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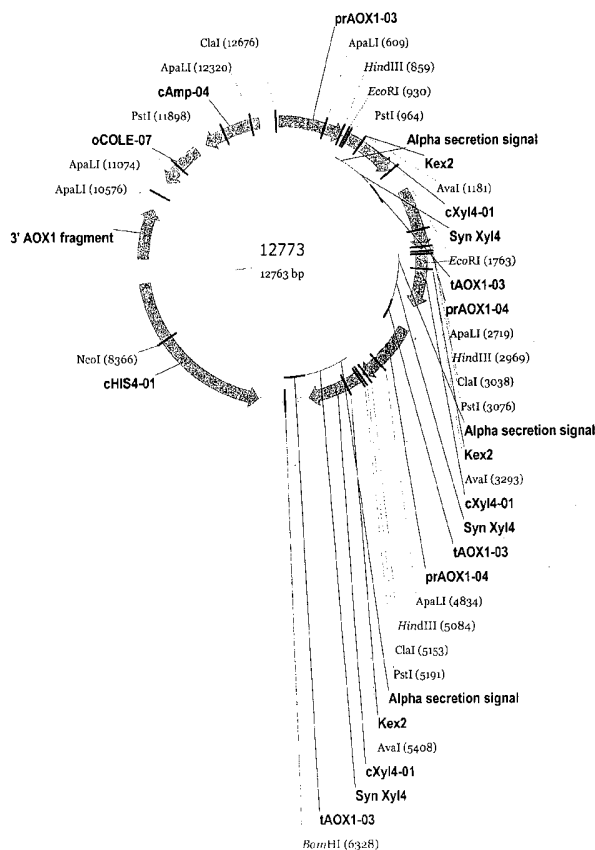
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(54) Title: MICROBIALLY EXPRESSED XYLANASES AND THEIR USE AS FEED ADDITIVES AND OTHER USES



(57) Abstract: The present invention relates to codon-optimized xylanase coding sequences and the expression of xylanases in microbes and yeast. The invention further relates to using multiple copies of the xylanase expression construct for high levels of protein expression. The invention also relates to the use of xylanases as feed or food additives. The invention also relates to methods of expression of enzymes to increase thermotolerance by expressing them in organisms that glycosylate proteins compared to expression that the same enzyme without the glycosylation. Further, the invention relates to methods of preparing feed, enzyme feed additives, and methods of reducing the feed conversion ration or increasing weight gain of animals.



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MICROBIALY EXPRESSED XYLANASES AND THEIR USE AS FEED ADDITIVES AND OTHER USES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of US Provisional Patent Application no.
5 60/531,404 filed December 19, 2003, incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to codon-optimized xylanase coding sequences and the expression of xylanases in microbes and yeast. The invention further relates to using multiple copies of the xylanase expression construct for high levels of protein expression. The
10 invention also relates to the use of xylanases as feed or food additives. The invention also relates to methods of expression of enzymes to increase thermotolerance by expressing them in organisms that glycosylate proteins compared to expression that the same enzyme without the glycosylation. Further, the invention relates to methods of preparing feed, enzyme feed additives, and methods of reducing the feed conversion ratio or increasing weight gain of
15 animals.

BACKGROUND OF THE INVENTION

Non-starch polysaccharides (NSP) have been implicated in the variability of the nutritional quality of cereals for chickens, associated with changes in viscosity of digesta (Bedford, M.R. & H.L. Classen (1993) "An in vitro Assay for Prediction of Broiler Intestinal
20 Viscosity and Growth When Fed Rye-Based Diets in the Presence of Exogeneous Enzymes" *Poult. Sci.* 72, 137-143). Arabinoxylans are the major NSP of wheat and several commercially available xylanase enzyme products, produced from *Trichoderma*, *Humicola* and *Aspergillus* spp have been shown to reduce digesta viscosity and usually to improve the nutritive value of diets.

25 Xylans are linear polysaccharides formed from beta-1,4 -linked D-xylopyranoses. In cereals, xylans frequently contain side chains of alpha-1,2, alpha-1,3, or alpha-1,2 and alpha-1,3 linked L-arabinofuranoside. These substituted xylans are commonly referred to as arabinoxylans. Xylanases (e.g., endo-1,4-beta-xylanase, EC 3.2.2.8) hydrolyze internal beta-1,4-xylosidic linkages in xylan to produce smaller molecular weight xylo-oligomers

30 Xylanases can be used, e.g., in animal feed compositions which are rich in arabinoxylans and glucoxylans, in baking, in brewing, and in pulp and paper applications, e.g. to improve the bleachability of pulps. When added to feeds (e.g. for monogastric animals, including poultry or swine) which contain cereals (e.g. barley, wheat, maize, rye, triticale or oats) or cereal by-products, a hemicellulolytic enzyme improves the break-down of plant cell
35 walls which leads to better utilization of the plant nutrients by the animal. This leads to

improved growth rate and feed conversion. Also, the viscosity of the feeds containing xylan can be reduced.

In many of the practical applications, physical conditions (e.g., temperature and pH) hinder the use of xylanases; the xylanases must be active in the temperature and pH conditions of the process in which they are used. Formulation of commercial feed using pelleting, extrusion or expanding, often contains steps involving high temperatures (70-180°C). Enzymes added to the formulation process should withstand these conditions. On the other hand, the corresponding temperature in the intestine of animals is about 40°C. Thus, ideal xylanases for feed compositions should withstand the above-mentioned extreme temperatures. In bleaching applications, xylanase application is not as simple as adding a xylanase treatment step. Because the bleaching process, and even the sequence of the steps used in the bleaching process varies in different pulp mills, there is thus a continuous need to find new xylanases active in different temperature and pH conditions.

Most commercial xylanases designed for feed applications are not very thermotolerant, especially when neutral or alkaline pH conditions are used. In practice, xylanases are generally inefficient or inactive at temperatures higher than 60°C and often these enzymes work under acidic conditions. Generally, there are differences in the physical characteristics of xylanases of fungi and bacteria (for review, see Wong et al., Microbiol. Rev. 52:305-317 (1988)). Typically, fungal xylanases have a temperature optimum at about 50°C and lower pH optimum than those of bacterial origin. Xylanases of bacterial origin generally have a temperature optimum in the range of 50 to 70°C. Numerous xylanases from fungal and bacterial microorganisms have been identified and characterized. (See, e.g., U.S. Pat. No. 5,437,992; Coughlin, M. P.; Biely, P. et al., "Proceedings of the second TRICEL symposium on *Trichoderma reesei* Cellulases and Other Hydrolases," Espoo 1993, P. Souminen and T. Reinikainen eds., Foundation for Biotechnical and Industrial Fermentation Research 8:125-135 (1993) and WO03/16654). In particular, three specific xylanases (XYL-I, XYL-II, and XYL-III) have been identified in *T. reesei* (Tenkanen, et al., Enzyme Microb. Technol. 14:566 (1992); Torronen, et al., Bio/Technology 10:1461 (1992); and Xu, et al., Appl. Microbiol. Biotechnol. 49:718 (1998)). Although numerous xylanases have been described in the literature, the need still exists to identify novel xylanases that are effective in applications such as those relating to animal feed and grain processing, biofuels, cleaning, fabric care, chemicals, plant processing, and delignifying and brightening of pulp and paper.

SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acid molecules having the sequence of SEQ ID NOS:1, 3, 5 or 7, and expression cassettes, vectors and recombinant host cells comprising these sequences. The invention also provides methods of preparing a

thermotolerant xylanase, comprising the steps of: expression in a microbial host cell an expression cassette comprising a promoter operably linked to a nucleic acid molecule encoding a xylanase which retains at least 40% activity after 30 minutes at 60°C and has a specific activity of greater than 400U/mg at a pH of less than pH 5.0 and greater than pH 1.5.

- 5 The invention further provides a method of preparing a thermotolerant xylanase wherein the xylanase is glycosylated. The invention also provides an isolated thermotolerant xylanase produced by such methods. The invention also provides enzyme feed additives and animal feeds comprising a thermotolerant xylanase. The invention further provides methods of preparing a pelleted animal feed comprising a thermotolerant xylanase and pelleted animal
- 10 feeds made by these methods. Also, the invention provides methods of decreasing the feed conversion ratio and increasing the weight gain of an animal, comprising the steps of feeding the animal an animal feed comprising the thermotolerant xylanase in an effective amount to decrease the feed conversion ratio in the animal. It also provides methods of improving the apparent metabolizable energy of animal feed comprising the step of formulating the animal
- 15 feed with one or more thermotolerant xylanases in an effective amount to improve the apparent metabolizable energy of the feed. The invention also provides a method improving the nutritive value of animal feed or human food comprising the step of adding a thermotolerant xylanase during the preparation of animal feed or human food.

20

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is the vector map of plasmid pBCS12771.

FIGURE 2 is the vector map of plasmid pBCS12772.

FIGURE 3 is the vector map of plasmid pSYN12773.

FIGURE 4 is a graph of gas production rate for selected xylanase enzymes for 18 h of fermentation.

- 25 Each point represents the mean of 6 observations.

FIGURE 5 is a graph of the enzyme activity of *Pichia* expressed xylanase PP6002 compared with pH.

FIGURE 6 is a graph of thermal tolerance of PP6002 Quantum® xylanase.

FIGURE 7 is a graph comparing the thermostability or thermotolerance of various xylanases.

30

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 is the nucleotide sequence of the xylanase coding region of PP6002 (without the kex 2 protease cleavage site or *Ava*I restriction site). Codons optimized for expression in *Pichia*.

- 35 SEQ ID NO:2 is the amino acid sequence of the xylanase coding region of PP6002 (also the same as BD6002 without the secretion signal).

SEQ ID NO:3 is the nucleotide sequence of the xylanase coding region of PP6016 (without the kex2 protease cleavage site or *Ava*I restriction site). Codons optimized for expression in *Pichia*.

5 SEQ ID NO:4 is the amino acid sequence of the xylanase coding region of PP6016 (also the same amino acid sequence as BD6016 without the secretion signal).

SEQ ID NO:5 is the nucleotide sequence of the xylanase coding region of PP6407 (without the kex2 protease cleavage site or *Ava*I restriction site). Codons optimized for expression in *Pichia*.

10 SEQ ID NO:6 is the amino acid sequence of the xylanase coding region of PP6407 (also the same amino acid sequence as BD6407 without the secretion signal).

SEQ ID NO:7 is the nucleotide sequence of the xylanase coding region of PP7436 (without the kex2 protease cleavage site or *Ava*I restriction site). Codons optimized for expression in *Pichia*.

15 SEQ ID NO:8 is the amino acid sequence of the xylanase coding region of PP7436 (also the same amino acid sequence as BD7436 without the secretion signal).

SEQ ID NO:9 is the nucleotide sequence of the PP6002 comprising an *Ava*I restriction site, a kex2 protease cleavage site and a *Pichia* codon optimized xylanase.

SEQ ID NO:10 is the nucleotide sequence of PP6016 comprising an *Ava*I restriction site, a kex2 protease cleavage site and a *Pichia* codon optimized xylanase.

20 SEQ ID NO:11 is the nucleotide sequence of PP6407 comprising an *Ava*I restriction site, a kex2 protease cleavage site and a *Pichia* codon optimized xylanase.

SEQ ID NO:12 is the nucleotide sequence of PP7436 comprising an *Ava*I restriction site, a kex2 protease cleavage site and a *Pichia* codon optimized xylanase.

25 SEQ ID NO:13 is the nucleotide sequence of xylanase XylA1A (also called BD6002 without the secretion signal).

SEQ ID NO:14 is the amino acid sequence of XylA1A (also referred to as BD6002) without the original secretion signal sequence.

SEQ ID NO:15 is the nucleotide sequence of xylanase XylA1B lacking a secretion signal sequence coding region (the full length sequence is also known as BD7436).

30 SEQ ID NO:16 is the amino acid sequence of xylanase XylA1B (BD7436) lacking the original secretion signal region.

SEQ ID NO:17 is the nucleotide sequence of xylanase XylA1C lacking a secretion signal sequence coding region (the full length sequence is also known as BD2230).

35 SEQ ID NO:18 is the amino acid sequence of xylanase XylA1C (BD2230) lacking a secretion signal sequence region.

SEQ ID NO:19 is the nucleotide sequence of xylanase XylA1D lacking a secretion signal sequence coding region (the full length sequence is also known as BD6016).

SEQ ID NO:20 is the amino acid sequence of xylanase XylA1D (BD6016) lacking a secretion signal sequence region.

SEQ ID NO:21 is the nucleotide sequence of xylanase XylA1E lacking a secretion signal sequence coding region (the full length sequence is also known as BD6407).

5 SEQ ID NO:22 is the amino acid sequence of xylanase XylA1E (BD6407) lacking a secretion signal sequence region.

SEQ ID NO:23 is the nucleotide sequence of Primer 1.

SEQ ID NO:24 is the nucleotide sequence of Primer 2.

SEQ ID NO:25 is the nucleotide sequence of Primer 3.

10 SEQ ID NO:26 is the nucleotide sequence of Primer 4.

SEQ ID NO:27 is the nucleotide sequence of Primer 5.

SEQ ID NO:28 is the nucleotide sequence of Primer 6.

SEQ ID NO:29 is the nucleotide sequence of Primer 7.

SEQ ID NO:30 is the nucleotide sequence of Primer 8.

15 SEQ ID NO:31 is the nucleotide sequence of Primer 9.

SEQ ID NO:32 is the nucleotide sequence of Primer 10.

SEQ ID NO:33 is the nucleotide sequence of plasmid pBCS12771.

SEQ ID NO: 34 is the nucleotide sequence of plasmid pBCS12772.

SEQ ID NO:35 is the nucleotide sequence of plasmid pSYN12773.

20 SEQ ID NO:36 is the nucleotide sequence encoding the kex2 protease cleavage site.

SEQ ID NO:37 is the amino acid sequence of the kex2 protease cleavage site.

SEQ ID NO:38 is the nucleotide sequence encoding the *Saccharomyces cerevisiae* α -mating factor pre-pro-peptide secretion signal.

25 SEQ ID NO:39 is the amino acid sequence of the *Saccharomyces cerevisiae* α -mating factor pre-pro-peptide secretion signal peptide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of a xylanase or xylanases, as well as the mutants and variants thereof, as an additive to animal feed, and to feedstuff that contains a xylanase.

30 The invention also relates to a method for improving the feed conversion ratio and/or the apparent metabolizable energy of feedstuffs using a xylanase or xylanases.

The invention provides a method of improving animal feed nutritive value. In this method, feed is formulated with one or more xylanases, such as for example, XylA1A, XylA1B, XylA1C, XylA1D, and XylA1E (SEQ ID NOS:13-22, respectively), for the
35 purposes of improving feed nutrient utilization. The feed comprises any cereal grains since

all contain xylans, but in particular wheat, rye, triticale, rice or corn, at an inclusion rate of less than 1.0%, more particularly, less than 0.1% w/w. The enzyme(s) is/are added to the feed using an inclusion rate of between 1 and 10000U/kg. The enzyme(s) may be added to the feed prior to or following feed processing, which encompasses pelleting, expansion and extrusion although other methods exist, or simply added to unprocessed (mash) feed. Addition of one or more of these enzymes results in an increase in apparent metabolizable energy (AME) and/or a decrease in the feed conversion ratio (FCR) compared with unsupplemented diets.

The invention also provides methods of preparing and using a nucleic acid molecule (i.e., a polynucleotide) that encodes a xylanase. The xylanase may be thermotolerant, but it is not necessarily thermotolerant. Hence, the invention also relates to methods of preparing and using a nucleic acid molecule that encodes a thermotolerant xylanase. A thermotolerant xylanase includes those which retain at least 40% activity after 30 minutes at about 60°C and which has a high specific activity, i.e., at least about 200 U/mg at 37°C and at acid pH, e.g., pH 5.3. In another embodiment, the xylanase retains at least 40% activity after 30 minutes at 70°C, or retains at least 40% activity after 30 minutes at 80°C, or retains at least 40% activity after 30 minutes at 85°C. The method expresses xylanase that is a thermotolerant xylanase in the absence of glycosylation. Alternatively, the method encompasses expressing a thermotolerant xylanase that is glycosylated by the host.

The invention also provides methods of preparing xylanases, including thermotolerant xylanases. The method comprises expressing in a microbial host cell an expression cassette comprising a promoter operably linked to a nucleic acid molecule encoding a xylanase. The microbial host cell may be a prokaryotic cell, such as a bacterial cell (e.g., *Escherichia*, *Pseudomonas*, *Lactobacillus*, and *Bacillus*), yeast (e.g., *Saccharomyces*, *Schizosaccharomyces*, *Pichia* or *Hansenula*) or fungal (e.g., *Aspergillus* or *Trichoderma*) cell. In particular, the host cell is *Pichia pastoris*. The microbial cell employed to prepare the recombinant xylanase may yield a glycosylated form of the recombinant xylanase.

The invention also provides the method of preparing a thermotolerant xylanase wherein the xylanase is encoded by the nucleotide sequence of SEQ ID NOS: 1, 3, 5 or 7. Further, the nucleic acid molecule encodes a fusion polypeptide comprising the xylanase. The fusion protein can further comprise a secretion signal peptide operably linked to the xylanase.

The invention further comprises a polynucleotide encoding the xylanase operably linked to at least one regulatory sequence, such as a promoter, an enhancer, an intron, a termination sequence, or any combination thereof, and, optionally, to a second polynucleotide

encoding a signal sequence, which directs the enzyme encoded by the first polynucleotide to a particular cellular location e.g., an extracellular location. Promoters can be constitutive promoters or inducible (conditional) promoters. As described herein, mutagenesis of a parent polynucleotide encoding a xylanase was employed to prepare variant (synthetic) DNAs
5 encoding a xylanase having improved properties relative to the xylanase encoded by the parent polynucleotide. In an embodiment, xylanase enzymes are screened for improved activity at acidic or basic pH, improved intestinal stability, or improved expression level in host organisms. In another embodiment, the mutations in a number of the variant DNAs were combined to prepare a synthetic polynucleotide encoding a xylanase with enhanced
10 thermotolerance and gastric stability and having a similar or a higher specific activity relative to the xylanase encoded by the parent polynucleotide. A parent polynucleotide may be obtained from any source including plant, bacterial or fungal nucleic acid, and any method may be employed to prepare a synthetic polynucleotide of the invention from a selected parent polynucleotide, e.g., combinatorial mutagenesis, recursive mutagenesis and/or DNA
15 shuffling.

Thus, in one embodiment of the invention, the thermotolerant xylanase has one or more amino acid substitutions relative to a corresponding xylanase, which substitutions are associated with the retention of activity at temperatures equal to or greater than 60°C.

In another embodiment, the thermotolerant xylanase has at least 40% activity at about
20 60°C for 30 minutes, or at least 40% activity at about 65°C for 30 minutes, or at least 35% activity at 70°C for 30 minutes, and which has a specific activity of at least 400 U/mg, more preferably at least 600 U/mg, and or at least 800 U/mg, at 37°C and at acid pH, e.g., less than pH 6.0 or at less than pH 4.0 and greater than pH 1.5. One xylanase unit (XU) is the quantity of enzyme that liberates 1 μ mol of reducing ends (xylose equivalents) per minute from
25 WAXY (wheat arabinoxylan) at 37°C, pH 5.3, under standard conditions.

In another embodiment, the invention provides a method to make an enzyme thermotolerant due to glycosylation comprising the step of expressing the enzyme in *Pichia pastoris*. In a particular embodiment, the enzyme is a xylanase.

In an embodiment of the invention, is an isolated nucleic acid molecule having the
30 sequence of SEQ ID NOS:1, 3, 5, or 7. The invention also provides an expression cassette comprising a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NOS: 1, 3, 5 or 7. The expression cassette can further comprise a proteolytic cleavage site such as the KEX2 protease cleavage site. In a more particular embodiment, the expression cassette is encoded by the nucleic acid sequence of SEQ ID NOS: 9, 10, 11 or 12. The expression

cassette can further comprise an isolated nucleotide sequence encoding a secretion signal peptide, such as the *Saccharomyces cerevisiae* α -mating factor pre-pro-peptide secretion signal. The expression cassette can further comprise at least one nucleic acid molecule encoding a xylanase of the invention operably linked to a promoter.

5 The invention also provides recombinant host cells comprising at least one nucleic acid molecule of SEQ ID NOS; 1, 3, 5 or 7. The recombinant host cell can be a bacteria, yeast or fungal cell. In particular the host cell is *Escherichia*, *Pseudomonas*, *Lactobacillus*, *Bacillus*, *Saccharomyces*, *Schizosaccharomyces*, *Pichia*, *Hansenula*, *Aspergillus* or *Trichoderma* cell. In particular, the host cell is *Pichia pastoris*. In a more particular
10 embodiment, the host cell comprises the vector pBCS12771, pBCS12772 or pSYN12773. In particular the host cell is *Pichia pastoris* comprising the vector pSYN12773.

 Also provided by the invention are vectors comprising the expression cassette or polynucleotide of the invention, and transformed microbial cells comprising the polynucleotide, expression cassette or vector of the invention. A vector of the invention can
15 encode more than one polypeptide including more than one xylanase or may encode a fusion polypeptide comprising the xylanase of the invention, and a transformed microbial cell may comprise one or more vectors of the invention. The transformed cells of the invention are useful for preparing the recombinant xylanase of the invention. Accordingly, the invention provides xylanase isolated from the transformed microbial cells of the invention, as well as
20 synthetically prepared enzyme. In particular, the vectors comprise the plasmids designated pBCS12771 (SEQ ID NO:33), pBCS12772 (SEQ ID NO:34), or pSYN12773 (SEQ ID NO:35).

 The invention also provides an isolated thermotolerant xylanase made by the method of the invention. Further, the isolated thermotolerant xylanase is glycosylated.

25 Further provided by the invention are methods for formulation of xylanases, xylanase formulations or formulated enzyme mixtures. The feed additives comprising thermotolerant xylanase comprise a thermotolerant xylanase of the invention. The feed additive formulations further comprise a stabilizing compound, such as but not limited to sorbitol. The recombinant xylanase or formulations thereof may be added as a supplement to food or animal feed or to
30 components of food and feed prior to, during, or after food or feed processing. Preferably, the recombinant xylanase of the invention is added to a mixture of feed components prior to and/or during heat (e.g., steam) conditioning in a pellet mill. Thus, the invention includes methods of making and using a xylanase.

Further, as a xylanase of the invention is capable of surviving the heat-conditioning step encountered in a commercial pellet mill during feed formulation, the invention provides a method of making animal feed, e.g., hard granular feed pellets comprising the xylanase. To make feed, the formulated xylanase may be mixed with feed components, the mixture steam conditioned in a pellet mill such that at least 50% of the pre-heat treated enzymatic activity is retained, and the feed extruded through a pellet die. In another embodiment, greater than 70% of enzyme activity is recovered after pelleting at 85C, or more particularly, greater than 90% of enzyme activity is recovered after pelleting at 85C. In a particular embodiment, at least 80% of pre-treatment enzymatic activity is recovered after pelleting at 90°C.

The xylanase may thus be used as a supplement in animal feed by itself, in addition with vitamins, minerals, other feed enzymes, agricultural co-products (e.g., wheat middlings or corn gluten meal), or in a combination therewith. The enzyme may also be added to mash diets, i.e., diets that have not been through a pelletizer.

Some benefit of using xylanases in feed is produced during the feed manufacturing process, for example, during heat treatment when the temperatures would range from 75 to 95°C as in traditional pelleting (expansion will be 105-125°C). Such treatments can decrease the viscosity of the feed in the gut of the animal. See Silversides and Bedford, Poultry Sci. 78:1184-1190 (1999).

