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(57) **Abrégé/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.

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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* or *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*

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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:43); Ak - *Aspergillus kawachii* xynC (SEQ ID NO:44); At - *Aspergillus tubingensis* (SEQ ID NO:29); Tr1 - *Trichoderma reesei* xyn1 (SEQ ID NO:2); Aa - *Aspergillus awamori* var. *kawachi* xyn B (SEQ ID NO:28); Fs - *Fibrobacter succinogenes* xyn II (SEQ ID NO:44); Ss - *Streptomyces* sp. 36a (SEQ ID NO:37); SIB - *Streptomyces lividans* xynB (SEQ ID NO:38); SIC - *Streptomyces lividans* xyn C (SEQ ID NO:39); TI - *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)TX_n, where "n" is a descriptor, for example "13" or "18", and the vector may comprise a 131 N mutation (i.e., pALT-ITX_n), or the vector may not comprise a 131N mutation (i.e., pALT-HTX_n). 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)TX_n-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/xITX_n-TV), or the vector may not comprise a 131N mutation (i.e., pc/xHTX_n-TV). For example when "n" is 18, and the vector does comprise the 131N mutation, the vector is pc/XITX_n-TV.

[0027a] 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.

[0027b] 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*, *Actinomadura*, *Chaetomium*, or *Thermatoga*.

[0037] Modification of *T. reesei* xylanase I (TrX I) to introduce an equivalent mutation at position 131, as determined by comparison with the sequence of *T. reesei* 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. reesei* 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 "S1C" 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, 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*
<i>Aspergillus niger</i>	Xyn A	SEQ ID NO:43	-
<i>Aspergillus awamori</i> var. <i>kawachi</i>	Xyn B	SEQ ID NO:28	P48824
<i>Aspergillus kawachii</i>	Xyn C	SEQ ID NO:43	-
<i>Aspergillus tubigenensis</i>	Xyn A	SEQ ID NO:29	-
<i>Bacillus circulans</i>	Xyn A	SEQ ID NO:30	P09850
<i>Bacillus pumilus</i>	Xyn A	SEQ ID NO:31	P00694
<i>Bacillus subtilis</i>	Xyn A	SEQ ID NO:32	P18429
<i>Cellulomonas fimi</i>	Xyn D	-	P54865
<i>Chainia</i> spp	Xyn	-	-
<i>Clostridium acetobutylicum</i>	Xyn B	SEQ ID NO:33	-
<i>Clostridium stercorarium</i>	Xyn A	SEQ ID NO:34	P33558
<i>Fibrobacter succinognes</i>	Xyn II	SEQ ID NO:44	-
<i>Neocallimasterix patriciarum</i>	Xyn A	-	P29127
<i>Nocardiopsis dassonvillei</i>	Xyn II	-	-
<i>Ruminococcus flavefaciens</i>	Xyn A	SEQ ID NO:35	P29126
<i>Schizophyllum commune</i>	Xyn A	SEQ ID NO:36	P35809
<i>Streptomyces</i> sp. No. 36a	Xyn	SEQ ID NO:37	
<i>Streptomyces lividans</i>	Xyn B	SEQ ID NO:38	P26515
<i>Streptomyces lividans</i>	Xyn C	SEQ ID NO:39	P26220
<i>Streptomyces thermoviolaceus</i>	Xyn II	-	-
<i>Thermomonospora fusca</i>	Xyn A	SEQ ID NO:40	-
<i>Thermomyces lanuginosus</i>	Xyn A	SEQ ID NO:45	O43907
<i>Trichoderma harzianum</i>	Xyn	SEQ ID NO:41	P48793
<i>Trichoderma reesei</i>	Xyn I	SEQ ID NO:2	P36218
<i>Trichoderma reesei</i>	Xyn II	SEQ ID NO:1	P36217
<i>Trichoderma viride</i>	Xyn	SEQ ID NO:42	-

*Amino acid sequences shown in the SEQ ID NO: do not contain the secretion signal peptides provided in the Swiss Prot entries.

[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 thereof. 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 example 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 T165H
ITX1	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, T131N, H144R, N157D, Q161R, Q162H and T165H
ITX2	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, T131N, Y135R, H144R, N157D, Q161R, Q162H and T165H
ITX3	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, T165H and F180N
ITX3'	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, T165H, F180N and S182T
ITX4	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, T165H and S182N
ITX4'	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H, T165H, S182N and S184T
ITX5	TrX with N10H, Y27M, N29L, Q34N, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and T165H
ITX5'	TrX with N10H, Y27M, N29L, Q34N, S36T, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and T165H
HTX18-R135Y	TrX with N10H, Y27M, N29L, S75A, L105H, S125A, I129E, H144R, N157D, Q161R, Q162H and T165H
Xln1(131N)	<i>T. reesei</i> xylanase I with mutation T118N
<i>S. lividans</i> xlnC(131N)	<i>S. lividans</i> 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), 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*, *egl*, *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 -phosphotransferase 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:27	Q34N,S36T
SEQ ID NO:4	L105H	SEQ ID NO:28	<i>Aspergillus awamori</i> <i>var.kawachi</i>
SEQ ID NO:5	S125A	SEQ ID NO:29	<i>Aspergillus tubigenis</i>
SEQ ID NO:6	I129E	SEQ ID NO:30	<i>Bacillus circulans</i>
SEQ ID NO:7	Y135R	SEQ ID NO:31	<i>Bacillus pumilus</i>
SEQ ID NO:8	H144R	SEQ ID NO:32	<i>Bacillus subtilis</i>
SEQ ID NO:9	N157D	SEQ ID NO:33	<i>Clostridium</i> <i>acetobutylicum</i>
SEQ ID NO:10	Q161R	SEQ ID NO:34	<i>Clostridium</i> <i>stercorarium</i>
SEQ ID NO:11	Q162H	SEQ ID NO:35	<i>Ruminococcus</i> <i>flavefaciens</i>
SEQ ID NO:12	T165H	SEQ ID NO:36	<i>Schizophyllum commune</i>
SEQ ID NO:13	T131N,R135Y	SEQ ID NO:37	<i>Streptomyces sp. No. 36a</i>
SEQ ID NO:14	R135Y	SEQ ID NO:38	<i>Streptomyces lividans</i> Xyn B
SEQ ID NO:15	T128N	SEQ ID NO:39	<i>Streptomyces lividans</i> Xyn C
SEQ ID NO:16	XynC-5F (Nhe)	SEQ ID NO:40	<i>Thermomonospora fusca</i>
SEQ ID NO:17	XynC-3R (Kpn)	SEQ ID NO:41	<i>Trichoderma harzianum</i>
SEQ ID NO:18	T118N	SEQ ID NO:42	<i>Trichoderma viride</i>
SEQ ID NO:19	Xyn1-F	SEQ ID NO:43	<i>Aspergillus niger</i>
SEQ ID NO:20	Xyn1-R (BamHI)	SEQ ID NO:44	<i>Aspergillus kawachii</i> Xyn C
SEQ ID NO:21	T131N	SEQ ID NO:45	<i>Fibrobacter</i> <i>succinogenes</i>
SEQ ID NO:22	F180N	SEQ ID NO:46	<i>Thermomyces</i> <i>lanuginosus</i>
SEQ ID NO:23	F180N,S182T	SEQ ID NO:47	modified <i>T. reesei</i> xylanase I (T131N)
SEQ ID NO:24	S182N	SEQ ID NO:48	modified <i>S. lividans</i> xylanase C (T131N)

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

Examples

[0076] Example 1 describes the isolation of genomic DNA from *Trichoderma reesei* 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 supernatant 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 *HindIII*, *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 ampicillin.

