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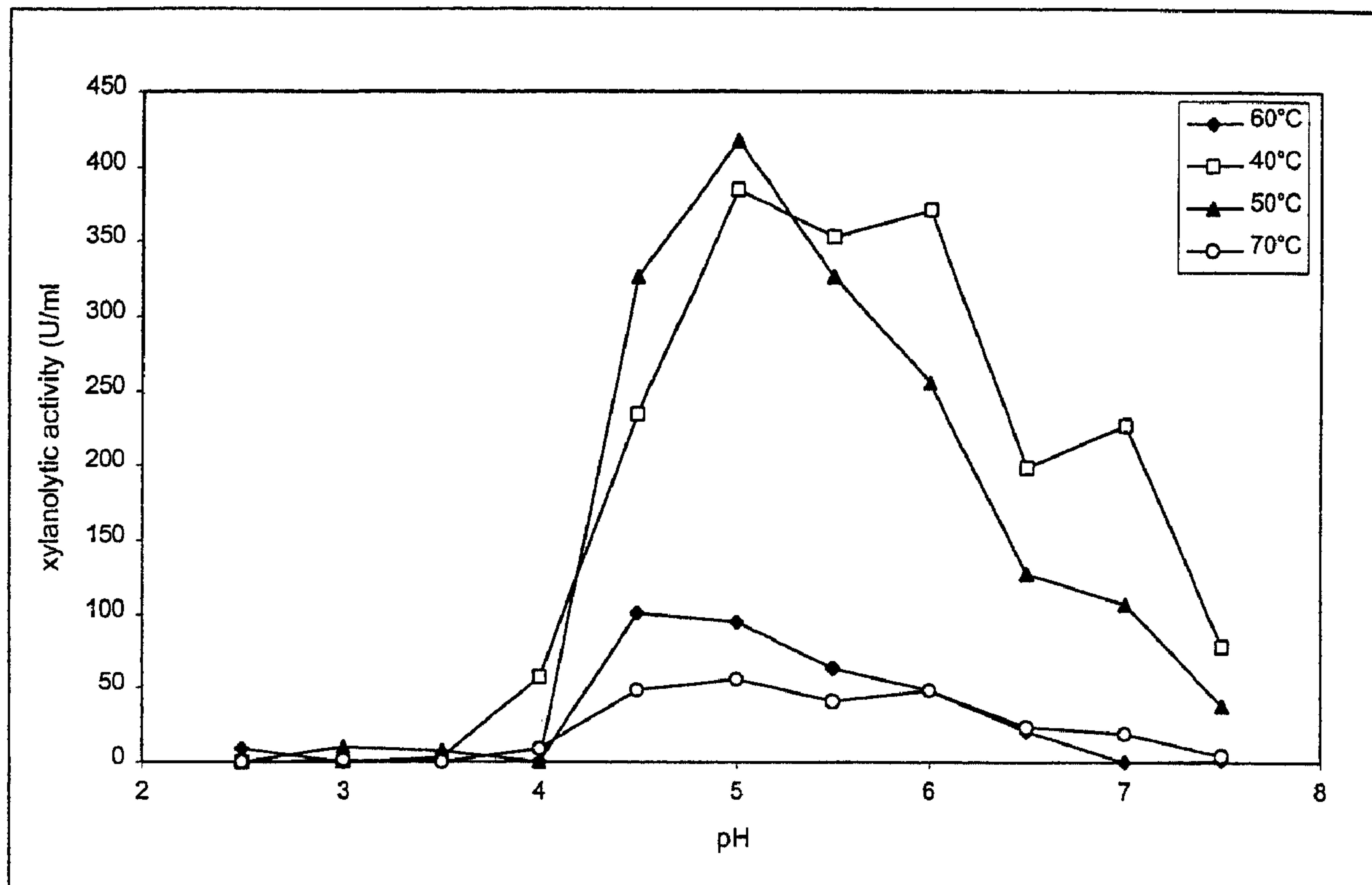
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(57) Abrégé/Abstract:

The present invention is related to an isolated and purified enzyme with xylanolytic activity having more than 70% homology with the amino acid sequence SEQ ID NO 11.

30

ABSTRACT

ENZYME WITH XYLANASE ACTIVITY

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The present invention is related to an isolated and purified enzyme with xylanolytic activity having more than 70% homology with the amino acid sequence SEQ ID NO 11.

10

(Figure 4)

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Xylanase

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ENZYME WITH XYLANASE ACTIVITY

Field of the invention

10 [0001] The present invention relates to an enzyme with xylanase activity identified by its amino acid and nucleotide sequences and variants thereof.

[0002] The present invention relates also to their uses in the agrofood and in the pulp and paper industries.

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Background of the invention

[0003] Xylans are heteropolysaccharides which form the major part of the hemicellulose present in the plant biomass.

20 [0004] The backbone of these polysaccharides is a chain of β 1-4 linked xylopyranosyl residues. Many different side groups could bind to these residues like acetyl, arabinosyl and glucuronosyl residues. Phenolic compounds such as ferulic or hydroxycinnamic acids are also 25 involved through ester binding in the cross linking of the xylan chains or in the linkage between xylan and lignin chains for example.

[0005] Endoxylanases hydrolyse specifically the backbone of the hemicellulose. In some cases, the side 30 groups may mask the main chain by steric hindrance. Different xylanase activities already described are characterised by their specificity towards their substrate and the length of the oligomers produced.

[0006] These differences between the xylanases concerning their properties seem to be partly related to their respective amino acid sequences. Endoxylanases have been classified into two families (F or 10 and G or 11) 5 according to their sequence similarities (Henrissat & Bairoch, Biochem. J., Vol. 293, p. 781 (1993)). The F family of xylanases are larger, more complex as compared to the G family of xylanases. Moreover the F family xylanases produce small oligosaccharides, while the G 10 family xylanases show a higher affinity for unsubstituted xylan.

[0007] Xylanases are used in various industrial areas such as the pulp, paper, feed and bakery industries. Other applications lie in the juice and beer sectors. 15 Xylanases could also be used in the wheat separation process. The observed technological effects are, among others, improved bleachability of the pulp, decreased viscosity of the feed or changes in dough characteristics.

[0008] Many different microbial genera have been 20 described to produce one or several xylanases. These microbial genera comprise bacteria as well as eukaryotic organisms like yeast or fungi.

Detailed description of the invention

25 [0009] A first aspect of the present invention is related to an isolated and purified (from possible contaminants) xylanase amino acid sequence presenting more than 50%, preferably more than 70, 80 or 85%, more preferably more than 90% homology (or sequence identity) 30 with the amino acid sequence SEQ ID NO 11.

[0010] Advantageously, the isolated and purified xylanase amino acid sequence according to the invention has a molecular weight comprised between 22 and 26 kD, preferably a molecular weight about 24 kD.

[0011] Said xylanase amino acid sequence or peptide is extra-cellular or intra-cellular expressed and/or secreted by the recombinant host cell according to the invention.

5 [0012] According to another preferred embodiment of the present invention, the isolated and purified xylanase amino acid sequence has the amino acid sequence of SEQ ID NO 11 or a smaller portion of said amino acid sequence (of more than 30 or 50 amino-acids, preferably more than 100 10 amino-acids), which has at least more than 80% of the xylanase activity of the complete amino acid sequence SEQ ID NO 11, preferably more than 95% of the xylanase activity or the complete xylanase activity of the complete amino acid sequence SEQ ID NO 11 (see also example 1). In other 15 words, the isolated and purified xylanase amino acid sequence according to the invention may be deleted partially while maintaining its enzymatic activity, which may be measured by methods well known by the person skilled in the art.

20 [0013] The purified xylanase enzyme according to the invention is also characterised by an optimum pH around pH 5.0 and temperature profile having its maximum activity at about 50 °C. More generally, the maximum activity of the enzyme is comprised between pH 4.5 and 7.0, at a 25 temperature comprised between 35 and 55 °C (see Fig. 4).

[0014] The present invention is also related to an isolated and purified nucleotidic sequence from a micro-organism origin, encoding a xylanase. Preferably, said micro-organism is selected from the group consisting 30 of bacteria or fungi (including yeast), preferably the *Penicillium* species fungi, more specifically the *Penicillium griseofulvum*.

[0015] According to a preferred embodiment of the present invention, said micro-organism is *Penicillium griseofulvum* having the deposit number MUCL-41920.

[0016] According to the invention, said nucleotide sequence presents more than 50%, preferably more than 70%, more preferably more than 90% homology (or sequence identity) with the sequence SEQ ID NO 8 described hereafter.

[0017] According to a preferred embodiment of the present invention, said isolated and purified nucleotide sequence corresponds to the nucleotide sequence SEQ ID NO 8 or a portion thereof encoding a peptide having a xylanase activity.