Xylanase enzymes that are not thermotolerant are often applied post pelleting, generally via spraying an enzyme solution onto pelleted feed. Some of the problems associated with spraying methods are that only a low percentage of the pellets are contacted with enzyme, the enzyme is only present on the surface of the coated pellets, and feed mills need to invest in and operate complex spraying machinery. In contrast, a thermotolerant xylanase of the invention, which may be added prior to pelleting, thereby facilitating production of a feed with an improved distribution of the enzyme. Moreover, feed comprising a thermotolerant xylanase of the invention may have a longer shelf life than feed sprayed with xylanase, as the spraying process introduces moisture which can support fungal and bacterial growth during storage. Current xylanases that are not thermotolerant can be made to survive high processing temperatures by coating with a wax layer to keep moisture out. This process is expensive, however, and reduces the efficacy of the product in low temperature pelleted diets or mash diets since the wax coating is slow to release the enzyme once introduced into the gut of the animal.

The invention thus provides a method of preparing animal feed comprising providing a mixture comprising one or more feed components and a preparation comprising a xylanase of the invention, such as a thermotolerant xylanase, and treating the mixture under appropriate

conditions of temperature and moisture so as to hydrolyze xylan which is present in the mixture. Also provided is animal feed prepared by such a method. The animal feeds include, but are not limited to, poultry feed, swine feed or ruminant feeds.

Further provided is a method of preparing a xylanase containing composition for feed
5 formulation comprising combining a liquid solution comprising the thermotolerant xylanase of the invention and meal flour, *e.g.*, soy meal flour, to yield a mixture; and drying the mixture to yield a dried composition. Drying the mixture may be accomplished by techniques routinely used in the art, including but not limited to lyophilising and/or heating.

The invention further provides a method in which a mixture comprising animal feed
10 components and a preparation comprising the xylanase of the invention is treated with heat so as to yield a heat-treated animal feed mixture. Heat-treated animal feed prepared by the method is also provided. The xylanase preparation may be a liquid or a solid preparation. In one embodiment, a liquid solution comprising a xylanase of the invention is combined with soy meal flour to yield a mixture and the mixture is then lyophilized. The mixture may also
15 comprise at least one vitamin, mineral, an enzyme other than a xylanase, an organic acid, a probiotic product, an essential oil or a grain-processing co-product. The heat-treated feed may be further processed, for example, by extruding the heat-treated feed through a pellet mill to yield pelletized animal feed. Also provided is an animal feed composition comprising the xylanase of the invention, and an enzyme feed additive or a food additive comprising such
20 a xylanase.

The invention further provides a pelletized animal feed. In one embodiment, the pelletized animal feed is steam conditioned in a pellet mill at about 85C such that greater than 70% of the pre-heate treated enzymatic activity is retained, and the feed extruded through a pellet dye. In another embodiment, the pelletized animal feed is steam conditioned in a pellet
25 mill at about 90C such that at least 80% of the pre-heated enzymatic activity is retained, and the feed extruded through a pellet dye.

Also provided is a method of decreasing the feed conversion ratio and increasing the weight gain of an animal comprising the step of feeding to an animal a feed comprising a thermotolerant xylanase of the invention in an effective amount to decrease the feed
30 conversion ratio in the animal and increase the weight gain of the animal.

The invention provides a method of improving the nutritive value of animal feed or human food. The method comprises the step of adding the xylanase of the invention during the preparation of animal feed or human food. Also provided is a method of preparing human food comprising providing a mixture of a food component and a preparation comprising the

xylanase of the invention; and treating the mixture under appropriate conditions of temperature and moisture to facilitate hydrolysis of xylan.

The invention also provides a method for improving the apparent metabolizable energy (AME) of animal feed comprising the step of formulating the animal feed with one or more thermotolerant xylanases of the invention with the animal feed in an effective amount to improve the apparent metabolizable energy of the feed.

Animals within the scope of the invention include polygastric animals, *e.g.*, calves, as well as monogastric animals including but not limited to, swine, poultry (*e.g.*, chickens, turkeys, geese, ducks, pheasant, grouse, quail and ostrich), equine, ovine, caprine, canine and feline, as well as fish and crustaceans. Further, ruminant animals such as cows are included in the scope of the invention. The levels of xylanase in feed or food are added at an inclusion rate of between about 1 to 10000 U/kg, more particularly 50 to 5000 U/kg, or 200 to 3,200 U/kg.

The xylanase enzyme, as well as the enzyme mixtures described above, can in principle be added to all feedstuffs. Suitable and preferred examples are those which comply with the provisions of the feedstuffs legislation, such as complete feed, supplementary feed and mineral feed.

The invention is a method of improving animal feed nutritive value. In this method, feed is formulated with one or more of the enzymes, for example, XylA1A, XylA1B, XylA1C, XylA1D, and XylA1E, for the purposes of improving feed nutrient utilization. The feed may be composed of any cereal grains since all contain xylans, but in particular wheat, rye, triticale, rice or corn, at an inclusion rate of less than 1.0%, more particularly, less than 0.1% w/w. The enzyme(s) is/are added to this feed using an inclusion rate of between 1 and 10000U/kg. The enzyme(s) may be added to the feed prior to or following feed processing, which encompasses pelleting, expansion and extrusion although other methods exist, or simply added to unprocessed (mash) feed. Addition of one or more of these enzymes results in an increase in apparent metabolizable energy (AME) and/or a decrease in the feed conversion ratio (FCR) compared with unsupplemented diets, but also in comparison with diets supplemented with the current commercial standard xylanases.

The enzyme dosed is in the range from 0.01 to 10,000 ppm, or alternatively in the range from 20 to 1000ppm. The activity of xylanase enzyme product is normally stated in units (U).

The enzyme product, alone or in a mixture, is mixed with the feed batch wise in appropriate ratios by weight. In this connection, it is important that the active substances in the feed are homogeneously distributed.

5 Feedstuffs which contain a xylanase enzyme can be employed for feeding all livestock, but particularly advantageously for feeding agricultural livestock used for the production of foodstuffs, in particular broilers, turkeys, pigs and cattle.

10 Addition of the enzyme product or of the mixture to the feedstuffs brings about a considerable improvement in the utilization thereof and, connected with this, an improvement in growth of the livestock. The use thereof as additive to feedstuffs has, by comparison with additives having antibiotic activity, the great advantage that there is no risk of resistance development on use over a prolonged period.

Other Uses

15 Xylanases of the present invention can be used in any application for which other xylanases are used, such as but not limited to, grain processing, biofuels, cleaning, fabric care, chemicals, plant processing, and delignifying and brightening of pulp and paper.

Constructs and Host Cells of the Invention

20 The invention preferably provides an expression cassette comprising a nucleic acid sequence (promoter) capable of directing expression of a polynucleotide encoding a xylanase either *in vitro* or *in vivo*. Methods to prepare and/or identify a xylanase include mutagenesis, e.g., recursive mutagenesis, and/or selection or screening, e.g., for xylanases having activity at temperatures greater than 60°C. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel, 1985; Kunkel et al., 1987; U.S. Pat. No. 4,873,192; Walker and Gaastra, 1983 and the references cited therein; and Arnold et al., 1996.

25 A. DNA and Host Cells for Transformation

Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes) BACs (bacterial artificial chromosomes) and isolated nucleic acid molecules for use in transforming cells will generally comprise the xylanase encoding nucleic acid molecules, as well as other nucleic acid molecules such as cDNA, gene or genes which one desires to introduce into the cells.
30 These nucleic acid constructs can further comprise nucleic acid molecules such as promoters, enhancers, polylinkers, or even regulatory genes as desired. One of the nucleic acid

molecules or genes chosen for cellular introduction will often encode a protein which will be expressed in the resultant transformed (recombinant) cells, such as will result in a screenable or selectable trait and/or which will impart an improved phenotype to the transformed cell. However, this may not always be the case, and the present invention also encompasses transformed cells incorporating non-expressed transgenes.

Isolated nucleic acid molecules useful for introduction into cells comprise that which has been derived or isolated from any source, that may be subsequently characterized as to structure, size and/or function, chemically altered, and later introduced into cells. An example of an isolated nucleic acid molecule "derived" from a source, would be a nucleic acid sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such a nucleic acid molecule "isolated" from a source would be a useful nucleic acid molecule sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. Such a nucleic acid molecule is commonly referred to as "recombinant." Therefore useful nucleic acid molecules comprise completely synthetic nucleic acid molecules, semi-synthetic nucleic acid molecules, nucleic acid molecules isolated from biological sources, and nucleic acid molecules derived from introduced RNA. Generally, the introduced nucleic acid molecule is not originally resident in the genotype which is the recipient of the nucleic acid molecule, but it is within the scope of the invention to isolate a gene from a given genotype, and to subsequently introduce multiple copies of the gene into the same genotype, e.g., to enhance production of a given gene product.

The introduced nucleic acid molecule comprises, but is not limited to, nucleic acid molecules isolated from genes such as those from bacteria, yeasts, fungi, or viruses. The introduced nucleic acid molecule includes modified or synthetic genes, portions of genes, or chimeric genes, including genes from the same or different genotype. The term "chimeric gene" or "chimeric nucleic acid molecule" is defined as a gene or nucleic acid molecule sequence or segment comprising at least two nucleic acid sequences or segments from species which do not combine nucleic acid under natural conditions, or which nucleic acid sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of the untransformed cell.

The introduced nucleic acid molecule used for transformation herein is circular or linear, double-stranded or single-stranded. Generally, the isolated nucleic acid molecule is in the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by regulatory sequences which promote the expression of the recombinant DNA

present in the transformed cell. For example, the nucleic acid molecule comprises or consists of a promoter that is active in a cell which is derived from a source other than that cell, or may utilize a promoter already present in the cell that is the transformation target.

Generally, the introduced nucleic acid molecule will be relatively small, i.e., less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the nucleic acid molecule increases. The number of proteins, RNA transcripts or mixtures thereof, which is introduced into the cell is customarily pre-selected and defined, e.g., from one to about 5-10 such products of the introduced DNA may be formed.

The selection of an appropriate expression vector will depend upon the host cells. Typically an expression vector comprises (1) prokaryotic nucleic acid molecule elements coding for a bacterial origin of replication and an antibiotic resistance gene to provide for the amplification and selection of the expression vector in a bacterial host; (2) nucleic acid molecules that control initiation of transcription such as a promoter; (3) nucleic acid molecules that control the processing of transcripts such as introns, transcription termination/polyadenylation sequence; and (4) a nucleic acid molecule or gene of interest that is operatively linked to the nucleic acid molecule to control transcription initiation. In a particular embodiment, the xylanase gene and operable elements would not replicate autonomously in a host cell. The expression vector used may be one capable of autonomously replicating in the above host or capable of integrating into the chromosome, originally containing a promoter at a site enabling transcription of the linked xylanase gene.

If prokaryotes such as bacteria are used as the host, the expression vector for the xylanase is preferably one capable of autonomously replicating in the micro-organism and comprising a promoter, a ribosome-binding sequence, the novel xylanase gene, and a transcription termination sequence. The vector may also contain a gene for regulating the promoter.

Yeast or fungal expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences.

Suitable vectors include by way of example: for bacteria, pQE70, pQE60, pQE-9 (Qiagen), pBluescript II (Stratagene), pTRC99a, pKK223-3, pDR540, pRIT2T (Pharmacia); for eukaryotic cells: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVLSV40

(Pharmacia). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEMI (Promega Biotec, Madison, Wis., USA). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

As representative examples of appropriate hosts, there may be mentioned: bacterial
5 cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; and various species within the genera *Escherichia*, *Pseudomonas*, *Serratia*, *Streptomyces*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Microbacterium*, and *Staphylococcus*, although others may also be employed as a matter of choice; fungal cells belonging to the genera *Aspergillus*, *Rhizopus*, *Trichoderma*, *Neurospora*, *Mucor*, *Penicillium*, etc., such as yeast belonging to the genera *Kluyveromyces*,
10 *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Schwanniomyces*, *Pichia* and the like.

The construction of vectors which may be employed in conjunction with the present invention will be known to those of skill of the art in light of the present disclosure (see, e.g., Sambrook et al., Molecular Cloning, Cold Spring Harbor Press, 1989; Gelvin et al., Plant Molecular Biology Manual, 1990). The expression cassette of the invention may contain one
15 or a plurality of restriction sites allowing for placement of the polynucleotide encoding a xylanase under the regulation of a regulatory sequence. The expression cassette may also contain a termination signal operably linked to the polynucleotide as well as regulatory sequences required for proper translation of the polynucleotide. The expression cassette containing the polynucleotide of the invention may be chimeric, meaning that at least one of
20 its components is heterologous with respect to at least one of the other components. Expression of the polynucleotide in the expression cassette may be under the control of a constitutive promoter, inducible promoter, regulated promoter, viral promoter or synthetic promoter.

The expression cassette may include in the 5'-3' direction of transcription, a
25 transcriptional and translational initiation region, the polynucleotide of the invention and a transcriptional and translational termination region functional in vivo and/or in vitro. The termination region may be native with the transcriptional initiation region, may be native with the polynucleotide, or may be derived from another source. The regulatory sequences may be located upstream (5' non-coding sequences), within (intron), or downstream (3' non-coding
30 sequences) of a coding sequence, and influence the transcription, RNA processing or stability, and/or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, enhancers, promoters, repressor binding sites, translation leader sequences, introns, and polyadenylation signal sequences. They may include natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural
35 sequences.

The vector, used in the present invention may also include appropriate sequences for amplifying expression.

B. Regulatory Sequences

A promoter is a nucleotide sequence that controls the expression of a coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter includes a minimal promoter, consisting only of all basal elements needed for transcription initiation, such as a TATA-box and/or initiator that is a short DNA sequence comprised of a TATA-box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. A promoter may be derived entirely from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may contain DNA sequences that are involved in the binding of protein factors that control the effectiveness of transcription initiation in response to physiological or developmental conditions. A promoter may also include a minimal promoter plus a regulatory element or elements capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence contains of proximal and more distal elements, the latter elements are often referred to as enhancers.

Representative examples of promoters include, but are not limited to, promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. Particular bacterial promoters include *E. Coli* lac or trp, the phage lambda P_L, lacI, lacZ, T3, T7, gpt, and lambda P_R promoters.

Any promoter capable of expressing in yeast hosts can be used as the promoter. Examples thereof include promoters for genes of hexokinase and the like in the glycolytic pathway, and promoters such as gal 1 promoter, gal 10 promoter, heat shock protein promoter, MFa-1 promoter and CUP 1 promoter.

Any promoter capable of expressing in filamentous fungi may be used. Examples are a promoter induced strongly by starch or cellulose, e.g., a promoter for glucoamylase or α-amylase from the genus *Aspergillus* or cellulase (cellobiohydrazase) from the genus *Trichoderma*, a promoter for enzymes in the glycolytic pathway, such as phosphoglycerate kinase (pgk) and glyceraldehyde 3-phosphate dehydrogenase (gpd), etc.

Two principal methods for the control of expression are known, viz.: over-expression and under-expression. Over-expression can be achieved by insertion of one or more than one extra copy of the selected gene. For under-expression there are two principle methods which

are commonly referred to in the art as "antisense down-regulation" and "sense down-regulation". Generically these processes are referred to as "gene silencing". Both of these methods lead to an inhibition of expression of the target gene.

Several inducible promoters are known in the art. Many promoters are described in a review by Gatz, (Current Opinion in Biotech. 7(2):168-172 (1996) (see also Gatz, Annual Rev. Plant. Physiol. and Plant Mol. Biol. 48:89-108, 1997)). Examples include tetracycline repressor system, Lac repressor system, copper-inducible systems, salicylate-inducible systems (such as the PR1a system), glucocorticoid-inducible (Aoyama T. et al., Plant Journal 11(3):605-612, 1997) and ecdysone-inducible systems. Also included are the benzene sulphonamide-inducible system (U.S. Pat. No. 5364,780), alcohol-inducible (WO 97/06269 and WO 97/06268) inducible systems and glutathione S-transferase promoters.

Regulated expression of a chimeric transacting viral replication protein can be further regulated by other genetic strategies. For example, Cre-mediated gene activation as described by Odell et al., (Molecular and General Genetics 223:369-378 1990). Thus, a DNA fragment containing 3' regulatory sequence bound by lox sites between the promoter and the replication protein coding sequence that blocks the expression of a chimeric replication gene from the promoter can be removed by Cre-mediated excision and result in the expression of the trans-acting replication gene. In this case, the chimeric Cre gene, the chimeric trans-acting replication gene, or both can be under the control of developmental-specific or inducible promoters. An alternate genetic strategy is the use of tRNA suppressor gene. For example, the regulated expression of a tRNA suppressor gene can conditionally control expression of a trans-acting replication protein coding sequence containing an appropriate termination codon as described by Ulmasov et al., Plant Mol. Biol. 35(4):417-424, 1997. Again, either the chimeric tRNA suppressor gene, the chimeric transacting replication gene, or both can be under the control of developmental-specific or inducible promoters.

In addition to the use of a particular promoter, other types of elements can influence expression of transgenes. In particular, introns have demonstrated the potential for enhancing transgene expression.

Other elements include those that can be regulated by endogenous or exogenous agents, e.g., by zinc finger proteins, including naturally occurring zinc finger proteins or chimeric zinc finger proteins. See, e.g., U.S. Pat. No. 5,789,538, WO 99/48909; WO 99/45132; WO 98/53060; WO 98/53057; WO 98/53058; WO 00/23464; WO 95/19431; and WO 98/54311.

An enhancer is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a particular promoter. An enhancer is capable of operating in both orientations (5' to 3' and 3'-5' relative to the gene of interest coding sequences), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects.

Vectors for use in accordance with the present invention may be constructed to include an enhancer element. Constructs of the invention will also include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the polyadenylation of the resultant mRNA.

As the DNA sequence between the transcription initiation site and the start of the coding sequence, i.e., the untranslated leader sequence, can influence gene expression, one may also wish to employ a particular leader sequence. Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, i.e., to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure.

20 C. Marker Genes

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., an antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening'. Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen

that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA and small active enzymes detectable in extracellular solution.

5 Selectable markers for use in prokaryotes include a tetracycline resistance or an ampicillin resistance gene. Screenable markers that may be employed include, but are not limited to, a β -glucuronidase or uidA gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; a beta-lactamase gene (Sutcliffe, Proc. Natl. Acad. Sci. USA 75(8):3737-3741, 1978), which encodes an enzyme for which various
10 chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a xylE gene (Zukowsky et al., Proc. Natl. Acad. Sci. USA 80:1101, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an alpha-amylase gene (Ikuta et al., Bio-technology 8(3):241-242, 1990); a tyrosinase gene (Katz et al., J. General Microbiol. 129(Pt. 9):2703-14, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA
15 and dopaquinone which in turn condenses to form the easily detectable compound melanin; a beta-galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (lux) gene (Ow et al., Science 234:856-859, 1986), which allows for bioluminescence detection; or even an aequorin gene (Prasher et al., Biochem Biophys Res Commun. 126(3):1259-68, 1985), which may be employed in calcium-sensitive
20 bioluminescence detection, or a green fluorescent protein gene (Niedz et al., Plant Cell Reports 14(7):403-406, 1995). The selectable marker may also be a negative selectable marker such as, but not limited to, transforming the gene into an organism that is ura3-genotype and using the ura3 system +/- 5FOA, +/- uracil in the medium.

Transformation

25 The expression cassette, or a vector construct containing the expression cassette, may be inserted into a cell. The expression cassette or vector construct may be carried episomally or integrated into the genome of the cell, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However,
30 any vector may be used as long as it is replicable and viable in the host.

A variety of techniques are available and known to those skilled in the art for introduction of constructs into a cellular host. Transformation of microbial cells may be accomplished through use of polyethylene glycol, calcium chloride, viral infection, DEAE dextran, phage infection, electroporation and other methods known in the art. Transformation

of fungus, in particular *Pichia*, may be accomplished according to "Pichia Protocols", in Methods Mol. Biol., Higgins, David R. and Cregg, James M.; Eds. (Humana, Totowa, N. J.) (1998). Introduction of the recombinant vector into yeasts can be accomplished by methods including electroporation, use of spheroplasts, lithium acetate, and the like. Any method
5 capable of introducing DNA into animal cells can be used: for example, electroporation, calcium phosphate, lipofection and the like.

Recombinant Enzyme

For preparation of recombinant xylanase, following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, e.g., a bacterial or yeast
10 host, a selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and cells cultured for an additional period to yield recombinant enzyme. Cells are then typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Alternatively, the recombinant protein may be produced as a fusion to a signal peptide that facilitates export of
15 the recombinant protein from the host cell. In this situation, cells are harvested by centrifugation and the supernatant is retained. The recombinant protein may then be purified from the supernatant.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use
20 of cell lysing agents, such methods are well known to those skilled in the art.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin
25 chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzymes of the present invention may be a product of chemical synthetic procedures, or produced by recombinant techniques from a microbial host (for example, by
30 bacterial, yeast, and fungal cells in culture). Depending upon the host employed in a recombinant production procedure, the enzyme of the present invention may or may not be covalently modified via glycosylation. In eukaryotic cells, glycosylation of secreted proteins serves to modulate protein folding, conformational and thermostability stability, and

resistance to proteolysis. Given a specific application of xylanase use, a glycosylated version of the enzyme may be preferable over a non-glycosylated form. For example, the use of a glycosylated xylanase in animal feed helps protect the enzyme from thermal denaturation during feed pelleting and from proteolytic inactivation as it passes through the stomach of the animal, helping deliver active enzyme to the intestinal tract and site of action. For food processing applications where enzyme activity is desired only during processing and not in the final product a non-glycosylated, thermolabile, and proteolytic susceptible xylanase is preferred. By producing the xylanase of this invention in various microbial hosts, both thermotolerance and susceptibility to proteolytic degradation are altered.

The enzymes of this invention may be employed for any purpose in which such enzyme activity is necessary or desired. In a preferred embodiment, the enzyme is employed for catalyzing the hydrolysis of xylan in animal feed. In another preferred embodiment, the enzyme is employed for catalyzing the hydrolysis of xylan in food.

Xylanase Compositions

Generally, xylanase compositions are liquid or dry. Liquid compositions need not contain anything more than the xylanase enzyme, preferably in a highly purified form. However, a stabilizer such as glycerol, sorbitol or mono propylene glycol may be added. The liquid composition may also comprise other additives, such as salts, sugars, preservatives, pH-adjusting agents, and proteins. Typical liquid compositions are aqueous or oil-based slurries. The liquid compositions may be added to a food or feed before or after an optional pelleting thereof.

Dry compositions may be freeze-dried or spray dried compositions, in which case the composition need not contain anything more than the enzyme in a dry form. Dry compositions may be granulates which may readily be mixed with, e.g., food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient means of incorporating enzymes into, e.g., processed food or animal feed.

For example, a stable xylanase enzyme formulation can be prepared by freezing a mixture of liquid enzyme solution with a bulking agent such as ground soybean meal, and then lyophilizing the mixture. The reduction in moisture and the binding interactions of the xylanase with the bulking agent protect the enzyme from external environmental factors such as the temperature extremes experienced during compound feed manufacture. Dry

formulations can further enhance stability by minimizing the activity of potential proteolytic enzymes that may be present as by-products in the liquid fermentation mixture used to manufacture the target enzyme. The resulting dry enzyme-soy flour mixture of the present invention can withstand high extremes of temperature. This formulated enzyme mixture can
5 be used as a feed supplement for use in poultry and swine production.