[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-saturated phenol:chloroform: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 μ l 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 3×10^5 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-*Bam*HI or -*Eco*RI libraries were identified by colony lift hybridization: $1-3 \times 10^4$ 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 *Bam*HI or *Eco*RI fragments, respectively, in a labelling reaction containing 10-50 ng target DNA, 0.2 mM each d(GCT)TP, 0.5 μ M dATP, 20-40 μ Ci α - ^{32} P-dATP, 10 pmole oligonucleotide primers and 0.5 units Taq polymerase in a total volume of 20 μ l. 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 μ g/ml denatured sheared salmon sperm DNA. Hybridizations were performed in heat-sealed bags in the same buffer with only 50 μ g/ml denatured sheared salmon sperm DNA and 5×10^6 - 5×10^7 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-*BamHI* 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 *BamHI* 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 *EcoRI* 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-*EcoRI* 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 *EcoRI* 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] Digoxigenin-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, 1×10^4 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 digoxigenin-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 digoxigenin-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:chloroform: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 digoxigenin-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 *Bam*HI 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 *Bam*HI 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 λ DASH library (Example 2) with a *xln2* probe comprising bp 100-783 of the published *xln2* sequence (Saarelainen et al., 1993). A 5.7 kb *KpnI* 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 λ DASH *xln2* clone. This fragment was subcloned into the *KpnI* site of pUC119 to generate the plasmid pXYN2K-2. Clones carrying the *pgk* gene were identified by colony lift hybridization of the λ DASH library (Example 2) with a *pgkI* probe comprising bp 4-1586 the published *pgk* sequence (Vanhanen et al., 1989). A 5.0 kb *EcoRI* 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 λ DASH *pgk* clone. This fragment was subcloned into the *EcoRI* site of pUC119 to generate the plasmid pGK5.0.

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

[0088] Xylanase C (*xylC*; SEQ ID NO:39) was amplified from genomic DNA isolated from *Streptomyces lividans* using primers that introduced a *NheI* site upstream and a *KpnI* site downstream of the coding sequence. Megaprimer PCR was used to introduce the T128N mutation into *xylC*. The mutagenic primer was used in conjunction with the reverse primer to introduce a *KpnI* site downstream. The resulting PCR product was isolated and used as a reverse primer in conjunction with the forward primer to introduce a *NheI* 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 (*xynI*; SEQ ID NO:2) was amplified from genomic DNA isolated from *T. reesei* strain M2C38 using primers that introduced a *NheI* site upstream and a *BamHI* site downstream of the coding sequence. Megaprimer PCR was used to introduce the T118N mutation into *xynI*. The mutagenic primer was used in conjunction with the reverse primer to introduce a *BamHI* site downstream. The resulting PCR product

was isolated and used as a reverse primer in conjunction with the forward primer to introduce a *NheI* site upstream. The sequence of the modified *T. reesei* 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). Specifically, a double-stranded *ApaI*/*PinAI* fragment comprising codons 8-33, in which codons 10, 27 and 29 of SEQ ID NO: 1 were altered, 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 *SsrI* 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 *SsrI* 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 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: AACCCTTCG 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: GGT TACTTTA GCAACGGCTC TGCTTCCATC (SEQ ID NO: 24)

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

Q34N: GGTCCCGGCG GGA ACTTCTC CGTCAACTGG (SEQ ID NO: 26)

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

to generate the plasmids pALT-ITX_n and pALT-ITX_n' (where n equals 2 through 5). A generic map of these plasmids, "pALT-H(I)TX_n", 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 *PinAI* 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 *KpnI* 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 *EcoRI* (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 *SphI*-*NotI*-*SalI* adaptor between the original *SphI* and *SalI* 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 *cbhI* promoter which was isolated by *HindIII* digestion of pCOR132; this generated the plasmid pBR322LXC. Finally, the *EcoRI* site of pBR322LXC was then

blunted with Klenow and *SpeI* 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 *EcoRI* site at the 3' end of *cbh2* in pZUK600. This fragment was digested at the engineered *XbaI* and *EcoRI* sites and inserted into the corresponding sites of pUC119 to generate pCB219. An *EcoRI-NotI* adaptor (Cat. No. 35310-010, Gibco/BRL) was inserted into the unique *EcoRI* 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 *SphI* and *KpnI* 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 *SphI* and *KpnI* 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 *cbhI*

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 *KpnI* site at bp 1877-1882 and the pUC reverse primer (Cat. No., 18432-013, Gibco/BRL) which anneals downstream of the *EcoRI* site at the 3' end of the *cbh1* terminator in pCB1Ta. The *cbh1* terminator PCR product was digested with *KpnI* and inserted into the unique *KpnI* 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 *KpnI* 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 *KpnI* and ligated to a 5.1 kb *KpnI* fragment from pHPT136 containing the *pgk* promoted-*hph* gene in pUC119 to generate the selection cassette plasmid pHPT136X, thus maintaining the unique *NotI* 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*^o fragment was inserted between the *XbaI* and *XhoI*^o 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 *xynI* PCR products (described in example 3, above) were digested with *Nhe*I and *Bam*HI 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 *Xba*I site at bp -1399 of the *cbh1* promoter and an *Nhe*I site directly downstream of the Gln codon comprising the first amino acid of the mature xylanase II protein. This PCR product was digested with *Xba*I and *Nhe*I 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 *EcoRI*. The *EcoRI*-*xynI* expression cassette-*XbaI*^o fragment was inserted between the *EcoRI* and *NotI*^o 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*^o fragment was inserted between the *XbaI* and *XhoI*^o 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 10⁶ 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 pNCBgINSNB(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 pNCBgINSNB(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 μ m, 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 1×10^6 spores on minimal 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 MIRACLOTHTM and collected into a sterile microcentrifuge tube.

[00109] For transformation, 0.1 ml of resuspended protoplasts (approximately 5×10^6 protoplasts) are combined with 2 μ g vector DNA and 25 μ l 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
$(\text{NH}_4)_2\text{SO}_4$	6 g
$\text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O}$	3 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5 mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.6 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.4 mg
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2 mg
Agar	20 g
20% Glucose f.s.	50 ml
1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 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 μ l of an appropriate dilution of culture filtrate is pre-incubated at 65°C for 5 min. Subsequently, 300 μ l 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:

<u>Component</u>	<u>g/L</u>
(NH ₄) ₂ SO ₄	6.35
KH ₂ PO ₄	4.00
MgSO ₄ -7H ₂ O	2.02
CaCl ₂ -2H ₂ O	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 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.6 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 1.4 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

** 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 $(\text{NH}_4)_2\text{SO}_4$, 2.0 g/l KH_2PO_4 , 0.31 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.53 g/l CaCl_2 ; 5.1 g/l dry corn steep, 5 mg/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.8 mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.7 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) 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 $(\text{NH}_4)_2\text{SO}_4$, 1.39 g/l KH_2PO_4 , 0.7 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.185 g/l CaCl_2 , 6 g/l dry corn steep, 3.75 mg/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.2 mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.05 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. 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 ^c	--
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 I	No	31.0	23.2	136 ^a	-
P279A	Xyn I-131N	118N	31.75	25.5	2042 ^a	15.0 x (1400%)
P348C	xlnC-131N	128N	35.2	23.8	2365 ^c	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% β-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 ammonium 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.