[0018] It is meant by "a portion of the nucleotide sequence SEQ ID NO 8", a fragment of said sequence SEQ ID NO 8 having more than 90 nucleotides, preferably more than 100 nucleotides or more than 120 nucleotides, of said nucleotide sequence and encoding a protein characterised by a xylanase enzymatic activity similar to the xylanase activity of the complete amino-acid sequence SEQ ID NO 11. Preferably, said portion has a xylanase enzymatic activity of more than 80% of the initial xylanase enzymatic activity of the complete enzyme defined by its amino acid sequence SEQ ID NO 11, preferably has a xylanase enzymatic activity corresponding the one of the amino acid sequence SEQ ID NO 11.

[0019] Another aspect of the present invention is related to a recombinant nucleotide sequence comprising, operably linked to the nucleotide sequence according to the invention and above-described, one or more adjacent regulatory sequence(s), preferably originating from homologous micro-organisms.

[0020] However, said adjacent regulatory sequences may also be originating from heterologous micro-organisms.

[0021] These adjacent regulatory sequences are specific sequences such as promoters, secretion signal sequences and terminators.

[0022] Another aspect of the present invention is 5 related to the vector comprising the nucleotide sequence(s) according to the invention, possibly operably linked to one or more adjacent regulatory sequence(s) originating from homologous or from heterologous micro-organisms.

[0023] It is meant by "a vector", any biochemical 10 construct which may be used for the introduction of a nucleotide sequence (by transduction, transfection, transformation, infection, conjugation, etc.) into a cell. Advantageously, the vector according to the invention is selected from the group consisting of plasmids, viruses, 15 phagemids, chromosomes, transposons, liposomes, cationic vesicles or a mixture thereof. Said vector may comprise already one or more of the above-described adjacent regulatory sequence(s) (able to allow its expression and its transcription into a corresponding peptide by said 20 micro-organism). Preferably, said vector is a plasmid incorporated in *E. Coli* and having the deposit number LMBP-39987.

[0024] The present invention is also related to the host cell, preferably a recombinant host cell, 25 "transformed" by the nucleotide sequence or the vector according to the invention above-described.

[0025] It is meant by "a host cell "transformed" by the nucleotide sequence or the vector according to the invention", a cell having incorporated said nucleotide 30 sequence or said vector and which does not comprise naturally (originally) said nucleotide sequence. The transformed host cell may be also a cell having incorporated said vector or said nucleotide sequence by

genetic transformation, preferably by homologous recombination or other method (recombinant microorganism).

[0026] A "host cell" may be also the original cell comprising the nucleotide sequence encoding the enzyme according to the invention and genetically modified (recombinant host cell) to overexpress or express more efficiently said enzyme (better pH profile, higher extra cellular expression,...).

[0027] Preferably, said host cell is also capable of overexpressing (higher expression than the expression observed in the initial microorganism) said nucleotide sequence or said vector and allows advantageously a high production of an amino acid sequence encoded by said nucleotide sequence or by said vector. The isolated and purified nucleotide sequence according to the invention may be either integrated into the genome of the selected host cell or present on an episomal vector in said host cell.

[0028] Advantageously, the recombinant host cell according to the invention is selected from the group consisting of the microbial world, preferably bacteria or fungi, including yeast.

[0029] Preferably, said recombinant host cell is modified to obtain an expression of the xylanase enzyme at high level obtained by the use of adjacent regulatory sequences being capable of directing the overexpression of the nucleotide sequence according to the invention in the recombinant host cell or by increasing the number of nucleotide copies of the sequences according to the invention.

[0030] The following description describes also the conditions (culture media, temperature and pH conditions, etc.) for the culture of the host selected for the expression of the xylanase according to the invention. For this purpose, the original production species and/or a

suitable host cell transformed with a DNA construct designed to express the said enzyme are present in a suitable growth medium.

[0031] According to the present invention, said 5 protein with xylanolytic activity may be isolated from the medium and/or purified. The culture, isolation and purification conditions are derived from conventional methods well-known to persons skilled in the art.

[0032] The xylanase enzyme according to the 10 invention may be used in different kinds of industries.

[0033] The enzyme with xylanolytic activity of the present invention, purified or not purified, is particularly suited as a bread-improving agent. Bread-improving agents are products which could improve or 15 increase texture, flavour, anti-staling effect, softness, crumb softness upon storage, freshness and machinability, volume of a dough and/or of a final baked product. Preferably, said enzyme with xylanolytic activity increases the specific volume of the final baked product.

20 [0034] "Baked product" intends to include any product prepared from dough, in particular a bread product. Dough is obtained from any type of flour or meal (e.g. based on rye, barley, oat or maize), preferably prepared with wheat or with mixes including wheat.

25 [0035] A further aspect of the present invention relates to the additive effect of said enzyme having xylanolytic activity with other enzymes, in particular with an alpha-amylase, preferably an alpha-amylase from *Aspergillus oryzae*. Said enzyme with xylanolytic activity 30 may be used in combination with other bread-improving agents like enzymes, emulsifiers, oxidants, milk powder, fats, sugars, amino acids, salts, proteins (gluten, cellulose binding site) well known to the person skilled in the art.

[0036] According to the present invention, the enzyme with xylanolytic activity, purified or not, shows hydrolytic activities in presence of plant cell wall components. Particularly, said enzyme degrades the wheat 5 cell wall components. Particularly, the degradation activities lead to a decrease of the flour viscosity in the presence of water. Said enzyme may thus advantageously be used in the separation of components of plant cell materials such as cereal components. Particularly, said 10 enzyme may be used to improve the separation of the wheat into gluten and starch by the so-called batter process.

[0037] According to the present invention, said enzyme may be used to improve the filtrability and/or decrease the viscosity of glucose syrups obtained from 15 impure cereal starch by subjecting the impure starch first to the action of an alpha-amylase, then to the action of said xylanase. It may also be used in beer brewing when cereal has to be degraded to improve the filtrability of the wort or to reuse the residuals from beer production for 20 e.g. animal feed. Said enzyme may be used in feed to improve the growth rate or the feed conversion ratio of animals such as poultry.

[0038] Another application resides in the oil extraction where oil has to be extracted from the plant 25 material such as the corn oil from corn embryos. The enzyme with xylanolytic activity of the present invention may be used in fruit and vegetable juice processing to improve the yield. According to the present invention, said enzyme may be used in all processes involving plant materials or waste 30 materials, e.g. from paper production, or agricultural wastes such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet, and the like.

[0039] The effect of the enzyme with xylanolytic activity of the present invention may be further improved by adding other enzymes in combination with said enzyme. Such enzymes may belong but are not restricted to 5 hydrolytic enzymes families such as glucanases, proteases, cellulases, hemicellulases, pectinases. Other enzymes are transglutaminases, oxido-reductases, isomerases, etc.

[0040] The enzyme with xylanolytic activity according to the invention may be used under several forms. 10 Cells expressing the enzyme, such as yeast, fungi, archaea bacteria or bacteria, may be used directly in the process. Said enzyme may be used as a cell extract, a cell-free extract (i.e. portions of the host cell that has been submitted to one or more disruption, centrifugation and/or 15 extraction steps) or as a purified protein. Any of the above-described forms may be used in combination with one or more other enzyme(s) under any of the above-described forms. These whole cells, cell extracts, cell-free extracts or purified enzymes may be immobilised by any conventional 20 means on a solid support to allow protection of the enzyme, continuous hydrolysis of substrate and/or recycling of the enzymatic preparation. Said cells, cell extracts, cell-free extracts or enzymes may be mixed with different ingredients (e.g. in the form of a dry powder or a granulate, in 25 particular a non-dusting granulate, in a form of a liquid, for example with stabilizers such as polyols, sugars, organic acids, sugar alcohols according to well-established methods).

[0041] The invention will be described in further 30 details in the following examples by reference to the enclosed drawings, without limiting its scope.

Brief description of the drawings

[0042] Figure 1 shows a SDS-polyacrylamide gel of the proteins recovered after the successive purification steps of the enzyme with xylanolytic activity.

5 [0043] Figure 2 show a Southern blot analysis of the *Penicillium griseofulvum* A160 genomic DNA.

[0044] Figure 3 represents the complete genetic sequence of the xylanase according to the invention.

10 [0045] Figure 4 shows the effect of the temperature and of the pH on the xylanase activity.

[0046] Figure 5 represents the increase of a bread volume according to the enzymatic activity of the xylanase according to the invention.

15 Example 1: Purification of an enzyme with xylanolytic activity from *Penicillium griseofulvum* A160

Strain

[0047] 5 g of a commercial Belgian wheat flour were suspended in 50 ml saline solution (NaCl 0.9%). Aliquots of 20 100 μ l of this suspension were spread on AMAM plates (Aspergillus Minimum Agar Medium: glucose 1%, NaNO₃ 0.6%, KCl 7 mM, KH₂PO₄ 11 mM, MgSO₄ 2 mM, ZnSO₄ 76 μ M, H₃BO₃ 178 μ M, MnCl₂ 25 μ M, FeSO₄ 18 μ M, CoCl₂ 7.1 μ M CuSO₄, 6.4 μ M, Na₂MoO₄ 6.2 μ M, EDTA 174 μ M, pH 6.5 (Pontecorvo et al., 25 1953, Adv. Genet. Vol 5, p 142) supplemented with 1.5% bacto-agar and 100 μ g/ml ampicilline.