Once a dry enzyme preparation is obtained, agglomeration granulates are prepared using agglomeration techniques in a high shear mixer during which a filler material and the enzyme are co-agglomerated to form granules. Absorption granulates are prepared by having
10 cores of a carrier material to absorb/be coated by the enzyme. Typical filler materials are salts such as disodium sulphate. Other fillers include kaolin, talc, magnesium aluminium silicate and cellulose fibres. Optionally, binders such as dextrans are also included in agglomeration granulates.

Typical carrier materials include starch, e.g., in the form of cassava, corn, potato, rice and wheat. Salts may also be used.

15 Optionally, the granulates are coated with a coating mixture. Such a mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired, other additives such as calcium carbonate or kaolin.

Additionally, xylanase compositions may contain other substituents such as coloring agents, aroma compounds, stabilizers, vitamins, minerals, other feed or food enhancing
20 enzymes and the like. This is so in particular for the so-called pre-mixes.

A "food or feed additive" is an essentially pure compound or a multi component composition intended for or suitable for being added to food or feed. In particular it is a substance that by its intended use is becoming a component of a food or feed product or affects any characteristics of a food or feed product. Thus, a xylanase additive is understood
25 to mean a xylanase which is not a natural constituent of the main feed or food substances or is not present at its natural concentration therein, e.g., the xylanase is added to the feed separately from the feed substances, alone or in combination with other feed additives. A typical additive usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients.

30 A "ready for use" xylanase additive is herein defined as an additive that is not produced in situ in animal feed or in processed food. A ready for use xylanase additive may be fed to humans or animals directly or, preferably, directly after mixing with other feed or food constituents. For example, a feed additive according to this aspect of the present

invention is combined with other feed components to produce feed. Such other feed components include one or more other (preferably thermostable) enzyme supplements, vitamin feed additives, mineral feed additives and amino acid feed additives. The resulting (combined) feed additive including possibly several different types of compounds can then be
5 mixed in an appropriate amount with the other feed components such as cereal and protein supplements to form an animal feed. Processing of these components into an animal feed can be performed using any of the currently used processing apparatuses such as a double-pelleting machine, a steam pelleter, an expander or an extruder.

Similarly, a food additive according to this aspect of the present invention is
10 combined with other food components to produce processed food products. Such other food components include one or more other (in particular thermostable) enzyme supplements, vitamin food additives and mineral food additives. The resulting (combined) food additive, including possibly several different types of compounds can then be mixed in an appropriate amount with the other food components such as cereal and plant proteins to form a processed
15 food product. Processing of these components into a processed food product can be performed using any of the currently used processing apparatuses.

In another embodiment, the xylanase compositions of the invention additionally comprises an effective amount of one or more feed or food enhancing enzymes, in particular feed or food enhancing enzymes selected from the group consisting of alpha-galactosidases, beta-galactosidases, in particular lactases, other xylanases, beta-glucanases, in particular
20 endo-beta-1,4-glucanases and endo-beta-1,3(4)-glucanases, cellulases, xylosidases, galactanases, in particular arabinogalactan endo-1,4-beta-galactosidases and arabinogalactan endo-1,3-beta-galactosidases, endoglucanases, in particular endo-1,2-beta-glucanase, endo-1,3-alpha-glucanase, and endo-1,3-beta-glucanase, pectin degrading enzymes, in particular
25 pectinases, pectinesterases, pectin lyases, polygalacturonases, arabinanases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases, rhamnogalacturonan-alpha-rhamnosidase, pectate lyases, and alpha-galacturonisidases, mannanases, beta-mannosidases, mannan acetyl esterases, xylan acetyl esterases, proteases, xylanases, arabinoxylanases and lipolytic enzymes such as lipases, phospholipases, phytases and cutinases.

The animal feed additive of the invention is supplemented to the animal before or
30 simultaneously with the diet. Preferably, the animal feed additive of the invention is supplemented to the animal simultaneously with the diet.

An effective amount of xylanase in food or feed is from about 1 to 10,000 U/kg; more particularly from about 50 to 5,000 U/kg, more particularly from about 500 to 4,000 U/kg or from about 250 to 3200 U/kg.

Also within the scope of this invention is the use of xylanase for processing and manufacturing human foods and animal feeds. Grains and flours destined for human foods can be enzymatically treated with xylanase to reduce the xylan content of the material. The reduced levels of xylan enhance the quality of the food by increasing the nutrient availability of essential minerals such as iron, calcium, and zinc. In addition to increasing the nutritional quality of food, xylanase used during food processing can improve the overall efficiency of the food production method. During food manufacture the xylanase is active during manufacture and processing only, and is not active in the final food product. This aspect is relevant for instance in dough making and baking. Similarly, animal feed grains such as toasted soybean meal or canola meal may be pre-processed with xylanase prior to compound feed manufacture. Removal of the anti-nutritive factors in animal feed components prior to compound feed manufacture produces a nutritionally higher quality and more valuable animal feed ingredient. In this processing method the xylanase is active during feed manufacturing, and may or may not be active in the digestive tract of the animal upon ingestion of the treated feed.

In addition to using xylanase as a food processing aid, the scope of this invention encompasses the use of xylanase as a human supplemental digestive aid. Xylanase in tablet form can be ingested at the time of food consumption to deliver active enzyme to the gastrointestinal tract of the recipient. Nutritional gains for the consumer would be experienced in vivo and may be taken with foods that cannot be treated with a xylanase during food processing.

Also within the scope of the invention is the use of a xylanase of the invention during the preparation of food or feed preparations or additives, i.e., the xylanase is active during the manufacture only and is not active in the final food or feed product. This aspect is particularly relevant, for instance, in dough making and baking and the production of other ready-to-eat cereal based products.

The xylanase may also be used advantageously in monogastrics as well as in polygastrics, especially young calves. Diets for fish and crustaceans may also be supplemented with xylanase to further improve feed conversion ratio. The feed according to the present invention may also be provided to animals such as poultry, e.g., turkeys, geese, ducks, as well as swine, equine, bovine, ovine, caprine, canine and feline, as well as fish and

crustaceans. It is however, particularly preferred that the feed is provided to pigs or to poultry, including, but not limited to, broiler chickens, hens, in particular laying hens, turkeys and ducks.

Feed Compositions and Methods of Use

5 The xylanases (formulated as described above) of the current invention may be combined with other ingredients to result in novel feed compositions with particular advantages.

 The xylanases of the present invention are so active that they can be used to create novel animal feed formulations that allow superior feed conversion efficiency and improved
10 weight gain relative to normal diets.

 Specifically, the animal feed of the invention comprises the combination of a xylanase of the present invention in combination with animal feed ingredients to form a feed that has substantially lowered intact xylan content. In a preferred embodiment, the feed compositions of the invention comprises typical feed ingredients, micronutrients, vitamins,
15 etc. and an effective amount of thermostable xylanase where the amounts of the xylanase is from about between the levels of 1–10,000 units of xylanase per kg of feed; more particularly between the levels of 50–5,000 units of xylanase per kg of feed.

 Also, within the scope of the invention are methods of improving weight gains, and feed conversions ratios (FCR) associated with production of farm animals. A xylanase of the
20 present invention allows improved weight gains and FCR. Specifically the method of the present invention is to improve the FCR, or weight gain by feeding a diet to an animal comprising a xylanase of the present invention.

 The animal feed of the present invention can be used on monogastric or polygastric animals. The animal feed of the present invention can be feed for poultry, or swine, or calves,
25 or companion animals such as dogs, cats, or horses. The animal feed can also be used on ruminants such as cows.

 The invention will be further described by the following examples, which are not intended to limit the scope of the invention in any manner.

EXAMPLE 1

30 XylA1A Expression Constructs

pBSC12771 (pPIC9 harboring the Synthetic XylA1A Xylanase Gene). A synthetic gene encoding the XylA1A xylanase amino acid sequence was constructed at Entelechon

(Regensburg, Germany) utilizing *Pichia pastoris* preferred codons and was designated PP6002 (for *Pichia pastoris* optimized codon version of XylA1A) (SEQ ID NO:1). The synthetic gene sequence was designed to include the *KEX2* protease cleavage signal (Glu-Lys-Arg) (SEQ ID NO: 37 and nucleotide sequence SEQ ID NO:36) in front of the mature peptide coding sequence. The synthetic gene was supplied in the pPIC9 vector (Invitrogen, Carlsbad, CA) and was designated pBSC12771 by Syngenta Quality Control (Figure 1 and SEQ ID NO:33). This cloning strategy produced a fusion protein in which the *Saccharomyces cerevisiae* α -mating factor pre-pro-peptide secretion signal (nucleotide and amino acid sequences, SEQ ID NOS: 38 and 39, respectively) is fused in frame to the N-terminus of the PP6002 gene sequence. The fusion peptide encoded by this gene is secreted from the cell after production. During the secretion process, the α -factor peptide portion of the fusion protein is cleaved by the Kex2 protease and XylA1A xylanase is released into the extracellular environment.

This facilitates the isolation and purification of XylA1A enzyme. The PP6002 gene in this construct is under the control of the *P. pastoris* alcohol oxidase-1 (AOX1) promoter that is inducible with methanol. The synthetic gene was confirmed by using plasmid specific 5AOX and 3AOX sequencing primers supplied by the manufacturer (Invitrogen, Carlsbad, CA). After sequence confirmation, the pBSC12771 plasmid was retransformed into chemically competent *E. coli* TOP10 cells as previously described and a glycerol stock was prepared using methods described by Sambrook J, Russel DW. 2001. "Molecular Cloning: A Laboratory Manual," 3rd Ed. (Cold Spring Harbor Press, Cold Spring Harbor, NY).

Construction of pCR4Blunt_5AOX-PP6002-3AOXTT Intermediate. The yeast multi-copy expression vector pAO815 (Invitrogen, Carlsbad, CA) was used to make multimers of the PP6002 expression cassette. In order to make the pAO815 multimerization expression vector, a series of intermediate vectors were constructed. The parent vector pBSC12771 was linearized by *Bam*HI digestion. The *Bam*HI restriction site was then backfilled using T4 polymerase (NEB, Beverly, MA). The backfilled *Bam*HI site was then ligated upon itself using T4 quick ligase (NEB, Beverly MA). Any remaining, unmodified parent vector was removed by a second *Bam*HI digestion. The *Bam*HI-digested, backfilled, and religated DNA was transformed into chemically-competent *E. coli* TOP10 cells and selected on LB_{amp100} overnight at 37°C. Single isolated colonies were grown in selective media and DNA was purified by methods described by Qiagen (Qiagen mini-prep purification kit, Valencia, CA). The elimination of the *Bam*HI site was confirmed by restriction digestion with *Bam*HI. The modified vector was designated pPIC9mod-PP6002 and was used as template for PCR. A GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) and Advantage cDNA Polymerase (Clontech, Palo Alto, CA) were used to amplify the target DNA using the oligos and thermocycling parameters below:

Primer1: 5'- **AGATCT**AACATCCAAAGACGAAAGGTTGAATGAAAC-3'

(SEQ ID NO: 23)

Primer2: 5'- **CATTAGGATCCGCACAAACGAACGTCTCACTTAATC** -3'(SEQ ID NO:24)

5 Table 1. Thermocycler parameters

Step	Temp(C)	Time	Cycles
1	94	5 min	1
2	94	30 sec	25
3	65	30 sec	
4	72	30 sec	
5	72	5 min	1
6	4	5 min to about 24 hrs	

Primers 1 and 2 were designed to amplify from the 5' end of the AOX1 promoter to the 3' end of the AOX1 transcription terminator, including the alpha-secretion factor-xylanase ORF. Also, these primers were designed to incorporate a *Bgl*III site on the 5' end of the product and a *Bam*HI site on the 3' end of the product for subsequent multimerization of the expression cassette. The resulting PCR product was cloned directly into the topoisomerase-I activated vector pCR4Blunt-TOPO by methods described by Invitrogen (Invitrogen, Carlsbad, CA). This vector was designated pCR4Blunt_5AOX-PP6002-3AOXTT. SP6, T7 and gene specific sequencing primers confirmed the fidelity of the amplification and cloning.

15 **Construction of pBSC12772 (pAO815_1x PP6002) Expression Vector.**

Oligonucleotides (see primers 3 and 4 below) were designed to amplify the ORF containing the *Saccharomyces cerevisiae* α -mating factor and PP6002 from pBSC12771 by PCR. A GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) and *PfuUltra* Hotstart Polymerase (Statagene, La Jolla, CA) were used to amplify the target DNA.

20 Primer3: 5'-GGGGCCGGGAATTCCGATGAGATTCCTTCAATTTT -3' (SEQ ID NO:25)

Primer4: 5'-GCCGGGGGAATTCCGCGGCCCTATTACCAGACAGTAACATTTGA -3' (SEQ ID NO:26)

The primers incorporated *Eco*RI sites onto the ends of the PCR product allowing for subsequent insertion of the product into the pAO815 recipient vector. Amplification by thermocycle reactions was performed using the following parameters:

Table 2. Thermocycle parameters

Step	Temp(C)	Time	Cycles
1	94	5 min	1

2	94	30 sec	25
3	65	30 sec	
4	72	1 min	
5	72	7 min	1
6	4	5 min to about 24 hrs	

The PCR product was digested with *EcoRI*. The restriction reaction was separated by electrophoresis through a 0.8% TAE gel and a 0.8 kb fragment was gel purified by methods described by Qiagen (Qiaquick Gel extraction Kit; Qiagen, Valencia, CA). In a parallel restriction digest reaction, the 7.7 kb *Pichia pastoris* multicopy expression vector pAO815 (Invitrogen; Carlsbad, CA) was digested with *EcoRI* and its cohesive ends were dephosphorylated by calf intestine phosphatase treatment (New England Biolabs, Beverly, MA). The vector fragment was separated by electrophoresis through a 0.8% TAE gel and gel purified as above. The *EcoRI*-digested and gel-purified PCR fragment was ligated directly into the linearized, dephosphorylated pAO815 using T4 DNA ligase (Quick Ligation Kit, New England Biolabs; Beverly, MA). The ligation reaction was transformed into chemically competent *E. coli* TOP10 cells and spread onto LB plates containing ampicillin (100 µg/mL). Single isolated colonies were grown in the selective media and DNA was purified by methods described by Qiagen (Qiagen mini-prep purification kit, Valencia, CA). The gene orientation and sequence were confirmed using plasmid specific 5AOX and 3AOX sequencing primers supplied by the manufacturer (Invitrogen, Carlsbad, CA). After sequence confirmation, the pAO815_1x PP6002 plasmid was retransformed into chemically competent *E. coli* TOP10 cells and a glycerol stock was prepared using methods described by Sambrook, *et al.* 2001. The pAO815_1x PP6002 construct was submitted to Syngenta Biotechnology, Inc. Quality Control and was designated pBSC12772 (See Figure 2 and SEQ ID NO:34).

Multimerization of the PP6002 Expression Cassette. The expression cassette (5AOX-PP6002-3AOXTT) was removed from pCR4Blunt_5AOX-PP6002-3AOXTT by double-digestion with *Bgl*II and *Bam*HI (New England Biolabs; Beverly, MA) and the 2.1 kb fragment was gel purified. In a separate reaction, the pBSC12772 was digested with *Bam*HI and its cohesive ends dephosphorylated by CIP treatment as previously described. The *Bam*HI-digested, CIP-treated pBSC12772 was electrophoresed through a 0.8% TAE gel and gel purified. The gel purified *Bam*HI-*Bgl*II expression fragment was ligated into the *Bam*HI-linearized, CIP-treated pBSC12772 vector using T4 DNA ligase (Quick Ligation Kit, New England Biolabs; Beverly, MA) and transformed into chemically competent *E. coli* TOP10 cells (Invitrogen; Carlsbad, CA). The transformed cells were spread onto LB plates containing ampicillin (100 µg/mL) and the plates incubated at 37°C overnight. Single

isolated colonies were grown in selective media and DNA was prepared by methods described by Qiagen (Valencia, CA). The two copy xylanase construct was confirmed by restriction analysis and DNA sequence analysis at the junctions using vector specific primers. The resulting pAO815 vector containing two copies of PP6002 was designated pAO815_2x PP6002.

Construction of pSYN12773 (pAO815_3x PP6002). A construct containing three copies of the synthetic XylA1A expression cassette was constructed by digesting pAO815_2x PP6002 with *Bam*HI and CIP treating the cohesive ends. The purified expression cassette fragment from the *Bam*HI-*Bgl*II double digest reaction pCR4Blunt_5AOX-PP6002-3AOXTT was ligated into the *Bam*HI-digested, CIP-treated pAO815_2x PP6002 vector using T4 DNA ligase (Quick Ligation Kit, New England Biolabs; Beverly, MA) and subsequently transformed into chemically competent *E. coli* TOP10 cells as previously described. The transformed cells were spread onto LB plates containing ampicillin (100 µg/mL) and the plates incubated at 37°C overnight. Single isolated colonies were grown in selective media and DNA was prepared by methods described by Qiagen (Valencia, CA). The 3x copy expression construct was confirmed by restriction fragment analysis. Furthermore, the complete nucleotide sequence of pAO815_3x PP6002 was confirmed by Syngenta Biotechnology Inc. Quality Control. The pAO815_3x PP6002 construct was designated pSYN12773 (see Figure 3 and SEQ ID NO:35) by Syngenta QC.

EXAMPLE 2

Preparation of pSYN12773 DNA for Transformation of *P. pastoris*. A 50 mL culture of TB broth supplemented with ampicillin (100 µg/mL) was inoculated with the glycerol stock of *E. coli* TOP10 cells harboring pSYN12773, and grown over-night at 37°C. DNA was purified from the culture by methods described by Qiagen (Qiaprep Midiprep protocol, Qiagen, Valencia, CA). The isolated plasmid DNA was digested over-night with *Bgl*II endonuclease (New England Biolabs, Beverly, MA). The digestion mix was electrophoresed through a 0.8% Tris Acetate EDTA (TAE) agarose gel and the 10.4 kb fragment corresponding to the XylA1A integration cassette purified from the gel by methods described by Qiagen (QiaQuick gel purification protocol, Valencia, CA). A portion of the purified fragment was electrophoresed through a 0.8% TAE gel to confirm complete digestion and its relative concentration. In addition, a portion of the purified fragment was transformed into chemically competent *E. coli* DH5α cells to confirm that no residual circularized plasmid harboring the ampicillin marker contaminated in the sample. The entire transformation mix was spread on an LB_{Amp100} plate and incubated at 37°C for 16 hours. No colonies grew on the plate.

EXAMPLE 3

Construction of *P. pastoris* XylA1A Expression Host

Preparation of *P. pastoris* GS115 Cells for Transformation. All microbiological manipulations were conducted in a laminar flow hood using aseptic techniques. *Pichia pastoris* GS115 yeast cells (Invitrogen, Carlsbad, CA) were prepared by streaking the cells onto YPD agarose plates. Following overnight growth at 30°C, a single yeast colony from the YPD agarose plate was transferred to 5 ml of YPD broth and grown at 30°C overnight. A portion of this "seed culture" was used to inoculate a sterile 2-liter, baffled flask containing 500 mL of YPD broth. This culture was grown with vigorous shaking overnight at 30°C to an optical density OD₆₀₀ = 1.5. The cells were harvested by centrifugation at 4000Xg, 4°C, 5 minutes, and resuspended in 80 mL of sterile double distilled (sdd) water. Ten milliliters of 10X TE buffer (10 mM Tris-HCl, 0.1 mM EDTA), pH 7.5 was added to the suspension followed by 10 mL of 1M lithium acetate (LiAc). The cell suspension was incubated at 30°C with gentle swirling. After 45 minutes of incubation, 2.5 mL of 1 M dithiothreitol (DTT) was added and the cell suspension returned to incubate at 30°C for an additional 15 minutes. The cells were then washed in a series of water washes and finally resuspended in 5 mL of ice-cold 1 M sorbitol.

Transformation of pSYN12773 DNA into *Pichia pastoris* GS115. Purified DNA (100 ng) of the XylA1A expression cassette from the *Bgl*II digested pSYN12773 plasmid was mixed with 80 µL of LiAc/sorbitol-treated *Pichia pastoris* GS115 cells in a 0.2 cm electroporation cuvette (Gene Pulser Cuvettes, BioRad, Hercules, CA) and incubated on ice for 5 minutes. The electroporation cuvette was placed into a BioRad Gene Pulser II instrument and pulsed using settings of 1.5 kV, 25 µF, and 200 Ω. Ice-cold sorbitol (0.5 mL) was added to the electroporation mix which was then plated onto histidine deficient, minimal media-dextrose (MD) agar plates. *P. pastoris* strain GS115 is a histidine auxotroph and is unable to grow in the absence of histidine, but stable transformants containing the *his4* gene on the XylA1A expression cassette are restored to histidine prototrophy and are capable of growth on histidine-free media. Growth at 30°C for 3 days produced a number of histidine prototrophic transformants. LiAc/Sorbitol washed GS115 cells electroporated in the absence of transforming DNA were plated onto MD and MD/histidine agar plates as controls. The GS115 cells with no transforming DNA present during electroporation generated no colonies capable of growth on MD plates lacking histidine.

Identification of *P. pastoris* Transformants Producing Xylanase. From the primary transformants on MD plates, 256 single, *his*⁺ prototrophic, isolated colonies were picked and replica plated onto a MD master plate. These colonies were subsequently replica-plated to histidine-deficient, minimal-media with 1.0% methanol (MM) agar plates containing 0.1% Azo-wheat arabinoxylan (*abbrev.* AzoWAXY; Megazyme, County Wicklow, Ireland). After 16 hours incubation at 30°C, clearing zones surrounding colonies on the MM AzoWAXY

plate identified 92 transformants producing xylanase activity. Twenty-four (24) of the 92 xylanase expressing clones were inoculated into 3 mL of BMMY induction broth contained in a 24 position, deep-well block and the clones expressed for 5 days. After induction, the supernatants were analyzed by xylanase activity assay and ELISA. Eight (8) transformants produced xylanase at levels putatively higher than yields of the controls. The expression levels for these 8 transformants were further investigated in 50 mL shake flasks. Following 5 days of induction, events 5501, 5517, and 5520 gave statistically significant improvements in yield and productivity.

Preparation of Glycerol Stocks for Long-Term Storage of *P. pastoris* Harboring pSYN12273 Transformants. Glycerol freezer stocks were prepared by inoculating 7 mL of sterile liquid MD media in a capped 16x150 mm glass tube with each of the 8 putative high-expressing xylanase positive clone from the MD master plate. These were grown overnight at 30°C on a rotating culture wheel. One milliliter (1 mL) of sterile glycerol was mixed into each culture to yield a 15% (v/v) mixture of glycerol to culture. Each culture was aliquoted into a sterile cryo-vials and stored at -80°C.

Characterization of *XylA1AP. pastoris* Expression Host

Screening for MutS Phenotype. In order to identify the MutS clones, the xylanase-positive clones were streaked onto histidine-deficient, minimal-media containing 1.0% methanol (MM) agar plates along side a MutS positive control (GS115 harboring pPIC9-secHSA; Invitrogen, Carlsbad, CA) and a Mut⁺ control (GS115 harboring pPIC3-βGal; Invitrogen, Carlsbad, CA). The plates were incubated at 30°C for 4 days and the growth on MM recorded. Thirty-nine (39) of the original 92 his⁺ xyn⁺ events identified previously exhibited slow growth on MM media comparable to the controls. Of these thirty nine events, eight had previously been identified as high expressors by activity and ELISA.