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DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS

1. A modified fungal Family 11 xylanase comprising, a sequence that introduces a functional consensus N-glycosylation site consisting of the amino acid sequence Asn-Xaa-Ser/Ihr (N-X-S/T) that is otherwise not found in a corresponding fungal Family 11 xylanase from which the modified fungal Family 11 xylanase is derived, the N-glycosylation site produced by substitution of an amino acid (X) to an asparagine (N) 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, the position determined from sequence alignment of the modified fungal Family 11 xylanase with the amino acid sequence of *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1, wherein the modified fungal Family 11 xylanase, when expressed from a genetic construct comprising a promoter and secretion signal for expressing the modified fungal Family 11 xylanase from a *Trichoderma* host strain, exhibits an increase in expression efficiency of at least 40% when compared to the expression efficiency of the corresponding fungal Family 11 xylanase when expressed from a genetic construct comprising the same promoter and the same secretion signal as a genetic construct used to express the modified fungal Family 11 xylanase from the *Trichoderma* host strain.
2. The modified fungal Family 11 xylanase of Claim 1, wherein the N-glycosylation site is produced by substitution of an amino acid (X) to asparagine (N) at position 131 (X131N).
3. The modified fungal Family 11 xylanase of Claim 1, wherein the amino acid substitution is selected from the group consisting of: substitution at position 34 (X34N) and further comprising a substitution to a threonine at position 36 (S36T); substitution at position 180 (X180N) and further comprising a substitution to a threonine at position 182 (S182T); substitution at position 182 (X182N) and further comprising a substitution to a threonine at position 184 (S184T); and a combination thereof.

4. The modified fungal Family 11 xylanase of any one of claims 1 through 3, wherein the corresponding fungal Family 11 xylanase from which the modified fungal Family 11 xylanase is derived is a *Trichoderma* xylanase.
5. The modified fungal Family 11 xylanase of Claim 4, wherein the *Trichoderma* xylanase is xylanase I or xylanase II from *Trichoderma reesei*.
6. The modified fungal Family 11 xylanase of Claim 1 or 2, wherein the modified fungal Family 11 xylanase is *Trichoderma reesei* xylanase I as defined in SEQ ID NO:2 comprising an amino acid substitution of T131N, the position determined from sequence alignment of the *Trichoderma reesei* xylanase I with the amino acid sequence of *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1.
7. A modified fungal Family 11 xylanase comprising a sequence that introduces a functional consensus N glycosylation site consisting of the amino acid sequence Asn-Xaa-Ser/Ihr (N-X-S/T) that is otherwise not found in a corresponding fungal Family 11 xylanase from which the modified fungal Family 11 xylanase is derived, wherein the modified fungal Family 11 xylanase is selected from the group consisting of:
 - (a) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, T131N, H144R, N157D, Q161R, Q162H, and T165H,
 - (b) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, T131N, Y135R, H144R, N157D, Q161R, Q162H, and T165H;
 - (c) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H, T165H and F180N;
 - (d) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H, T165H, F180N and S182T;

- (e) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H, T165H and S182N;
 - (f) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions. N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H, T165H, S182N and S184T;
 - (g) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions, N10H, Y27M, N29L, Q34N, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H and T165H; and
 - (h) *Trichoderma reesei* xylanase II as defined in SEQ ID NO:1 with amino acid substitutions N10H, Y27M, N29L, Q34N, S36T, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H and T165H.
8. A modified Family 11 xylanase genetic construct comprising a promoter operatively linked to a secretion signal that is operatively linked to a coding region that encodes the modified fungal Family 11 xylanase as defined in claim 1, the modified Family 11 xylanase genetic construct resulting in an increase in expression efficiency of the encoded modified fungal Family 11 xylanase of at least 40% when compared to the expression efficiency of a corresponding fungal Family 11 xylanase from which the encoded modified fungal Family 11 xylanase is derived when said corresponding fungal Family 11 xylanase is expressed from a genetic construct comprising the same operatively linked promoter and secretion signal as said modified Family 11 xylanase genetic construct.
9. The modified Family 11 xylanase genetic construct of claim 8, wherein the secretion signal is a *Trichoderma* secretion signal.
10. The modified Family 11 xylanase genetic construct of claim 9, wherein the *Trichoderma* secretion signal is a xylanase secretion signal.

11. The modified Family 11 xylanase genetic construct of claim 10, wherein the xylanase secretion signal is a *Trichoderma* xylanase I secretion signal or a *Trichoderma* xylanase II secretion signal.
12. The modified Family 11 xylanase genetic construct of claim 8, wherein the coding region encodes a modified fungal Family 11 xylanase that is derived from *Trichoderma reesei* xylanase II as defined in SEQ ID NO: 1, which xylanase further comprises amino acid substitutions N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, H144R, N157D, Q161R, Q162H, and T165R.
13. The modified Family 11 xylanase genetic construct of claim 8, wherein the promoter is selected from the group consisting of a *Trichoderma cbh1* promoter, a *Trichoderma cbh2* promoter, a *Trichoderma eg1* promoter, a *Trichoderma eg2* promoter, a *Trichoderma eg3* promoter, a *Trichoderma eg5* promoter, a *Trichoderma xln1* promoter, a *Trichoderma xln2* promoter, and a combination of two or more than two of these promoters.
14. A genetically modified microbe produced by introducing the modified Family 11 xylanase genetic construct of any one of claims 8 through 13 into a host microbe.
15. The genetically modified microbe of claim 14, wherein the microbe comprises a member of the genus of *Trichoderma*.
16. The use of the modified xylanase in any one of claims 1 through 7 in an industrial or food process.
17. The use as defined in claim 16 wherein the industrial process is pulp manufacturing.
18. The use as defined in claim 16 wherein the food process is baking or brewing.

19. The use of the modified xylanase in any one of claims 1 through 7 as a poultry feed additive or a swine feed additive.

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Ca	23				S	AFNTQAAP	31
Cs	1					G	1

Tr2#			10	20	30	40	
Bp	1	RTITNNEMGN	HSGYD YELWK	DYGNT-SMTL	NNGGAF SAGW	N-- NIGNA	45
Ca	32	KTITSNEIGV	NGGYD YELWK	DYGNT-SMTL	KNGGAF SCQW	S-- NIGNA	76
Fs	1	NSSVTGNVG	SSPYH YEIWY	QGG-NNSMTF	YDNG TYKASW	N--GTN DF	44
Cs	2	RIIYDNETGT	HGGYD YELWK	DYGNT-IMEL	NDGG TFSCQW	S-- NIGNA	46
Rf	1	SAADQQTRGN	VGGYD YEMWN	QNGQGQASMN	PGAG SFTCSW	S-- NIENF	46
Tr2	1	QTIQPGTGY	NNGYF YSYWN	DGHGGV TYTN	GPGG QFSVNW	S-- NSGNF	45
Tv	1	QTIQPGTGF	NNGYF YSYWN	DGHGGV TYTN	GPGG QFSVNW	S-- NSGNF	45
Th	1	QTIGPGTGY	SNGYY YSYWN	DGHAGV TYTN	GGG GSFTVNW	S-- NSGNF	45
Sc	1	SGTPSSSTGT	DGGYY YSWWT	DGAGDATYQN	NGGG SYTLTW	SG-- NNGNL	46
An	1	S	AGINYVQNYN	GNLGDFTY-D	ESAG TFSMYW	EDGVSS DF	38
Ak	1	S	AGINYVQNYN	GNLADFTY-D	ESAG TFSMYW	EDGVSS DF	38
AT	1	S	AGINYVQNYN	QNLGDFTY-D	ESAG TFSMYW	EDGVSS DF	38
Tr1	1		ASINYDQNYQ	TGG-QVSY-	PSNTGF SVNW	N--TQDD DF	34
Aa	1	RSTPSSTGE	NNGYY YSFWT	DGGGDV TYTN	GNAGSY SVEW	S-- NVGNF	45
Ss	1	ATTIT-NETGY	D-GMY YSFWT	DGGGSV SMTL	NGGGSY STRW	T-- NCGNF	45
SlB	1	DTVVTTNQEGT	NNGYY YSFWT	DSQGT VSMTL	GSGG QYSTSW	R-- NTGNF	47
SlC	1	ATTITTNQTGT	D-GMY YSFWT	DGGGSV SMTL	NGGGSY STQW	T-- NCGNF	46
Tl	1	QTTPNSEGW	HDGYY YSWWS	DGGAQATYTN	LEG GTYEISW	G--DGG NL	45
Tf	1	AVTSNETGY	HDGYF YSFWT	DAPGTV SMEL	GPGG NYSTSW	R-- NTGNF	45
Bc	1		ASTDYWQ NWT	DGGGIVNAVN	GSGG NYSVNW	S-- NTGNF	36
Bs	1		ASTDYWQ NWT	DGGGIVNAVN	GSGG NYSVNW	S-- NTGNF	36