[0048] Among the strains that appeared on the plates after incubation at 30 °C, a particular strain was isolated and identified as *Penicillium griseofulvum* with the 30 isolation reference A160 (MUCL-41920).

Determination of the xylanolytic activity

[0049] The xylanolytic activity was determined by measuring the reducing sugars formed from the Beechwood xylan (Sigma). The reducing sugars were revealed with the 5 2,3-dinitrosalicylic acid (Bailey et al., J. Biotechnol. Vol 23, p 257 (1992)). The reaction was carried out at 30°C in a 100 mM acetate buffer at pH 4.5. The xylanolytic activity was expressed in µmole xylose/min.

[0050] For rapid identification of the enzyme, the 10 xylanolytic activity was assayed using Azo-xylan (Megazyme) as substrate following the supplier instructions with the exception that the reaction was carried out at 35°C in a 100 mM citrate-phosphate buffer at pH 6.0.

[0051] In this case, one xylanase unit was 15 arbitrarily defined as the amount of enzyme required to increase the optical density by one unit at 595 nm in 10 min.

Purification of the xylanolytic enzyme

20 [0052] The strain of *Penicillium griseofulvum* A160 was cultivated in 2 litres of Aspergillus Minimal Medium pH 6.5 (Ponteverco et al. (1953)), supplemented with 1% xylan from oat spelt (Sigma) at 30°C. After 72 hours, the culture was filtered through a Miracloth filter (Calbiochem) to 25 remove the mycelium. The filtrate was concentrated by ultrafiltration in a Pellicon device with a 10kDa Biomax 10 cassette (Millipore) to a final volume of 170 ml. The concentrate was diluted 3 times to reach a final concentration of 50 mM in sodium acetate pH 4.2.

30 [0053] This solution was loaded at 2 ml/min on a Pharmacia XK16/20 column filled with 30 ml of the Bio-Rad Macro High S resin equilibrated in 50 mM sodium acetate pH 4.2. Proteins were eluted with a linear increasing NaCl

gradient from 0 to 0.6 M in 50 mM sodium acetate pH 4.2. Xylanase activity was determined in the eluted fractions. Active fractions were pooled and equilibrated in 1.2 M ammonium sulfate, 50 mM Na acetate pH 5.0 in a final volume 5 of 65 ml.

[0054] These were applied on a Phenyl Sepharose HP column (Pharmacia) and eluted at 2.5 ml/min with a 1.2M-0M ammonium sulfate linear gradient in a 50 mM Na acetate pH 5.0 buffer. Xylanase activity was determined in the eluted 10 fractions. The xylanase activity was collected as one peak at 0.8 M ammonium sulfate.

[0055] One major protein is present in this peak as shown by SDS-polyacrylamide gel (Figure 1).

15 Example 2: Determination of the amino acid sequence of the enzyme with xylanolytic activity

[0056] General procedures were followed to perform the N-terminal sequencing of the protein after electrophoresis on a 12% SDS-polyacrylamide gel and 20 electroblotting on a PVDF Immobilon-P membrane (Millipore). An automatic 477A Protein Sequencer coupled to a HPLC 120A Analyser (Applied Biosystem) was used.

[0057] The following sequence has been obtained with the protein with an apparent molecular weight of 24 kDa:

25 SEQ ID NO 1:

D I T Q N E R G T N N G Y F Y S F W T X G G G N V Y

Example 3: Cloning of a gene coding for a enzyme with xylanolytic activity

30

Cloning of internal DNA fragments

[0058] The genomic DNA from *Penicillium griseofulvum* A160 was isolated according to Boel et al. (EMBO J., vol 7, p. 1581 (1984)). The strain was grown in 50 ml *Aspergillus*

Minimum Medium supplemented with 0.5% Yeast Extract (Difco). After 24 hours, the mycelium was harvested by filtration on a Miracloth filter and washed twice with water. 1 g mycelium was incubated in 10 ml solution A (sorbitol 1 M, EDTA 25 mM, pH 8.0) for 30 min at 30°C. The cells were then centrifuged and suspended in 10 ml solution B (Novozym 234 20 mg, sorbitol 1 M, Na citrate 0.1 M, EDTA 10 mM, pH 5.8). After 30 min at 30°C, the cells were centrifuged and lysed with 15 ml of solution C (phenol 40%, SDS 1%). DNA was separated from the contaminating material by successive extractions with phenol and phenol-chloroform, followed by ethanol precipitation.

[0059] The degenerated synthetic oligonucleotides mixtures SEQ ID NO 2 and SEQ ID NO 3 were designed based on the N-terminal sequence. A third synthetic oligonucleotides mixture SEQ ID NO 4 has been designed based on a hypothetical degenerated sequence coding for the amino acid sequence EYYIVD, conserved among the family G xylanases.

SEQ ID NO 2: GGY TAY TTY TAY AAY TTY TGG AC
 20 SEQ ID NO 3: GGY TAY TAY TAY TCI TTY TGG AC ~
SEQ ID NO 4: TCG ACR AYG TAG TAY TC

[0060] In these sequences, Y stands for T or C, R for A or G, I for inosine.

[0061] The PCR reaction was carried out with 10 ng gDNA of *Penicillium griseofulvum* A160 in the presence of 5 pmole of each synthetic oligonucleotides mixture SEQ ID NO 2 and SEQ ID NO 3 and 10 pmole synthetic oligonucleotides mixture SEQ ID NO 4. The reaction mix contained also 1 unit rTAQ polymerase (Pharmacia), 200µM dNTP, 50 mM KCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl pH 9.0 in a final volume of 25 µl. After 4 min of denaturation at 94°C, 25 cycles of [30 s 94°C, 30 s 50°C and 45 s 72 °C] were performed followed by 7 min of elongation at 72°C. Only one fragment of 0.3 kb

length was amplified as revealed by agarose gel electrophoresis.

[0062] 1 μ l of the PCR reaction described above was directly sequenced on a ABI 377 sequencer (Applied Biosystem) with either 3 pmoles synthetic oligonucleotides mixtures SEQ ID NO 2 or SEQ ID NO 3 as primers. The sequencing with the oligonucleotides SEQ ID NO 2 and SEQ ID NO 3 gave the nucleotide sequences SEQ ID NO 5 and SEQ ID NO 6, respectively.

10

SEQ ID NO 5:

CNAGTACAACACAGNNAAGNCCGGCNAATACAGNGTGNANTGGAAGAACTGCGGNTATT
TCACCTCTGGCAAGGGCTGGANNACTGGTAGNGCCGGTAAGT

SEQ ID NO 6:

15 CGGCNAATACAAGGGTGTNANTGGAAGAACTGCGGNNATTCACCTCNGGCAAGGGCTG
GACTACTGGTAGTGCCCCGGTAAGTGCAA

[0063] An homology search with those sequences against the NCBI proteins database (05-jan-99) using the BLASTX 2.0.8 software found the best homology with the 20 endo- β 1,4-xylanase A from *Chaetomium* (accession number: dbj|BAA08649).

Southern blotting of the *Penicillium griseofulvum* A160 genomic DNA

25 [0064] Genomic DNA (0.5 μ g) was digested overnight at 37°C with either 2 units of the restriction enzyme EcoRI (Pharmacia), or 2 units of each restriction enzymes BamHI and EcoRI (Pharmacia), or 2 units of each restriction enzymes EcoRI and XbaI in a final volume of 20 μ l (buffer: 1 30 x One-Phor-All buffer PLUS (Pharmacia)). The digested DNAs were loaded on a 0.8% agarose gel in 1 x TBE buffer. After electrophoresis, the restricted fragments were transferred onto a Hybond-N+ membrane (Amersham). The PCR fragments

described above (1 μ l) were labelled with digoxigenin using the DIG High Prime DNA Labelling and Detection Starter Kit II (Boehringer Mannheim). The membrane was hybridised overnight at 42°C in the presence of a standard 5 hybridisation buffer (SSC 5 x, formamide 50%, N-lauroylsarcosine 0.1%, SDS 0.02%, Blocking reagent) and a probe concentration of ca. 10 ng/ml (denatured during 5 min at 97°C). After the hybridisation, the membrane was first washed at 55°C with 2 x SSC, 0.1% SDS (2 x 15 min) followed 10 with 3 washes with a 0.5x SSC, 0.1% SDS solution (30 min). After immunological detection, the hybridising bands were identified by a 4 hours exposition to a Kodak X-OMAT AR film at room temperature.

[0065] The results of the hybridisation experiment 15 are shown on the figure 2. It revealed that under the hybridisation conditions tested, one DNA fragment hybridised with the probe.

Construction of a gDNA restriction fragments library of
20 *Penicillium griseofulvum* A160

[0066] Genomic DNA (5 μ g) was digested overnight at 37°C with 10 units of each restriction enzyme EcoRI and BamHI (Pharmacia) in a final volume of 100 μ l. The restriction fragments were separated by electrophoresis on 25 a 0.8% agarose gel, 1 x TBE. A piece of the gel corresponding to fragments between 3.5kb and 2.5 kb in length was cutted off. The DNA was purified out of this piece of agarose gel using the Qiaquick gene extraction kit (QIAGEN) in a final volume of 30 μ l.

