18 production strain, P67AB, respectively.

[00121] Strains P304B and P321H, comprising modified xylanase genetic constructs containing the N-glycosylation motif N-X-S/T at positions 180-182 of the *T. reesei* xylanase II sequence in addition to the mutations present in HTX18, produce up to two-fold higher xylanase activity than strain P67AB expressing the unmodified HTX18.

[00122] Strains P322B and P323B, comprising modified xylanase genetic constructs containing the N-glycosylation motif N-X-S/T at positions 182-184 of the *T. reesei* xylanase II sequence in addition to the mutations present in HTX18, produce up to 3.5-fold higher xylanase activity than strain P67AB expressing the unmodified HTX18.

[00123] Strains P331B and P336B, comprising modified xylanase genetic constructs containing the N-glycosylation motif N-X-S/T at positions 34-36 of the *T. reesei* xylanase II sequence in addition to the mutations present in HTX18, produce 1.6- and 2.1-fold higher xylanase activity than strain P67AB expressing the unmodified HTX18, respectively.

[00124] Strain P279A, comprising a modified xylanase genetic construct containing the N-glycosylation motif N-X-T at positions 118-120 of the native *T. reesei* xylanase I sequence, produces 15.0 times higher xylanase activity than strain P300A expressing the unmodified xylanase I. This mutation is equivalent to X131N of *T. reesei* xylanase II (see Figure 1).

[00125] Strain P348C, comprising a modified xylanase genetic construct containing the N-glycosylation motif N-X-T at positions 128-130 of the native *S. lividans* xylanase C sequences produces similarly high levels of xylanase activity as strain P279A, comprising a modified xylanase genetic construct containing the N-glycosylation motif N-X-T at positions 118-120 of the native xylanase I sequence, which is commercially significant for the

manufacturing of xylanase for industrial applications. This mutation is equivalent to X131N of *T. reesei* xylanase II (see Figure 1).

[00126] Strains P321H, P323B and P336B, comprising modified xylanase genetic constructs containing N-X-T glycosylation motifs produce higher amounts of xylanase activity than strains P304B, P322B and P331B, comprising modified xylanase genetic constructs containing N-X-S glycosylation motifs in the same respective positions within the xylanase II sequence.

Example 11: Comparison of the alkalophilicity and thermophilicity of the modified xylanase ITX1 with its native counterpart HTX18

[00127] Activity measurements were determined as described in Example 8. To determine alkalophilicity (Figure 6), the incubation temperature of the assay was reduced to 55°C and the phosphate buffer containing Tween-20 and the NSP substrate solutions were adjusted to the desired pH for activity measurements. To determine thermophilicity (Figure 7), the incubation temperature was adjusted to the desired temperature for activity measurements.

[00128] The modified ITX1 xylanase produced by strain P210A, comprising the same mutations as HTX18 but without the Y135R mutation and the T131N mutation (see Table 2), has a similar pH and temperature activity profile as the HTX18 xylanase (Figures 6 and 7). Thus the addition of the T131N mutation does not alter the desirable biophysical and biochemical properties of the xylanase due to the other mutations. This is significant for the utility of increasing the expression of any target Family 11 from *Trichoderma* via the introduction of the X131N mutation (TrX numbering).

Example 12: Mass-spectral analysis of the ITX1 enzyme comprising the T131N mutation

[00129] The fermentation filtrate produced by strain P210A and containing the modified xylanase ITX1 were diluted in 2X Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% ß-mercaptoethanol), boiled for 5 min and cooled. The proteins were separated by SDS-PAGE using a resolving gel containing 12% acrylamide (37.5:1 acrylamide: bisacrylamide, BioRad Cat. No. 161-0122) using a Mini-PROTEAN® 3 Electrophoresis Cell (BioRad Cat. No. 165-3301) running at 200V (constant) for 40 min. The proteins in the gel were visualized with staining using Bio-Safe™ Coomassie Stain

(BioRad Cat. No.161-0786). The protein band at 20 kDa was excised from the gel, destained and in-gel digested with trypsin as per standard protocols. Briefly, the gel bands were rinsed with 30% acetonitrile in 100 mM ammonium bicarbonate for approximately 10 minutes and the supernatant was discarded. This procedure was repeated until the stain was completely removed. The gel bands were then washed with deionized water and followed by acetonitrile. Approximately, 20 mL of 50 mM ammomium bicarbonate containing 200 ng of trypsin was added to each gel band. The gel bands were allowed to re-swell for 10 minutes and were topped off with approximately 30 mL of 50 mM ammonium bicarbonate (enough to ensure that the gel pieces were completely submerged during the digestion). The digestion was allowed to continue for 4 hours after which the liquid from each sample was transferred to a fresh vial. The solutions were evaporated on a Savant to a final volume of approximately 10 mL.

[00130] The digestion solutions were analyzed by nanoHPLC-tandem mass spectrometry (nanoLC-MS/MS) using a CapLC system (Waters) coupled with a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Waters). 3 mL of the 10 mL digests were injected onto a 0.3 x 5 mm C18 micro precolumn cartridge (Dionex/LC Packings). The peptides were retained while the salts and other solution components were washed away. The trap was then brought on-line with a 75 mm x 150 mm C18 nano-Series column (Dionex/LC-Packings) and the peptides were separated by gradient elution (3-45% acetonitrile, 0.2% formic acid in 35 minutes followed by a rapid increase to 85% at 38.5 minutes). The mass spectrometer was set to acquire MS/MS spectra in automated mode for doubly and triply charged ions. Priority was given to multiply charged ions from the tryptic peptide 123-141 with and without a HexNAc residue attached. The MS/MS spectra were analyzed manually.

Table 4: Detection of glycosylation at 131N by LC-MS/MS

Peptide	LC retention time	M/z of doubly-charged ion	Amino Acid sequence	Distribution
aa 123-141	25.4 min	1203.5367	VNAPSIEGN*ATFYQYWSVR + N-acetyl hexosamine	~80%
aa 123-141	26.7 min	1102.009	VNAPSIEGNATFYQYWSVR	~20%

[00131] These results confirm that the *Trichoderma* host strain recognizes the NAT consensus N-glycosylation motif introduced via mutation of T131N and that the introduction

of this functional glycosylation motif facilitates high level expression of the modified xylanase from *Trichoderma*.

[00132] In summary, modified xylanase demonstrating increased expression efficiency from *Trichoderma* can be constructed through mutation of X131N (TrX numbering). A similar increase in expression efficiency may also be obtained by introducing other N-X-S/T N-glycosylation motifs into a Family 11 xylanase at positions X34N, X180N, X182N, X34N-S36T, X180N-S182T, X182N-S184T, or a combination thereof, at equivalent positions when the Family 11 xylanase is aligned with TrxII, as described herein.

[00133] The present invention has described mutant xylanases that exhibit increased expression and secretion from a *Trichoderma* host. These mutant xylanases may be used in industrial processes such as pulp and paper processing, as animal feed additives, or in baking and brewing applications.

[00134] The present invention has been described with regard to one or more embodiments. However, it will be apparent to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims.

[00135] All references and citations are herein incorporated by reference.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A modified Family 11 xylanase comprising, a sequence that introduces a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived.

2. The modified Family 11 xylanase of claim 1, where in the modification results from direct substitution of one or more amino acids within the primary sequence of the Family 11 xylanase from which the modified Family 11 xylanase is derived.

3. The modified Family 11 xylanase of claim 2 comprising, a substitution of an amino acid at a position selected from the group consisting of position 34 (X34N), position 131 (X131N), position 180 (X180N), position 182 (X182N), and a combination thereof, to an asparagine, the position determined from sequence alignment of the Family 11 xylanase with the amino acid sequence of *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1.

4. The modified Family 11 xylanase of claim 3 comprising, X131N.

5. The modified Family 11 xylanase of claim 3 further comprising, a substitution of an amino acid at a position selected from the group consisting of position 36 (X34N-S36T), position 182 (X180N-S182T), position 184 (X182N-S184T), and a combination thereof, to a threonine.

6. The modified Family 11 xylanase of claim 1, wherein the modified xylanase when expressed in a *Trichoderma* host strain exhibits an increase in expression efficiency of at least 40% when compared to the expression efficiency of a Family 11 xylanase from which the modified xylanase is derived.