FIGURE 1

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Tr2#		50		60		70		80	
							*		
Bp	46	LFRK- G KKFD	ST-RTHHQLG	NISINYNASF	N- P SGNSYLC	V Y GW	TQSP	90	
Ca	77	LFRK- G KKFN	DT-QTYKQLG	NISVNYNCNY	Q- P YGN SYLC	V Y GW	TSSP	121	
FS	45	LARV- G FKYD	EK-HTYEELGPIDAYYKWSKQ	GSAGGYN YIG	I Y GW	TVDP	91		
Cs	47	LFRK- G RKFN	SD-KTYQELG	DIVVEYGC DY	N- P NGNSYLC	V Y GW	TRNF	91	
Rf	47	LARM- G KNYD	SQKKNYKAFG	NIVLTYDVEY	T- P RGNSYMC	V Y GW	TRNP	92	
Tr2	46	V G GK- G WQPG	TKNKV-----	---INFS-GS	YN P NGNSYLS	V Y GW	SRNP	83	
Tv	46	V G GK- G WQPG	TKNKV-----	---INFS-GS	YN P NGNSYLS	V Y GW	SRNP	83	
Th	46	V G GK- G WQPG	TKNKV-----	---INFS-GS	YN P NGNSYLS	I Y GW	SRNP	83	
Sc	47	V G GK- G WNPG	AASRS-----	---ISYS-GT	YQ P NGNSYLS	V Y GW	TRSS	84	
An	39	V V GL- G WTTG	SSNA-----	---ITYSAEY	SASGSSSYLA	V Y GW	VNYP	76	
Ak	39	V V GL- G WTTG	SSNA-----	---ITYSAEY	SASGSSSYLA	V Y GW	VNYP	76	
At	39	V V GL G WTTG	SSNA-----	---ITYSAEY	SASGSASYLA	V Y GW	VNYP	76	
Tr1	35	V V GV- G WTTG	SSAP-----	---INFGGSF	SVNSGTGLLS	V Y GW	STNP	72	
Aa	46	V G GK- G WNPG	SAKD-----	---ITYSGNF	T- P SGNGYLS	V Y GW	TTDP	83	
Ss	46	V A GK- G WANG	GR-RT-----	---VRYT-GW	FN P SGNGYGC	L Y GW	TSNP	82	
SlB	48	V A GK- G WANG	GR-RT-----	---VQYS-GS	FN P SGNAYLA	L Y GW	TSNP	84	
SlC	47	V A GK- G WSTG	DGN-----	---VRYN-GY	FN P VGNGYGC	L Y GW	TSNP	82	
Tl	46	V G GK- G WNPG	LNARA-----	---IHFE-GV	YQ P NGNSYLA	V Y GW	TRNP	83	
Tf	46	V A GK- G WATG	GR-RT-----	---VTYS-AS	FN P SGNAYLT	L Y GW	TRNP	82	
Bc	37	V V GK- G WTTG	SPFRT-----	---INYNAGV	WAP P NGNGYLT	L Y GW	TRSP	75	
Bs	37	V V GK- G WTTG	SPFRT-----	---INYNAGV	WAP P NGNGYLT	L Y GW	TRSP	75	

Tr2#		90		100		110		120		130
					*					
Bp	91	LA E YYIVDSW	GT Y R-PT--G	AYK G SFYADG	GT Y DIYETTR	VN O PSIIG	135			
Ca	122	LV E YYIVDSW	GSWRPP--GG	TSK G TITVDG	GI Y DIYETTR	IN O PSIOG	167			
Fs	92	LV E YYIVDDW	FNKPGANLLG	QRK G EFTVDG	DT Y EIWQNTTR	VQ O PSIKG	139			
Cs	92	LV E YYIVESW	GSWRPP--GA	TPK G TITQWMAGT Y EIYETTR	VN O PSIDG	138				
Rf	93	LM E YYIVEGW	GDWRPPGNDG	EVK G TVSANG	NT Y DIRKTMR	YN O PSLDG	140			
Tr2	84	LI E YYIVENF	GT Y N-PSTGA	TKL G EVTS DG	SV Y DIYRTOR	VN O PSIIG	130			
Tv	84	LI E YYIVENF	GT Y N-PSTGA	TKL G EVTS DG	SV Y DIYRTOR	VN O PSIIG	130			
Th	84	LI E YYIVENF	GT Y N-PSTGA	TKL G EVTS DG	SV Y DIYRTOR	VN O PSIIG	130			
Sc	85	LI E YYIVESY	GS Y D-PSSAA	SHK G SVTCNG	AT Y DILSTWR	YN A PSIDG	131			
An	77	GA E YYIVEDY	GD Y N-PCSSA	TSL G TVYS DG	ST Y QVCTDTR	IN E PSITG	123			
Ak	77	QA E YYIVEDY	GD Y N-PCSSA	TSL G TVYS DG	ST Y QVCTDTR	TN E PSITG	123			
At	77	QA E YYIVEDY	GD Y N-PCSSA	TSL G TVYS DG	ST Y QVCTDTR	IN E PSITG	123			
Tr1	73	LV E YYIMEDN	H N Y--PAQ-G	TVK G TVTS DG	AT Y TIWENTR	VN E PSIOG	117			
Aa	84	LI E YYIVESY	GD Y N-PGSGG	TTR G NVSS DG	SV Y DIYTATR	TN A PSIDG	130			
Ss	83	LV E YYIVDNW	GS Y R-PT--G	ETR G TVHSDG	GT Y DIYKTTR	YN A PSVEA	127			
SlB	85	LV E YYIVDNW	GT Y R-PT--G	EYK G TVTS DG	GT Y DIYKTTR	VN K PSVEG	129			
SlC	83	LV E YYIVDNW	GS Y R-PT--G	TYK G TVSS DG	GT Y DIYQTTR	YN A PSVEG	127			
Tl	84	LV E YYIVENF	GT Y D-PSSGA	TDL G TVECDG	SI Y RLGKTTR	VN A PSIDG	130			
Tf	83	LV E YYIVESW	GT Y R-PT--G	TYM G TVTT DG	GT Y DIYKTTR	YN A PSIEG	127			
Bc	76	LI E YYVVDSW	GT Y R-PT--G	TYK G TVKSDG	GT Y DIYTTR	YN A PSIDG	120			
Bs	76	LI E YYVVDSW	GT Y R-PT--G	TYK G TVKSDG	GT Y DIYTTR	YN A PSIDG	120			