Preliminary Hybridization Screen for the *XylA1A* Expression Cassette. A series of hybridization experiments were conducted. In the preliminary hybridization screen, genomic DNA was prepared using standard techniques (Miles JD, Busser K, Stalder C, and Higgins DR (1998) Isolation of nucleic acids, in *Pichia Protocols*, vol. 103 (Higgins DR & Cregg JM, eds.), Humana Press, Totowa, NJ, pp. 73-80) for the eight putative high-expressing events. Two micrograms of genomic DNA was digested using BglIII restriction endonuclease. The digests were run through a 0.8% agarose gel then transferred bi-directionally onto two nitrocellulose membranes generating duplicate blots. DNA hybridization probes specific for PP6002 CDS, *PP6002* and the ampicillin gene (*cAmp-04*) were prepared. The xylanase and *amp* probes were generated by polymerase chain reaction using gene specific primers (see primers 5 and 6 for PP6002 and primers 7 and 8 for amp).

Primer5: 5'-GCATCTACTGACTACTGGCAG-3' (SEQ ID NO:27)

Primer6: 5'-CCAGACAGTAACATTTGAATAACC-3' (SEQ ID NO:28)

Primer7: 5'-GGGCGACACGGAAATGTTGAATACTCAT-3' (SEQ ID NO:29)

Primer8: 5'-TTACCAATGCTTAATCAGTGAGGCACC-3' (SEQ ID NO:30)

The products were gel purified and radiolabelled with [³²P]-dCTP using the Rediprime-II labeling system (Amersham Biosciences, Piscataway, NJ). Following stringent hybridization

5 with the [³²P]dCTP-*amp* probe in hybridization buffer at 65°C, the first blot did not show any hybridizing bands, with the exception of the positive control, *Bgl*II digested pSYN12773. This experiment indicated that the ampicillin gene did not integrate into the genome of *P. pastoris* in any of these events. The duplicate blot was probed with [³²P]dCTP-PP6002 using the high stringency methods previously described and produced a single band of

10 approximately 10.4 kb for events 5517 and 5520, indicating that three copies of the xylanase gene had successfully integrated into these events.

Detailed Hybridization Screen for Event 5520. In support of the preliminary hybridization screen, additional restriction digestions of the event 5520 genomic DNA were performed. Two micrograms of event 5520 genomic DNA were digested with *Bam*HI, *Bgl*II, *Eco*RI x

15 *Not*I *Pst*I, *Pvu*II, and *Xho*I x *Not*I restriction endonucleases. The digests were run through a 0.8% agarose gel then transferred bi-directionally onto two nitrocellulose membranes generating duplicate blots. DNA hybridization probes specific for the cXyl4-01 CDS, *αss*-PP6002 and the vector backbone genes (*cAmp*-04 and *oCOLE*-10) were prepared. The *xylanase* and *backbone* probes were generated by polymerase chain reaction using gene

20 specific primers (primers 3 and 6 for cXyl4-01 and primers 9 and 10 below for backbone).

Primer9: 5'-GCTGCCTCGCGCGTTTCGGTGATGA-3' (SEQ ID NO:31)

Primer10: 5'-GGGAACACTGAAAAATAACAGTTAT-3' (SEQ ID NO:32)

The products were gel purified and radiolabelled with [³²P]-dCTP using the Rediprime-II labeling system (Amersham Biosciences, Piscataway, NJ). Following stringent hybridization

25 with the [³²P]dCTP-*Backbone* probe in PerfectHyb™ Plus hybridization buffer (Sigma Chemical Co., St. Louis MO) at 65°C, the first blot did not show any hybridizing bands, with the exception of the positive control, *Bgl*II digested pSYN12773. This experiment indicated that the vector backbone did not integrate into the genome of *P. pastoris* in event 5520. The duplicate blot was probed with [³²P]dCTP-*cXyl4-01* using the high stringency methods

30 previously described and produced a single band of approximately 10.4 kb in the *Bgl*II digested sample, indicating that three copies of the xylanase gene had successfully integrated into event 5520. The *Bam*HI, *Eco*RI x *Not*I, *Pst*I, *Pvu*II and *Xho*I x *Not*I digested samples produced bands with the expected migration profile for integration at the AOX1 locus of *P. pastoris*. In summary, all characterizations of *P. pastoris* PP6002 expression event 5520 by

35 Southern blotting and growth characteristics on methanol containing minimal media demonstrates that the event has a His⁺, Mut^S phenotype, contains three copies of the PP6002

expression cassette inserted into the AOX1 locus, and does not contain the ampicillin resistance gene or other components of the vector backbone.

EXAMPLE 4

Preparation of the PP6002 *P. pastoris* Master Cell Bank

- 5 From the MD glycerol freezer stock of event 5520, a master cell bank was made; hereafter named SYN12773. Under aseptic conditions, a sample from the SYN12773 glycerol freezer stock was revived by streaking onto a MD plate and incubated at 30°C until the appearance of colonies. A single colony was picked from the MD plate and inoculated into a culture tube containing 5 mL of MD liquid media. The tube was incubated over night on a rotating wheel
- 10 at 30°C. The following day, the SYN12773 starter culture was inoculated into a 2.8 L baffled flask containing 350 mL of YPD medium. The culture was grown at 30°C on a shaker at 250 rpm until an $OD_{600} = 2.0-3.0$, 3.0×10^7 cells was reached. Under aseptic conditions, 150 mL of sterile glycerol was added to the 350 mL of YPD culture resulting in a 30% (v/v) ratio of glycerol to YPD. Aliquots (1.0 mL) of culture were transferred to 243 sterile 2.0 mL
- 15 polypropylene cryogenic tubes with screw caps and O-ring seal attachments (Fisher Science, Cat No. 056698). The 243 cryo-vials were placed into 3 cryo-boxes and stored at -80°C.

- Purity of the *P. pastoris* Xylanase Master Cell Bank.** A one microliter sample from one of the vials in the master cell bank was streaked onto a YPD agar plate. The plate was incubated overnight at 30°C to generate individual colonies. These colonies were examined visually
- 20 and were found to have a homogenous colony morphology that was identical to that of the parent strain *P. pastoris* GS115. A single colony from the YPD plate was streaked to a MD and a MM AzoWAXY agar plate and grown at 30°C until the appearance of single colonies. The resulting colonies were able to grow on both MD and MM agar that lack histidine, indicating that like event SYN12773, but unlike the parent strain GS115, they all had a His⁺
- 25 phenotype. Furthermore, all colonies grew slowly on MM agar containing methanol as a source of carbon, indicating that like event SYN12773, but unlike strain GS115, they have a Mut^S phenotype that is expected of AOX1 mutants. Additionally, all colonies on the MM AzoWAXY plate produced clearing zones indicating expression of active xylanase. The results of these analyses indicate that the MCB described herein is pure and uncontaminated
- 30 with other microbes.

- Genetic stability of *P. pastoris* xylanase clone.** The genetic stability of the multicopy PP6002 expression cassette in event SYN12773 was tested by conducting 20 consecutive plating experiments on MD agar. Cells from one of the MCB cryogenic vials were revived by streaking onto a MD agar plate and grown up for 48 hours at 30°C (plate 1). From plate 1, a
- 35 single colony was picked and replated onto a second MD plate. This cycle of single colony picking and replating was conducted 20 consecutive times. Genomic DNA was purified from

a single colony from plates 1 and 20, and used for Southern analysis. The hybridization profiles were compared between the generations 1 and 20. Southern analysis was performed as previously described and no differences were observed between generations 1 and 20. Liquid cultures were prepared from single colonies of plates 1 and 20 for protein expression analysis. Three individual colonies from each of these plates were used to inoculate 50 mL of BMGY media. Cells were grown up overnight at 30°C, spun down and resuspended in 50 mL of BMMY. Cultures were incubated at 30°C for 5 days with the addition of methanol (MeOH) every day to a final concentration of 1.0% (v/v). At the end of the fermentation period, clarified supernatant was analyzed for xylanase activity.

Clones from both plates produced active xylanase at statistically indistinguishable levels ($p < 0.05$) among cultures from same plate as well as between cultures from plates 1 and 20. Analyses of genomic DNA by Southern analysis and by protein expression for cells from plates 1 and 20 demonstrate the stability of the integrated PP6002 expression cassette in the genome of *Pichia pastoris* GS115 and expression of the xylanase gene within it.

EXAMPLE 5

Determination of the Xylanase Activity

Enzymatic activity was determined using wheat arabinoxylan as substrate and measuring the release of reducing ends by reaction of the reducing ends with either 3,5-dinitrosalicylic acid (DNS) or 2,2'-bicinchoninic acid (BCA). The substrate was prepared as a 1.4% w/w solution of wheat arabinoxylan (Megazyme P-WAXYM) in 100 mM sodium acetate buffer pH5.30 containing 0.02% sodium azide. The DNS reagent consisted of 0.5% w/w, 15% sodium potassium tartrate, and 1.6% w/w sodium hydroxide. This solution was stable and was stored for up to 3 months at room temperature. The BCA reagent was prepared by combining 50 parts reagent A with 1 part reagent B (reagents A and B were from Pierce, product numbers 23223 and 23224, respectively). These reagents were combined no more than four hours before use.

In the DNS assay, 500 microliters of substrate were combined with 200 microliters of enzyme sample. After incubation at the desired temperature for the desired length of time, 700 microliters of DNS reagent was added. The contents were mixed and placed at 100°C for 10 minutes. The contents were allowed to cool and then transferred to cuvettes and the absorbance at 540nm was measured relative to known concentrations of xylose. The choice of enzyme dilution, incubation time, and incubation temperature could be varied by one skilled in the art.

In the BCA assay, 200 microliters of substrate were combined with 80 microliters of enzyme sample. After incubation at the desired temperature for the desired length of time, 2.80 milliliters of BCA reagent was added. The contents were mixed and placed at 80°C for

35 minutes. The contents were allowed to cool and then transferred to cuvettes and the absorbance at 560nm was measured relative to known concentrations of xylose. The choice of enzyme dilution, incubation time, and incubation temperature could be varied by one skilled in the art. Enzymatic activity was determined in accordance with the procedure of
 5 Miller G.L. (1959) *Anal. Chem.* 31 426-428.

EXAMPLE 6

Trials with Chickens

Standard poultry diets were used containing wheat, rye and soybean meal as the main ingredients. An exemplary diet is set forth in Table 3. XylA1A xylanase, produced in
 10 recombinant *P. pastoris* was used. Six (6) replicate pens of 6 chickens for each diet were grown until 21 days of age, and final weights determined by subtracting the weight of the one-day-old chicks. Records were kept of the amount of feed consumed by each pen of chickens, and an average feed consumption was determined. The XylA1A xylanase was formulated by lyophilising the active enzyme preparation then reconstituting with water at the
 15 trial site. This formulation was added directly to the diets. Avizyme 1300 was used according to as a commercial standard at similar dosages as the XylA1A xylanase.

Table 3. 3.2 Diet formulation

Ingredient	Starter
Rye	20.00%
Wheat - Feed	37.49%
Soybean meal 48	33.65%
Soy oil	5.31%
Salt	0.39%
DL Methionine	0.22%
Lysine HCl	0.04%
Limestone	1.13%
Dicalcium Phos	1.28%
VIT/MIN	0.49%
Crude protein %	22.88
Poult ME kcal/kg	3,000.00
Pig DE Kcal	3,487.48
Calcium %	0.85
Phos %	0.67
Avail Phos %	0.40
Fat %	6.46

Fibre %	2.52
Met %	0.56
Cys %	0.39
Me+Cys %	0.95
Lys %	1.25
His %	0.56
Tryp %	0.28
Thr %	0.84
Arg %	1.52
Iso %	0.95
Leu %	1.69
Phe %	1.07
Tyr %	0.75
Val %	1.05
Gly %	0.93
Ser %	1.07
Phe+Tyr %	1.82
Na %	0.18
Cl %	0.29
K %	0.96
Linoleic acid %	2.54
Na+K-Cl	241.72
DUA	396.57
Sulphur%	0.22
Magnesium	0.16
Betaine	0.47
Choline	1,378.97
Poult ME MJ/kg	12.55
Total soya	0.34

- Table 4 illustrates the effect of dietary inclusion of XylA1A xylanase on poultry growth performance, represented by feed conversion ratios (FCR). Feed conversion ratio (FCR) refers to the amount of feed consumed divided by the net weight gain of the chicken.
- 5 A lower ratio indicates that a chicken gained more weight per unit of feed consumed. A lower ratio indicates that a chicken more efficiently utilized the feed that was consumed.

The control diets (with no enzyme supplementation) clearly showed poorer performance than addition of even the lowest dose of either enzyme. FCR of the control

- improved in concert with each incremental dose of enzyme, from 1.671 to an optimum of 1,449 at an inclusion rate of 400 units of XylA1A xylanase. Whilst both xylanases improved performance as dosage increased, it is clear that the XylA1A xylanase was superior to that of Avizyme 1300 as shown by the significant enzyme statistical term. Thus, the use of this
- 5 XylA1A Xylanase of the invention to supplement such feeds reduces the amount of feed required to produce each unit of weight of broiler chicken compared with the unsupplemented diet and diets containing the commercial standard.

Table 4. Data from Feeding Studies of chickens

Enzyme	Dose	Intake	Gain	FCR	FCRc	Mortality
None	0	1088	653	1.671	1.648	4.46
Az 1310	50	1125	716	1.571	1.541	2.38
Az 1310	100	1079	710	1.521	1.521	0.00
Az 1310	200	1068	682	1.570	1.570	0.00
Az 1310	400	1079	699	1.544	1.544	0.00
Az 1310	800	1069	690	1.552	1.552	0.00
Az 1310	1600	1104	709	1.558	1.535	2.38
6002	50	1063	712	1.493	1.493	0.00
6002	100	1097	733	1.497	1.497	0.00
6002	200	1097	707	1.554	1.545	2.38
6002	400	1074	741	1.449	1.449	0.00
6002	800	1072	730	1.469	1.469	0.00
6002	1600	1097	753	1.457	1.457	0.00
	0	1088	653	1.671	1.648	4.46
	50	1094	714	1.532	1.517	1.19
	100	1088	722	1.509	1.509	0.00
	200	1083	694	1.562	1.558	1.19
	400	1077	720	1.497	1.497	0.00
	800	1070	710	1.510	1.510	0.00
	1600	1101	731	1.508	1.496	1.19
Az 1310		1087	694	1.569	1.559	1.32
6002		1084	718	1.513	1.508	0.98

Statistical p values						
Enzyme		0.7119	0.0001	0.0001	0.0001	0.6801
Dose		0.6719	0.0000	0.0000	0.0000	0.0627
Enz*Dose		0.2444	0.1804	0.2595	0.3358	0.7516
R-Square		0.1495	0.5615	0.5702	0.5577	0.1886
RMSE		42.99	27.86	0.06277	0.05728	3.73

EXAMPLE 7**Thermostability of Xylanase Enzyme Produced in Different Hosts**

The xylanase protein, BD6002 (also called XylA1A, SEQ ID NO:14), was expressed in the hosts *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Pseudomonas fluorescens*. Purified enzyme was obtained and residual activity at 85°C was measured to determine if there was a difference in thermostability of the xylanase due to expression in different hosts. These hosts differ in the ability to glycosylate the proteins expressed.

Residual Xylanase Assay

This assay procedure is specific for *endo*-1, 4-beta-D-xylanase activity. On incubation of Azo-Wheat Arabinoxylan with *endo*-xylanase, the substrate is depolymerised by an *endo*-mechanism to produce low-molecular weight dyed fragments, which remain in solution on addition of industrial methylated spirits (IMS) or 95% ethanol to the reaction mixture. High molecular-weight material is removed by centrifugation, and the color of the supernatant is measured. *Endo*-Xylanase in the assay solution is determined by reference to a Standard Curve. Each enzyme has its own Standard Curve. The same enzyme produced in different hosts will have different Standard Curves.

Solutions

A. 0.1M citric acid. Dissolve 21.02g citric acid monohydrate (EM Sciences CX1725-1) in 900mL demineralized water in glass beaker with stirring. Transfer solution to 1L volumetric flask and make to 1L with demineralized water. Filter sterilize and store at room temperature.

B. 0.2M sodium phosphate, dibasic

Dissolve 53.6g sodium phosphate dibasic heptahydrate (Sigma S9390) in 900mL demineralized water in glass beaker with stirring. Transfer solution to 1L volumetric flask and make to 1L with demineralized water. Filter sterilize and store at room temperature.

C. 50mM citrate phosphate buffer pH 5.4 (abbrev 50CPB54)

Combine 22.2mL 0.1M citric acid (§3.A.) with 27.8mL 0.2M sodium phosphate in 100mL volumetric flask. Make volume to 100mL with demineralized water.

D. 1% w/v wheat arabinoxylan in 50mM citrate phosphate buffer pH 5.4

- Add 90ml of 50CPB54 buffer in a 200ml beaker with stir bar. Cover beaker with aluminum foil. The beaker and contents are placed on a hot-plate stirrer and the water is brought to a boil with vigorous stirring. The aluminum foil is carefully removed, powdered substrate (1.000 g) is added, and the aluminum foil is replaced. The solution is allowed to stir for 10 min while boiling and the heat is turned off. The solution is stirred until it has cooled to room temperature, no clumps of substrate should be observed. The solution is transferred to a 100mL volumetric flask with stopper. Sodium azide (2% w/v, 1ml) is added to the volumetric flask. The sides of the beaker are twice washed with a small amount (3-4ml) of 50CPB54 to remove residual substrate. The washes are combined with the contents in the volumetric flask. The volume is adjusted to 100ml with 50CPB54 and stopper is inserted. The flask is shaken vigorously. This substrate solution should be stored the solution at 4°C between uses. Under these conditions and excluding contamination, the substrate is stable for at least several months.

Assay

- A. Place 16x100mm glass tubes in rack in 37°C water bath.
- B. Add 500uL of 1%w/v WAXY to each tube and equilibrate to 37°C for at least 5 minutes.
- C. Place 600µL+ sample at appropriate dilution in 1.5mL eppendorf tube and place in 37°C water bath for at least 5 minutes.
- Typically, it is desirable to target ~0.1U/mL following dilution to give a signal within the linear range for this assay.
- D. Initiate reaction by adding 500uL sample to substrate in glass tubes. Vortex immediately and start timer.
- E. Incubate for exactly 10 minutes at 37°C.
- F. Add 2.5mL 95% ethanol with repeat pipettor and vortex immediately. Transfer tube to rack at room temperature.
- G. Let stand 10 minutes at room temperature then vortex again.
- H. Centrifuge 10 minutes at 1,000g, 22°C in Eppendorf 5810R with acceleration and brake at maximal speed.
- I. Remove supernatant to 1.5mL polystyrene cuvettes.
- J. Measure absorbance at 590nm.

The results from the assays on residual activity of xylanase BD6002 are set forth in Table 5. The results show that the xylanase BD6002 expressed in *Pichia pastoris* had the greatest amount of residual activity after 30 minutes at 85°C. The difference in thermotolerance is expected to be due to difference in the glycosylation of the protein expressed in different host organisms. It is known that prokaryotic hosts, such as *E. coli* and *Pseudomonas* strains do not glycosylate proteins.

Table 5. Residual Activity at 85°C of BD6002 Produced in Different Hosts

Time	<i>E. coli</i>	<i>P. pastoris</i>	<i>S. cerevisiae</i>	<i>P. fluorescens</i>
0	100.0	100.0	100.0	100.0
5	9.1	70.9	43.8	6.5
10	2.4	59.3	26.3	4.3
15	0.8	43.0	14.4	1.9
20	0.1	36.6	7.3	1.4
25	0.5	30.7	5.7	1.4
30	0.3	26.7	4.0	1.0

EXAMPLE 8

10 Effects of xylanase enzyme addition on the cumulative gas production in Ruminant fluid

This example illustrates the difference of additional xylanase enzymes added to ruminant feed as measured by the difference in the gas production when incubated with ruminal fluid.

The objective of this experiment is to assess the effect of xylanase supplementation on ruminal degradation with alfalfa hay. Based on the results of endoglucanase activity and manufacturer's recommendation, dose level 2 and 3 were chosen for enzymes. On the other hand, four commercial products were applied at a rate of 1.0 mg/g DM alfalfa hay. One gram DM of alfalfa hay was ground for 10 sec using a Knifetec 1095 sample mill (Foss Tecator, Höganäs, Sweden) and weighed into fermentation bottles (125 ml capacity). All enzymes were resuspended by adding 10 ml of H₂O, and appropriate volume of each enzyme was added to corresponding bottles in six replications. Enzymes were applied at 20 h prior to inoculation with ruminal fluid. Three hours later, 40 ml anaerobic buffer medium, prepared as outlined by Goering and Van Soest (1970) and adjusted to pH 6.0 using 1 M *trans*-aconitic acid (Sigma Chemicals), was added, and the bottles stored at 20°C overnight. Ruminal fluid was obtained 4 h post feeding (1100 h) from a lactating dairy cow fed a TMR diet composed of barley silage, chopped alfalfa hay, rolled corn grain, and concentrate for early lactation. Strained ruminal fluid was transported to the laboratory in sealed, preheated containers and kept at 39°C in a water bath. The inoculum was dispensed (10 ml per bottle) into culture bottles that had been warmed to 39°C in an incubator and flushed with oxygen-free CO₂. The bottles were then sealed with a 14 mm butyl rubber stopper plus aluminum crimp cap

immediately after loading and incubated for 18 h. Negative controls (ruminal fluid plus buffer alone and ruminal fluid plus buffer and enzyme product) were also incubated. These controls were used to correct for gas release and fermentation residues resulting directly from the inoculum. These treatments and controls were included in 6 replications. Headspace gas produced by substrate fermentation was measured at 2, 6, 12, and 18 h post inoculation by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer (type T443A, Bailey and Mackey, Birmingham, UK) connected to a visual display (Data Track, Christchurch, UK). The transducer was then removed leaving the needle in place to permit venting. Pressure values, corrected by the amount of substrate OM incubated and for gas release from negative controls, were used to generate volume estimates using the quadratic equation (gas volume = $0.18 + 3.697 \times \text{gas pressure} + 0.0824 \times \text{gas pressure}^2$) reported by Mauricio et al., Anim. Feed Sci. Technol. 79:321-330 (1999). On removal, the bottles were placed in the refrigerator at 4°C to stop fermentation, and filtered through a commercial coffee filter paper with vacuum application. Apparent DM degradation (DMD) was determined by drying residues at 100°C for 24 h and OM degradation (OMD) was determined by difference after ashing the dry residues at 500°C overnight.

The results from the assays are set forth below in Tables 6 and 7. These results demonstrate that xylanases differ in their ractivity in ruminal fluid.

Table 6. Effects of xylanase enzyme addition on the cumulative gas production (mL/g OM) on alfalfa hay for 18 h of incubation with ruminal fluid.

Treatment	Incubation time, h				Ranking ¹
	2	6	12	18	
Control	7.19	42.0	105.1	166.4	14
BD2230	11.2 ^c	56.5 ^a	124.0 ^a	189.8 ^a	4
BD2236	14.8 ^a	64.3 ^a	133.9 ^a	199.5 ^a	2
BD2248	12.0 ^b	53.9 ^a	117.5 ^c	179.3	9
BD6002	11.4 ^b	54.5 ^a	119.1 ^b	183.3 ^b	7
BD6004	13.9 ^a	57.7 ^a	122.5 ^a	188.2 ^a	6
BD6405	15.4 ^a	60.2 ^a	122.6 ^a	183.2 ^b	8
BD6407	12.5 ^a	52.4 ^b	112.4	170.7	12
BD6890	10.0	45.9	101.4	157.0	15
BD7150	13.6 ^a	52.9 ^b	109.9	167.8	13
BD7182	13.9 ^a	55.1 ^a	114.0	173.8	11
A	16.1 ^a	59.6 ^a	119.5 ^b	179.1	10
B	22.7 ^a	73.1 ^a	136.4 ^a	197.7 ^a	3
C	20.5 ^a	69.1 ^a	130.9 ^a	189.0 ^a	5
D	24.5 ^a	79.3 ^a	148.5 ^a	210.8 ^a	1
SEM	0.8	1.9	2.7	3.5	

¹Relative ranking according to the cumulative gas production measured at 18 h of incubation.