7. The modified Family 11 xylanase of claim 2, wherein the modified xylanase when expressed in a *Trichoderma* host strain exhibits an increase in expression efficiency of at least 40% when compared to the expression efficiency of a Family 11 xylanase from which the modified xylanase is derived.

8. The modified Family 11 xylanase of claim 3, wherein the modified xylanase when expressed in a *Trichoderma* host strain exhibits an increase in expression efficiency of at least

40% when compared to the expression efficiency of a Family 11 xylanase from which the modified xylanase is derived.

9. The modified Family 11 xylanase of claim 1, wherein the Family 11 xylanase is a *Trichoderma* xylanase.

10. The modified Family 11 xylanase of claim 2, wherein the Family 11 xylanase is a *Trichoderma* xylanase.

11. The modified Family 11 xylanase of claim 3, wherein the Family 11 xylanase is a *Trichoderma* xylanase.

12. The modified Family 11 xylanase of claim 9, wherein the *Trichoderma* xylanase is xylanase 1 or xylanase 2 from *Trichoderma reesei*.

13. The modified Family 11 xylanase of claim 10, wherein the *Trichoderma* xylanase is xylanase 1 or xylanase 2 from *Trichoderma reesei*.

14. The modified Family 11 xylanase of claim 11, wherein the *Trichoderma* xylanase is xylanase 1 or xylanase 2 from *Trichoderma reesei*.

15. A modified Family 11 xylanase selected from the group consisting of: ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5', Xln1-131N, and *S. lividans* xlnC-T131N.

16. A modified Family 11 xylanase genetic construct comprising a promoter operatively linked to a secretion signal that is operatively linked to a coding region, the coding region comprising a functional consensus N-glycosylation site that is otherwise not found in the Family 11 xylanase from which the modified Family 11 xylanase is derived, the modified xylanase genetic construct resulting in an increase in expression efficiency of an encoded modified xylanase when compared to the expression efficiency of an encoded Family 11 xylanase from which the encoded modified xylanase was derived.

17. The modified Family 11 xylanase genetic construct of claim 16, wherein the modification results from direct substitution of one or more amino acids within the primary sequence of the Family 11 xylanase from which the modified Family 11 xylanase is derived.

18. The modified Family 11 xylanase genetic construct of claim 16, wherein the expression efficiency for the encoded modified xylanase is increased by at least 40% compared to the expression efficiency of the encoded Family 11 xylanase from which the encoded modified xylanase was derived.

19. The modified Family 11 xylanase genetic construct of claim 17, wherein the expression efficiency for the encoded modified xylanase is increased by at least 40% compared to the expression efficiency of the encoded Family 11 xylanase from which the encoded modified xylanase was derived.

20. The modified Family 11 xylanase genetic construct of claim 16, wherein the secretion signal is a *Trichoderma* secretion signal.

21. The modified Family 11 xylanase genetic construct of claim 16, wherein the *Trichoderma* secretion signal is a xylanase secretion signal.

22. The modified Family 11 xylanase genetic construct of claim 21, wherein the xylanase secretion signal is a *Trichoderma* xylanase I secretion signal or a *Trichoderma* xylanase II secretion signal.

23. The modified Family 11 xylanase genetic construct of claim 16, wherein the coding region encodes a modified xylanase selected from the group consisting of: ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5', Xln1-131N, and *S. lividans* xlnC-T131N.

24. The modified Family 11 xylanase genetic construct of claim 23, wherein the coding region encodes ITX1.

25. The genetic construct of claim 16, wherein the promoter is selected from the group consisting of a *Trichoderma cbh1* promoter, a *cbh2* promoter, an *eg1* promoter, an *eg2* promoter, an *eg3* promoter, an *eg5* promoter, a *xln1* promoter, a *xln2* promoter, and a combination of two or more than two of these promoters.

26. A genetically modified microbe comprising the modified Family 11 xylanase genetic construct of claim 16.

27. A genetically modified microbe comprising the modified Family 11 xylanase genetic construct of claim 17.

28. The genetically modified microbe of claim 26, wherein the expression efficiency for the encoded modified xylanase is increased by at least 40% compared to the expression efficiency of the encoded Family 11 xylanase from which the encoded modified xylanase was derived.

29. The genetically modified microbe of claim 26, wherein the microbe comprises a member of the genus of *Trichoderma* or *Hypocrea*.

30. The genetically modified microbe of claim 26, wherein the secretion signal is a *Trichoderma* secretion signal.

31. The genetically modified microbe of claim 30, wherein the *Trichoderma* secretion signal is a *Trichoderma* xylanase secretion signal.

32. The genetically modified microbe of claim 31, wherein the *Trichoderma* xylanase secretion signal is a *Trichoderma* xylanase I secretion signal, or a *Trichoderma* xylanase II secretion signal.

33. The genetically modified microbe of claim 26, wherein the coding region encodes a modified xylanase selected from the group consisting of: ITX1, ITX2, ITX3, ITX3', ITX4, ITX4', ITX5, ITX5', Xln1-131N, and *S. lividans* xlnC-T131N.

34. The genetically modified microbe of claim 26, wherein the promoter is selected from the group consisting of a *Trichoderma cbh1* promoter, a *cbh2* promoter, an *eg1* promoter, an *eg2* promoter, an *eg3* promoter, an *eg5* promoter, a *xln1* promoter, a *xln2* promoter, and a combination of two or more than two of these promoters.

35. A method of processing food or feed comprising, treating the food or feed with an additive comprising the modified Family 11 xylanase of claim 1.

36. The method as defined in claim 35 wherein the food or feed additive is selected from the group consisting of a poultry feed additive, a swine feed additive, an additive used in baking, or an additive used in brewing.

37. A method of paper pulp manufacturing comprising treating the pulp with the modified Family 11 xylanase of claim 1.

38. The use of the modified xylanase in claim 1 in an industrial feed or food process.

39. The use as defined in claim 38 wherein the industrial process is paper pulp manufacturing.

```
Ca     23                                                    S AFNTQAAP  31
Cs      1                                                          G   1

Tr2#                        10         20         30         40
                            |          |          |          |
Bp      1      RTITNNEMGN HSGYDYELWK DYGNT-SMTL NNGGAFSAGW N--NIGNA  45
Ca     32      KTITSNEIGV NGGYDYELWK DYGNT-SMTL KNGGAFSCQW S--NIGNA  76
Fs      1       NSSVTGNVG SSPYHYEIWY QGG-NNSMTF YDNGTYKASW N--GTNDF  44
Cs      2      RIIYDNETGT HGGYDYELWK DYGNT-IMEL NDGGTFSCQW S--NIGNA  46
Rf      1      SAADQQTRGN VGGYDYEMWN QNGQGQASMN PGAGSFTCSW S--NIENF  46
Tr2     1       QTIQPGTGY NNGYFYSYWN DGHGGVTYTN GPGGQFSVNW S--NSGNF  45
Tv      1       QTIQPGTGF NNGYFYSYWN DGHGGVTYTN GPGGQFSVNW S--NSGNF  45
Th      1       QTIGPGTGY SNGYYYSYWN DGHAGVTYTN GGGGSFTVNW S--NSGNF  45
Sc      1       SGTPSSTGT DGGYYYSWWT DGAGDATYQN NGGGSYTLTW SG-NNGNL  46
An      1               S AGINYVQNYN GNLGDFTY-D ESAGTFSMYW EDGVSSDF  38
Ak      1               S AGINYVQNYN GNLADFTY-D ESAGTFSMYW EDGVSSDF  38
AT      1               S AGINYVQNYN QNLGDFTY-D ESAGTFSMYW EDGVSSDF  38
Tr1     1                 ASINYDQNYQ TGG-QVSYS- PSNTGFSVNW N--TQDDF  34
Aa      1      RSTPSSTGE NNGYYYSFWT DGGGDVTYTN GNAGSYSVEW S--NVGNF  45
Ss      1    ATTIT-NETGY D-GMYYSFWT DGGGSVSMTL NGGGSYSTRW T--NCGNF  45
SlB     1    DTVVTTNQEGT NNGYYYSFWT DSQGTVSMNM GSGGQYSTSW R--NTGNF  47
SlC     1    ATTITTNQTGT D-GMYYSFWT DGGGSVSMTL NGGGSYSTQW T--NCGNF  46
Tl      1      QTTPNSEGW HDGYYYSWWS DGGAQATYTN LEGGTYEISW G--DGGNL  45
Tf      1      AVTSNETGY HDGYFYSFWT DAPGTVSMEL GPGGNYSTSW R--NTGNF  45
Bc      1                ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW S--NTGNF  36
Bs      1                ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW S--NTGNF  36
```