30 [0067] The purified fragments were inserted by ligation between the EcoRI and BamHI restriction sites of the pBluescript II SK(+) vector (Stratagene). 1 μ g of pBluescript SK(+) plasmid DNA was first digested with 5 units each of EcoRI and BamHI restriction enzymes

(Pharmacia) in 50 μ l (37°C, 16 h) and subsequently purified from both enzymes using the Qiaquick gene extraction kit. The ligation was performed using 3 μ l of purified genomic DNA fragments, 0.25 μ g of digested pBluescript SK(+) DNA, 3 5 units of T4 DNA ligase (Pharmacia), 1 mM ATP in a final volume of 30 μ l (1 x One-Phor-All buffer PLUS, 16°C, 16 h). The ligation mixture was then dialysed on a VSWP 013 membrane (Millipore) against water during 20 min. 1 μ l of this mixture was electroporated into 40 μ l electrocompetent 10 *Escherichia coli* DH10b cells (BRL-Gibco) according to the BRL-Gibco protocol. After electroporation, cells were plated on LB plates supplemented with 100 μ g/ml ampicillin to select the transformed cells.

[0068] The above-described library was screened 15 progressively using PCR reactions on pools of transformants of decreasing sizes. The PCR reaction conditions were the same as described above with the exception that the template DNA was the plasmids from the pooled *Escherichia coli* transformants purified from 3 ml cultures with the 20 High Pure Plasmid Isolation Kit (Boehringer Mannheim). A 0.3 kb fragment was amplified in one clone out of ca. 1000 clones analysed. The plasmid (LMBP-39187) recovered from this clone (pPGXYNA) contained one EcoRI-BamHI insert of 3 kb length. A partial sequence of the pPGXYNA plasmid 25 comprising the xylanolytic enzyme coding sequence was determined on both strands by primer walking using among others the oligonucleotide with the sequence SEQ ID NO 7 as primer.

SEQ ID NO 7: TAT TTC ACC TCT GGC AAG GGC T

30 [0069] The nucleotide sequence SEQ ID NO 8 according to the invention codes for an amino acid sequence SEQ ID NO 11 and the localisation of an intron was deduced from alignments of the *Penicillium griseofulvum* A160 sequence with the most homologous xylanase protein sequences

obtained from a homology search in GENBANK with the BLASTP 2.0.8 software (Altschul et al., Nucl. Ac. Res., vol 25, p 3389 (1997)). This localisation was confirmed by the presence of the putative lariat-formation internal sequence 5 and with the definition of the consensus 5' and 3' splice-junction sequences ('GT-AG' rule). The sequences SEQ ID NO 9 and SEQ ID NO 10 are the sequences encoding the two exons of the enzyme with xylanolytic activity. The sequence SEQ ID NO 11 is the amino acid sequence of the *Penicillium griseofulvum* A160 enzyme. A signal sequence driving the secretion of the enzyme covers the 27 first amino acids of the sequence (Fig. 3).

Example 4: Expression of the xylanolytic enzyme gene in
15 *Aspergillus oryzae*

Construction of expression vectors

[0070] A DNA fragment covering the coding region as well as its terminator region was amplified by PCR. The first synthetic oligonucleotide SEQ ID NO 12 was chosen to 20 contain the ATG codon corresponding to the first methionine of the coding region of the polypeptide gene as well as a recognition site for the restriction enzyme *Eco*RI. The second oligonucleotide SEQ ID NO 13 corresponded to the sequence located 250 bp downstream of the last codon and 25 contained a *Xba*I restriction site.

SEQ ID NO 12: GGAATTCCATAATGGTCTCTTCT

SEQ ID NO 13: GCTCTAGAGCCACTTGTGACATGCT

[0071] Both primers (40 pmoles) were used for a PCR reaction with ca. 40 ng of pPGXYNA plasmid DNA as template. 30 The 100 μ l PCR reaction contained also 2.5 units *Pfu* DNA polymerase (Stratagene) and 1 μ g BSA in the following buffer: Tris-HCl pH8.0 20 mM, KCl 10 mM, $MgCl_2$ 2 mM, $(NH_4)_2SO_4$ 6 mM and Triton X-100 0.1%. After denaturation of *

the DNA during 4 min at 94°C, 20 cycles of elongation were performed [30s at 94°C, 30s at 55°C and 60s at 72°C] followed by an elongation step of 7 min at 72°C. The amplified DNA fragment was purified with the QiaQuick PCR 5 purification kit (Qiagen) according to the manufacturer's protocol and recovered in a final volume of 50 µl. The extremities of the fragment were removed by digestion with the EcoRI and XbaI restriction enzymes (5 units of XbaI and 5 units of EcoRI enzymes (Pharmacia), 1 x One-Phor-All 10 buffer PLUS, final volume 60 µl, 37°C, overnight). The fragment was then purified with the QIAquick gel extraction kit (Qiagen) after separation by electrophoresis on an agarose gel and recovered in 30 µl water.

[0072] The PCR DNA fragment was inserted between the 15 EcoRI and XbaI restriction sites of the pBluescript II SK(+) vector (Stratagene). The vector was prepared as follows: 0.5 µg pBluescript SK(+) DNA was digested with 5 units EcoRI and 5 units XbaI restriction enzymes (Pharmacia) (final volume 20 µl, 2x One-Phor-All buffer 20 PLUS, 37°C, overnight). After separation by electrophoresis in an agarose gel, it was purified with the QIAquick gel extraction kit (Qiagen) and recovered in 30 µl water.

[0073] 2 µl of PCR DNA fragment were ligated with this vector (1 µl) in the presence of ATP (1 mM), 1 unit of 25 T4 DNA ligase (Pharmacia) and 1x One-Phor-All buffer PLUS (final volume 10 µl, 16°C, overnight). 1 µl of the ligation mixture was electroporated in competent *Escherichia coli* DH10b cells (BRL-Gibco) after dialysis against water. A right clone was selected after analysis of a number of 30 transformants plasmids by extraction, digestion with appropriate restriction enzymes and separation by electrophoresis on an agarose gel using standard procedures. The new plasmid was termed pPGXYN1E-X.

[0074] The promoter of the glyceraldehyde-3-P dehydrogenase gene from *Aspergillus nidulans* was cloned in front of the xylanolytic enzyme gene. This promoter allows a strong constitutive transcription of the genes located 5 downstream of it (Punt et al., 1990, Gene, vol 93, p 101; Punt et al., 1991, J. Biotechnol., vol 17, p 19). The plasmid pFGPDGLAT2 contains this promoter between two restriction sites: *Eco*RI and *Nco*I. This promoter was inserted into the pBluescript II SK(+) plasmid between two 10 *Eco*RI restriction sites to give the pSK-GPDp plasmid using standard procedures. This plasmid (1 μ g) as well as pPGXYN1E-X (1 μ g) were digested by the *Eco*RI restriction enzyme (5 units) in the presence of 1x One-Phor-All buffer PLUS (final volume 10 μ l, 37°C, overnight). The DNA 15 fragments of interest were then separated by electrophoresis on an agarose gel and purified with the QIAquick gel extraction kit (Qiagen) and collected in 30 μ l water. The purified promoter DNA fragment (1 μ l) was inserted by ligation between the *Eco*RI recognition sites of 20 pPGXYN1E-X (1 μ l) in the presence of ATP (1 mM), 1 unit of T4 DNA ligase (Pharmacia) and 1 x One-Phor-All buffer PLUS (final volume 10 μ l, 16°C, overnight). 1 μ l of the ligation mixture was electroporated into competent *Escherichia coli* DH10b cells (BRL-Gibco) after dialysis against water. A 25 right clone was selected after analysis of a number of transformants plasmids by extraction, digestion with appropriate restriction enzymes and separation by electrophoresis on an agarose gel using standard procedures. The new plasmid was termed pGPDp-PGXYN1.

30

Transformation of *Aspergillus oryzae*

[0075] The strain *Aspergillus oryzae* MUCL 14492 was transformed by generating protoplasts according to the protocol described by Punt et al. (Meth. Enzymol, vol 216,

p 447 (1992)). The pGPDp-PGXYN1 plasmid was cotransformed with the p3SR2 plasmid that contains a selection marker used to recover transformants (the *Aspergillus nidulans* acetamidase gene - Hynes et al., Mol. Cell. Biol., vol 3, 5 p 1430 (1983)). Transformants were selected on minimum medium plates containing acetamide as sole nitrogen source.