^{a,b,c}Different from the control within columns at $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

Table 7. Effects of xylanase enzyme addition on the apparent degradability of DM (DMD), OM (OMD), and partitioning factor of fermentation (PF) on alfalfa hay after 18 h of incubation with ruminal fluid.

Treatment	Added activity of xylanase ¹	DMD, %	Rank ²	OMD, %	Rank ²	PF ³	Rank ²
Control	-	41.1	14	39.3	14	2.36	4
BD2230	874	44.2 ^b	5	42.5 ^a	5	2.24	10
BD2236	666	45.9 ^a	3	44.3 ^a	3	2.22	11
BD2248	755	43.7 ^b	6	42.3 ^a	6	2.36	4
BD6002	1695	42.1	10	40.6	10	2.22	11
BD6004	787	43.4 ^c	7	41.2	9	2.19 ^c	13
BD6405	582	40.8	15	38.9	15	2.12 ^b	14
BD6407	643	41.5	12	39.8	12	2.33	6
BD6890	1050	47.5 ^a	1	45.7 ^a	1	2.99 ^a	1
BD7150	988	41.7	11	39.8	12	2.38	3
BD7182	596	41.5	12	40.1	11	2.31	8
A	54.5	42.3	9	41.5 ^c	7	2.32	7
B	-	43.4 ^c	7	41.5 ^c	7	2.10 ^a	15
C	213	44.6 ^a	4	42.9 ^a	4	2.27	9
D	15.1	46.3 ^a	2	44.4 ^a	2	2.41	2
SEM		0.6		0.5		0.04	

¹Xylanase activity was expressed as nanomoles of glucose released per minute.

²Relative ranking according to DMD, OMD, and FE.

³Partitioning factor = mg OM degraded/mL gas produced.

^{a,b,c}Different from the control within columns at $P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively.

EXAMPLE 9. Characterization of the enzyme

Purification from test article material (TAM) (without separation of the isoforms)

Preparation of the xylanase affinity column:

Lyophilized xylanase, approximately 10 mg of *Pichia pastoris* produced BD6002 (rXylPP6002, lot Xvl-BD6002-PB206) was resuspended in 1.25ml distilled water and brought up to 5 mls with 0.1M NaHCO₃ pH8.3. This solution was dialyzed against 4L of 0.1M NaHCO₃ for 5.5 hr at 4°C and then added to distilled water-washed affigel-10 (Bio-Rad) according to the manufacturer's instruction. The xylanase-coupled affigel-10 was poured into a 2ml column.

Goat antiserum:

Antibodies to xylanase were generated from purified xylanase protein BD7346 and prepared in goats (American Alpine). The goat was injected with 500 µg of xylanase in complete Freund's adjuvant, were allowed to rest for 3 weeks and then injected with 500 µg of xylanase in incomplete Freund's adjuvant. The goats were bled 10 days after each
 5 injection.

Immuno-affinity purification of xylanase antibodies:

The xylanase affinity column was pre-eluted with 1 ml of 0.1M glycine-HCl pH 2.5 followed by equilibration in phosphate buffered saline, pH 7.3 (PBS). Five mls of goat anti-xylanase serum was applied to the column by gravity. The column was then washed with
 10 PBS until baseline was reached. Bound xylanase antibodies were eluted using 1 ml of 0.1M glycine-HCl pH 2.5 followed by PBS. Two ml fractions were collected. Absorbance at 280nm was recorded for each fraction. The recovered antibodies were dialyzed against 0.1M NaHCO₃ pH 8.3.

Preparation of the goat anti-xylanase affinity column:

15 Goat anti-xylanase (~13 mg) was added to distilled water-washed affigel-10 (Bio-Rad) according to the manufacturer's instruction. The xylanase antibody-coupled affigel-10 was poured into a 2ml column.

Immuno-affinity purification of xylanase:

The xylanase antibody affinity column was pre-eluted with 1 ml of 0.1M glycine-HCl pH 2.5 followed by equilibration in phosphate buffered saline, pH 7.3 (PBS). One gram of lyophilized *Pichia* xylanase (XYL-PP6002-PD064) was suspended in 5 ml dH₂O plus 5 mls PBS and mixed for 30 min at 4°C. The entire suspension was applied to the column by gravity and the unbound solution was collected (flow-through). The column was then washed with PBS until baseline was reached. Bound xylanase was eluted using 1 ml of 0.1M glycine-
 25 HCl pH2.5 followed by PBS. Two ml fractions were collected. Absorbance at 280nm was recorded for each fraction. Fractions containing protein (A₂₈₀ > 0.09) were pooled. The flow-through was repeatedly passed over the column until no more xylanase was recovered.

EXAMPLE 10. Protein concentration of purified enzyme

30 Two different methods were used to measure the concentration of the purified *Pichia* xylanase (XYL-PP6002-PD064). First a BCA protein assay (Pierce Chemicals), was used with Bovine Serum Albumin (BSA) as a reference protein. Two different conditions (37°C, 30 minutes, room temperature for 2 hours) gave very similar results.

The protein concentration was estimated to be 0.403 mg / mL (see below Tables 8 and 9).

35 Table 8.

37°C, 30 minutes

sample	microg/mL	Abs	Abs	Average	Adjusted	Slope	981.9888
--------	-----------	-----	-----	---------	----------	-------	----------

		562 nm	562 nm	Abs	abs	R ²	0.995803
BSA	0	0.0695	0.0755	0.0725	0.0000		
BSA	5	0.0849	0.0743	0.0796	0.0071		
BSA	25	0.0936	0.0914	0.0925	0.0200		
BSA	125	0.2334	0.2361	0.2348	0.1623		
BSA	250	0.4012	0.393	0.3971	0.3246		
BSA	500	0.6718	0.6784	0.6751	0.6026		
BSA	750	0.9008	0.9212	0.911	0.8385		
BSA	1000	1.1868	1.206	1.1964	1.1239		
BSA	1500	1.5761	1.6613	1.6187	1.5462		
BSA	2000	2.0807	2.0882	2.08445	2.0120		
xyn	407.1653	0.4818		0.487133	0.414633333		
xyn		0.4791					
xyn		0.5005					

Table 9.

Room Temperature 2 hours

sample	microg/mL	Abs	Abs	Average	Adjusted	Slope	932.3376
		562 nm	562 nm	Abs	abs	R ²	0.99865
BSA	0	0.0596	0.0601	0.0599	0.0000		
BSA	5	0.071	0.062	0.0665	0.0067		
BSA	25	0.0788	0.0759	0.0774	0.0175		
BSA	125	0.2271	0.2211	0.2241	0.1643		
BSA	250	0.3771	0.3577	0.3674	0.3076		
BSA	500	0.6796	0.6417	0.6607	0.6008		
BSA	750	0.928	0.8889	0.90845	0.8486		
BSA	1000	1.1788	1.1864	1.1826	1.1228		
BSA	1500	1.6548	1.6566	1.6557	1.5959		
BSA	2000	2.271	2.1562	2.2136	2.1538		
xyn	398.3723	0.4818		0.487133	0.4273		
xyn		0.4791					
xyn		0.5005					

The second method consists of measuring the pixel intensity of protein run in as SDS-PAGE and stained with the protein specific dye SYPRO Tangerine (Invitrogen). Since the *Pichia* produced xylanase contains multiple isoforms in addition to the 20 kD native protein, all bands were selected for Pixel quantitation. Concentration of the enzyme is compared to the standard Carbonic anhydrase that migrates at about 33 kDa. The concentration in the purified xylanase sample is about 0.125 mg/mL.

Table 10.

Carbonic Anhydrase		
micro g / mL	Pixel intensity	Adjusted

	(mean)	
1000	167.5	43.5
500	164.5	40.5
250	155	31
125	144.23	20.23
background	124	

Xylanase PP6002 PD064

all bands selected lane	Pixel intensity (mean)	Adjusted	Average	Concentration
1	136.7	18.7	20.55	125 microgram / mL
2	140.4	22.4		
background	118			

EXAMPLE 11. Specific Activity of the purified enzyme

The antibody purified sample was assayed for its specific activity on wheat arabinoxylan. An aliquot was diluted to about 1000 fold and assayed with the DNS protocol.

This protocol describes a method to measure reducing ends produced during the hydrolysis of carbohydrates, especially xylans and glucans. The assay is based on the reaction of reducing ends with dinitrosalicylic acid (DNS). The DNS assay has several advantages over other reducing sugar assays, such as the Somogyi-Nelson, including a lower detection limit, slightly faster analysis time, and use of less toxic reagents (*i.e.*, sodium arsenate used in Somogyi-Nelson protocol). The method presented here is based on a protocol in Miller G.L. (1959) Anal. Chem. 31 426-428.

Reagents

Use 18 mΩ water for the preparation of all reagents and test solutions

0.4 M Sodium Hydroxide. Dissolve 16.0 g of anhydrous sodium hydroxide (Fisher S318) in 900 mL of water with stirring in a 1 L beaker. Transfer to a 1 L volumetric flask, and adjust volume to 1 L with water. Label and store at 4°C for not more than 90 days.

DNS Reagent. Dissolve 5.0 g 3,5-dinitrosalicylic acid (Sigma D0550) and 150. g sodium potassium tartrate tetrahydrate (Fisher S387) in 900 ml of 0.4 M Sodium Hydroxide. Transfer to a 1 L volumetric flask and adjust volume to 1 L with 0.4 M Sodium Hydroxide. Filter through 0.2 μm filter. Store at room temperature. Note: this reagent is a yellow-orange color.

200 mM Sodium Acetate Buffer, pH 5.30 (2x SAB). Dissolve 27.2 g of sodium acetate trihydrate (Fisher S209-3) in 900 mL of water with stirring in a 1 L beaker. Adjust pH to 5.30

± 0.05 with Glacial Acetic Acid (Fisher A38) and transfer to a 1 L volumetric flask. Add 20. mL 2% w/v Sodium Azide in Water and adjust volume to 1 L. Filter through 0.2 µm filter. Label and store at 4°C. Solution is stable for several months.

5 **2% w/v Sodium Azide in Water.** Dissolve 2.00 g sodium azide (Sigma S8032) in 90 mL water in a glass beaker with stirring. Transfer to 100 mL volumetric flask. Wash beaker with water and combine in volumetric flask. Bring volume to 100 mL with water.

10 **100 mM Sodium Acetate Buffer, pH 5.30 (1x SAB).** Add 500 mL 2x SAB to a 1 L volumetric flask. Adjust volume to 1 L with water. Label and store at 4°C. Solution is stable for several months.

15 **Substrate Solution: 1.40% w/v wheat arabinoxylan in 1x SAB.** Accurately weigh 1.40 g wheat arabinoxylan (Megazyme P-WAXYM) into a 120 ml dry pyrex beaker. Wet the sample with 8 mL of 95% ethanol. Add a magnetic stirrer bar followed by 50 mL of 2x SAB and 30 mL of water. Cover and immediately place the slurry on a magnetic stirrer plate with vigorous stirring overnight or until dissolved. Transfer to a 100 mL volumetric flask. Wash the beaker with ~10 mL water and combine with contents of volumetric flask. Adjust volume to 100 mL with water. Label and store at 4°C. Solution is stable for several months.

20 **Xylose Concentrated Stock Solution, 10.0 mg/mL D (+) xylose in 1x SAB**
Dissolve 250.0 mg D (+) xylose (Sigma X1500) in 20 mL 1x SAB in a 50 mL glass beaker with stirring. Transfer solution to 25 mL volumetric flask. Wash beaker with 4 mL 1x SAB and combine in volumetric flask. Adjust volume to 25 mL with 1x SAB.

Xylose Working Stock Solution, 1.00 mg/mL D (+) xylose in 1x SAB

Add 10.0 mL Xylose Concentrated Stock Solution to 100 mL volumetric flask. Add 1x SAB to 100 mL.

25 **Xylose Standards**

Use the table below to prepare the appropriate dilutions for the *Xylose Standards* in 15 mL conicals where Solution A = Xylose Working Stock Solution and Solution B = 1x SAB. A positive displacement pipettor should be used to transfer the Solutions A and B. Xylose Standards should be stored at 4°C between uses.

30 Table 11.

			Total
Xylose	Volume A	Volume B	Volume

(μ mol)	(μ L)	(μ L)	(μ L)
0	0	500	500
0.20	75	425	500
0.40	150	350	500
0.60	225	275	500
0.80	300	200	500
1.00	375	125	500
1.20	450	50	500
1.33	500	0	500

Larger aliquots of Xylose Standards may be prepared and stored at 4°C for up to 90 days.

Sample Preparation

- For those new to the DNS Assay for Measurement of Xylanase Activity, a single vial of control xylanase is prepared as described below. For the interlaboratory cross-over study, five vials of control xylanase are prepared as described below.

Note: Only glassware should be used to prepare xylanase solutions as plasticware has been shown to bind xylanase and decrease activity.

Control Xylanase (including for Cross-Over Study)

- Record the weight of solid contained in the vial (from the label). Add 10.0 g $\pm$ 0.100 g of water to one vial of the control xylanase (lot XYL-PP6002-PD014R) and record the weight. Each vial of Control Xylanase contains 100. mg $\pm$ 1.0 mg of solid. Vortex gently until completely dissolved.

Working Control Xylanase Solution

- As very high dilution rates are encountered in this assay, detail to sample preparation is very important. All dilutions are made using 1x SAB. For specific activity determination, make dilutions for each vial of Control Xylanase as described below.

- For the initial dilution, weigh ~0.100 g Control Xylanase into a tared 16 x 100 mm glass tube and record weight. Add ~10 mL of 1x SAB and record weight, generating a 1:100 dilution (see dilution table below).

The second serial dilution is made by weighing ~0.100 g of the 1:100 dilution into a tared 16 x 100 mm glass tube and record weight. Add ~10 mL of 1x SAB and record weight, generating a 1:10,000 dilution (see dilution table below).

Table 12.

Serial Dilutions for Control Xylanase				
Starting Dilution	Enzyme Sample	1x SAB	Final Volume (mL)	Final Dilution

Xylanase Standard Stock Solution	0.100 g	~10 mL	~10.0	~1:100
1:100	0.100 g	~10 mL	~10.0	~1:10,000

Preparation of Liquid Xylanase Samples

Liquid xylanase samples are without modification used as a stock solution for preparing the Working Xylanase Solution as described below.

5 Preparation of Solid (e.g., freeze-dried) Xylanase Samples

Add 1.00 mL of water to 100 mg of Solid Xylanase Sample. Vortex gently until completely dissolved.

Working Xylanase Solution for Liquid or Solid Xylanase Samples

10 Due to variable expected expression levels in non-standard samples, the appropriate dilution rate may vary among samples. Prepare initial dilution (1:100) as described above for Working Control Xylanase Solution.

If desired dilution is between 1:100 and 1:10,000, then prepare a final working dilution and record weights of sample and 1x SAB used for dilution.

15 If desired dilution is higher than 1:10,000, then a 1:10,000 dilution is first prepared as described above for the Working Xylanase Solution. Then, a final working dilution is prepared by weight as above for the Control Xylanase.

Overview of Specific Activity Determination Procedure

20 For the accurate determination of specific activity using the DNS Assay for Measurement of Xylanase Activity, it is required that each Working Xylanase Solution be tested in triplicate. For this assay protocol, data is taken at 0 and 15 minutes. Note that only a single replicate of the zero time may be sufficient when doing many samples. In this case, 4 data points are generated for each Working Xylanase Solution. Also, it is recommended that the Xylose Standards be included in at least duplicate.

25 **Note:** The 0 µg/mL Xylose Standard is also considered to be the Reagent Blank, i.e., this sample represents the background produced by the Substrate Solution alone.

The table below is intended to show how the samples, standards, and control reactions are derived:

Table 13.

Set-up of Controls, Samples, and Standards				
	<i>Substrate</i>	<i>1x</i>	<i>Working Xylanase</i>	<i>Xylose</i>

Reaction	Solution	SAB	Solution	Standard
Enzyme + Substrate	500 μ L	----	200 μ L	----
Substrate Control	500 μ L	200 μ L	----	----
Standard	500 μ L	----	----	200 μ L

Pipettor Calibration

Measure and record the mass of five 0.2 mL aliquots of 1x SAB delivered to a beaker tared on an analytical balance using a P-1000. The spreadsheet calculates the average mass of a 0.2 mL aliquot of 1x SAB. Use this calibrated P-1000 pipetman to conduct the protocol.

Specific Activity Determination Procedure

•Dispense 500 μ L aliquots of **Substrate Solution** into 13x100 mm glass tubes using a positive displacement dispenser with a 5 mL repeat pipettor tip.

Reminder: Each enzyme is tested with three replicates for each time point (0 and 15 minutes; therefore, 6 aliquots of substrate are required for each Working Xylanase Solution).

•Place tubes containing Substrate Solution in 37° C water bath for at least 5 minutes.

•Aliquot 5mL of each Working Xylanase Solution to be tested into 16 x 100 mm glass tube and equilibrate in 37° C water bath for at least 5 minutes

•Aliquot 500 μ L of each Xylose Standards into 1.5 mL eppendorf tubes and equilibrate in 37° C water bath for at least 5 minutes.

•For the zero time point samples, add 700 μ L of DNS Reagent to these tubes using a positive displacement pipettor with a 5 mL tip.

•Initiate the reaction by adding 200 μ L Working Xylanase Solution to an aliquot of Substrate Solution with a 5 mL repeat pipettor tip. Mix solution by vortexing and return to 37° C water bath.

•Following the initiation of all the enzyme reactions, add 200 μ L of Xylose Standards to the appropriately labeled tubes containing Substrate Solution.

•At 15 minutes, add 700 μ L DNS Reagent to the appropriate-labelled tubes using a positive displacement pipettor with a 5 mL tip and remove from water bath. Vortex vigorously.

•After termination of all enzyme reactions, add 700 μ L DNS Reagent to each aliquot of Xylose Standard in Substrate Solution and remove samples from bath and vortex vigorously.

•Place tubes in vigorously boiling water bath or oil bath for exactly 10 minutes.

Note: It is essential that the bath be at exactly 100°C and that the incubation be exactly 10 minutes.

•Remove tubes from boiling water bath and place in a room temperature water bath. Allow to cool at room temperature for at least 5 minutes.

- Transfer at least 1 mL of the contents to 1.5 mL polystyrene cuvettes and measure absorbance at 540 nm.

Calculation of Specific Activity

- Enter the absorbances for the standards and samples on the appropriate cells in the embedded worksheet. The worksheet automatically generates a xylose standard curve by plotting the absorbance readings (OD_{540}) vs. the xylose concentration ($\mu\text{mol}/\text{assay}$). This standard curve is then used to convert the sample absorbances into specific activity using the equation below.

$$\begin{aligned} \text{Activity} &= \frac{DF}{(t_2 - t_1) \cdot V_s} \cdot \left\{ \left\{ \frac{(OD_{540} \text{ sample}_2 - OD_{540} \text{ Reagent Blank})}{\text{slope}} - \text{intercept} \right\} - \left\{ \frac{(OD_{540} \text{ sample}_1 - OD_{540} \text{ Reagent Blank})}{\text{slope}} - \text{intercept} \right\} \right\} \\ &= \frac{DF}{(t_2 - t_1) \cdot V_s} \cdot \left\{ \frac{(OD_{540} \text{ sample}_2 - OD_{540} \text{ sample}_1)}{\text{slope}} \right\} \end{aligned}$$

[=] $\mu\text{moles xylose equivalents}/\text{min}/\text{g}$ of original enzyme

[=] Units/g of original undiluted enzyme sample

- where DF = dilution factor (calculated from First, Second, and Additional Dilutions)

t_2 = second time point (e.g., 15 minutes)

t_1 = first time point (e.g., 0 minutes)

V_s = sample weight (e.g., 0.2g)

- The results of three independent experiments conducted during three different days is reported in Table 14. The activity for the TAM material PD064 is also reported to compare the two fractions.

Table 14.

PP6002 PDO64 lot	unpurified 10 mg / mL			Antibody purified		
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3
Dilution factor	10100	10317	10018	994	955	1001
time (min)	15	15	15	15	15	15
Absorbance: $OD_{15\text{min}} - OD_{0\text{min}}$	0.957	1.003	1.035	0.697	0.731	0.683
weight of enzyme in original stock	0.2	0.207	0.2	0.201	0.207	0.2
slope (xylose calibration curve)	0.86	0.893	0.869	0.882	0.893	0.869
Activity (Units per g)	3746	3731	3975	262	252	262

Formula used is:

Activity (Units / g) = (dilution factor / time x sample weight) x ($OD_{15\text{min}} - OD_{0\text{min}}$ / slope)

Activity (Units / g) = (dilution factor / time x sample weight) x (OD_{15min} - OD_{0min} / slope)

The average activity of the purified enzyme is 259 units per gram of enzyme solution, and there is very little variation between experiments. The unpurified lot shows an activity of more than 10 fold higher because its concentration is much higher (10 mg/mL versus 0.400 mg/mL in the purified sample as measured by the BCA method). When one corrects for enzyme concentration, the activity per grams of dry material is now 650 U per mg of solid in the purified fraction, versus 373 U per mg of solid in the TAM sample.

EXAMPLE 12. Optimal reaction conditions

The activity of Quantum Xylanase was measured over a broad range of pH values to determine the optimal pH, but also whether the enzyme display significant activity under acidic condition of pH 2 to 3 that mimics conditions in the digestive tract of a monogastric animal.

The PP6002 lot PD064 Quantum® Xylanase (10 mg/mL) was diluted at 1:9187 and submitted to a range of pH conditions from 1.5 to 9. Both reaction conditions and wheat arabinoxylan substrate were prepared in the buffer components indicated below in the Table 15.

Table 15.

Reaction pH	Buffer Composition and Concentration
1.5	0.031 M HCl + 0.05 M KCl
2.0	0.01 M HCl + 0.05 M KCl
2.5	0.1 M Glycine + 0.1 M HCl
3.0	0.1 M Glycine + 0.1 M HCl
3.5	0.1 M Glycine + 0.1 M HCl
4.0	0.1 M Sodium Acetate
4.5	0.1 M Sodium Acetate
5.0	0.1 M Sodium Acetate
5.5	0.1 M Sodium Acetate
6.0	0.1 M Tris-HCl
6.5	0.1 M Tris-HCl
7.0	0.1 M Tris-HCl
8.0	0.1 M Tris-HCl
9.0	0.1 M Tris-HCl

The enzyme stock (10 mg/mL) was diluted in the pH buffer solution and added to the substrate also prepared in the same buffer. All reactions were conducted at 37C for 15 min using the DNS protocol. A calibration curve for xylose (in 0.1 M Na Acetate, pH 5.3) was established with a slope of 0.8582, also corrected for an intercept of 0 value. ($R^2 = 0.9982$).

The results are from two experiments and represent the average of 3 absorbance readings, corrected to the $t = 0$ min absorbance (DNS added just prior to reaction).

The activity of Quantum® Xylanase is then recorded as a percentage of the value corresponding to the maximum activity, observed here at pH = 5.5. At higher pH the activity drops and becomes marginal at pH 9.0 (about 5% of the activity at pH 6.0). There is residual activity at acidic pH, but only 11% at pH = 3.0, and none at lower pH (1.5; 2; 2.5). Results in Figure 5.