FIGURE 1

```
Tr2#                50                  60                  70                  80
                    |                   |                   |       *           |
Bp       46  LFRK-GKKFD ST-RTHHQLG NISINYNASF N-PSGNSYLC VYGWTQSP  90
Ca       77  LFRK-GKKFN DT-QTYKQLG NISVNYNCNY Q-PYGNSYLC VYGWTSSP 121
FS       45  LARV-GFKYD EK-HTYEELGPIDAYYKWSKQ GSAGGYNYIG IYGWTVDP  91
Cs       47  LFRK-GRKFN SD-KTYQELG DIVVEYGCDY N-PNGNSYLC VYGWTRNF  91
Rf       47  LARM-GKNYD SQKKNYKAFG NIVLTYDVEY T-PRGNSYMC VYGWTRNP  92
Tr2      46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS VYGWSRNP  83
Tv       46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS VYGWSRNP  83
Th       46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS IYGWSRNP  83
Sc       47  VGGK-GWNPG AASRS----- ---ISYS-GT YQPNGNSYLS VYGWTRSS  84
An       39  VVGL-GWTTG SSNA------ ---ITYSAEY SASGSSSYLA VYGWVNYP  76
Ak       39  VVGL-GWTTG SSNA------ ---ISYSAEY SASGSSSYLA VYGWVNYP  76
At       39  VVGLGGWTTG SSNA------ ---ITYSAEY SASGSASYLA VYGWVNYP  77
Tr1      35  VVGV-GWTTG SSAP------ ---INFGGSF SVNSGTGLLS VYGWSTNP  72
Aa       46  VGGK-GWNPG SAKD------ ---ITYSGNF T-PSGNGYLS VYGWTTDP  83
Ss       46  VAGK-GWANG GR-RT----- ---VRYT-GW FNPSGNGYGC LYGWTSNP  82
S1B      48  VAGK-GWANG GR-RT----- ---VQYS-GS FNPSGNAYLA LYGWTSNP  84
S1C      47  VAGK-GWSTG DGN------- ---VRYN-GY FNPVGNGYGC LYGWTSNP  82
Tl       46  VGGK-GWNPG LNARA----- ---IHFE-GV YQPNGNSYLA VYGWTRNP  83
Tf       46  VAGK-GWATG GR-RT----- ---VTYS-AS FNPSGNAYLT LYGWTRNP  82
Bc       37  VVGK-GWTTG SPFRT----- ---INYNAGV WAPNGNGYLT LYGWTRSP  75
Bs       37  VVGK-GWTTG SPFRT----- ---INYNAGV WAPNGNGYLT LYGWTRSP  75

Tr2#                90                 100                 110                 120                 130
                    |                   |       *           |                   |                   |
Bp       91  LAEYYIVDSW GTYR-PT--G AYKGSFYADG GTYDIYETTR VNQPSIIG 135
Ca      122  LVEYYIVDSW GSWRPP--GG TSKGTITVDG GIYDIYETTR INQPSIQG 167
Fs       92  LVEYYIVDDW FNKPGANLLG QRKGEFTVDG DTYEIWQNTR VQQPSIKG 139
Cs       92  LVEYYIVESW GSWRPP--GA TPKGTITQWMAGTYEIYETTR VNQPSIDG 138
Rf       93  LMEYYIVEGW GDWRPPGNDG EVKGTVSANG NTYDIRKTMR YNQPSLDG 140
Tr2      84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Tv       84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Th       84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Sc       85  LIEYYIVESY GSYD-PSSAA SHKGSVTCNG ATYDILSTWR YNAPSIDG 131
An       77  GAEYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 123
Ak       77  QAEYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR TNEPSITG 123
At       78  QAEYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 124
Tr1      73  LVEYYIMEDN HNY--PAQ-G TVKGTVTSDG ATYTIWENTR VNEPSIQG 117
Aa       84  LIEYYIVESY GDYN-PGSGG TTRGNVSSDG SVYDIYTATR TNAPSIDG 130
Ss       83  LVEYYIVDNW GSYR-PT--G ETRGTVHSDG GTYDIYKTTR YNAPSVEA 127
S1B      85  LVEYYIVDNW GTYR-PT--G EYKGTVTSDG GTYDIYKTTR VNKPSVEG 129
S1C      83  LVEYYIVDNW GSYR-PT--G TYKGTVSSDG GTYDIYQTTR YNAPSVEG 127
Tl       84  LVEYYIVENF GTYD-PSSGA TDLGTVECDG SIYRLGKTTR VNAPSIDG 130
Tf       83  LVEYYIVESW GTYR-PT--G TYMGTVTTDG GTYDIYKTTR YNAPSIEG 127
Bc       76  LIEYYVVDSW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR YNAPSIDG 120
Bs       76  LIEYYVVDSW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR YNAPSIDG 120
```

FIGURE 1 CONT'D

```
Tr2#                      140                 150         160
                           |                   |           |
Bp      136  -IATFKQYWS VRQTKRTS-- ------GTVS VSAHFRKWES LGMPM-GK 174
Ca      168  -NTTFKQYWS VRRTKRTS-- ------GTIS VSKHFAAWES KGMPL-GK 206
Fs      140  -TQTFPQYFS VRKSARSC-- ------GHID ITAHMKKWEE LGMKM-GK 178
Cs      139  -TATFQQYWS VRTSKRTS-- ------GTIS VTEHFKQWER MGMRM-GK 177
Rf      141  -TATFPQYWS VRQTSGSANN QTNYMKGTID VSKHFDAWSA AGLDMSGT 187
Tr2     131  -TATFYQYWS VRRNHR-S-S ------GSVN TANHFNAWAQ QGLTL-GT 168
Tv      131  -TATFYQYWS VRRTHR-S-S ------GSVN TANHFNAWAQ QGLTL-GT 168
Th      131  -TATFYQYWS VRRNHR-S-S ------GSVN TANHFNAWAS HGLTL-GT 168
Sc      132  -TQTFEQFWS VRNPKKAPGG SIS---GTVD VQCHFDAWKG LGMNLGSE 175
An      124  -TSTFTQYFS VRESTRTS-- ------GTVT VANHFNFWAQ HGFGN-SD 162
Ak      124  -TSTFTQYFS VRESTRTS-- ------GTVT VANHFNFWAQ HGFGN-SD 162
At      125  -TSTFTQYFS VRESTRTS-- ------GTVT VANHFNFWAH HGFHN-SD 163
Tr1     118  -TATFNQYIS VRNSPR-T-S ------GTVT VQNHFN-WAS LGLHLGQM 155
Aa      131  -TQTFSQYWS VRQNKR-VG- ------GTVT TSNHFNAWAK LGMNL-GT 168
Ss      128  -PAAFDQYWS VRQSKVT--S ------GTIT TGNHFDAWAR AGMNMGNF 168
SlB     130  TR-TFDQYWS VRQSKR-TG- ------GTIT TGNHFDAWAR AGMPLGNF 168
SlC     128  TK-TFQQYWS VRQSKVTSGS ------GTIT TGNHFDAWAR AGMNMGQF 168
Tl      131  TQ-TFDQYWS VRQDKR-T-S ------GTVQ TGCHFDAWAR AGLNVNGD 169
Tf      128  TR-TFDQYWS VRQSKRTS-- ------GTIT AGNHFDAWAR HGMHLGTH 166
Bc      121  DRTTFTQYWS VRQSKRPTGS N-----ATIT FTNHVNAWKS HGMNLGSN 163
Bs      121  DRTTFTQYWS VRQSKRPTGS N-----ATIT FSNHVNAWKS HGMNLGSN 163

Tr2#          170         180         190
                |           |           |
Bp      175  MYETAFTVEG YQSSGSANVM TNQLFIGN         201
Ca      207  MHETAFNIEG YQSSGKADVN SMSINIGK         233
Fs      179  MYEAKVLVEA GGGSGSFDV- TYFKMT           203
Cs      178  MYEVALTVEG YQSSGYANVY KNEIRIGANP....
Rf      188  LYEVSLNIEG YRSNGSANVK SVSV             211
Tr2     169  MDYQIVAVEG YFSSGSASI- TVS               190
Tv      169  MDYQIVAVEG YFSSGSASI- TVS               190
Th      169  MDYQIVAVEG YFSSGSASI- TVS               190
Sc      176  HNYQIVATEG YQSSGTATI- TVT               197
An      163  FNYQVMAVEA WSGAGSASV- TISS              184
Ak      163  FNYQVMAVEA WSGAGSASV- TISS              184
At      164  FNYQVVAVEA WSGAGSAAV- TISS              185
Tr1     157  MNYQVVAVEG WGGSGSASQ- SVSN              178
Aa      169  HNYQILATEG YQSSGSSSI- TIQ               190
Ss      167  RYYMINATEG YQSSGSSTI- TVSG              189
SlB     169  SYYMINATEG YQSSGTSSI- NVGG..........
SlC     169  RYYMINATEG YQSSGSSNI- TVSG              191
Tl      170  HYYQIVATEG YFSSGYARI- TVADVG            194
Tf      167  D-YMIMATEG YQSSGSSNVT LGTS..........
Bc      164  WAYQVMATEG YQSSGSSNV- TVW               185
Bs      164  WAYQVMATEG YQSSGSSNV- TVW               185
```