[0076] The strain *Aspergillus oryzae* MUCL 14492 was grown in 500 ml *Aspergillus* Minimum Liquid medium (Pontecorvo et al. (1992)) during 16 hours at 30°C. The 10 culture was filtered through a Miracloth filter to collect the mycelium. The mycelium was washed with the Osm solution (CaCl₂ 0.27 M, NaCl 0.6 M). and then incubated with 20 ml solution Osm/g mycelium supplemented with 20 mg Novozym 234 (Sigma). After 1 hour at 30°C with slow agitation (80 rpm), 15 the protoplasts were formed and the suspension was putted on ice. The protoplasts were separated from intact mycelium by filtration through a sterile Miracloth filter and diluted with 1 volume STC1700 solution (sorbitol 1.2 M, Tris-HCl pH 7.5 10 mM, CaCl₂ 50 mM, NaCl 35 mM). The 20 protoplasts were then collected by centrifugation at 2000 rpm, 10 min, 4°C and washed twice with STC1700 solution. They were resuspended in 100 µl of STC1700 (10⁸ protoplasts/ml) in the presence of 3 µg p3SR2 plasmid DNA and 9 µg pGPDp-PGXYN1 plasmid DNA. After 20 min at 20°C, 25 250, 250 and 850 µl PEG solution (PEG 4000 60%, Tris-HCl pH 7.5 10 mM and CaCl₂ 50 mM) were added successively and the suspension was further incubated for 20 min at 20°C. PEG treated protoplast suspensions were diluted by the addition of 10 ml STC1700 and centrifugated 10 min at 4°C, 2000 rpm. 30 The protoplasts were then resuspended in 200 µl STC 1700 and plated onto *Aspergillus* Minimum Agar Medium osmotically stabilized with 1.2 M sorbitol. To select the

transformants, the nitrogen sources in the plates were replaced by 10 mM acetamide and 12 mM CsCl.

Analysis of *Aspergillus oryzae* transformants

5 [0077] 48 transformants were analysed for the xylanolytic enzyme expression. They were grown in *Aspergillus* Minimum Liquid Medium supplemented with 3% sucrose as carbon source and 0.5% Bacto yeast extract (Difco). After 75 hours at 30°C and 130 rpm, the 10 supernatant of the cultures was assayed for xylanolytic activity. 10 of the transformants showed a significantly higher xylanolytic activity as compared to a control strain transformed only with the p3SR2 plasmid.

15 Example 5: Characterisation of the enzyme with xylanolytic activity from *Penicillium griseofulvum* A160

Purification of the enzyme with xylanolytic activity expressed in *Aspergillus oryzae*

20 [0078] The enzyme with xylanolytic activity expressed in *Aspergillus oryzae* was purified in order to separate it from the traces of α -amylase present in the culture supernatants of the transformants. 10 ml of a culture supernatant from a selected transformant were 25 diluted 3 times to reach a final concentration of 50 mM in sodium acetate pH 4.2.

[0079] This solution was loaded at 2 ml/min on a Pharmacia XK16/20 column filled with c.a. 30 ml of the Bio-Rad Macro High S resin equilibrated in 50 mM sodium acetate 30 pH 4.2. Proteins were eluted with a linear increasing NaCl gradient from 0 to 0.6 M in 50 mM sodium acetate pH 4.2. Xylanase and amylase activities were determined in the eluted fractions. The amylase activity was recovered in the

flow through fractions while the xylanase activity was eluted approximately at 0.1 M NaCl. The active fractions with xylanolytic activity were pooled and kept for further analysis.

5

Optimum pH and temperature

[0080] The pH and temperature dependance of the activity of the xylanolytic enzyme secreted by one *Aspergillus oryzae* transformant was analysed. The activity 10 was measured in a citrate/phosphate buffer (0.1M) at various pH (Fig. 4).

[0081] The maximum activity was observed around 50°C. At this temperature, the optimum pH was about 5.0.

[0082] These properties are similar to those of the 15 enzyme with xylanolytic activity purified from the *Penicillium griseofulvum* A160.

Example 6: Baking trials

[0083] Baking trials were performed to demonstrate 20 the positive effect of the *Aspergillus griseofulvum* A160 xylanase in baking. The positive effect was evaluated by the increase in bread volume compared to a reference not containing the enzyme.

[0084] The xylanase was tested in Belgian hard rolls 25 that are produced on a large scale every day in Belgium. The procedure described is well known to the craft baker and it is obvious to one skilled in the art that the same results may be obtained by using equipment from other suppliers.

[0085] The ingredients used are listed in the table below:

Ingredients (g)	RECIPE 1	RECIPE 2	RECIPE 3	RECIPE 4	RECIPE 5
Flour (Surbi – Molens van Deinze)	1500	1500	1500	1500	1500
Water	915	915	915	915	915
Fresh yeast (Bruggeman – Belgium)	90	90	90	90	90
Sodium chloride	30	30	30	30	30
Ascorbic acid	0.12	0.12	0.12	0.12	0.12
Multec Data 2720S™	3.5	3.5	3.5	3.5	3.5
Dextrose	10	10	10	10	10
Xylanase™ A160 (Megazyme units)	0	23	35	52	70

[0086] The ingredients were mixed for 2 min at low 5 and 7 min at high speed in a Diosna SP24 mixer. The final dough temperature as well as the resting and proofing temperatures were 25°C. After resting for 15 min at 25°C, the dough was reworked manually and rested for another 10 min. Afterwards, 2 kg dough pieces were made up and proofed 10 for 10 min. The 2 kg dough pieces were divided and made up using the Eberhardt Optimat. 66 gr round dough pieces were obtained. After another 5 min resting time, the dough pieces were cut by pressing and submitted to a final proofing stage for 70 min.

15 [0087] The dough pieces were baked at 230°C in a Miwe Condo™ oven with steam (Michael Wenz - Arnstein - Germany). The volume of 6 rolls was measured using the commonly used rapeseed displacement method.

[0088] The results are presented on the table below:

Xylanase units	Volume (ml)
0	2125
23	2475
35	2550
52	2675
70	2775

[0089] A graphical representation of the effect of
5 the xylanase on bread volume is shown on figure 5.

Example 7: Effect of the enzyme on the flour viscosity
in the presence of water

[0090] Purified xylanase was used for the test. The
10 enzyme was purified as described in example 5. 100 g of
wheat flour (Surbi, Molens van Deinze) were mixed manually
with 117 ml water containing 25 xylanase units of the
enzyme with xylanolytic activity from *Penicillium*
15 *griseofulvum* A160. After 15 min at 35°C, the viscosity was
measured (Programmable DV-II + Viscometer, Helipath system,
Spindel F, Brookfield). The speed was maintained at 4 rpm
and the viscosity value was measured after 10 s. The
viscosity of a blank sample was obtained in the same way
with untreated flour. The same experiment was also carried
20 out with 10 units of the best performing enzyme available
actually on the market (*Aspergillus aculeatus* xylanase
available from Novo Nordisk (Shearzyme™ L)). Each
experiment was performed in triplicate. The viscosity
results presented below are expressed in centipoises.

Blank sample	116.000 +/- 2000
<i>Penicillium griseofulvum</i> enzyme	65.034 +/- 5047
<i>Aspergillus aculeatus</i> xylanase	68.959 +/- 2253

[0091] *Aspergillus aculeatus* xylanase was shown to give better results than xylanases from *Humicola insolens*, *Trichoderma reesei* (Spezyme CP, Genencor) and another 5 xylanase from *Aspergillus aculeatus* (xylanase I) (patent application WO 94/21785). Christophersen et al. (Christophersen et al, 1997, Starch/Stärke, vol 49, p 5)) also showed the better performance of *Aspergillus aculeatus* xylanase as compared to a xylanase from *Thermomyces lanuginosus* and two commercial hemicellulase cocktails sold 10 for wheat separation.

[0092] The results presented above showed that the enzyme with xylanolytic activity from *Penicillium griseofulvum* A160 has the biggest capacity of reducing the 15 viscosity of flour suspended in water.

Example 8: Wheat separation

[0093] When mixed with water, the flour may be separated into a starch, a gluten, a sludge and a soluble 20 fraction by centrifugation. A decrease of the sludge fraction leads to a better wheat separation. The performances of a pure xylanase could therefore be evaluated by measuring the decrease of the solid sludge fraction after centrifugation.

25 [0094] Such experiment has been carried out with the purified enzyme with xylanolytic activity from *Penicillium griseofulvum* A160 of example 5 compared to Shearzyme™ L.

[0095] 100 g of wheat flour (Surbi, Molens of Deinze) were mixed manually with 117 ml water containing 30 different concentrations of enzyme. After 15 min at 35°C,

the mixture was centrifugated for 10 min at 4000 g (Varifuge 3.0R, Heraeus Sepatech). The liquid phase was weighted.

[0096] The table below shows the results of a 5 typical experiment, by reporting the relative increase of the liquid phase induced by the presence of the xylanolytic enzyme. The enzyme from *Penicillium griseofulvum* A160 allowed to reach a higher liberation of liquid than Shearzyme™ L.

10

Enzyme (units/test)	P. griseofulvum enzyme (%)	Shearzyme (%)
0	100	100
3.125	110	127
6.25	129	130
12.5	134	134
25	166	137

[0097] The applicant has made a deposit of micro-organism for the strain *Penicillium griseofulvum* Diercks A160 according to the invention under the deposit number 15 MUCL 41920 on 13/12/1999 at the BCCM/MUCL Culture Collection (Mycothèque de l'Université Catholique de Louvain, Place de la Croix du Sud 3, B-1348 LOUVAIN-LA-NEUVE, BELGIUM and the deposit of the micro-organism *Escherichia coli* DH10B (pPGXYNA) according to the invention 20 on 13/12/1999 under the deposit number LMBP 39987 at the Laboratorium voor Moleculaire Biologie BCCM/LMBP (K.L. Ledeganckstraat 35, B-9000 GENT, BELGIUM).