EXAMPLE 13. Thermal Tolerance

Thermal tolerance of enzyme by ELISA and activity

The activity of Quantum Xylanase was measured following thermal treatment from 37 to 99°C with a five degree increment. Solutions of enzyme of 10 mg/mL were prepared in 0.1 M NaOAc pH 5.3 and diluted to 1:100. Samples of 1 mL of 1:100 diluted enzyme were heat rated, then diluted further to 1:10,000 and assayed for activity using the DNS protocol.

A calibration curve was determined for xylose. The results are shown in Figure 6 as a percentage of the activity at 37°C. They are the average of two experiments done on two different days. Results in Figure 6.

PP6002 shows 100% activity until 55°C when it starts to decrease. Then its activity stays above 80% until 65°C, when it drops further down to about 45% at 80°C. Above this temperature the activity remains at the same level. This clearly indicates that PP6002 shows high specific activity at even higher temperatures.

Comparison of heat tolerance of pure protein with other xylanases by activity

The activity of Quantum xylanase was compared with the activity of *Thermomyces lanuginosus* (Megazyme®), and the *E.coli* produced BD6002 xylanase. Solutions of enzyme of 10 mg/mL were prepared in 0.1 M NaOAc pH.5.3 and diluted to various levels so that the absorbance measurements are in a similar range at the control temperature 37°C.

Temperatures assayed were in the range of 37°C to 95°C. A calibration curve was determined for xylose. The results are shown in the Figure 7 as a percentage of the activity at 37°C. As observed above, PP6002 shows some strong specific activity even at high temperature, and it shows more than 40% activity at 95°C.

BD6002 *E.coli* showed 100% activity until 55°C. Above this temperature it drops sharply until no activity is detected at or above 75°C.

A commercial enzyme from *Trichoderma Longibrachiatum* (Megazyme International) also shows no thermotolerance. Its activity dropped sharply above 55°C, to no activity above 65°C. These results demonstrate the thermotolerant property of Quantum® Xylanase, when produced in *Pichia pastoris*, and one possibility is that it is related to its isoforms, some of are glycosylation modifications. See Figure 7 for results.

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and further embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the scope of the present invention.

- 5 Numerous patents, applications and references are discussed or cited within this specification, and all are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule having the sequence of SEQ ID NOS: 1, 3, 5 or 7.
2. An expression cassette comprising a nucleic acid sequence of claim 1.
3. The expression cassette of claim 2, further comprising a proteolytic cleavage site.
4. The expression cassette of claim 3, wherein the proteolytic cleavage site is a KEX2 protease cleavage site.
5. The expression cassette of claim 4, encoded by the nucleic acid sequence of SEQ ID NOS: 9, 10, 11 or 12.
6. The expression cassette of claim 2 further comprising an isolated nucleotide sequence encoding a secretion signal peptide.
7. An expression cassette comprising at least one nucleic acid molecule of claim 1 operably linked to a promoter.
8. A vector comprising at least one expression cassette of claim 2.
9. The vector of claim 8, comprising the plasmid designated pBCS12771 (SEQ ID NO: 33), pBCS12772 (SEQ ID NO:34), or pSYN12773 (SEQ ID NO:35).
10. A recombinant host cell comprising at least one nucleic acid molecule of claim 1.
11. The recombinant host cell of claim 10 wherein the host cell is a bacteria, a yeast or a fungal cell.
12. The recombinant host cell of claim 11 wherein the bacteria, yeast or fungal cell is a *Kluyveromyces*, *saccharomyces*, *Shizosaccharomyces*, *Trichosporon*, *Schwanniomyces*, *Pichia*, *Hansuela*, *Eschericia*, *Psudomonas*, *Lactobacillus*, *Bacillus*, *Aspergillus*, *Rhizopus*, *Trichoderma*, *Neurospora*, *Mucor*, or *Penicillium* cell.
13. The recombinant host cell of claim 11, wherein the host cell is *Pichia pastoris*.
14. The recombinant host cell of claim 13, comprising the vector of claim 9.

15. A method to prepare a thermotolerant xylanase, comprising the steps of:

a) expressing in a microbial host cell an expression cassette comprising a promoter operably linked to a nucleic acid molecule encoding a xylanase which retains at least 40% activity after 30 minutes at 60°C and has a specific activity of greater than 400U/mg at a pH less than pH 5.0 and greater than pH 1.5.

16. The method of claim 15, wherein the xylanase retains at least 40% activity after 30 minutes at 70°C.

17. The method of claim 15, wherein the xylanase retains at least 40% activity after 30 minutes at 80°C.

18. The method of claim 15, wherein the xylanase retains at least 40% activity after 30 minutes at 85°C.

19. The method of claim 15, wherein the xylanase is a thermotolerant xylanase in the absence of glycosylation.

20. The method of claim 15, wherein the xylanase is thermotolerant when glycosylated by the host cell.

21. The method of claim 15 further comprising the step of isolating the thermotolerant xylanase.

22. The method of claim 15, wherein the host cell is a bacterial, yeast or fungal cell.

23. The method of claim 22, wherein the host cell is a *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Schwanniomyces*, *Pichia* or *Hansenula* cell.

24. The method of claim 22, wherein host cell is *Saccharomyces cerevisiae*, *Hansenula polymorpha* or a *Schizosaccharomyces pombe* cell.

25. The method of claim 22, wherein the host cell is *Pichia pastoris*.

26. The method of claim 22, wherein the host cell is *Eschericia*, *Pseudomonas*, *Lactobacillus*, or *Bacillus*.

27. The method of claim 22, wherein the host cell is an *Aspergillus*, *Rhizopus*, *Trichoderma*, *Neurospora*, *Mucor*, or *Penicillium* cell.
- 5 28. The method of claim 15, wherein the xylanase is encoded by the nucleic acid of claim 1.
29. The method of claim 15, wherein the nucleic acid molecule encodes a fusion polypeptide comprising the xylanase.
- 10 30. The method of claim 29, wherein the fusion polypeptide comprises a secretion signal sequence which is operably linked to the xylanase.
31. An isolated thermotolerant xylanase prepared and isolated by the method of claim 21.
- 15 32. The xylanase of claim 31, wherein the xylanase is glycosylated.
33. An enzyme feed additive comprising the thermotolerant xylanase of claim 31.
34. A method of preparing animal feed, comprising the steps of:
- 20 a) providing a mixture comprising animal feed components and a preparation comprising the thermotolerant xylanase of claim 31, and
- b) heating the mixture at a temperature greater than 75-90°C so as to yield a heat-treated animal feed mixture.
35. The method of claim 34, wherein the xylanase containing preparation is a liquid
- 25 preparation.
36. The method of claim 34, wherein the xylanase containing preparation is a solid preparation.
- 30 37. The method of claim 34, wherein the mixture in a) further comprises at least one vitamin, mineral, an enzyme other than a thermotolerant xylanase, an organic acid, a probiotic product, an essential oil, or a grain processing co-product.
38. A heat-treated animal feed mixture produced by the method of claim 34.
- 35 39. The method of claim 34, further comprising the step of extruding the heat-treated mixture through a pellet mill to yield pelletized animal feed.

40. A pelletized animal feed produced by the method of claim 39.

41. The pelletized animal feed of claim 40, wherein the mixture is steam-conditioned in a pellet mill at about 85°C such that greater than 70% of the pre-heat treated enzymatic activity is retained, and the feed extruded through a pellet dye.

42. The pelletized animal feed of claim 40, wherein the mixture is steam-conditioned in a pellet mill at about 85°C such that greater than 90% of the pre-heated treated enzymatic activity is retained, and the feed extruded through a pellet dye.

43. The pelletized animal feed of claim 40, wherein the mixture is steam conditioned in a pellet mill at about 90°C such that at least 80% of the pre-heated treated enzymatic activity is retained, and the feed extruded through a pellet dye.

44. An animal feed composition comprising the thermotolerant xylanase of claim 31.

45. The feed of claim 44, wherein the grain is wheat, rye, triticale, rice or corn.

46. The feed of claim 44, wherein one or more xylanase enzymes is added to the feed using an inclusion rate of between 1 and 10,000U/kg.

47. The feed of claim 44, wherein one or more xylanase enzymes is added to the feed using an inclusion rate of between 50 to 5,000 U/kg

48. The feed of claim 44, wherein one or more xylanase enzymes is added to the feed using an inclusion rate of between 200 to 3,200 U/kg.

49. A method for preparing a thermotolerant xylanase containing composition for feed formulation comprising the steps of:

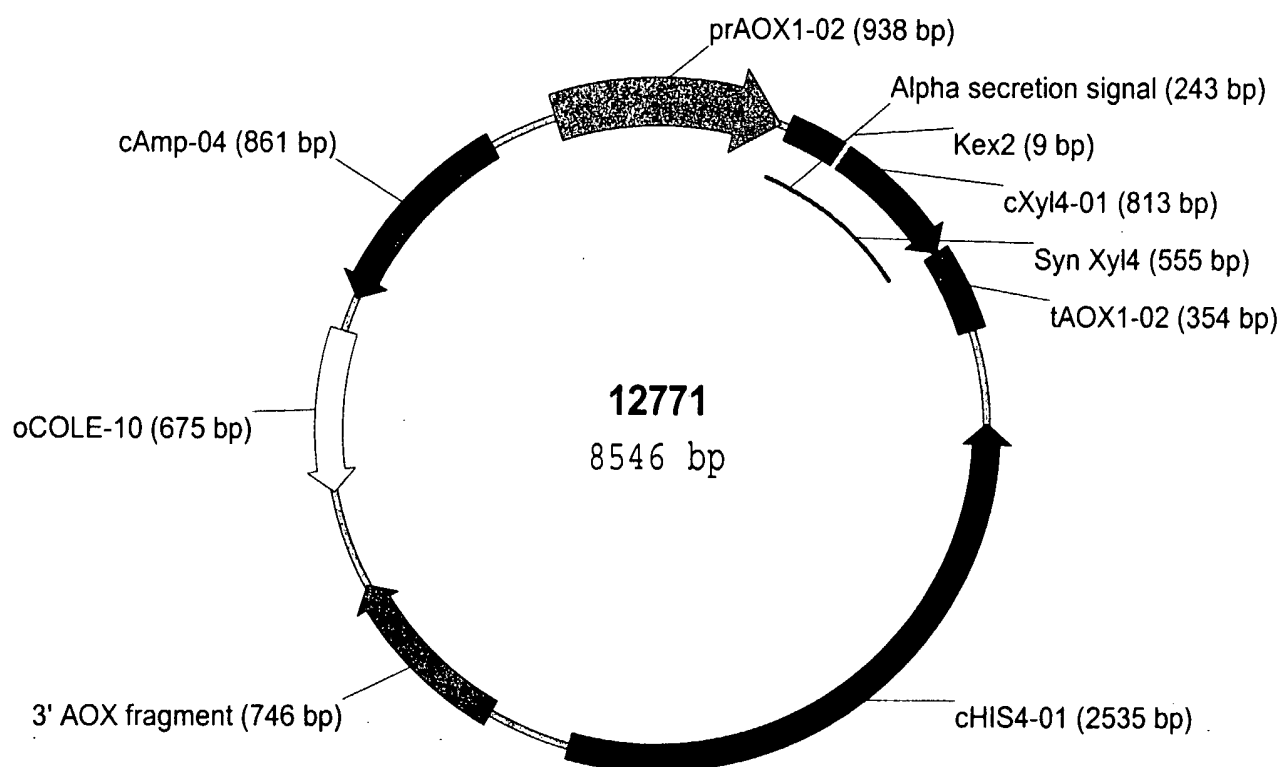
- a) combining a liquid solution comprising the thermotolerant xylanase of claim 31 and soy meal flour to yield a mixture; and
- b) lyophilizing the mixture to yield a thermotolerant xylanase containing lyophilized composition.

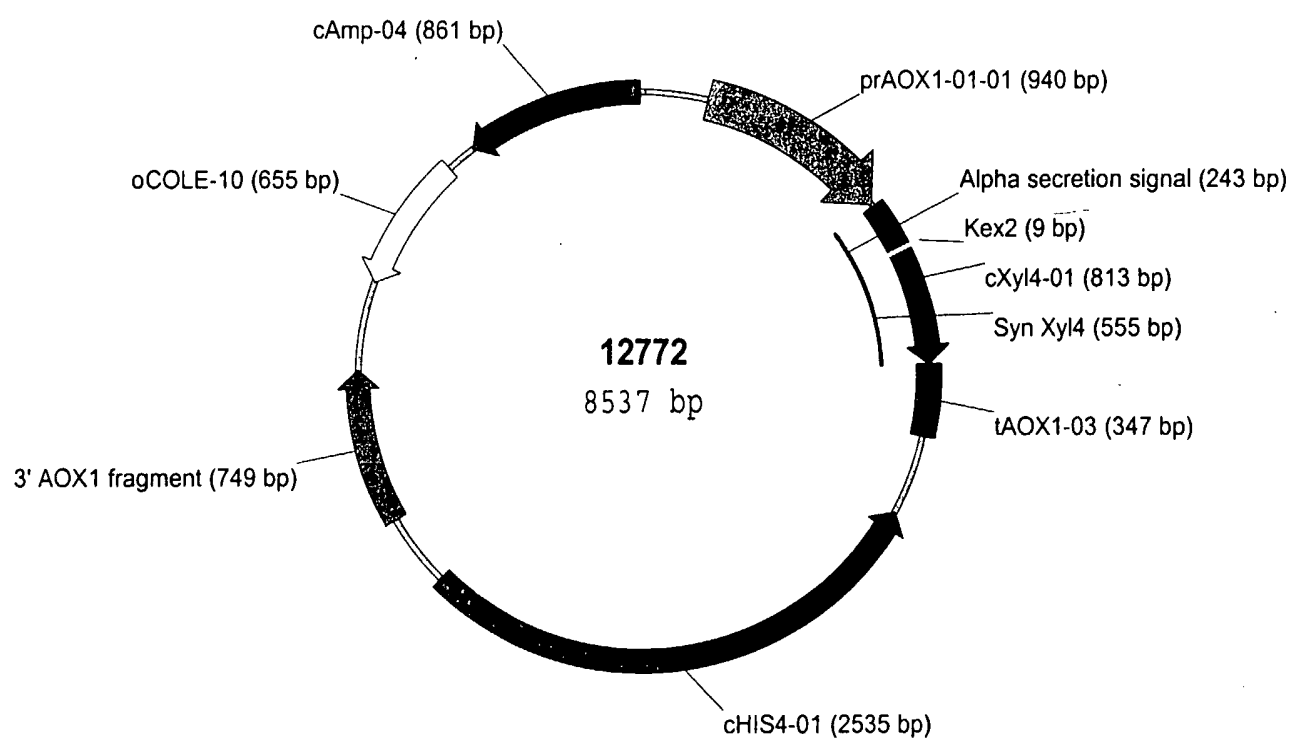
50. The method of claim 49, further comprising the step of combining the lyophilized composition with other feed components to yield a further mixture.

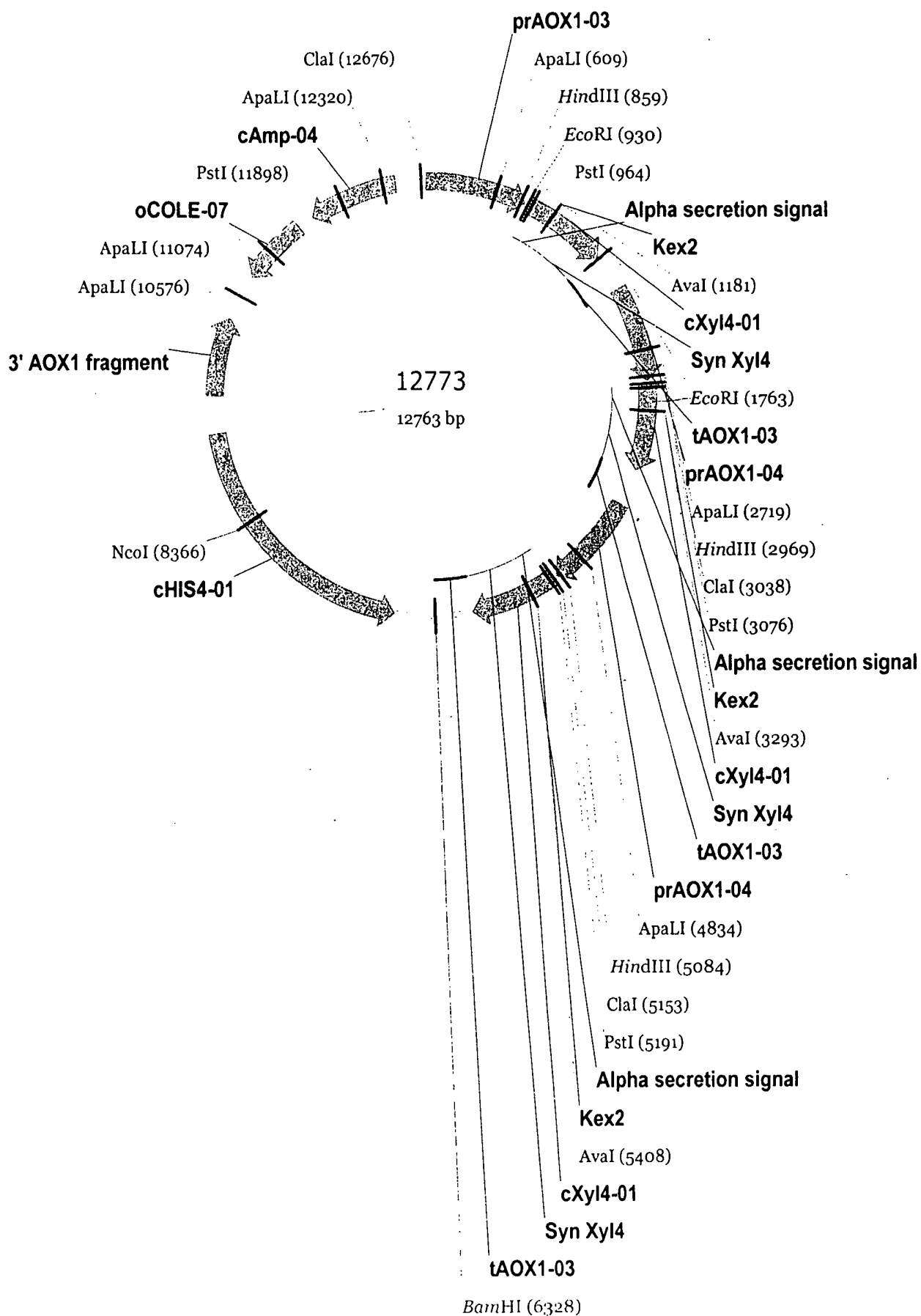
51. A lyophilized composition prepared by the method of claim 50.
52. A method of preparing a xylanase containing composition for feed formulation comprising the steps of:
- 5 a) combining a liquid solution comprising the thermotolerant xylanase of claim 31 and meal flour to yield a mixture;
- b) and drying the mixture the mixture to yield a dried composition.
53. A method of decreasing the feed conversion ratio and increasing the weight gain of an animal, comprising the step of:
- 10 feeding to an animal a feed comprising of the thermotolerant xylanase of claim 31 in an amount effective to decrease the feed conversion ratio in the animal.
54. A method of improving the apparent metabolizable energy of animal feed comprising the step of formulating the animal feed with one or more thermotolerant xylanases of claim 31 to the animal feed in an effective amount to improve the apparent metabolizable energy of the feed.
- 15 55. A method of improving the nutritive value of animal feed or human food comprising the step of adding the thermotolerant xylanase of claim 31 during the preparation of animal feed or human food.
- 20 56. The method of claim 55, further comprising the step of treating the mixture under appropriate conditions of temperature and moisture to facilitate hydrolysis of xylan.
- 25 57. A method of preparing animal feed comprising the steps of:
- a) providing a mixture comprising one or more feed components and a preparation comprising the thermotolerant xylanase of claim 31; and
- b) treating the mixture under appropriate conditions of temperature and moisture so as to hydrolyze xylan which is present in the mixture.
- 30 58. An animal feed prepared by the method of claim 57.
59. The method of any one of claims 52 to 57, wherein the feed is poultry feed.

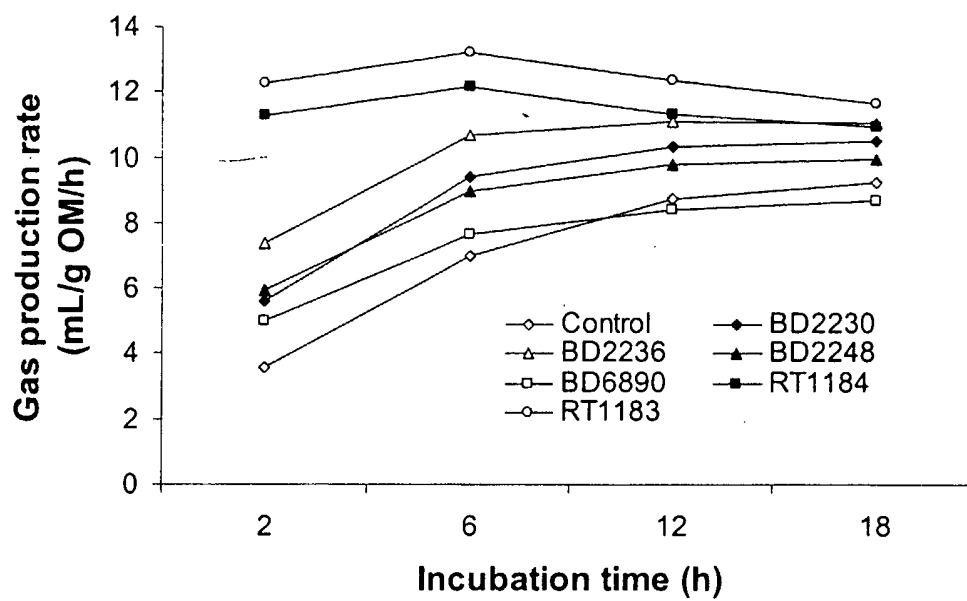
60. The method of any one of claims 52 to 57 wherein the feed is swine feed.

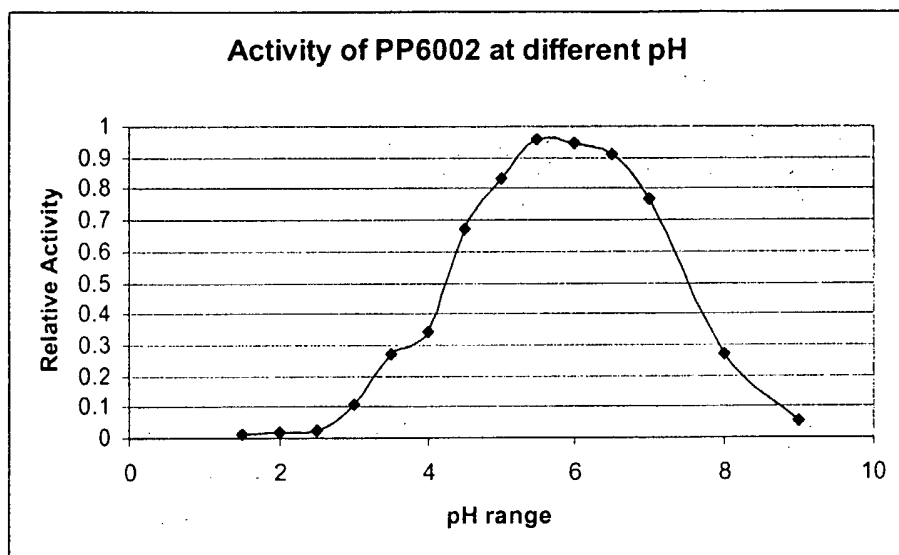
61. The method of any one of claims 52 to 57 wherein the feed is ruminant feed.