FIGURE 1 CONT'D

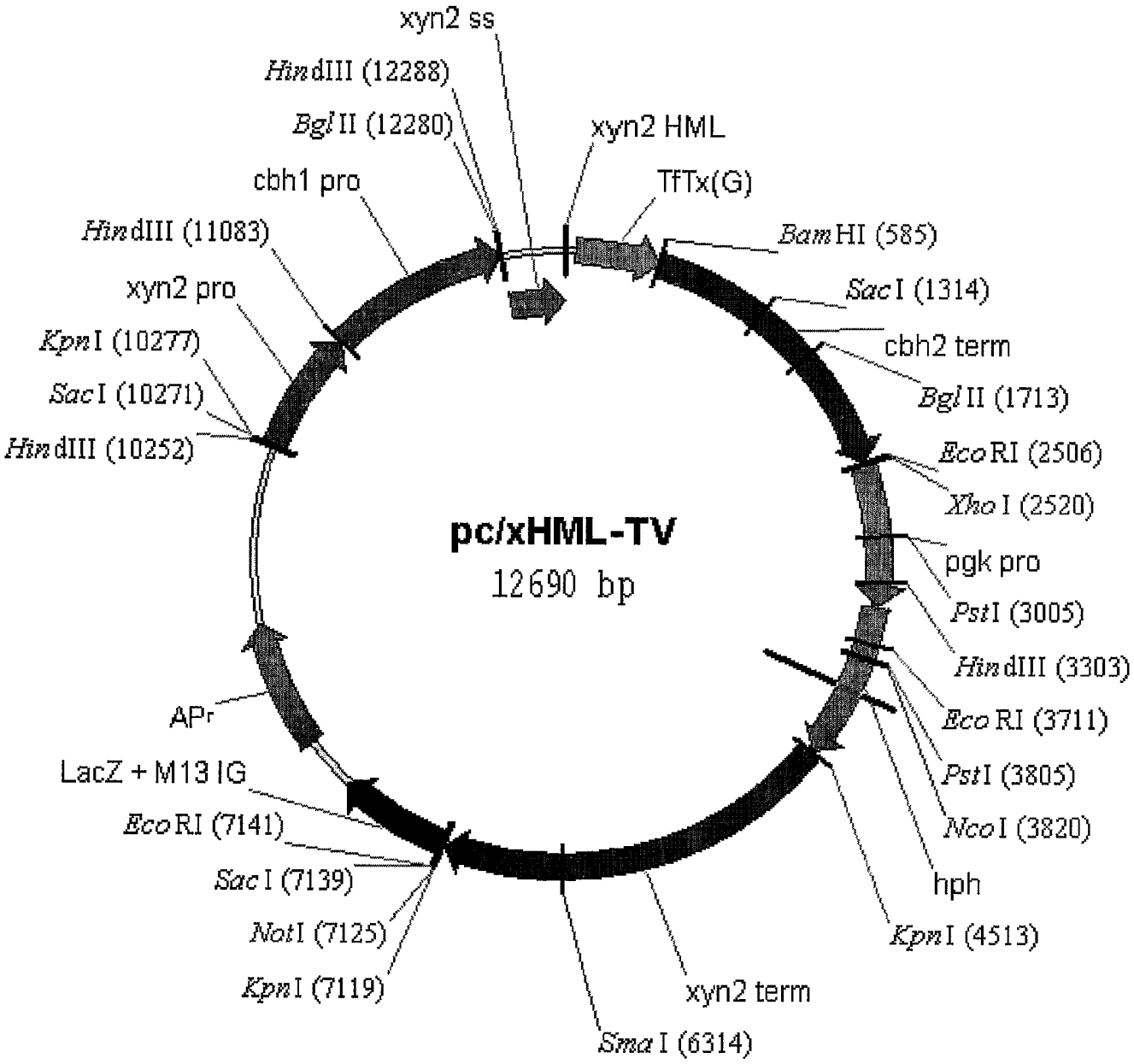

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xyn2 pro
SacI (1314)
KpnI (10277)
cbh2 term
SacI (10271)
BglII (1713)
HindIII (10252)
EcoRI (2506)
XhoI (2520)
pc/xHML-TV
12690 bp
pgk pro
PstI (3005)
HindIII (3303)
APr
EcoRI (3711)
LacZ + M13 IG
PstI (3805)
EcoRI (7141)
NcoI (3820)
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NotI (7125)
KpnI (4513)
KpnI (7119)
xyn2 term
SmaI (6314)

Figure 2

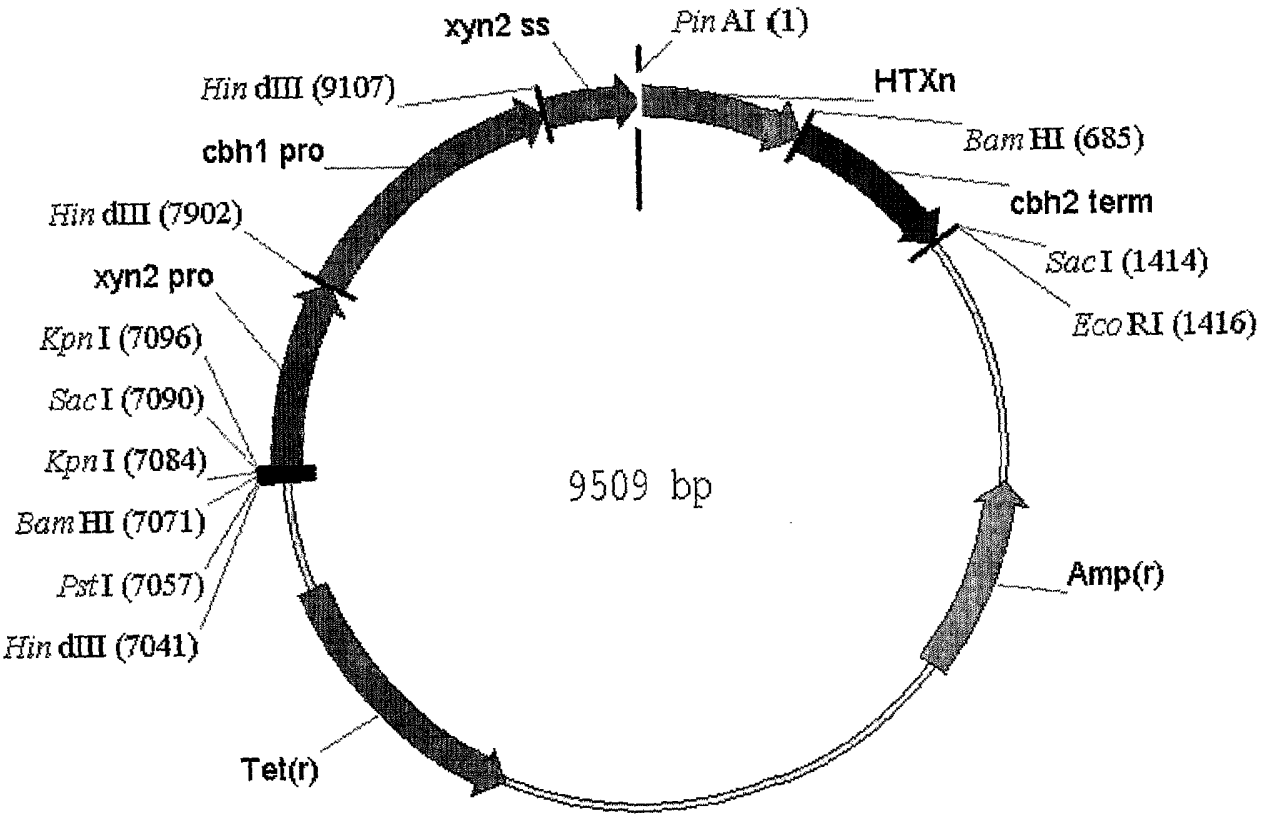

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xyn2 pro
Eco RI (1416)
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Sac I (7090)
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Amp(r)
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Hin dIII (7041)
Tet(r)