FIGURE 1 CONT'D

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Tr2#		140	150	160	
Bp	136	-IAT TFKQYWS VR QTKRTS--	----- GT VS	VSA HF RK W ES	L G M P M-GK 174
Ca	168	-NT TFKQYWS VR RTKRTS--	----- GT IS	VSK HF AA W ES	K G M P L-GK 206
Fs	140	-TQ TF P QYFS VR KSARSC--	----- GH ID	ITA HF MKK W EE	L G M K M-GK 178
Cs	139	-TAT TFQOYWS VR TSKRTS--	----- GT IS	VTE HF KQ W ER	M G M R M-GK 177
Rf	141	-TAT TF P QYWS VR QTS GS ANN	QTN Y M K GT ID	VSK HF DA W SA	A G L D M S GT 187
Tr2	131	-TAT TFYQYWS VR NRHR-S-S	----- GS VN	TAN HF NA W AQ	O G L T L-GT 168
Tv	131	-TAT TFYQYWS VR RTHR-S-S	----- GS VN	TAN HF NA W AQ	O G L T L-GT 168
Th	131	-TAT TFYQYWS VR NRHR-S-S	----- GS VN	TAN HF NA W AS	H G L T L-GT 168
Sc	132	-TQ TFE Q FWS VR NP KK APGG	SIS-- GT VD	VQ C HF DA W KG	L G M N L G SE 175
An	124	-TS TF T QYFS VR ESTRTS--	----- GT VT	VAN HF N F W AQ	H G F G N-SD 161
Ak	124	-TS TF T QYFS VR ESTRTS--	----- GT VT	VAN HF N F W AQ	H G F G N-SD 161
At	124	-TS TF T QYFS VR ESTRTS--	----- GT VT	VAN HF N F W AH	H G F H N-SD 161
Tr1	118	-TAT TF N OYIS VR NSPR-T-S	----- GT VT	VQ N HF N- W AS	L G L H L G QM 155
Aa	131	-TQ TF S QYWS VR Q N KR-VG-	----- GT VT	TS N HF NA W AK	L G M N L-GT 168
Ss	128	-PAA FDQYWS VR QSKVT--S	----- GT IT	TG N HF DA W AR	A G M N M G NF 168
SlB	130	TR- TFDQYWS VR QSKR-TG-	----- GT IT	TG N HF DA W AR	A G M P L G NF 168
SlC	128	TK- TFQOYWS VR QSKVTSGS	----- GT IT	TG N HF DA W AR	A G M N M G QF 168
Tl	131	TQ- TFDQYWS VR QDKR-T-S	----- GT VQ	TG C HF DA W AR	A G L N V N GD 169
Tf	128	TR- TFDQYWS VR QSKRTS--	----- GT IT	AG N HF DA W AR	H G M H L G TH 166
Bc	121	DRT TF T QYWS VR QSKRPTGS	N----- AT IT	FT N HF VNA W KS	H G M N L G SN 163
Bs	121	DRT TF T QYWS VR QSKRPTGS	N----- AT IT	FS N HF VNA W KS	H G M N L G SN 163

Tr2#		170	180	190	
Bp	175	MYETAFTV EG	YQSS GS ANVM	TNQLFIGN	201
Ca	207	MHETAFNI EG	YQSS G KADVN	SMSINIGK	233
Fs	179	MYEAKVLV EA	GGGS GS FDV-	TYFKMT	203
Cs	178	MYEVALTV EG	YQSS G YANVY	KNEIRIGANP....	
Rf	188	LYEVSLNI EG	YRSN GS ANVK	SVSV	211
Tr2	169	MDYQIVAV EG	YFSS GS ASI-	TVS	190
Tv	169	MDYQIVAV EG	YFSS GS ASI-	TVS	190
Th	169	MDYQIVAV EG	YFSS GS ASI-	TVS	190
Sc	176	HNYQIVAT EG	YQSS G TATI-	TVT	197
An	162	FNYQVMAV EA	WSGAG S ASV-	TISS	184
Ak	162	FNYQVMAV EA	WSGAG S ASV-	TISS	184
At	162	FNYQVVAV EA	WSGAG S AAV-	TISS	184
Tr1	157	MNYQVVAV EG	WGG S GSASQ-	SVSN	178
Aa	169	HNYQILAT EG	YQSS GS SSI-	TIQ	190
Ss	167	RYYMINAT EG	YQSS GS STI-	TVSG	189
SlB	169	SYMINAT EG	YQSS G TSSI-	NVGG.....	
SlC	169	RYYMINAT EG	YQSS GS SNI-	TVSG	191
Tl	170	HYYQIVAT EG	YFSS G YARI-	TVADVG	194
Tf	167	D-YMIMATE EG	YQSS GS SNVT	LGTS.....	
Bc	164	WAYQVMAT EG	YQSS GS SNV-	TVW	185
Bs	164	WAYQVMAT EG	YQSS GS SNV-	TVW	185

FIGURE 1 CONT'D

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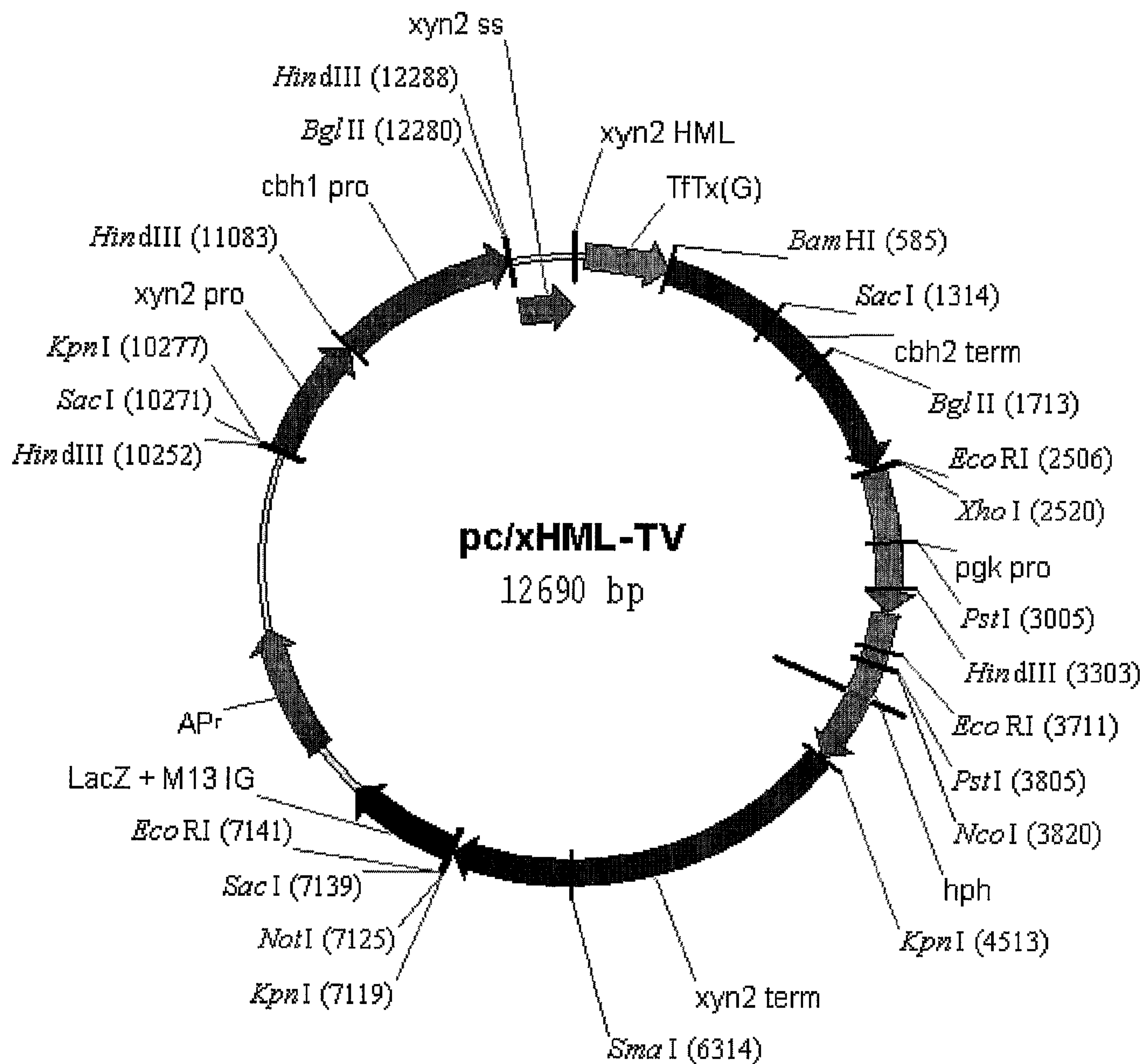