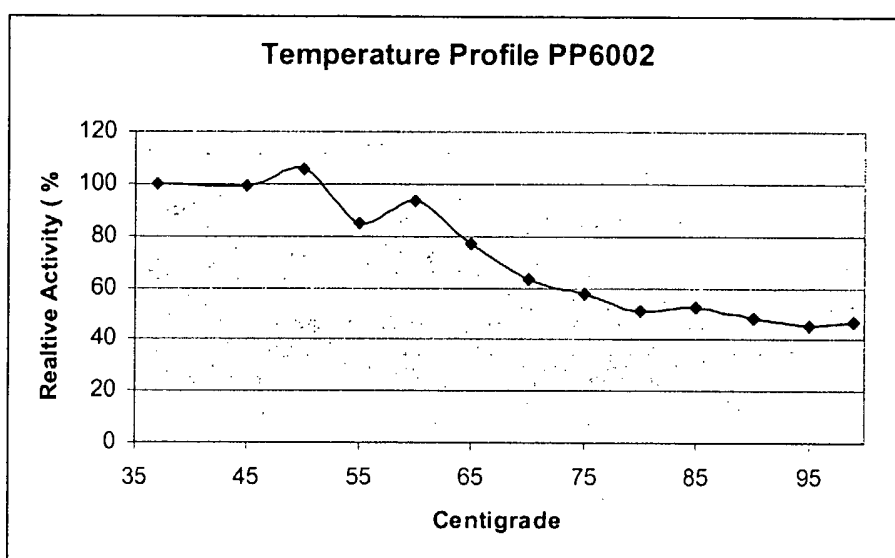


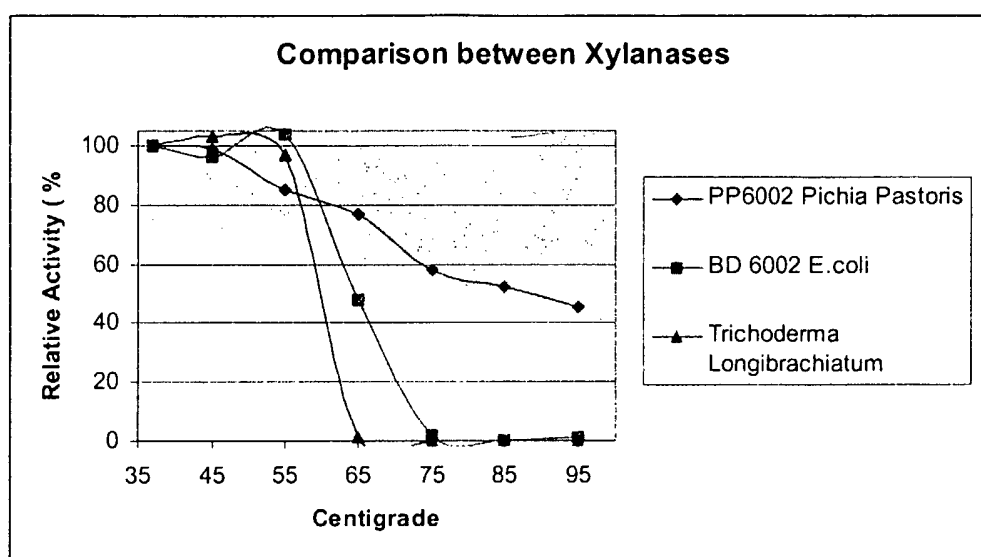












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Bauer, Michael
Bedford, Michael
Pulliam, Derrick

<120> Microbially Expressed Xylanases and Their Use as Feed Additives and Other Uses

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 tct att gat ggt acc aga act ttt tac caa tat tgg tcc gtt aga aca 432
 Ser Ile Asp Gly Thr Arg Thr Phe Tyr Gln Tyr Trp Ser Val Arg Thr
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 Ser Lys Arg Pro Thr Gly Ser Asn Gln Thr Ile Thr Phe Ala Asn His
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caa att atg gcc act gag ggt tat caa tct agt gga aat tct aat ttg 576
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 35 40 45

Cys Gly Lys Gly Trp Ser Thr Gly Ser Ser Ser Arg Arg Ile Gly Tyr
 50 55 60

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

Gly Ser Trp Arg Pro Pro Gly Gly Thr Ser Ala Gly Thr Val Asn Ser
 100 105 110

Asp Gly Gly Thr Tyr Asn Leu Tyr Arg Thr Gln Arg Val Asn Ala Pro
 115 120 125

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

Ser Lys Arg Pro Thr Gly Ser Asn Gln Thr Ile Thr Phe Ala Asn His
 145 150 155 160

Val Asn Ala Trp Arg Ser Lys Gly Trp Asn Leu Gly Ser His Val Tyr
 165 170 175

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

Thr Val Trp Ala Gln
 195

<210> 5
 <211> 972
 <212> DNA
 <213> artificial sequence

<220>
 <223> xylanase coding region of PP6407

<220>
 <221> CDS
 <222> (1)..(972)
 <220>
 <221> msic_feature
 <222> (1)..(972)

<400> 5
 gca caa act ttg aac aac aac tcc act gga act cac gac ggt ttt tat 48
 Ala Gln Thr Leu Asn Asn Asn Ser Thr Gly Thr His Asp Gly Phe Tyr
 1 5 10 15
 tac aca ttt tgg aaa gac tct ggt tct gct tcc atg acc ctt cac cct 96
 Tyr Thr Phe Trp Lys Asp Ser Gly Ser Ala Ser Met Thr Leu His Pro
 20 25 30
 ggt gga aga tat tca tca cag tgg aca tct aac act aac aat tgg gtt 144
 Gly Gly Arg Tyr Ser Ser Gln Trp Thr Ser Asn Thr Asn Asn Trp Val
 35 40 45
 ggt ggt aag ggt tgg aac cct gga ggt cct aga gta gtt aac tac agt 192
 Gly Gly Lys Gly Trp Asn Pro Gly Gly Pro Arg Val Val Asn Tyr Ser
 50 55 60
 gga tat tac ggt gtt aat aac tcc caa aac tcc tac ctt gcc ttg tac 240
 Gly Tyr Tyr Gly Val Asn Asn Ser Gln Asn Ser Tyr Leu Ala Leu Tyr
 65 70 75 80
 ggt tgg act cgt aat cct ttg gtg gaa tac tac gtt att gag agt tac 288
 Gly Trp Thr Arg Asn Pro Leu Val Glu Tyr Tyr Val Ile Glu Ser Tyr
 85 90 95
 ggt tct tac aat cca gca tcc tgt gct gga gga gtt gat tat ggt tcc 336
 Gly Ser Tyr Asn Pro Ala Ser Cys Ala Gly Gly Val Asp Tyr Gly Ser
 100 105 110
 ttc caa tct gac ggt gct acc tat aac gta aga agg tgc ttg aga caa 384
 Phe Gln Ser Asp Gly Ala Thr Tyr Asn Val Arg Arg Cys Leu Arg Gln
 115 120 125
 aat gct cca agt atc gaa ggt aat aac tct aca ttt tac cag tac ttt 432
 Asn Ala Pro Ser Ile Glu Gly Asn Asn Ser Thr Phe Tyr Gln Tyr Phe
 130 135 140
 tca gtt cgt aat cca aag aag ggt ttc ggt aat att tct ggt act att 480
 Ser Val Arg Asn Pro Lys Lys Gly Phe Gly Asn Ile Ser Gly Thr Ile
 145 150 155 160
 acc gtc gct aat cat ttt aat tat tgg gca tcc aga ggt ctg aac tta 528

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Thr Val Ala Asn His Phe Asn Tyr Trp Ala Ser Arg Gly Leu Asn Leu
 165 170 175

gga aac cat gat tat atg gtt ttc gcc act gaa ggt tat caa tct caa 576
 Gly Asn His Asp Tyr Met Val Phe Ala Thr Glu Gly Tyr Gln Ser Gln
 180 185 190

ggt tct agt gat att act gtg tca agt ggt act gga ggt ggt gga gga 624
 Gly Ser Ser Asp Ile Thr Val Ser Ser Gly Thr Gly Gly Gly Gly Gly
 195 200 205

gga gga aat acc gga tct aaa aca ata gtc gtc aga gcc aga ggt acc 672
 Gly Gly Asn Thr Gly Ser Lys Thr Ile Val Val Arg Ala Arg Gly Thr
 210 215 220

gca ggt gga gag aat ata tcc ttg aag gta aat aat gcc act atc gct 720
 Ala Gly Gly Glu Asn Ile Ser Leu Lys Val Asn Asn Ala Thr Ile Ala
 225 230 235 240

tca tgg acc ttg aca act tca atg gct aac tac aca gct act aca tct 768
 Ser Trp Thr Leu Thr Thr Ser Met Ala Asn Tyr Thr Ala Thr Thr Ser
 245 250 255

gct tct ggt gga tct ctg gtt gag ttc act aac gat gga ggt aat aga 816
 Ala Ser Gly Gly Ser Leu Val Glu Phe Thr Asn Asp Gly Gly Asn Arg
 260 265 270

gat gtt caa gtg gac tat tta tca gtc aat ggt gct gtc aga caa gca 864
 Asp Val Gln Val Asp Tyr Leu Ser Val Asn Gly Ala Val Arg Gln Ala
 275 280 285

gaa gat cag act tat aat act ggt gtg tat cag aac gga cag tgc gga 912
 Glu Asp Gln Thr Tyr Asn Thr Gly Val Tyr Gln Asn Gly Gln Cys Gly
 290 295 300

ggt gga aac gga agg tct gag tgg ttg cat tgt aac ggt gcc att gga 960
 Gly Gly Asn Gly Arg Ser Glu Trp Leu His Cys Asn Gly Ala Ile Gly
 305 310 315 320

ttt ggt aat ttg 972
 Phe Gly Asn Leu

<210> 6
 <211> 324
 <212> PRT
 <213> artificial sequence

<220>
 <223> xylanase coding region of PP6407

<400> 6

Ala Gln Thr Leu Asn Asn Asn Ser Thr Gly Thr His Asp Gly Phe Tyr
 1 5 10 15

Tyr Thr Phe Trp Lys Asp Ser Gly Ser Ala Ser Met Thr Leu His Pro
 20 25 30

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

Gly Gly Lys Gly Trp Asn Pro Gly Gly Pro Arg Val Val Asn Tyr Ser

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60

50

55

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

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

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

Phe Gln Ser Asp Gly Ala Thr Tyr Asn Val Arg Arg Cys Leu Arg Gln
115 120 125

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

Ser Val Arg Asn Pro Lys Lys Gly Phe Gly Asn Ile Ser Gly Thr Ile
145 150 155 160

Thr Val Ala Asn His Phe Asn Tyr Trp Ala Ser Arg Gly Leu Asn Leu
165 170 175

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

Gly Ser Ser Asp Ile Thr Val Ser Ser Gly Thr Gly Gly Gly Gly
195 200 205

Gly Gly Asn Thr Gly Ser Lys Thr Ile Val Val Arg Ala Arg Gly Thr
210 215 220

Ala Gly Gly Glu Asn Ile Ser Leu Lys Val Asn Asn Ala Thr Ile Ala
225 230 235 240

Ser Trp Thr Leu Thr Thr Ser Met Ala Asn Tyr Thr Ala Thr Thr Ser
245 250 255

Ala Ser Gly Gly Ser Leu Val Glu Phe Thr Asn Asp Gly Gly Asn Arg
260 265 270

Asp Val Gln Val Asp Tyr Leu Ser Val Asn Gly Ala Val Arg Gln Ala
275 280 285

Glu Asp Gln Thr Tyr Asn Thr Gly Val Tyr Gln Asn Gly Gln Cys Gly
290 295 300

Gly Gly Asn Gly Arg Ser Glu Trp Leu His Cys Asn Gly Ala Ile Gly
305 310 315 320

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Phe Gly Asn Leu

<210> 7
 <211> 579
 <212> DNA
 <213> artificial sequence

<220>
 <223> xylanase region of PP7436

<220>
 <221> CDS
 <222> (1)..(579)

<220>
 <221> misc_feature
 <222> (1)..(579)

<400> 7
 gag gct gaa gct gct ctg atg gct tcc act ttc tac tgg cat ctt tgg 48
 Glu Ala Glu Ala Ala Leu Met Ala Ser Thr Phe Tyr Trp His Leu Trp
 1 5 10 15
 act gat gga atc ggt aca gtt aac gcc acc aac ggt tcc gat gga aac 96
 Thr Asp Gly Ile Gly Thr Val Asn Ala Thr Asn Gly Ser Asp Gly Asn
 20 25 30
 tac tcc gtt tct tgg agt aac tgt gga aac ttt gtc gta ggc aag gga 144
 Tyr Ser Val Ser Trp Ser Asn Cys Gly Asn Phe Val Val Gly Lys Gly
 35 40 45
 tgg acc act ggc agt gca acc aga gtc att aac tac aat gcc cat gcc 192
 Trp Thr Thr Gly Ser Ala Thr Arg Val Ile Asn Tyr Asn Ala His Ala
 50 55 60
 ttt tct gtg gtt ggt aat gct tat tta gct ctc tat gga tgg aca aga 240
 Phe Ser Val Val Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp Thr Arg
 65 70 75 80
 aat tca cta atc gaa tac tat gtg gtt gat tct tgg ggt act tat aga 288
 Asn Ser Leu Ile Glu Tyr Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg
 85 90 95
 cca act ggt act tac aaa ggt act gtt acc tcc gac ggt ggc act tac 336
 Pro Thr Gly Thr Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr
 100 105 110
 gac att tac aca aca aca cgt act aac gct cct agt atc gac gga aat 384
 Asp Ile Tyr Thr Thr Thr Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn
 115 120 125
 aat aca acc ttt act cag ttc tgg tca gtt cga cag agt aag agg cca 432
 Asn Thr Thr Phe Thr Gln Phe Trp Ser Val Arg Gln Ser Lys Arg Pro
 130 135 140
 att ggt acc aac aat act att acc ttc tct aat cac gta aat gca tgg 480
 Ile Gly Thr Asn Asn Thr Ile Thr Phe Ser Asn His Val Asn Ala Trp
 145 150 155 160
 aaa tct aag ggt atg aac ttg gga tct tca tgg agt tac caa gtc ttg 528
 Lys Ser Lys Gly Met Asn Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu
 165 170 175
 gct act gag ggt tat caa tca tct ggt tat tct aat gta aca gtg tgg 576

70357WOPCT.ST25

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

taa

579

<210> 8
 <211> 192
 <212> PRT
 <213> artificial sequence

<220>
 <223> xylanase region of PP7436

<400> 8

Glu Ala Glu Ala Ala Leu Met Ala Ser Thr Phe Tyr Trp His Leu Trp
 1 5 10 15

Thr Asp Gly Ile Gly Thr Val Asn Ala Thr Asn Gly Ser Asp Gly Asn
 20 25 30

Tyr Ser Val Ser Trp Ser Asn Cys Gly Asn Phe Val Val Gly Lys Gly
 35 40 45

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

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

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

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

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

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

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

Lys Ser Lys Gly Met Asn Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu
 165 170 175

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

<210> 9
 <211> 598
 <212> DNA

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<213> artificial sequence

<220>

<223> PP6002 cassette

<220>

<221> misc_feature

<222> (1)..(598)

<400> 9

tttccctctc gagaagaggg catctactga ctactggcag aactggactg acggtggtgg	60
taccgtgaac gctactaatg gtagtgatgg taattactca gtttcttggt caaactgtgg	120
aaacttcgtc gttggttaagg gatggacaac cggatctgct actagagtaa tcaactacaa	180
tgccggagct ttttctcctt ctggtaatgg ttacttgccc ttgtatggat ggacaagaaa	240
ctctttgatt gaatattacg ttgtggattc ctgggggtact tatcgtccaa ctggaacata	300
taaagggtact gtaacttctg acggaggtac ctatgatatt tacacaacta caagaactaa	360
tgctccatct atcgacggaa ataacactac atttaccag ttttgggtctg tcagacaatc	420
taaaagacct attggaacta ataataccat aactttcagt aatcatgtta acgcttgga	480
gtcaaaagggt atgaacttgg gttcctcctg gtcctaccaa gttttggcaa ctgaggggta	540
ccaatctagt ggttattcaa atgttactgt ctggtaatag gcggccgcaa aaggaaaa	598

<210> 10

<211> 628

<212> DNA

<213> artificial sequence

<220>

<223> PP6016 exp. cassette

<220>

<221> misc_feature

<222> (1)..(628)

<400> 10

tttccctctc gagaagagag ctgagactat ctgttctaac cagactggta ctaataacgg	60
atatttttac tcatttttga aggacactgg ttctgcatgc atgacccttg gttccggagg	120
aaactattct gttaattgga acttggggtc tggtaatatg gtctgtggta agggatggtc	180
taccggatct tcatcaagaa gaatcgggta caacgcagga gtttgggccc caaacggtaa	240
cgcttacttg acattgtatg gttggactag gaaccctttg atagaatact acgtcgtaga	300
tagttgggga tcatggcgtc ctccagggtg aacttcagct ggtaccgtaa attccgatgg	360
agggtacttat aatttatata gaactcaaag ggtgaacgct ccatctattg atggtaccag	420
aactttttac caatattggt ccggttagaac aagtaaacgt cctactggat ctaatcagac	480
aattacattc gcaaatcatg ttaatgcttg gagatccaaa ggttggaatc tgggttctca	540
cgtgtaccaa attatggcca ctgaggggta tcaatctagt ggaaattcta atttgacagt	600
ttgggctcaa tagtaagcgg ccgcaaaa	628

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<210> 11
 <211> 1009
 <212> DNA
 <213> artificial sequence

<220>
 <223> PP6407 exp. cassette

<220>
 <221> misc_feature
 <222> (1)..(1009)

<400> 11
 tttccctctc gagaaaagag cacaaacttt gaacaacaac tccactggaa ctcacgacgg 60
 tttttattac acattttgga aagactctgg ttctgcttcc atgacccttc accctggtgg 120
 aagatattca tcacagtgga catctaacac taacaattgg gttggtggta agggttggaa 180
 ccctggaggt cctagagtag ttaactacag tggatattac ggtgttaata actcccaaaa 240
 ctctacctt gccttgtag gttggactcg taatcctttg gtggaatact acgttattga 300
 gagttacggt tcttacaatc cagcatcctg tgctggagga gttgattatg gttccttcca 360
 atctgacggt gctacctata acgtaagaag gtgcttgaga caaatgctc caagtatcga 420
 aggtaataac tctacatttt accagtactt ttcagttcgt aatccaaaga agggtttcgg 480
 taatattttct ggtactatta ccgtcgctaa tcattttaat tattgggcat ccagaggtct 540
 gaacttagga aaccatgatt atatggtttt cgccactgaa gggtatcaat ctcaaggttc 600
 tagtgatatt actgtgtcaa gtggtactgg aggtggtgga ggaggaggaa ataccggatc 660
 taaaacaata gtcgtcagag ccagaggtac cgcaggtgga gagaatatat ccttgaaggt 720
 aaataatgcc actatcgctt catggacctt gacaacttca atggctaact acacagctac 780
 tacatctgct tctggtggat ctctggttga gttcactaac gatggaggta atagagatgt 840
 tcaagtggac ttttatcag tcaatggtgc tgtcagacaa gcagaagatc agacttataa 900
 tactggtgtg tatcagaacg gacagtgcgg aggtggaaac ggaaggtctg agtggttgca 960
 ttgtaacggt gccattggat ttggttaattt gtaataggcg gccgcaaaa 1009

<210> 12
 <211> 619
 <212> DNA
 <213> artificial sequence

<220>
 <223> PP7436 exp. cassette

<220>
 <221> misc_feature
 <222> (1)..(619)

<400> 12
 tttccctctc gagaaaagag aggtgaagc tgctctgatg gttccactt tctactggca 60

70357WOPCT.ST25

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tcttttgact gatggaatcg gtacagttaa cgccaccaac gggtccgatg gaaactactc 120
cgtttcttgg agtaactgtg gaaactttgt cgtaggcaag ggatggacca ctggcagtgc 180
aaccagagtc attaactaca atgcccattgc cttttctgtg gttggtaatg cttatttagc 240
tctctatgga tggacaagaa attcactaat cgaatactat gtgggtgatt cttgggggtac 300
ttatagacca actggtactt acaaaggtac tgttacctcc gacggtggca cttacgacat 360
ttacacaaca acacgtacta acgctcctag tatcgacgga aataatacaa cttttactca 420
gttctggtca gttcgacaga gtaagaggcc aattggtacc aacaatacta ttaccttctc 480
taatcacgta aatgcatgga aatctaaggg tatgaacttg ggatcttcat ggagttacca 540
agtcttggtt actgaggggtt atcaatcatc tggttattct aatgtaacag tgtggtaata 600
ggcggccgca aaaggaaaa 619

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<210> 13
 <211> 561
 <212> DNA
 <213> artificial nucleotide sequence

<220>
 <223> XYLA1A (BD6002)

<220>
 <221> CDS
 <222> (1)..(561)

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<400> 13
atg gct tcg aca gac tac tgg caa aat tgg act gat ggt ggt ggg aca 48
Met Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Thr
1 5 10 15

gta aat gct acc aat gga tct gat ggc aat tac agc gtt tca tgg tca 96
Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser
20 25 30

aat tgc ggg aat ttt gtt gtt ggt aaa ggc tgg act acc gga tca gca 144
Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala
35 40 45

act agg gta ata aac tat aat gcc gga gcc ttt tcg ccg tcc ggt aat 192
Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn
50 55 60

gga tat ttg gct ctt tat ggg tgg acg aga aat tca ctc ata gaa tat 240
Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr
65 70 75 80

tac gtc gtt gat agc tgg ggg act tat aga cct act gga act tat aaa 288
Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys
85 90 95

ggc act gtg act agt gat gga ggg act tat gac ata tac acg act aca 336
Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr
100 105 110

cga acc aac gca cct tcc att gac ggc aat aat aca act ttc acc cag 384
Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln
115 120 125

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ttc tgg agt gtt agg cag tcg aag aga ccg att ggt acc aac aat acc 432
 Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr
 130 135 140
 atc acc ttt agc aac cat gtt aac gcc tgg aag agt aaa gga atg aat 480
 Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn
 145 150 155 160
 ttg ggg agt agt tgg tct tat cag gta tta gca aca gag ggc tat caa 528
 Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln
 165 170 175
 agt agt ggg tac tct aac gta acg gtc tgg taa 561
 Ser Ser Gly Tyr Ser Asn Val Thr Val Trp
 180 185

<210> 14
 <211> 186
 <212> PRT
 <213> artificial nucleotide sequence

<220>
 <223> xylA1A (BD6002)

<400> 14

Met Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Thr
 1 5 10 15
 Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser
 20 25 30
 Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala
 35 40 45
 Thr Arg Val Ile Asn Tyr Asn Ala Gly Ala Phe Ser Pro Ser Gly Asn
 50 55 60
 Gly Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr
 65 70 75 80
 Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys
 85 90 95
 Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr
 100 105 110
 Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln
 115 120 125
 Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr
 130 135 140
 Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn
 145 150 155 160

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

Ser Ser Gly Tyr Ser Asn Val Thr Val Trp
 180 185

<210> 15
 <211> 561
 <212> DNA
 <213> artificial nucleotide

<220>
 <223> xylA1B (BD7436)