What is claimed is:

1. An isolated and purified enzyme with xylanolytic activity comprising an amino acid sequence, which has more than 80% identity with the amino acid sequence of SEQ ID NO 11.
2. The enzyme according to claim 1, wherein said amino acid sequence has more than 90% identity with the amino acid sequence of SEQ ID NO 11.
3. An isolated and purified enzyme with xylanolytic activity comprising the amino acid sequence of SEQ ID NO 11.
4. The enzyme according to any one of claims 1 to 3, characterized in that it presents an optimum enzymatic activity at a pH between 4.5 and 7.0 and at a temperature comprised between 35 and 55°C.
5. An isolated and purified enzyme with xylanolytic activity comprising the amino acid sequence DITQNERGTNNGYFYSFWTXGGGNVY.
6. An isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the enzyme according to any one of claims 1 to 5.
7. An isolated and purified nucleic acid molecule comprising a nucleotide sequence which encodes a polypeptide with xylanolytic activity, wherein said nucleotide sequence has more than 80% identity with the nucleotide sequence of SEQ ID NO 8.
8. The isolated and purified nucleic acid molecule according to claim 7, wherein said nucleotide sequence has more than 90% identity with the nucleotide sequence of SEQ ID NO 8.

9. An isolated and purified nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO 8 encoding a polypeptide with a xylanolytic activity.

10. A recombinant nucleic acid molecule comprising, operably linked to the nucleic acid molecule according to any one of claims 6 to 9, one or more adjacent regulatory sequences.

11. A vector comprising the nucleic acid molecule according to any one of claims 6 to 10.

12. The vector according to claim 11, being a plasmid incorporated in *Escherichia coli* and having the deposit number LMBP-3987.

13. A recombinant host cell transformed by the nucleic acid molecule according to any one of claims 6 to 10 or by the vector according to claim 11 or 12.

14. The recombinant host cell according to claim 13, characterized in that it is selected from the group consisting of bacteria and fungi.

15. The recombinant host cell according to claim 13, characterized in that it is yeast.

16. A solid support fixing an element selected from the group consisting of:

- the cell according to any one of claims 13 to 15,
- a cell extract of the cell according to claim 13 or 14, said cell extract comprising the enzyme with xylanolytic activity according to any one of claims 1 to 5, and
- the isolated and purified enzyme with xylanolytic activity according to any one of claims 1 to 5.

17. Use of the recombinant host cell according to any one of claims 13 to 15 or of the enzyme with xylanolytic activity according to any one of claims 1 to 5, for the degradation of plant cell wall components.
18. Use according to claim 17 for decomposing plants and fruits.
19. Use according to claim 18 for the preparation of fruit juices, legume juices, beer, paper, starch, gluten or vegetable oil.
20. Use according to claim 17 in processes for decomposing wastes.
21. Use according to claim 20 for decomposing agricultural wastes or wastes from paper mills.
22. Use according to claim 17 in baking processes.
23. Use according to claim 22 for increasing the volume of baked products.
24. Use according to claim 17 in starch and gluten separation processes.

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	Cati on			
Phenyl sepharose fractions	exc han ger +	Sup erna tant		Mar kers

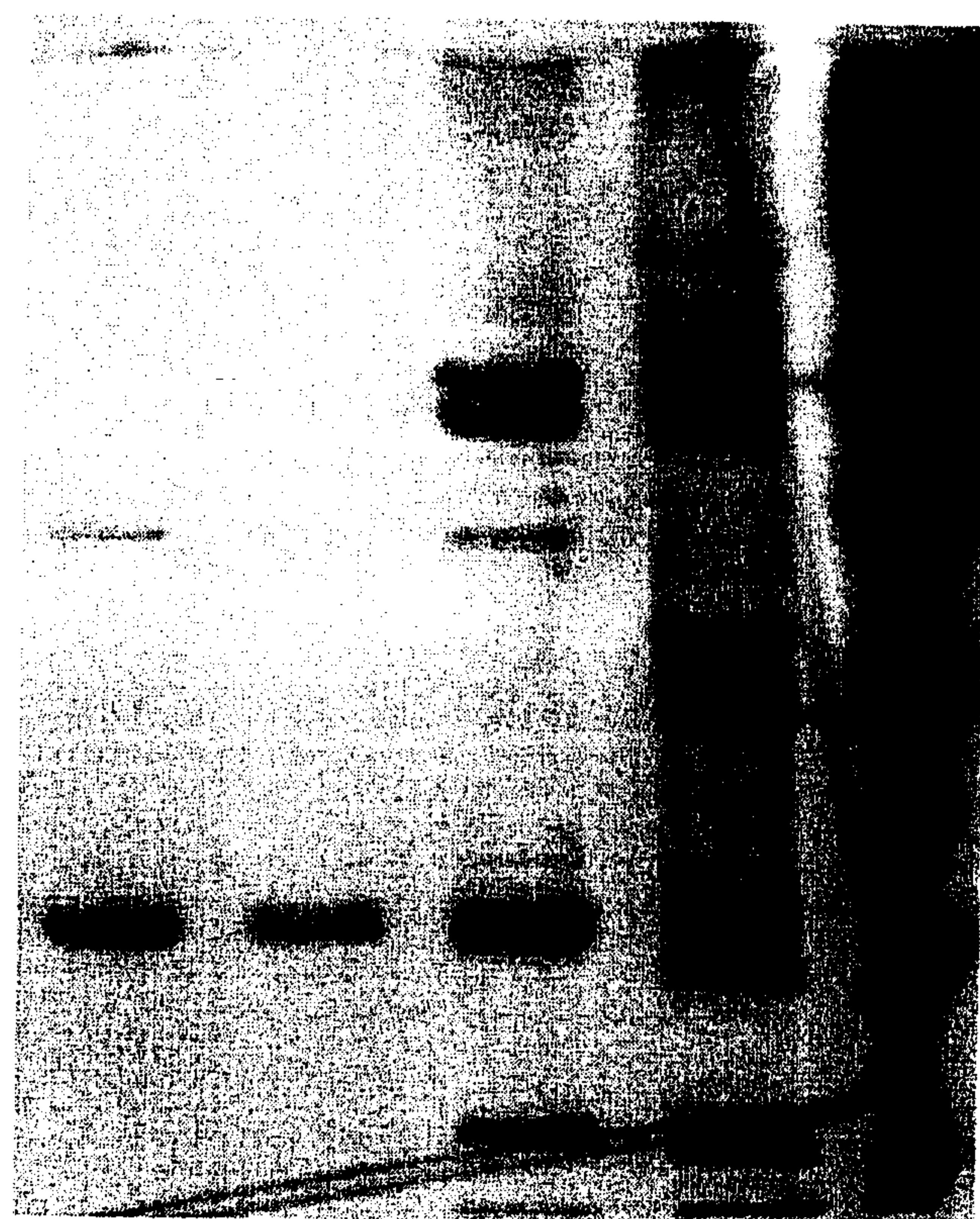


FIG. 1

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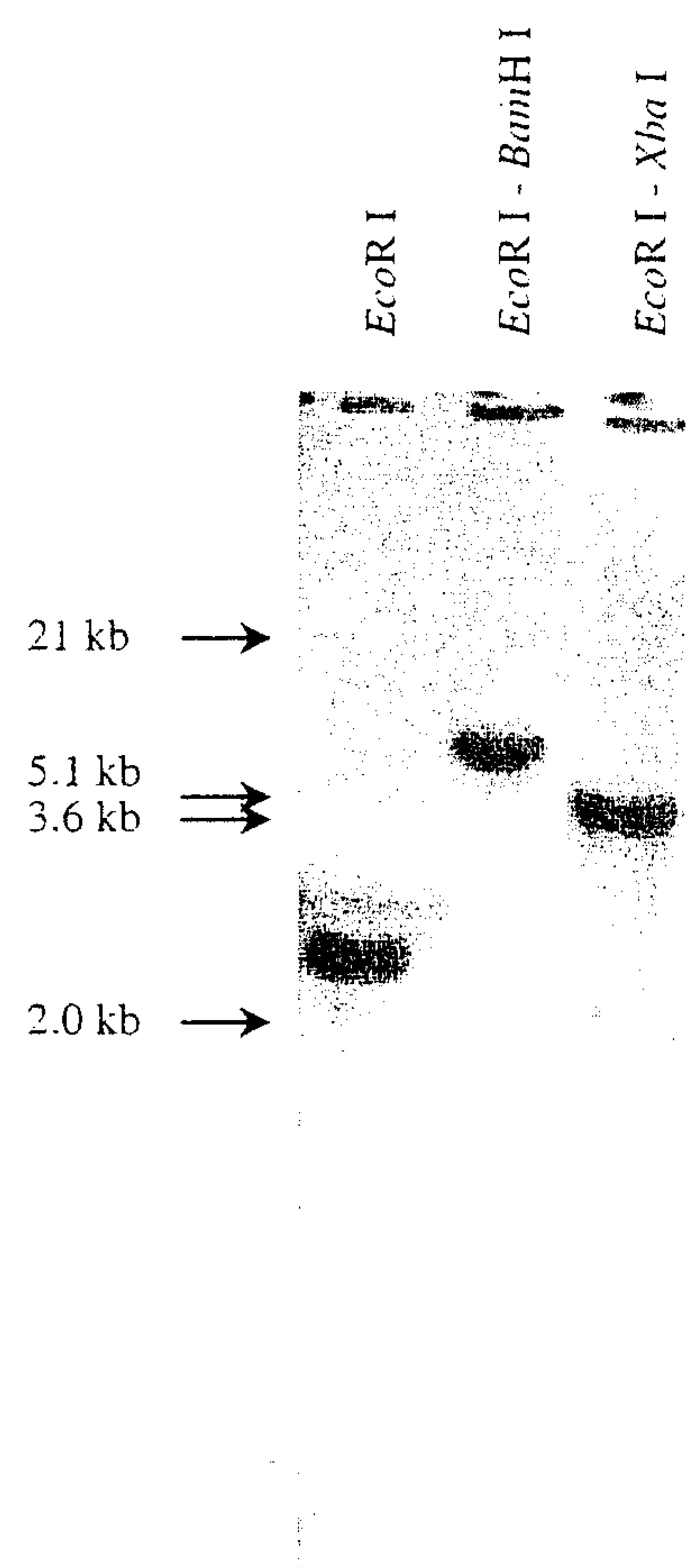