Figure 2

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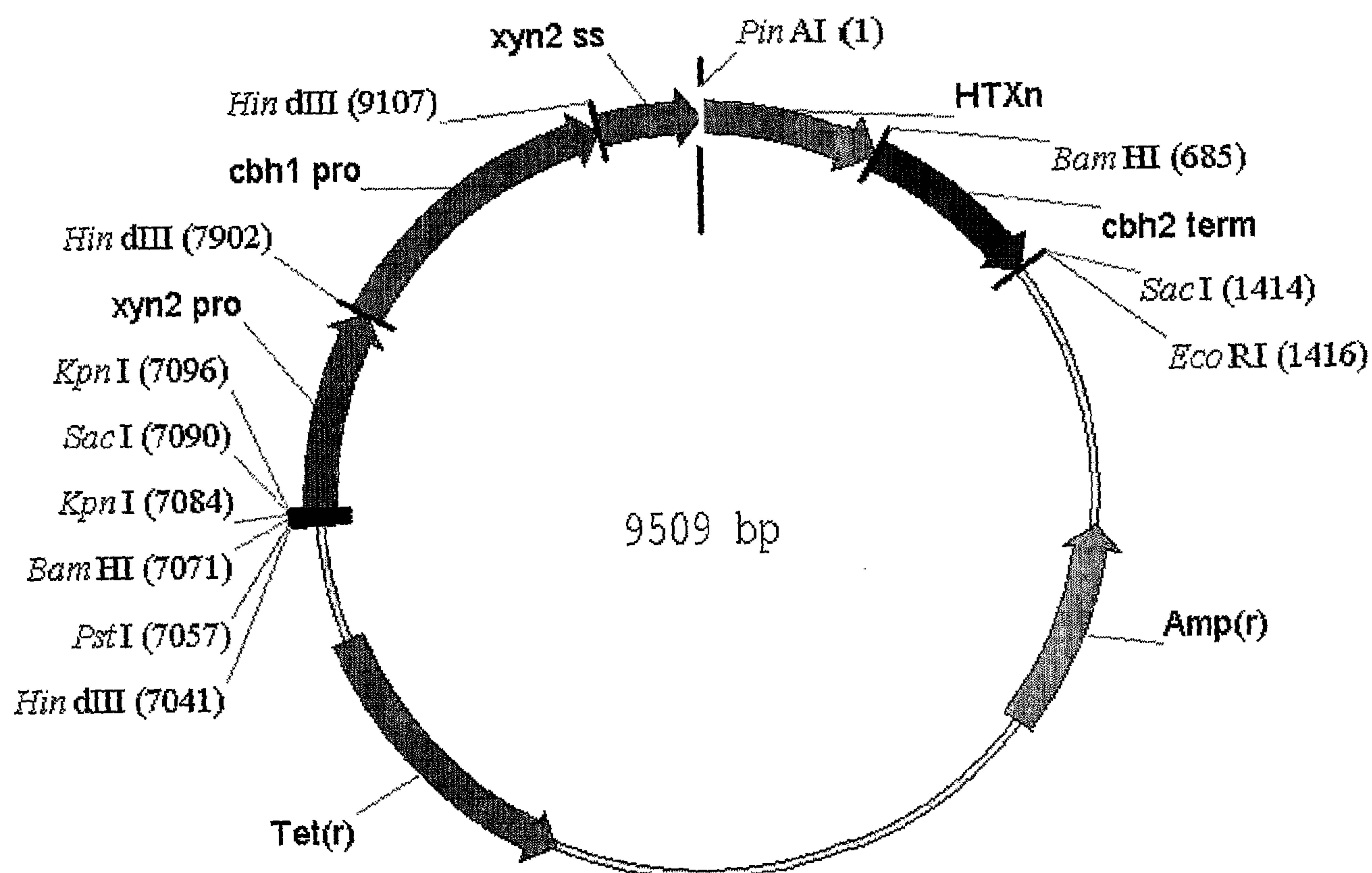


Figure 3

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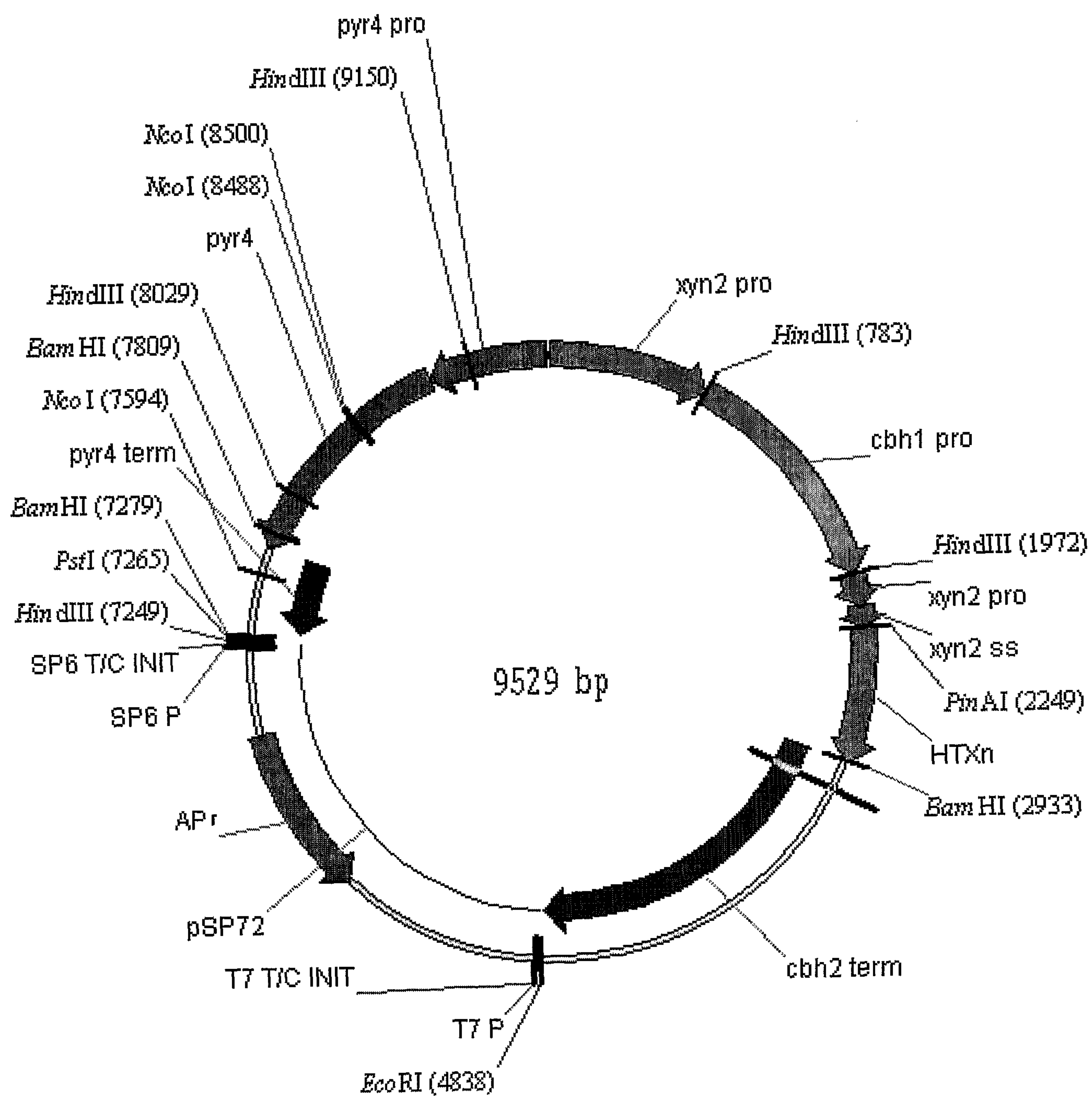