<220>
 <221> CDS
 <222> (1)..(561)

<400> 15
 atg gct tcg aca ttc tac tgg cac ttg tgg act gat ggt ata ggg aca 48
 Met Ala Ser Thr Phe Tyr Trp His Leu Trp Thr Asp Gly Ile Gly Thr
 1 5 10 15
 gta aat gct acc aat gga tct gat ggc aat tac agc gtt tca tgg tca 96
 Val Asn Ala Thr Asn Gly Ser Asp Gly Asn Tyr Ser Val Ser Trp Ser
 20 25 30
 aat tgc ggg aat ttt gtt gtt ggt aaa ggc tgg act acc gga tca gca 144
 Asn Cys Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Ala
 35 40 45
 act agg gta ata aac tat aat gcc cac gcc ttt tcg gta gtg ggt aat 192
 Thr Arg Val Ile Asn Tyr Asn Ala His Ala Phe Ser Val Val Gly Asn
 50 55 60
 gct tat ttg gct ctt tat ggg tgg acg aga aat tca ctc ata gaa tat 240
 Ala Tyr Leu Ala Leu Tyr Gly Trp Thr Arg Asn Ser Leu Ile Glu Tyr
 65 70 75 80
 tac gtc gtt gat agc tgg ggg act tat aga cct act gga act tat aaa 288
 Tyr Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys
 85 90 95
 ggc act gtg act agt gat gga ggg act tat gac ata tac acg act aca 336
 Gly Thr Val Thr Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr
 100 105 110
 cga acc aac gca cct tcc att gac ggc aat aat aca act ttc acc cag 384
 Arg Thr Asn Ala Pro Ser Ile Asp Gly Asn Asn Thr Thr Phe Thr Gln
 115 120 125
 ttc tgg agt gtt agg cag tcg aag aga ccg att ggt acc aac aat acc 432
 Phe Trp Ser Val Arg Gln Ser Lys Arg Pro Ile Gly Thr Asn Asn Thr
 130 135 140
 atc acc ttt agc aac cat gtt aac gcc tgg aag agt aaa gga atg aat 480
 Ile Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser Lys Gly Met Asn
 145 150 155 160
 ttg ggg agt agt tgg tct tat cag gta tta gca aca gag ggc tat caa 528
 Leu Gly Ser Ser Trp Ser Tyr Gln Val Leu Ala Thr Glu Gly Tyr Gln
 165 170 175
 agt agt ggg tac tct aac gta acg gtc tgg taa 561

70357WOPCT.ST25

Ser Ser Gly Tyr Ser Asn Val Thr Val Trp
 180 185

<210> 16
 <211> 186
 <212> PRT
 <213> artificial nucleotide

<220>
 <223> xylA1B (BD7436)

<400> 16

Met Ala Ser Thr Phe Tyr Trp His Leu Trp Thr Asp Gly Ile Gly Thr
 1 5 10 15

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

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

Thr Arg Val Ile Asn Tyr Asn Ala His Ala Phe Ser Val Val Gly Asn
 50 55 60

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

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

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

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

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

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

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

Ser Ser Gly Tyr Ser Asn Val Thr Val Trp
 180 185

<210> 17
 <211> 594
 <212> DNA
 <213> artificial nucleotide

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<220>

<223> xylA1C

<220>

<221> CDS

<222> (1)..(594)

<400> 17

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Met Ala Gln Thr Ile Cys Ser Asn Gln Thr Gly Thr Asn Asn Gly Tyr	
1 5 10 15	

ttc tac tcg ttc tgg aag gac acc ggg tcg gcg tgc atg aca ctg ggt	96
Phe Tyr Ser Phe Trp Lys Asp Thr Gly Ser Ala Cys Met Thr Leu Gly	
20 25 30	

tcc ggc ggc aac tac agc gtc aac tgg aac ctg ggt tcc ggg aac atg	144
Ser Gly Gly Asn Tyr Ser Val Asn Trp Asn Leu Gly Ser Gly Asn Met	
35 40 45	

gtc tgc ggc aaa ggc tgg agt acc gga tct tca agc cgc aga atc ggc	192
Val Cys Gly Lys Gly Trp Ser Thr Gly Ser Ser Ser Arg Arg Ile Gly	
50 55 60	

tac aac gcc ggc gtc tgg gcg ccg aac ggc aat gcc tac ctg act ctg	240
Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Ala Tyr Leu Thr Leu	
65 70 75 80	

tat ggg tgg acc agg aac ccg ctc atc gag tac tac gtg gtc gac agt	288
Tyr Gly Trp Thr Arg Asn Pro Leu Ile Glu Tyr Tyr Val Val Asp Ser	
85 90 95	

tgg gga agc tgg agg ccg cca ggc gga acc tcc gcg ggc acc gtc aat	336
Trp Gly Ser Trp Arg Pro Pro Gly Gly Thr Ser Ala Gly Thr Val Asn	
100 105 110	

agc gat ggc ggg acc tac aac ctc tat cgg acg cag cgg gtc aac gcg	384
Ser Asp Gly Gly Thr Tyr Asn Leu Tyr Arg Thr Gln Arg Val Asn Ala	
115 120 125	

cct tcc atc gac ggc acc ccg acg ttc tat cag tac tgg agt gtc ccg	432
Pro Ser Ile Asp Gly Thr Arg Thr Phe Tyr Gln Tyr Trp Ser Val Arg	
130 135 140	

acc tcg aag agg ccc acc ggg agc aac cag acc atc acc ttc gcg aac	480
Thr Ser Lys Arg Pro Thr Gly Ser Asn Gln Thr Ile Thr Phe Ala Asn	
145 150 155 160	

cac gtg aat gcg tgg agg agc aaa ggg tgg aat ctg ggg agt cac gtc	528
His Val Asn Ala Trp Arg Ser Lys Gly Trp Asn Leu Gly Ser His Val	
165 170 175	

tac cag ata atg gca aca gag gga tat caa agc agc ggg aat tcc aac	576
Tyr Gln Ile Met Ala Thr Glu Gly Tyr Gln Ser Ser Gly Asn Ser Asn	
180 185 190	

ctg acg gtg tgg gcg cag	594
Leu Thr Val Trp Ala Gln	
195	

<210> 18

<211> 198

<212> PRT

<213> artificial nucleotide

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<220>

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<400> 18

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Phe Tyr Ser Phe Trp Lys Asp Thr Gly Ser Ala Cys Met Thr Leu Gly
 20 25 30

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

Val Cys Gly Lys Gly Trp Ser Thr Gly Ser Ser Ser Arg Arg Ile Gly
 50 55 60

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

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

Trp Gly Ser Trp Arg Pro Pro Gly Gly Thr Ser Ala Gly Thr Val Asn
 100 105 110

Ser Asp Gly Gly Thr Tyr Asn Leu Tyr Arg Thr Gln Arg Val Asn Ala
 115 120 125

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

Thr Ser Lys Arg Pro Thr Gly Ser Asn Gln Thr Ile Thr Phe Ala Asn
 145 150 155 160

His Val Asn Ala Trp Arg Ser Lys Gly Trp Asn Leu Gly Ser His Val
 165 170 175

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

Leu Thr Val Trp Ala Gln
 195

<210> 19

<211> 972

<212> DNA

<213> artificial nucleotide

<220>

<223> xylA1D

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<221> CDS

<222> (1)..(972)

<400> 19

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tac acg ttc tgg aag gac tcg ggc agc gcc tcg atg acc ctc cat ccg	96
Tyr Thr Phe Trp Lys Asp Ser Gly Ser Ala Ser Met Thr Leu His Pro	
20 25 30	
ggc gga cgc tac agc tcc cag tgg acc agc aac acc aac aac tgg gtc	144
Gly Gly Arg Tyr Ser Ser Gln Trp Thr Ser Asn Thr Asn Asn Trp Val	
35 40 45	
ggc ggg aaa ggc tgg aat ccc ggt ggc ccg cgc gtg gtc aac tac tcg	192
Gly Gly Lys Gly Trp Asn Pro Gly Gly Pro Arg Val Val Asn Tyr Ser	
50 55 60	
ggc tac tac ggg gtc aac aac agc cag aac tcc tac ctg gcg ctg tac	240
Gly Tyr Tyr Gly Val Asn Asn Ser Gln Asn Ser Tyr Leu Ala Leu Tyr	
65 70 75 80	
ggc tgg acc cgc aat ccg ctg gtc gag tac tac gtg atc gag agc tac	288
Gly Trp Thr Arg Asn Pro Leu Val Glu Tyr Tyr Val Ile Glu Ser Tyr	
85 90 95	
ggc tcc tac aac ccg gcc agt tgc gcc ggc ggg gtg gac tac ggc agc	336
Gly Ser Tyr Asn Pro Ala Ser Cys Ala Gly Gly Val Asp Tyr Gly Ser	
100 105 110	
ttc cag agc gat ggc gcc acc tac aac gta cgc cgc tgc ctg cgc cag	384
Phe Gln Ser Asp Gly Ala Thr Tyr Asn Val Arg Arg Cys Leu Arg Gln	
115 120 125	
aac gcg ccg tcg atc gaa ggc aac aac agc acc ttc tac cag tac ttc	432
Asn Ala Pro Ser Ile Glu Gly Asn Asn Ser Thr Phe Tyr Gln Tyr Phe	
130 135 140	
agc gtg cgc aat ccc aag aag gga ttc ggc aac atc tcc ggc acg atc	480
Ser Val Arg Asn Pro Lys Lys Gly Phe Gly Asn Ile Ser Gly Thr Ile	
145 150 155 160	
acc gtc gcc aac cac ttc aac tac tgg gcc agc cgc ggc ctc aac ctc	528
Thr Val Ala Asn His Phe Asn Tyr Trp Ala Ser Arg Gly Leu Asn Leu	
165 170 175	
ggc aac cac gac tac atg gtg ttc gcc acc gag ggc tac cag agc cag	576
Gly Asn His Asp Tyr Met Val Phe Ala Thr Glu Gly Tyr Gln Ser Gln	
180 185 190	
ggc agc agc gac atc acc gtg agt tcg ggt acc ggc ggc ggc ggt ggc	624
Gly Ser Ser Asp Ile Thr Val Ser Ser Gly Thr Gly Gly Gly Gly Gly	
195 200 205	
ggc ggc aac acg ggc agc aag acc atc gtg gtg cgc gcg cgc ggc acc	672
Gly Gly Asn Thr Gly Ser Lys Thr Ile Val Val Arg Ala Arg Gly Thr	
210 215 220	
gcc gcc gga gag aac atc tcg ctc aag gtc aac aac gcc acc atc gcc	720
Ala Gly Gly Glu Asn Ile Ser Leu Lys Val Asn Asn Ala Thr Ile Ala	
225 230 235 240	
agc tgg acg ctc acc acc agc atg gcc aac tac acg gcc acc acc tcg	768
Ser Trp Thr Leu Thr Thr Ser Met Ala Asn Tyr Thr Ala Thr Thr Ser	
245 250 255	

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gca tcg ggc ggc tcg ctg gtg gag ttc acc aac gac ggc ggc aac cgc 816
 Ala Ser Gly Gly Ser Leu Val Glu Phe Thr Asn Asp Gly Gly Asn Arg
 260 265 270

gac gtg cag gtg gac tac ctc agc gtc aat ggc gcc gtc cgc cag gcc 864
 Asp Val Gln Val Asp Tyr Leu Ser Val Asn Gly Ala Val Arg Gln Ala
 275 280 285

gag gac cag acc tac aac acc ggc gtg tac cag aac ggc cag tgc ggc 912
 Glu Asp Gln Thr Tyr Asn Thr Gly Val Tyr Gln Asn Gly Gln Cys Gly
 290 295 300

ggc ggc aac ggc cgc agc gaa tgg ctg cac tgc aac ggt gcc atc ggc 960
 Gly Gly Asn Gly Arg Ser Glu Trp Leu His Cys Asn Gly Ala Ile Gly
 305 310 315 320

ttc gga aat ctc 972
 Phe Gly Asn Leu

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 <212> PRT
 <213> artificial nucleotide

<220>
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Tyr Thr Phe Trp Lys Asp Ser Gly Ser Ala Ser Met Thr Leu His Pro
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Gly Gly Arg Tyr Ser Ser Gln Trp Thr Ser Asn Thr Asn Asn Trp Val
 35 40 45

Gly Gly Lys Gly Trp Asn Pro Gly Gly Pro Arg Val Val Asn Tyr Ser
 50 55 60

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

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

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

Phe Gln Ser Asp Gly Ala Thr Tyr Asn Val Arg Arg Cys Leu Arg Gln
 115 120 125

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

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Ser Val Arg Asn Pro Lys Lys Gly Phe Gly Asn Ile Ser Gly Thr Ile
 145 150 155 160

Thr Val Ala Asn His Phe Asn Tyr Trp Ala Ser Arg Gly Leu Asn Leu
 165 170 175

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

Gly Ser Ser Asp Ile Thr Val Ser Ser Gly Thr Gly Gly Gly Gly
 195 200 205

Gly Gly Asn Thr Gly Ser Lys Thr Ile Val Val Arg Ala Arg Gly Thr
 210 215 220

Ala Gly Gly Glu Asn Ile Ser Leu Lys Val Asn Asn Ala Thr Ile Ala
 225 230 235 240

Ser Trp Thr Leu Thr Thr Ser Met Ala Asn Tyr Thr Ala Thr Thr Ser
 245 250 255

Ala Ser Gly Gly Ser Leu Val Glu Phe Thr Asn Asp Gly Gly Asn Arg
 260 265 270

Asp Val Gln Val Asp Tyr Leu Ser Val Asn Gly Ala Val Arg Gln Ala
 275 280 285

Glu Asp Gln Thr Tyr Asn Thr Gly Val Tyr Gln Asn Gly Gln Cys Gly
 290 295 300

Gly Gly Asn Gly Arg Ser Glu Trp Leu His Cys Asn Gly Ala Ile Gly
 305 310 315 320

Phe Gly Asn Leu

<210> 21
 <211> 978
 <212> DNA
 <213> artificial nucleotide

<220>
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<220>
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 <222> (1)..(978)

<400> 21
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 Met Ala Gln Thr Cys Ile Thr Ser Ser Gln Thr Gly Thr Asn Asn Gly
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48

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aat	tac	ttt	tcg	ttc	tgg	aaa	gac	agt	ccg	ggc	acg	gtg	aac	ttc	tgc	
Asn	Tyr	Phe	Ser	Phe	Trp	Lys	Asp	Ser	Pro	Gly	Thr	Val	Asn	Phe	Cys	96
			20					25					30			
atg	tat	gcg	aat	ggc	cgc	tat	acc	tcc	aac	tgg	agc	ggc	atc	aac	aac	144
Met	Tyr	Ala	Asn	Gly	Arg	Tyr	Thr	Ser	Asn	Trp	Ser	Gly	Ile	Asn	Asn	
		35					40					45				
tgg	gtg	ggc	ggc	aag	ggc	tgg	gct	acc	ggc	tcc	agc	cac	acg	atc	agc	192
Trp	Val	Gly	Gly	Lys	Gly	Trp	Ala	Thr	Gly	Ser	Ser	His	Thr	Ile	Ser	
	50					55					60					
tac	tcc	ggc	acg	ttc	aat	tcg	ccg	ggc	aac	ggg	tac	ctg	gcc	ctg	tat	240
Tyr	Ser	Gly	Thr	Phe	Asn	Ser	Pro	Gly	Asn	Gly	Tyr	Leu	Ala	Leu	Tyr	
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ggc	tgg	acc	acc	aat	cca	ttg	gtc	gag	tac	tac	atc	gtc	gac	agc	tgg	288
Gly	Trp	Thr	Thr	Asn	Pro	Leu	Val	Glu	Tyr	Tyr	Ile	Val	Asp	Ser	Trp	
				85					90					95		
ggg	acc	tac	cgt	ccg	ccg	ggc	ggc	cag	ggg	ttc	atg	ggc	acg	gta	gtt	336
Gly	Thr	Tyr	Arg	Pro	Pro	Gly	Gly	Gln	Gly	Phe	Met	Gly	Thr	Val	Val	
			100					105					110			
agc	gac	ggg	ggc	acg	tac	gac	gtg	tac	cgg	acg	caa	cgc	gtg	aac	cag	384
Ser	Asp	Gly	Gly	Thr	Tyr	Asp	Val	Tyr	Arg	Thr	Gln	Arg	Val	Asn	Gln	
		115					120					125				
cca	tcc	atc	atc	ggc	aac	gcc	acg	ttc	tac	cag	tac	tgg	agc	gtg	cgg	432
Pro	Ser	Ile	Ile	Gly	Asn	Ala	Thr	Phe	Tyr	Gln	Tyr	Trp	Ser	Val	Arg	
	130					135					140					
cag	tcg	aag	cgc	gtg	ggc	ggc	acc	atc	acc	atc	gcc	aac	cat	ttc	aac	480
Gln	Ser	Lys	Arg	Val	Gly	Gly	Thr	Ile	Thr	Ile	Ala	Asn	His	Phe	Asn	
145					150					155					160	
gcc	tgg	gcc	acg	ctg	ggc	atg	aac	ctg	ggc	cag	cac	aac	tac	cag	gtc	528
Ala	Trp	Ala	Thr	Leu	Gly	Met	Asn	Leu	Gly	Gln	His	Asn	Tyr	Gln	Val	
				165					170					175		
atg	gcc	acc	gag	ggg	tac	cag	agc	agc	ggc	agc	tcc	gac	atc	acc	gtg	576
Met	Ala	Thr	Glu	Gly	Tyr	Gln	Ser	Ser	Gly	Ser	Ser	Asp	Ile	Thr	Val	
			180					185					190			
acc	gaa	ggg	ggc	ggc	agc	tcc	tcg	tcg	tcc	tcg	ggc	ggc	ggc	agc	acc	624
Thr	Glu	Gly	Gly	Gly	Ser	Ser	Ser	Ser	Ser	Ser	Gly	Gly	Gly	Ser	Thr	
		195					200					205				
agc	agc	agt	ggg	ggc	ggc	ggc	aac	aag	agc	ttc	acg	gtg	cgt	gcg	cgc	672
Ser	Ser	Ser	Gly	Gly	Gly	Gly	Asn	Lys	Ser	Phe	Thr	Val	Arg	Ala	Arg	
	210					215					220					
ggc	acg	gcc	gga	ggc	gag	aac	atc	cag	ctg	cag	gtg	aac	aac	cag	acg	720
Gly	Thr	Ala	Gly	Gly	Glu	Asn	Ile	Gln	Leu	Gln	Val	Asn	Asn	Gln	Thr	
225					230					235					240	
gtc	gcg	agc	tgg	aac	ctc	acc	acc	agc	atg	cag	aac	tac	acc	gcc	tcg	768
Val	Ala	Ser	Trp	Asn	Leu	Thr	Thr	Ser	Met	Gln	Asn	Tyr	Thr	Ala	Ser	
				245					250					255		
acc	agc	ctg	agc	ggc	ggc	atc	acc	gtg	ctc	tac	acc	aac	gac	ggc	ggc	816
Thr	Ser	Leu	Ser	Gly	Gly	Ile	Thr	Val	Leu	Tyr	Thr	Asn	Asp	Gly	Gly	
			260					265					270			
agc	cgc	gac	gtg	cag	gtg	gac	tac	atc	atc	gtg	aac	ggc	cag	acc	cgc	864
Ser	Arg	Asp	Val	Gln	Val	Asp	Tyr	Ile	Ile	Val	Asn	Gly	Gln	Thr	Arg	
		275					280					285				

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cag tcc gaa gcg cag agc tac aac acc ggg ttg tat gcg aat gga cgc 912
 Gln Ser Glu Ala Gln Ser Tyr Asn Thr Gly Leu Tyr Ala Asn Gly Arg
 290 295 300

tgc ggc ggt ggc tcg aac agc gag tgg atg cat tgc aac ggc gcg atc 960
 Cys Gly Gly Gly Ser Asn Ser Glu Trp Met His Cys Asn Gly Ala Ile
 305 310 315 320

ggc tac ggc aat acg ccc 978
 Gly Tyr Gly Asn Thr Pro
 325

<210> 22
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<220>
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<400> 22

Met Ala Gln Thr Cys Ile Thr Ser Ser Gln Thr Gly Thr Asn Asn Gly
 1 5 10 15

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

Met Tyr Ala Asn Gly Arg Tyr Thr Ser Asn Trp Ser Gly Ile Asn Asn
 35 40 45

Trp Val Gly Gly Lys Gly Trp Ala Thr Gly Ser Ser His Thr Ile Ser
 50 55 60

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

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

Gly Thr Tyr Arg Pro Pro Gly Gly Gln Gly Phe Met Gly Thr Val Val
 100 105 110

Ser Asp Gly Gly Thr Tyr Asp Val Tyr Arg Thr Gln Arg Val Asn Gln
 115 120 125

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

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

Ala Trp Ala Thr Leu Gly Met Asn Leu Gly Gln His Asn Tyr Gln Val
 165 170 175

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

Thr Glu Gly Gly Gly Ser Ser Ser Ser Ser Gly Gly Gly Ser Thr
 195 200 205

Ser Ser Ser Gly Gly Gly Gly Asn Lys Ser Phe Thr Val Arg Ala Arg
 210 215 220

Gly Thr Ala Gly Gly Glu Asn Ile Gln Leu Gln Val Asn Asn Gln Thr
 225 230 235 240

Val Ala Ser Trp Asn Leu Thr Thr Ser Met Gln Asn Tyr Thr Ala Ser
 245 250 255

Thr Ser Leu Ser Gly Gly Ile Thr Val Leu Tyr Thr Asn Asp Gly Gly
 260 265 270

Ser Arg Asp Val Gln Val Asp Tyr Ile Ile Val Asn Gly Gln Thr Arg
 275 280 285

Gln Ser Glu Ala Gln Ser Tyr Asn Thr Gly Leu Tyr Ala Asn Gly Arg
 290 295 300

Cys Gly Gly Gly Ser Asn Ser Glu Trp Met His Cys Asn Gly Ala Ile
 305 310 315 320

Gly Tyr Gly Asn Thr Pro
 325

<210> 23
 <211> 36
 <212> DNA
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 <222> (1)..(36)

<400> 23
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36

<210> 24
 <211> 36
 <212> DNA
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<220>
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<220>
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<400> 24
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<210> 25
<211> 37
<212> DNA
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<220>
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<222> (1)..(37)

<400> 25
ggggccggga attccgatga gatttccttc aattttt 37

<210> 26
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<220>
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<222> (1)..(45)

<400> 26
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<222> (1)..(21)

<400> 27
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<210> 28
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<220>
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<222> (1)..(24)

<400> 28
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24

<210> 29
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<222> (1)..(28)

<400> 29
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28

<210> 30
<211> 27
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<222> (1)..(27)

<400> 30
ttaccaatgc ttaatcagtg aggcacc

27

<210> 31
<211> 25
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<400> 31
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25

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<400> 32
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25

<210> 33
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 agatttagaa ggggatttcg atgttgctgt tttgccattt tccaacagca caaataacgg 720
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 tattggaact aataatacca taactttcag taatcatgtt aacgcttgga agtcaaaagg 1260
 tatgaacttg gggtcctcct ggtcctacca agttttggca actgaggggtt accaatctag 1320

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aagaggatgt cagaatgcca ttgacctgag agatgcaggc ttcatttttg atactttttt	1500
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tggcaactgc acagcttggg tgtgaacagc atcttcaatt ctggcaagct ccttgtctgt	2580
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gtctgaggca acgaaatctg gatcagcgta tttatcagca ataactagaa cttcagaagg	2700
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