Figure 3

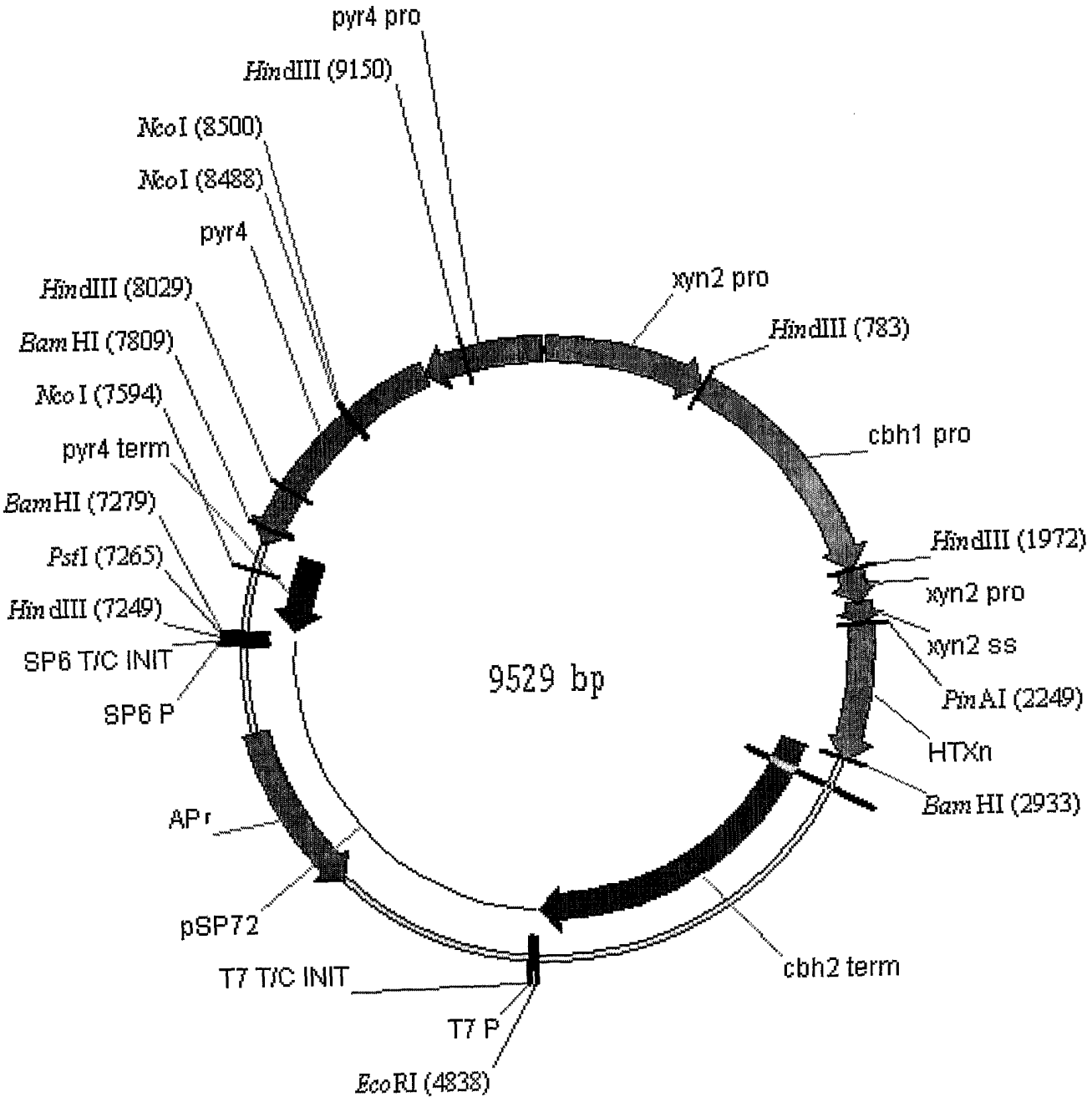

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pyr4
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BamHI (7809)
NcoI (7594)
pyr4 term
BamHI (7279)
PstI (7265)
HindIII (7249)
SP6 T/C INIT
SP6 P
APr
pSP72
T7 T/C INIT
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HindIII (783)
cbh1 pro
HindIII (1972)
xyn2 pro
xyn2 ss
PinAI (2249)
HTXn
BamHI (2933)
cbh2 term
9529 bp