FIG. 2

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5'	9	18	27	36	45	54
	GAA TTC TGC TTT GCC	AAG NTT CAA CGC GGA GAC	TCA CAG TCA CAT	TCT TCG AAT		
	63	72	81	90	99	108
	CTT CTT GGC ACG TGT	TCT TGG GTC CTT CGA GAA	ATC ATG GAT	CTG GAA AGT	TAA	
	117	126	135	144	153	162
	CCA GTA AGC CGG TTA	GAA GAC CCG GAT CAG CGA	CAA ATA GCC GGT	AGT AAA TTA		
	171	180	189	198	207	216
	CTT AAT CGT ATC GCT	AGA TCT GAT CAT CCG ATA	GAC AAA CAA ACA	AAC TTA GGC		
	225	234	243	252	261	270
	TAC CCT AGA GAT GAA	TCA TGA CAG TAG ACT ATT	TTA CCA AGG AAT	ATT TAG AAC		
	279	288	297	306	315	324
	AAG CAT ACC CCT CAC	TAA TTG GGT TGA CTA TAT	AAA TAC GGT TAA AAG CAT	GGG		
	333	342	351	360	369	378
	GGA CTT TCC CAA GGT	TGT TCC TGC CAA GCT TTG	AGA TAT ACA CCC GTT GAT	CCA		
	387	396	405	414	423	432
	TGG ATC ACC GAG GTT	GTC CCT GAG CTG TCT CAA	GCT TAC AAC AAC TTC CAA	GGT		
	441	450	459	468	477	486
	TCT CCA ATG TCT TAT	GAG AGC TGA TAA TCG AAA	TAA GAT CAA GTA GCC GAT	GTT		
	495	504	513	522	531	540
	TCC CCG GCT TTT AAA	CTG CCT GAT CTT GGG TTT	AGC CTG GCC AAG CTA CAT	CCA		
	549	558	567	576	585	594
	TTA TAG CCG TGA TGA	ATT TCC CCG CAT TTA CAC	AGC CGG TGG CTG AAG TGT	GCA		
	603	612	621	630	639	648
	ACA TGC TTA TTT TTA	CTT GAA GAA GTT TAG CCG	ACT CAA TAG TTT CTA CAT	GCT		
	657	666	675	684	693	702
	TAT TTA GCT ACT AAA	ATC TGA TTT TAG CCT GGT	TGG ATG ATA TAG GGA TAT	AGC		
	711	720	729	738	747	756
	TGT CGG TCC GAT GGA	CCA GTA ATA GTT CAT GGA	CAG TGA ACA TGA CCC GTG	TTT		
	765	774	783	792	801	810
	AAC GTA TAA TTA GTG	CAA TTG GAA CAG GGC AAG	GGG ATA AAT AGG TCG TTG	GCT		
	819	828	837	846	855	864
	AAA TTC ATT CGA GAC	ATG TGG AGG ACT ATG AAA	CTG TTT AAA CTC GCC CCA	CAC		
	873	882	891	900	909	918
	CCT CCG TCA ATA TAA	AAG AGG TCT TCT CCC CAA	GGA ATC ATC CAT CAC AAA	ACA		
	927	936	945	954	963	972
	CAC TCC AAT TCA TTC	CTC AAT TAC CAG CAT CTG	ACC TTT CAT AAT GGT	CTC TTT		
	<u>M</u>	<u>V</u>	<u>S</u>	<u>F</u>		
	981	990	999	1008	1017	1026
	CTC AAG CCT CTT TGT	CGC TGC ATG CGC CGC TGT	CAG TGC CCT CGC GCT	TCC CAG		
	<u>S</u>	<u>S</u>	<u>L</u>	<u>F</u>	<u>V</u>	<u>A</u>
	1035	1044	1053	1062	1071	1080
	TGA CGT GGA AAA GCG	CGA CAT CAC CCA GAA CGA	GCG AGG AAC CAA CGG	CGG CTA		
	<u>D</u>	<u>V</u>	<u>E</u>	<u>K</u>	<u>R</u>	<u>D</u>
	1089	1098	1107	1116	1125	1134
	CTT CTA CTC TTT CTG	GAC CAA CGG TGG CGG CAG	TGT CTC CTA CAA CAA CGG	CAA		
	<u>F</u>	<u>Y</u>	<u>S</u>	<u>F</u>	<u>W</u>	<u>T</u>

FIG. 3A

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1143	1152	1161	1170	1179	1188
TGC AGG CCA ATA CAG TGT CAA CTG GAA GAA TTG CGG ATC TTT CAC CTC TGG CAA					
A	G	Q	Y	S	K
1197	1206	1215	1224	1233	1242
GGG CTG GGC TAC AGG TAG CGC CCG <u>GTA AGT CCA GAC AAC ATA CTC AAT ATT GAT</u>					
G	W	A	T	G	S A R /
1251	1260	1269	1278	1287	1296
<u>AAA TAC TTA CGT CGT GTT AGA AAC ATC AAC TTT TCC GGA AAT TTC AAT CCC TCC</u>					
/	N	I	N	F	S G N F N P S
1305	1314	1323	1332	1341	1350
GGA AAT GCT TAC CTG GCT GTC TAC GGC TGG ACC AAG GGC CCC CTC GTT GAG TAC					
G	N	A	Y	L	A V Y G W T K G P L V E Y
1359	1368	1377	1386	1395	1404
TAC ATC ATG GAA AAC TAT GGC GAA TAC AAC CCA GGC GGC AGC ATG ACC TTC AAG					
Y	I	M	E	N	Y G E Y N P G G S M T F K
1413	1422	1431	1440	1449	1458
GGA ACA GTA ACC AGC GAT GGG TCC GTC TAT GAT ATC TAC AAG CAT ACT CAG GTC					
G	T	V	T	S	D G S V Y D I Y K H T Q V
1467	1476	1485	1494	1503	1512
AAC CAG CCT TCG ATC ATT TCG GAT TCT AGC ACC TTC GAC CAG TAC TGG TCT ATC					
N	Q	P	S	I	I S D S S T F D Q Y W S I
1521	1530	1539	1548	1557	1566
CGT CGG AAC AAG CGT AGC AGT GGA ACT GTC ACT ACT GGT AAC CAC TTC AAT GCT					
R	R	N	K	R	S S G T V T T G N H F N A
1575	1584	1593	1602	1611	1620
TGG GCT AAG CTT GGA ATG GGT CTT GGA TCT CAC GAC TAC CAG ATT GTT AAC ACT					
W	A	K	L	G	M G L G S H D Y Q I V N T
1629	1638	1647	1656	1665	1674
GAG GGT TAC CAA AGC AGT GGA TCT GCA ACC ATC ACT GTT TCA TAA GCG TGT GAA					
E	G	Y	Q	S	S G S A T I T V S *
1683	1692	1701	1710	1719	1728
TAC CCT GCA GTG GTT TCA TGC GAA ATG TCA CTT GCT GCT AGC AAG GGT TTG GAA					
1737	1746	1755	1764	1773	1782
GAG CTA TTG TTA TGA ACC TGT TAA CTG TAT ATG GAG CAA AGT TGT GTA CCG ATA					
1791	1800	1809	1818	1827	1836
CTT CAC TTC AAT CCG GTT CAT CGG GTG TTT AGC TTG TTG GTC TTC TCT TGG ATA					
1845	1854	1863	1872	1881	1890
TTT GCC TTG TTA GGA ATC AAT CCA TAT TTA CGC CCC AAA TTT AAG TTT CTA GGA					
1899	1908	1917	1926	1935	1944
GTA TCC ACA GGT GCT TGC CTT AGT ATG TTT CAG CCT GCG GAG TAG TAG TTT CTA					
1953	1962	1971	1980	1989	1998
ACA AAA GTA ATG AGA TGC GAT GTC TAT TTT GAA AAT TGC ATG TCG CAC CTA TAT					
2007	2016	2025	2034	2043	2052
GCA GAT ACT AAA AAG CAT GTC ACA AGT GGC TAT ATA TCG ACA ATA GTG GTT AGT					
2061	2070	2079	2088	2097	2106
ATA TCA CCG TTC CTA AAA GTG CAT TTC GCA TAA CTC ACA TTC TGT TGG GGA TCA					
2115	2124	2133	2142	2151	2160
GTG AAA CCA CAA CTA GGC CCA CTA CTT TTC TTC GGT ATC TTC CCG AAC TTC TTA					
2169	2178	2187	2196	2205	2214
CGC CCG CTA AGC GGC GCC TTG TGC GCC AAC GGA TAC CCA TAC CAA AAC CAC CAA					
2223					
CTT GGG GGG GA 3'					