Figure 4

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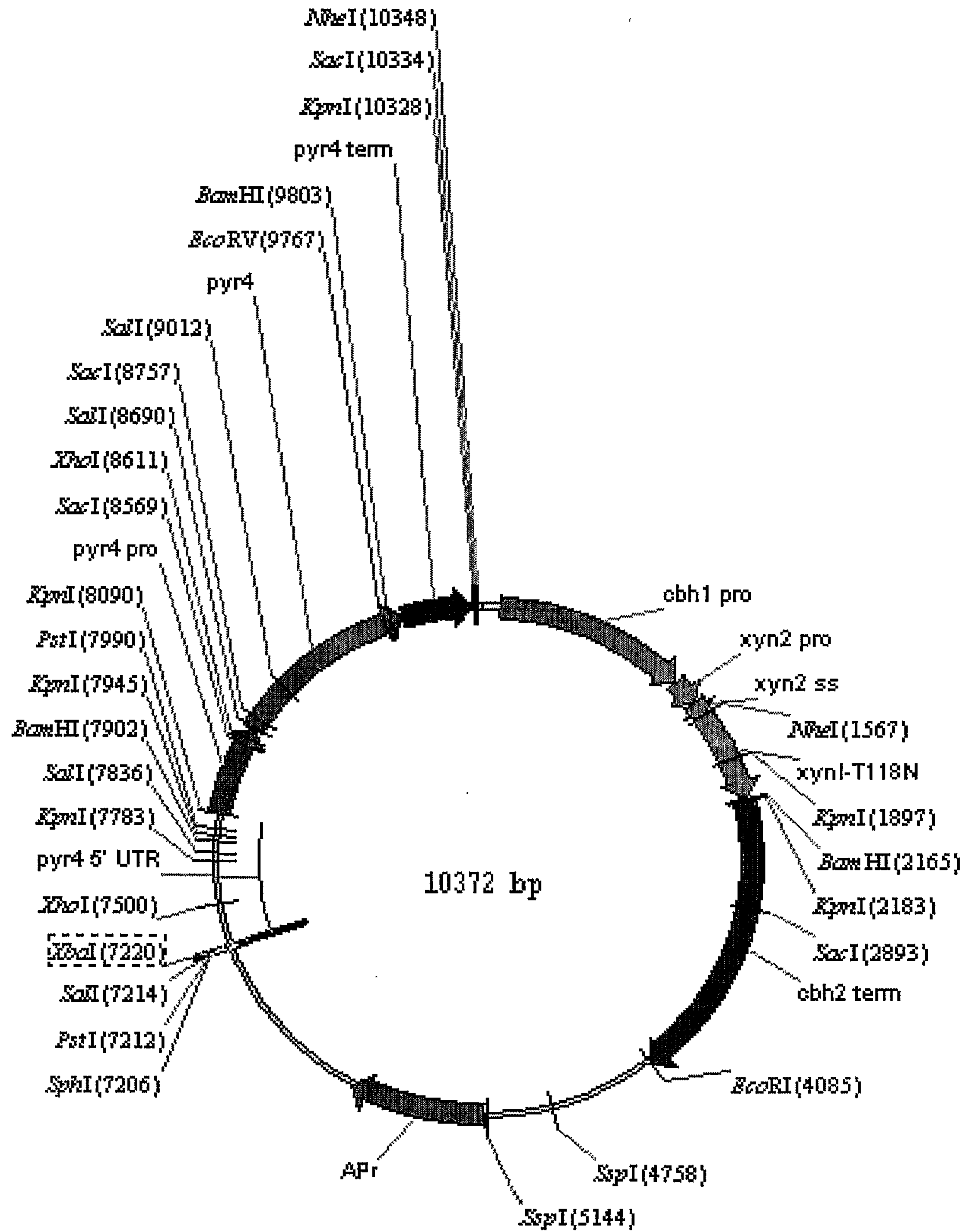


Figure 5A

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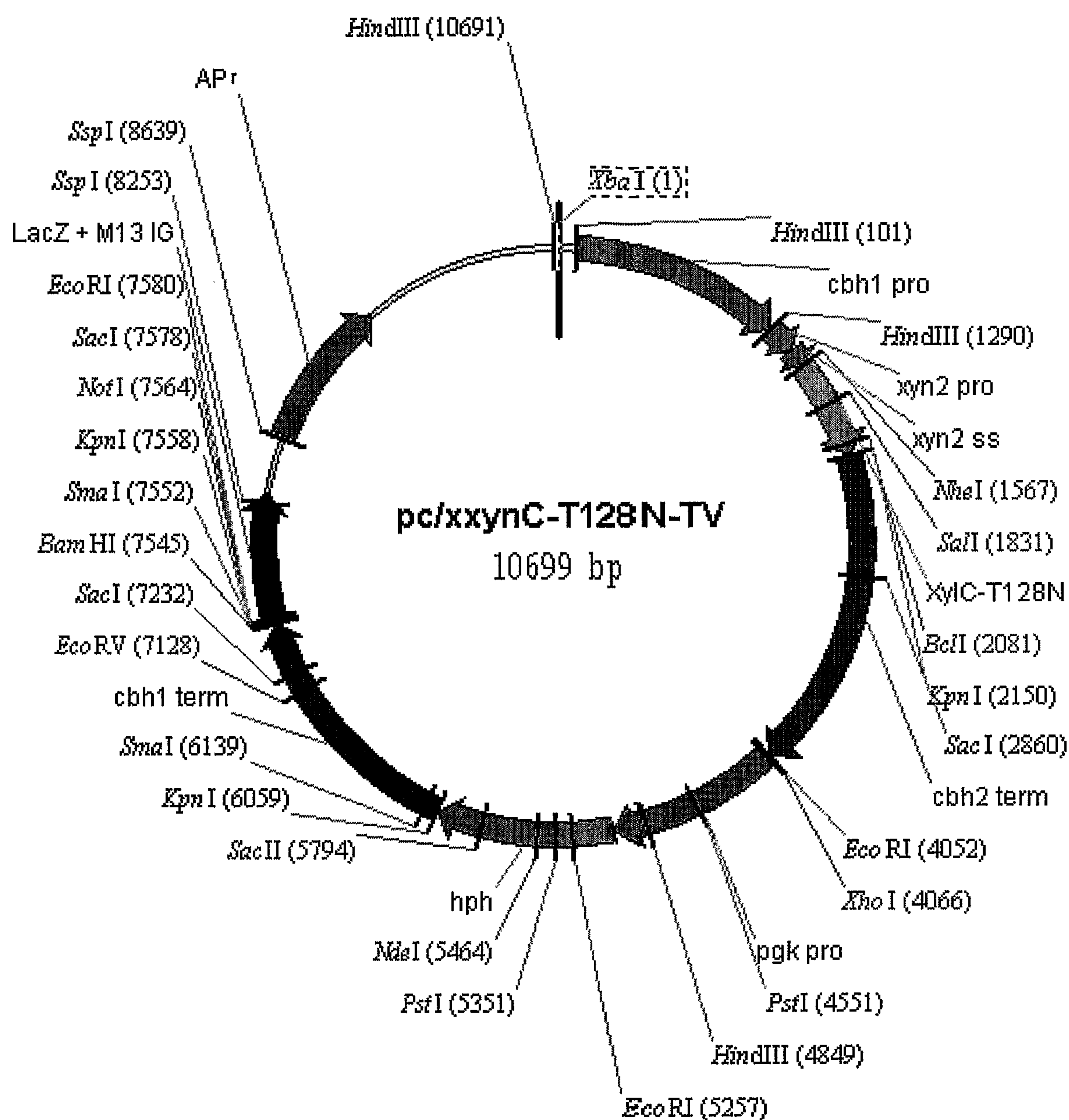