Figure 4

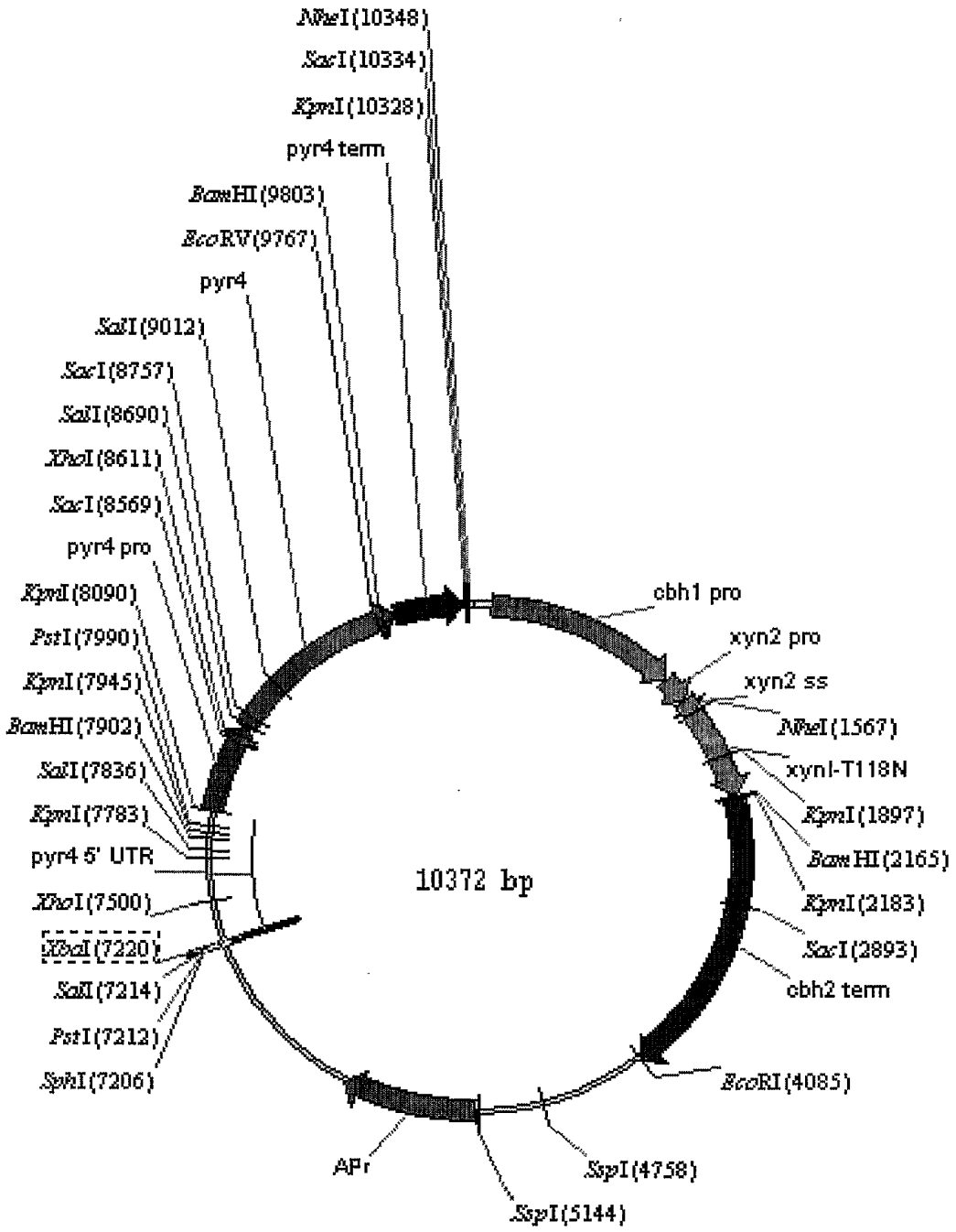

NheI(10348)
SacI(10334)
KpnI(10328)
pyr4 term
BamHI(9803)
EcoRV(9767)
pyr4
SalI(9012)
SacI(8757)
SalI(8690)
XhoI(8611)
SacI(8569)
pyr4 pro
KpnI(8090)
PstI(7990)
KpnI(7945)
BamHI(7902)
SalI(7836)
KpnI(7783)
pyr4 5' UTR
XhoI(7500)
XbaI(7220)
SalI(7214)
PstI(7212)
SphI(7206)
APr
SspI(5144)
SspI(4758)
EcoRI(4085)
cbh2 term
SacI(2893)
KpnI(2183)
BamHI(2165)
KpnI(1897)
xynI-T118N
NheI(1567)
xyn2 ss
xyn2 pro
cbh1 pro
10372 bp

Figure 5A

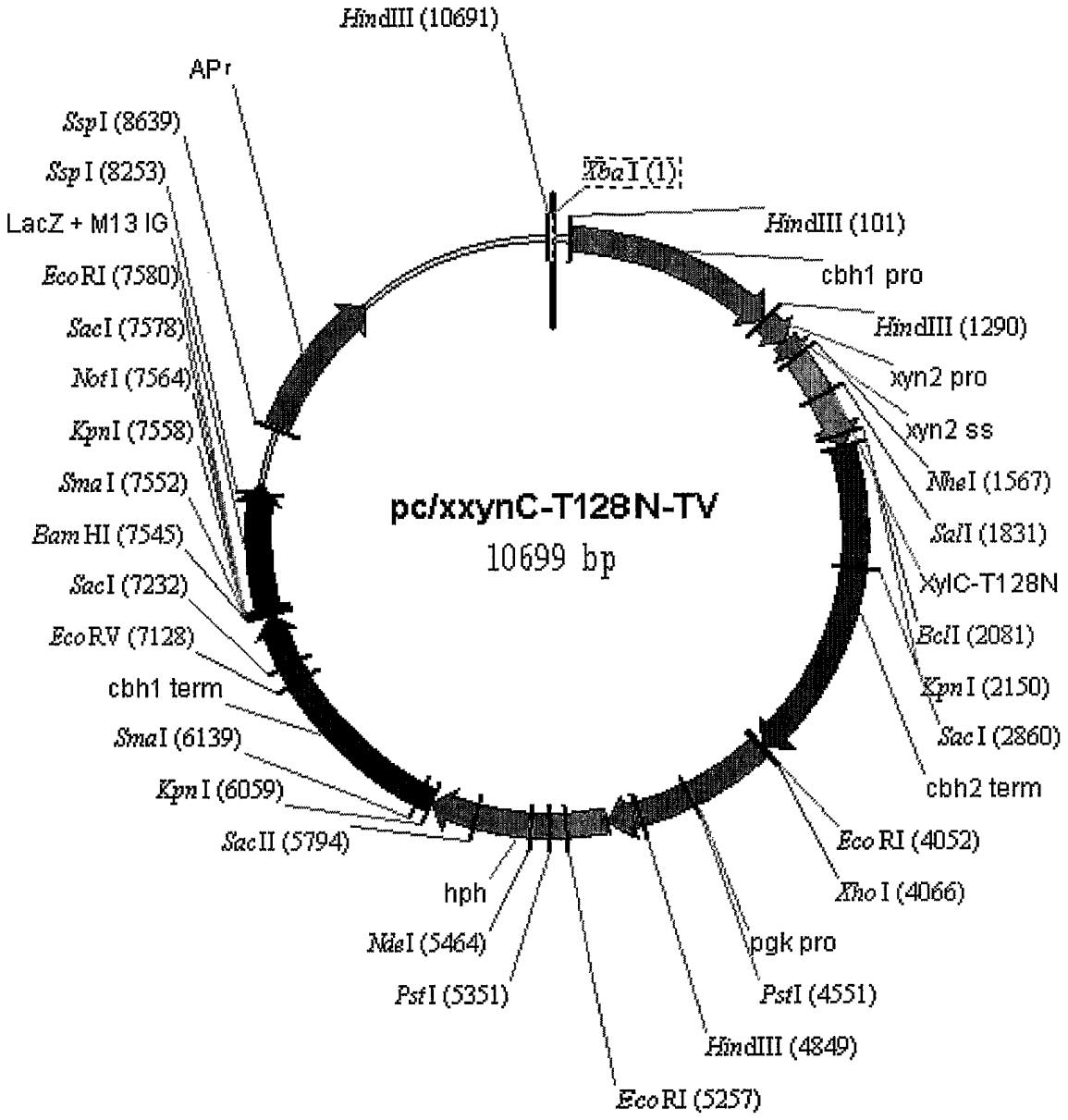

HindIII (10691)
APr
SspI (8639)
SspI (8253)
LacZ + M13 IG
EcoRI (7580)
SacI (7578)
NotI (7564)
KpnI (7558)
SmaI (7552)
BamHI (7545)
SacI (7232)
EcoRV (7128)
cbh1 term
SmaI (6139)
KpnI (6059)
SacII (5794)
hph
NdeI (5464)
PstI (5351)
EcoRI (5257)
HindIII (4849)
pgk pro
PstI (4551)
XhoI (4066)
EcoRI (4052)
cbh2 term
SacI (2860)
KpnI (2150)
BclI (2081)
XylC-T128N
SalI (1831)
NheI (1567)
xyn2 ss
xyn2 pro
HindIII (1290)
cbh1 pro
HindIII (101)
XbaI (1)
pc/xxynC-T128N-TV
10699 bp

Figure 5B

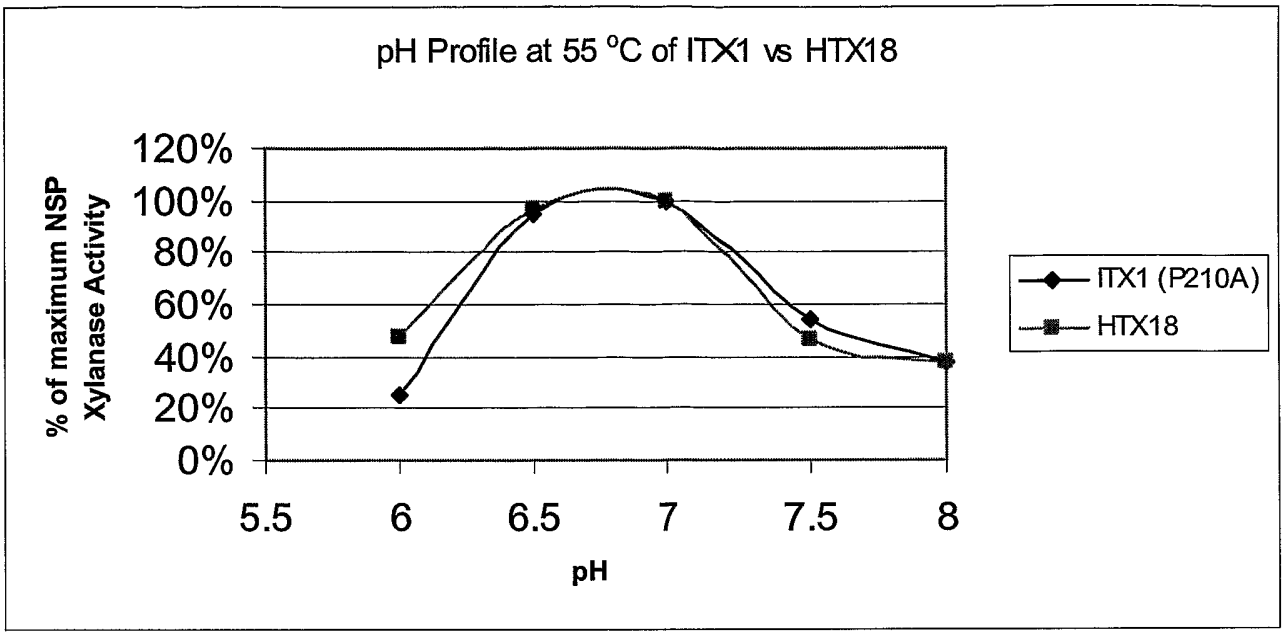

pH Profile at 55 °C of ITX1 vs HTX18
% of maximum NSP Xylanase Activity
120%
100%
80%
60%
40%
20%
0%
5.5
6
6.5
7
7.5
8
pH
ITX1 (P210A)
HTX18