FIG. 3B

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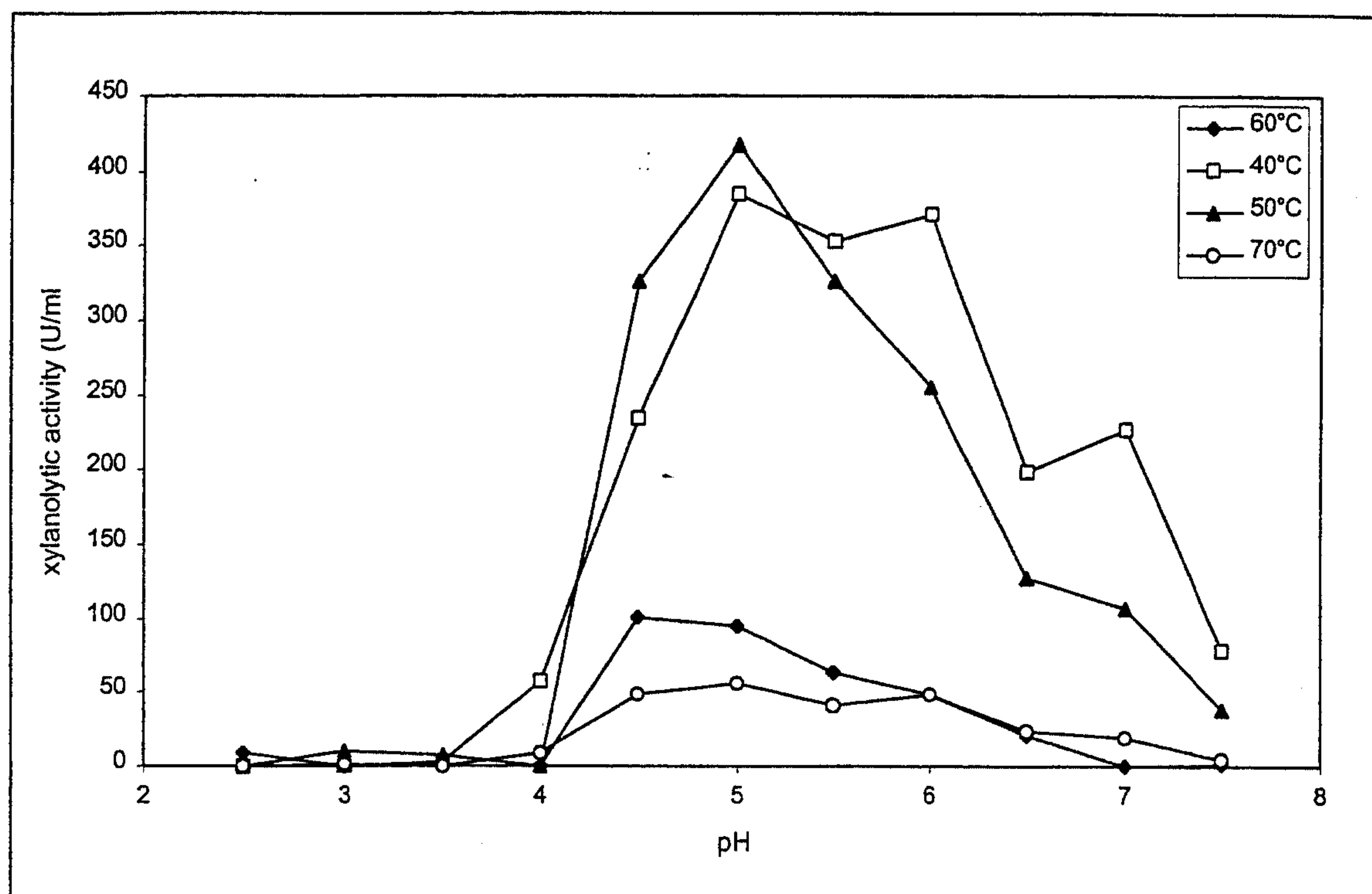


FIG. 4

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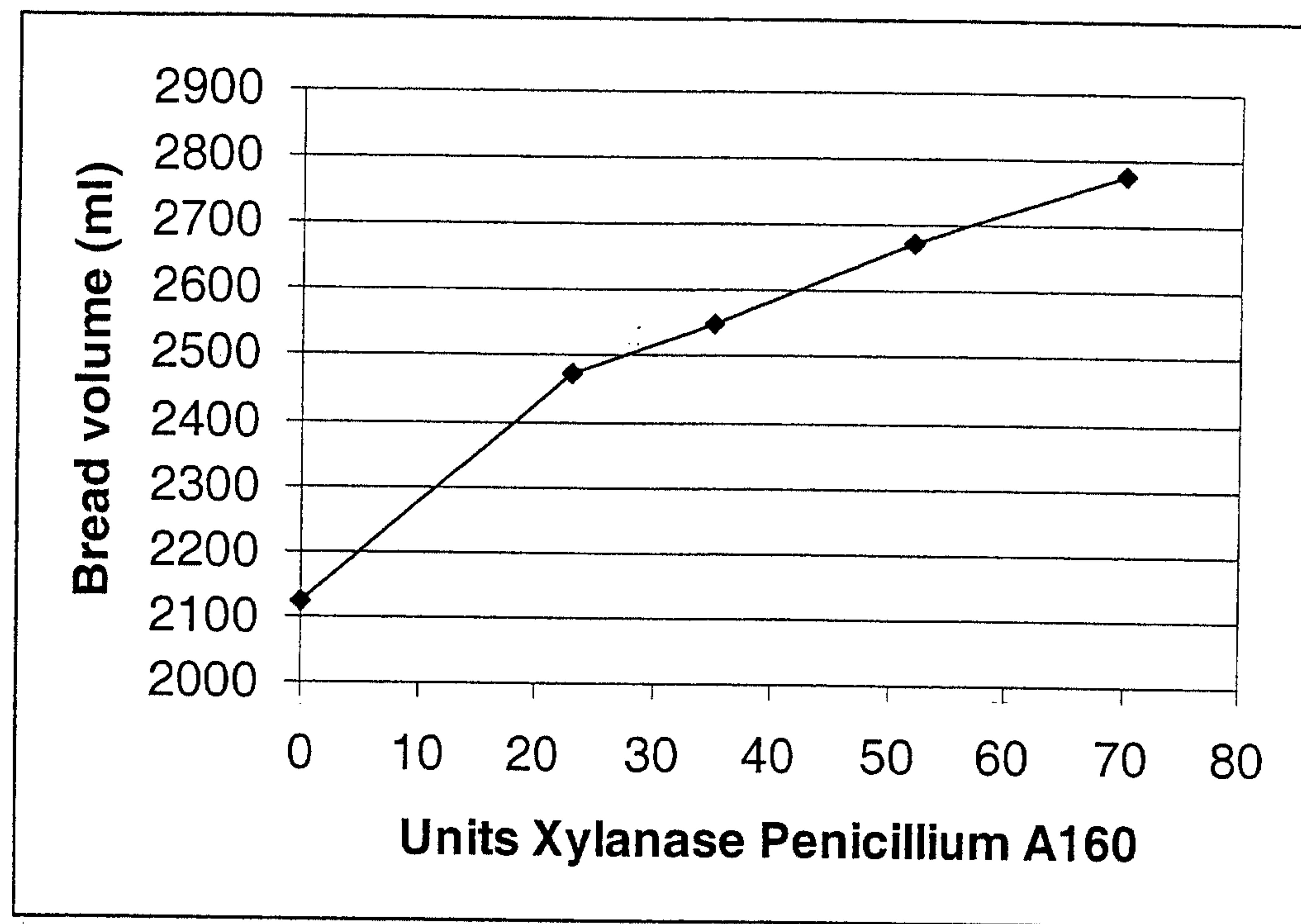


FIG. 5