Figure 5B

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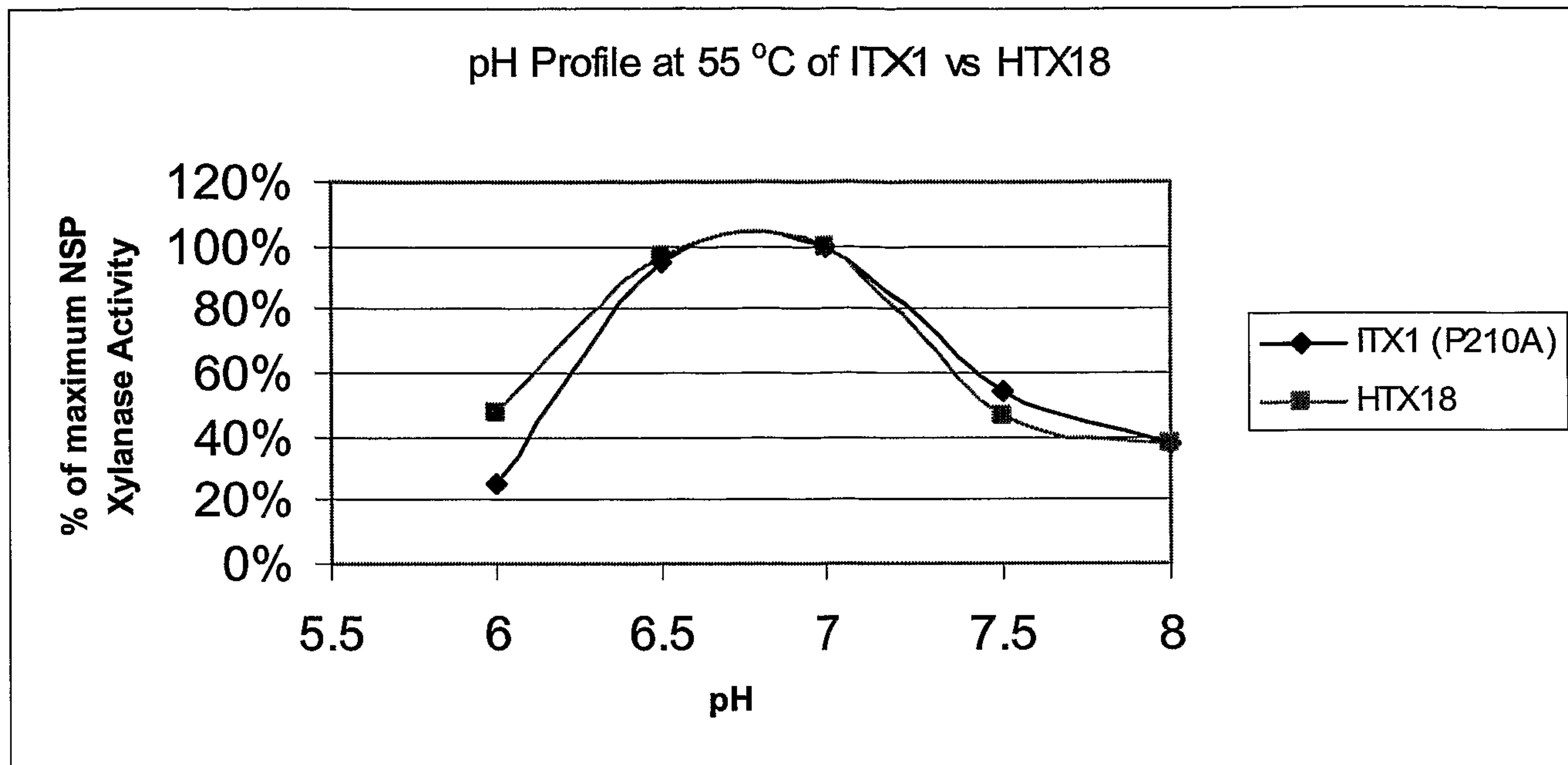
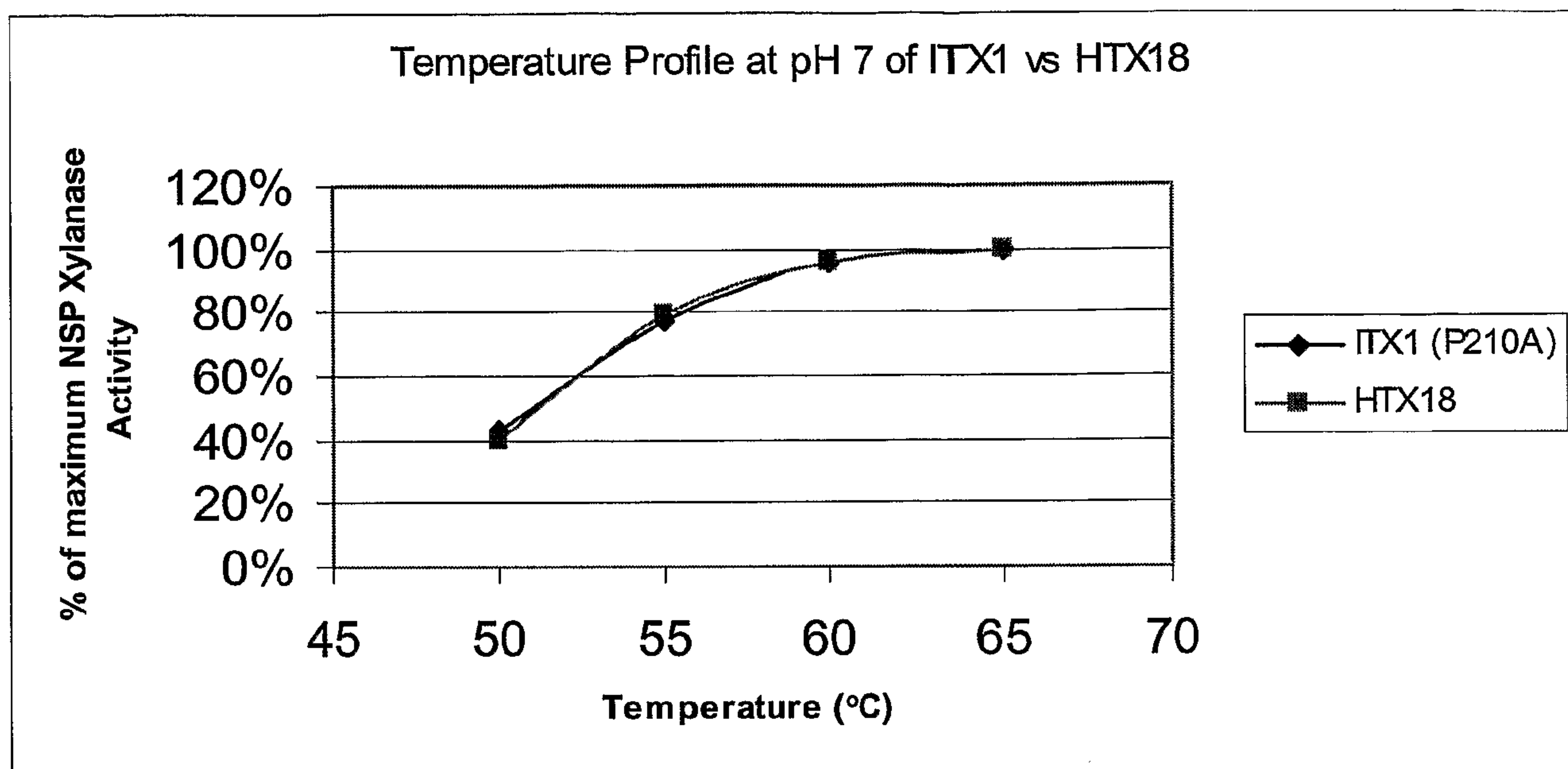


Figure 6

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**Figure 7**