Figure 6

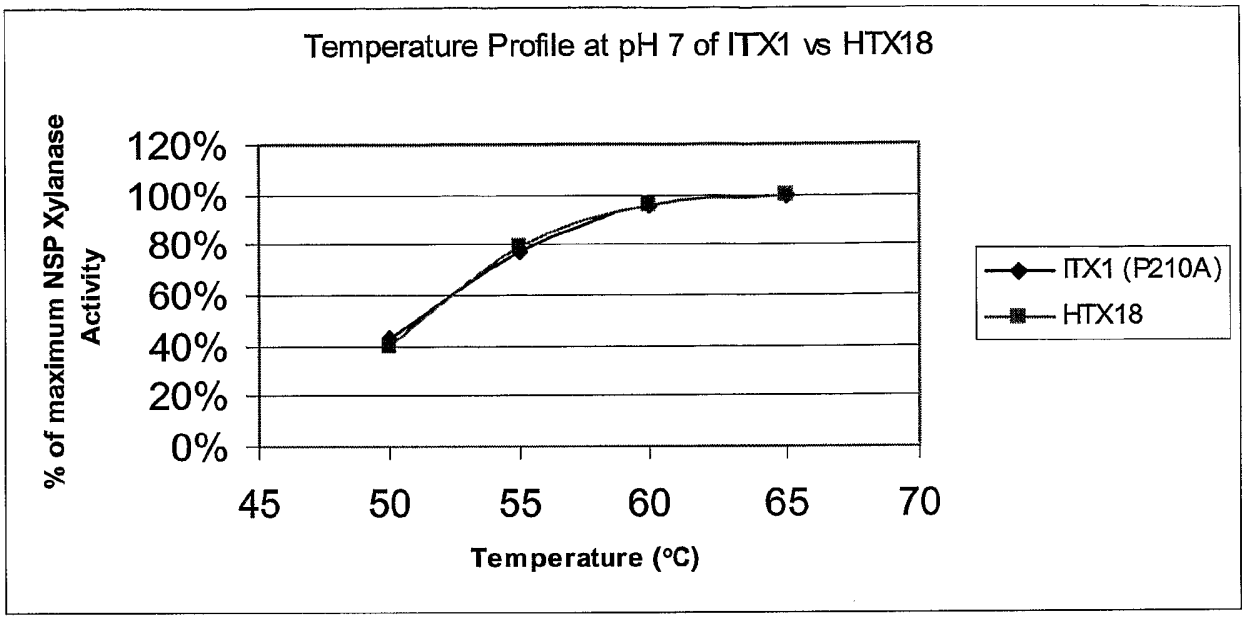

Temperature Profile at pH 7 of ITX1 vs HTX18
% of maximum NSP Xylanase Activity
120%
100%
80%
60%
40%
20%
0%
45
50
55
60
65
70
Temperature (°C)
ITX1 (P210A)
HTX18

Figure 7

SEQUENCE LISTING

<110> Iogen Bio-Products Corporation
White, Theresa C
Giroux, Genevieve R
Wallace, Katie E.A.

<120> Modified Xylanases Exhibiting Improved Expression

<130> 08899867WO

<140> not yet known
<141> 2005-03-24

<150> US 60/556,061
<151> 2004-03-25

<160> 44

<170> PatentIn version 3.2

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20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His

130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Glu Ile Val Ala Val
165 170 175

Arg Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

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Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
35 40 45

Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
50 55 60

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65 70 75 80

Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
85 90 95

Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
100 105 110

Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
115 120 125

Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
130 135 140

Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
145 150 155 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
165 170 175

Ser Asn

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<213> XynC-3R (Kpn)

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cgtccaccgg taccaggtca acc 23

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ccatccaggg caacgcgacc ttc 23

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cgtcgtgcta gcatcaacta cgac 24

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<213> Xyn1-R (BamHI)

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ccgtccatcg agggcaacgc caccttcgc 30

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<400> 22
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ggttacttta gcaacggcac cgcttccatc 30

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<400> 26
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Phe Trp Thr Asp Gly Gly Gly Asp Val Thr Tyr Thr Asn Gly Asn Ala
20 25 30

Gly Ser Tyr Ser Val Glu Trp Ser Asn Val Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Asn Pro Gly Ser Ala Lys Asp Ile Thr Tyr Ser Gly Asn

50 55 60

Phe Thr Pro Ser Gly Asn Gly Tyr Leu Ser Val Tyr Gly Trp Thr Thr
65 70 75 80

Asp Pro Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Asp Tyr Asn
85 90 95

Pro Gly Ser Gly Gly Thr Thr Arg Gly Asn Val Ser Ser Asp Gly Ser
100 105 110

Val Tyr Asp Ile Tyr Thr Ala Thr Arg Thr Asn Ala Pro Ser Ile Asp
115 120 125

Gly Thr Gln Thr Phe Ser Gln Tyr Trp Ser Val Arg Gln Asn Lys Arg
130 135 140

Val Gly Gly Thr Val Thr Thr Ser Asn His Phe Asn Ala Trp Ala Lys
145 150 155 160

Leu Gly Met Asn Leu Gly Thr His Asn Tyr Gln Ile Leu Ala Thr Glu
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Gly Tyr Gln Ser Ser Gly Ser Ser Ser Ile Thr Ile Gln
180 185

<210> 29
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<213> Aspergillus tubigensis

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Phe Thr Tyr Asp Glu Ser Ala Gly Thr Phe Ser Met Tyr Trp Glu Asp
20 25 30

Gly Val Ser Ser Asp Phe Val Val Gly Leu Gly Gly Trp Thr Thr Gly
35 40 45

Ser Ser Asn Ala Ile Thr Tyr Ser Ala Glu Tyr Ser Ala Ser Gly Ser
50 55 60

Ala Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gln Ala Glu
65 70 75 80

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Tyr Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala
                85                  90                  95

Thr Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys
            100                 105                 110

Thr Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe
        115                 120                 125

Thr Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val
    130                 135                 140

Thr Val Ala Asn His Phe Asn Phe Trp Ala His His Gly Phe His Asn
145                 150                 155                 160

Ser Asp Phe Asn Tyr Gln Val Val Ala Val Glu Ala Trp Ser Gly Ala
                165                 170                 175

Gly Ser Ala Ala Val Thr Ile Ser Ser
            180                 185
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<213> Bacillus circulans

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1               5                   10                  15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
            20                  25                  30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
        35                  40                  45

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

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65                  70                  75                  80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
                85                  90                  95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
```

100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
130 135 140

Thr Phe Thr Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

<210> 31
<211> 201
<212> PRT
<213> Bacillus pumilus

<400> 31

Arg Thr Ile Thr Asn Asn Glu Met Gly Asn His Ser Gly Tyr Asp Tyr
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Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ser Met Thr Leu Asn Asn Gly
20 25 30

Gly Ala Phe Ser Ala Gly Trp Asn Asn Ile Gly Asn Ala Leu Phe Arg
35 40 45

Lys Gly Lys Lys Phe Asp Ser Thr Arg Thr His His Gln Leu Gly Asn
50 55 60

Ile Ser Ile Asn Tyr Asn Ala Ser Phe Asn Pro Ser Gly Asn Ser Tyr
65 70 75 80

Leu Cys Val Tyr Gly Trp Thr Gln Ser Pro Leu Ala Glu Tyr Tyr Ile
85 90 95

Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Ala Tyr Lys Gly Ser
100 105 110

Phe Tyr Ala Asp Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Thr Arg Val
115 120 125

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Asn Gln Pro Ser Ile Ile Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser
    130                 135                 140

Val Arg Gln Thr Lys Arg Thr Ser Gly Thr Val Ser Val Ser Ala His
145                 150                 155                 160

Phe Arg Lys Trp Glu Ser Leu Gly Met Pro Met Gly Lys Met Tyr Glu
                165                 170                 175

Thr Ala Phe Thr Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val
            180                 185                 190

Met Thr Asn Gln Leu Phe Ile Gly Asn
        195                 200
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<210> 32
<211> 185
<212> PRT
<213> Bacillus subtilus

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1               5                   10                  15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
            20                  25                  30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
        35                  40                  45

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

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65                  70                  75                  80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
                85                  90                  95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
            100                 105                 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
        115                 120                 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
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130 135 140

Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

<210> 33
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<212> PRT
<213> Clostridium acetobutylicum

<400> 33

Ser Ala Phe Asn Thr Gln Ala Ala Pro Lys Thr Ile Thr Ser Asn Glu
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Ile Gly Val Asn Gly Gly Tyr Asp Tyr Glu Leu Trp Lys Asp Tyr Gly
20 25 30

Asn Thr Ser Met Thr Leu Lys Asn Gly Gly Ala Phe Ser Cys Gln Trp
35 40 45

Ser Asn Ile Gly Asn Ala Leu Phe Arg Lys Gly Lys Lys Phe Asn Asp
50 55 60

Thr Gln Thr Tyr Lys Gln Leu Gly Asn Ile Ser Val Asn Tyr Asn Cys
65 70 75 80

Asn Tyr Gln Pro Tyr Gly Asn Ser Tyr Leu Cys Val Tyr Gly Trp Thr
85 90 95

Ser Ser Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly Ser Trp
100 105 110

Arg Pro Pro Gly Gly Thr Ser Lys Gly Thr Ile Thr Val Asp Gly Gly
115 120 125

Ile Tyr Asp Ile Tyr Glu Thr Thr Arg Ile Asn Gln Pro Ser Ile Gln
130 135 140

Gly Asn Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg
145 150 155 160

Thr Ser Gly Thr Ile Ser Val Ser Lys His Phe Ala Ala Trp Glu Ser
165 170 175

Lys Gly Met Pro Leu Gly Lys Met His Glu Thr Ala Phe Asn Ile Glu
180 185 190

Gly Tyr Gln Ser Ser Gly Lys Ala Asp Val Asn Ser Met Ser Ile Asn
195 200 205

Ile Gly Lys
210

<210> 34
<211> 206
<212> PRT
<213> Clostridium stercocrarium

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Tyr Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ile Met Glu Leu Asn Asp
20 25 30

Gly Gly Thr Phe Ser Cys Gln Trp Ser Asn Ile Gly Asn Ala Leu Phe
35 40 45

Arg Lys Gly Arg Lys Phe Asn Ser Asp Lys Thr Tyr Gln Glu Leu Gly
50 55 60

Asp Ile Val Val Glu Tyr Gly Cys Asp Tyr Asn Pro Asn Gly Asn Ser
65 70 75 80

Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
85 90 95

Ile Val Glu Ser Trp Gly Ser Trp Arg Pro Pro Gly Ala Thr Pro Lys
100 105 110

Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
115 120 125

Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
130 135 140

Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val

145 150 155 160

Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
165 170 175

Met Tyr Glu Val Ala Leu Thr Val Glu Gly Tyr Gln Ser Ser Gly Tyr
180 185 190

Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
195 200 205

<210> 35
<211> 211
<212> PRT
<213> Ruminoccus flavefaciens

<400> 35

Ser Ala Ala Asp Gln Gln Thr Arg Gly Asn Val Gly Gly Tyr Asp Tyr
1 5 10 15

Glu Met Trp Asn Gln Asn Gly Gln Gly Gln Ala Ser Met Asn Pro Gly
20 25 30

Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
35 40 45

Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
50 55 60

Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
65 70 75 80

Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
85 90 95

Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
100 105 110

Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
115 120 125

Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
130 135 140

Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
145 150 155 160

Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
165 170 175

Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
180 185 190

Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
195 200 205

Val Ser Val
210

<210> 36
<211> 197
<212> PRT
<213> Schizophyllum cimmune

<400> 36

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Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
20 25 30

Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
35 40 45

Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
50 55 60

Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
65 70 75 80

Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
85 90 95

Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
100 105 110

Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
115 120 125

Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
130 135 140

Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys

145 150 155 160

His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
165 170 175

Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
180 185 190

Thr Ile Thr Val Thr
195

<210> 37
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<213> Streptomyces sp. No. 36a

<400> 37

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Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
20 25 30

Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
35 40 45

Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
50 55 60

Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
115 120 125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
130 135 140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
145 150 155 160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
165 170 175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
180 185

<210> 38
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<213> Streptomyces lividans Xyl B

<400> 38

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Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
20 25 30

Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
35 40 45

Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser
50 55 60

Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
65 70 75 80

Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
85 90 95

Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
100 105 110

Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
115 120 125

Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
130 135 140

Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145 150 155 160

Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
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Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly

180 185 190

<210> 39
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<212> PRT
<213> Streptomyces lividans Xyl C

<400> 39

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20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
115 120 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
130 135 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145 150 155 160

Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
165 170 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
180 185 190

<210> 40
<211> 189
<212> PRT

<213> Thermomonospora fusca

<400> 40

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Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
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Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
35 40 45

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
50 55 60

Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
115 120 125

Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
130 135 140

Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
145 150 155 160

Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
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Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
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<210> 41
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<212> PRT
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<400> 41

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Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
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Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
    50                  55                  60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
65                  70                  75                  80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
                85                  90                  95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
            100                 105                 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
        115                 120                 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
    130                 135                 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145                 150                 155                 160

Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
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Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
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<210> 42
<211> 190
<212> PRT
<213> Trichoderma viride

<400> 42

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Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
            20                  25                  30
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Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

<210> 43
<211> 178
<212> PRT
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<400> 43

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Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
20 25 30

Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
35 40 45

Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
50 55 60

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65 70 75 80

Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
85 90 95

Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
100 105 110

Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
115 120 125

Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
130 135 140

Ala Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Asn Tyr Gln Val
145 150 155 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
165 170 175

Ser Asn

<210> 44
<211> 191
<212> PRT
<213> Streptomyces lividans xylanase C

<400> 44

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Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

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Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
                85                  90                  95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
            100                 105                 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Asn
        115                 120                 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
    130                 135                 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145                 150                 155                 160

Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Met Ala Thr
                165                 170                 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
            180                 185                 190
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INTERNATIONAL SEARCH REPORT

XInternational application No.
PCT/CA2005/000448

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): C12N 15/56, C12N 9/24, C12N 1/15, A23K 1/165, A23L 1/03, D21H 17/22

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)
IPC(7): C12N 15/56, C12N 9/24, C12N 1/15, A23K 1/165, A23L 1/03, D21H 17/22

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, PubMed, GeneSeq, NCBI, CAplus
Search terms: xylanase, family 11, G xylanase, glycosylated, glycosylation, *Trichoderma reesei*, glycosyl hydrolase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9424270 A2 (NATIONAL RESEARCH COUNCIL OF CANADA) 27.10.1994 Figure 1	1 and 2 (in part), 3, 4
X	WO 03106654 A2 (DIVERSA CORPORATION) 24.12.2003 SEQ ID NOs:160, 184, 204, 318	1 and 2 (in part), 3, 4

[ ] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 June 2005 (21-06-2005)	18 July 2005 (18-07-2005)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Debora Fujimoto (819) 997-1855

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/000448

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

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

1. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. [X] Claim Nos. : 1 and 2 (in part), 6, 7, 9, 10, 11 (in part), 12-14, 15 (in part), 16-22, 23 (in part), 24 (in part), 25-32, and 34-39
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

The Family 11 xylanases of claims 1 and 2 (in part), 6, 7, 9, 10, 12-14, 16-22, 25-32, and 34-39 are defined only in vague and broad language, and thus, are undefined amino acid sequences that cannot be searched. Claims 11, 23, 24, and 33 refer to ITX1, ITX2, ITX3, ITX3', ITX4', ITX5, and ITX5' amino acid sequences that are defined in a vague manner that encompasses too many possible amino acid sequences and thus, these claims, when referring to said ITX sequences, cannot be searched.

3. [ ] Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/000448

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO9424270 A2	27-10-1994	US5405769 A	11-04-1995
WO03106654 A2	24-12-2003	AU2003251549 A1	31-12-2003
		CA2488916 A1	24-12-2003
		EP1516053 A2	23-03-2005