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(54) Title: XYLANASE, CORRESPONDING RECOMBINANT DNA SEQUENCE, XYLANASE CONTAINING AGENT, AND USE OF THE AGENT

(57) Abstract

The xylanase is characterized by several partial amino acid sequences and is immunoreactive with an antibody raised against a purified xylanase derived from Humicola insolens, DSM 1800. This xylanase preparation is practically free of cellulase and is well suited for treatment of paper pulp, as a baking agent and as an additive to fodder.

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XYLANASE, CORRESPONDING RECOMBINANT DNA SEQUENCE, XYLANASE CONTAINING AGENT, AND USE OF THE AGENT

The invention comprises a xylanase, a corresponding recombinant DNA sequence, a vector, a transformed host, a method for production of the xylanase, an agent containing the xylanase, and a use of the agent.

Xylan, a major component of plant hemicellulose, is a polymer of D-xylose linked by β-1,4-xylosidic bonds. Xylan can be degraded to xylose and xylo-oligomers by acid or enzymatic hydrolysis. Enzymatic hydrolysis of xylan produces free sugars without the by-products formed with acid (e.g. furans).

There are currently five major applications for xylanases; 1) enzymatic breakdown of agricultural wastes for production of alcohol fuels; 2) enzymatic modification of animal feeds or feed components or addition to animal feeds for *in vivo* breakdown of the hemicellulose fraction; 3) use as a baking agent; 4) manufacturing of dissolving pulps yielding cellulose; and 5) bio-bleaching of wood pulp. [Detroym R.W. In: Organic Chemicals from Biomass, (CRC Press, Boca Raton, FL, 1981) 19-41.; Paice, M.G., and L. Jurasek. J. Wood Chem. Technol. 4: 187-198.; Pommier, J.C., J.L. Fuentes, G. Goma. Tappi Journal (1989): 187-191.; Senior, D.J., et al., Biotechnol. Letters 10 (1988):907-912.]

The pulp and paper industry is using xylanase compositions in the bleaching process to enhance the brightness of bleached pulps, to decrease the amount of chemicals used in the bleaching stages, and to increase the freeness of pulps in the recycled paper process [Eriksson, K. E. L., Wood Science and Technology 24 (1990); 79-101.; Paice, M. G., R. Bernier, and L. Jurasek, Biotechnol. and Bioeng. 32 (1988): 235-239.; Pommier, J. C., J. L. Fuentes, and G. Goma, Tappi Journal (1989): 187-191.]

Kraft pulping, a process widely used in the pulp and paper industry, involves the alkaline sulfate cooking of pulp to remove 90-98% of the lignin. The remaining 2-10% of lignin gives the pulp a dark brown color which has the tendency to darken in UV light or by oxidation. In order to obtain a white pulp for high quality

paper, the brown color is removed by a multi-stage bleaching process using chemicals, e.g. chlorine, chlorine dioxide, ozone, oxygen or hydrogen peroxide.

Presently, there is much concern about the environmental impact of the chemicals generated from the bleaching process. Enzymes can aid in the removal of lignin from the pulp without any harmful side products. Reports show that lignin in wood is linked to xylan [Eriksson, O., et al., Wood Sci.Technol. 14 (1980); 267.; Takashi, N., and T. Koshijiima, Wood Sci.Technol. 22 (1988); 177-189]. By a limited hydrolysis of the xylan a greater release of lignin occurs during bleaching. Thus, by enzymatically treating the pulp prior to bleaching the amount of bleaching chemicals needed would in turn decrease. [Viikari, L., et al., Proceedings of the 3rd International Symposium on Biotechnology in the Pulp and Paper Industry (1986); 67.]

According to the technical literature, good results have been obtained by means of fungal preparations from Trichoderma [Paice, M. G., L. Jurasek, J. Wood Chem. Technol. 4 (1989): 187-198.; Senior, D.J., et al., Biotechnol. Letters. 10 (1988): 907-912], which require pH adjustment of the wood pulps below pH 6.0.

A *Trichoderma* xylanase preparation, Pulpzyme^{TM} HA (commercially available from Novo Nordisk A/S) can be used for delignification of kraft pulps at pH 5-7. At 50°C and pH higher than 7, the enzyme shows only little effect.

Also a *Bacillus pumilus* xylanase preparation, Pulpzyme HB (commercially available from Novo Nordisk A/S) can be used for delignification of Kraft pulps at pH 5-7. At 50°C and pH higher than 7, the enzyme shows only little effect.

Humicola insolens xylanases have been described (Yoshioka, H et al., Agric. Biol. Chem. 43(3) (1981) 579-586). In the crude preparation they have a pH-optimum of 6.0 and a temperature optimum of 60°C. At pH 9 they show 25% of the activity at pH 6. According to the information in the article the enzymes were not purified and will therefore contain significant cellulase activity, as *H. insolens* is known as a good cellulase producer, reference being made to US 4,435,307. Further the enzymes are not characterized with respect to cellulases, M_W, pI or amino acid composition and has not been tested on kraft pulps, in baking or for animal feed.

The prior art xylanase preparation from *Humicola insolens* YH-8 described in Agric. Biol. Chem. and the other prior art xylanase preparations indicated above are not as well suited for use in delignification of kraft pulp, partly due to the relatively high cellulase content.

Thus, it is the purpose of the invention to provide a xylanase, which can be produced as a preparation with very small amounts of other enzyme activities, especially cellulase activities and other xylanase activities, and which is well suited for use in delignification of kraft pulp, as a baking agent and as an additive to animal fodder.

The xylanase according to the invention is characterized by the fact that it has the following partial amino acid sequences

1 Thr-Asn-Thr-Gly-Asn-Phe-Val-Gly-Gly-Lys-Trp-Asn-Pro-Gly-Thr-Gly-Arg-Thr-Lys-Asn-Tyr-,

2 Thr-Ala-Asn-Pro-Leu-Val-Glu-Tyr-Tyr-,

3 Ser-Trp-Trp-Ser-Asp-Gly-Gly-Gly-Gln-Val-Gln-Tyr-,

4 Val-Ser-Thr-Arg-Tyr-Asn-Gln-Pro-Ser-Ile-Asp-Gly-Thr-Arg-Thr-Phe-Gln-Gln-Tyr-Trp-Ser-Ile-Arg-Lys-,

5 Tyr-Val-Ile-Glu-Ser-Tyr-Gly-Thr-Tyr-Asn-Pro-Gly-Ser-Gln-Ala-Gln-Tyr-Lys-Gly-Thr-Phe-Tyr-Thr-Asp-Gly-Asp-Gln-Tyr-Asp-,

and

6 Gln-Val-Thr-Pro-Asn-Ala-Glu-Gly-Trp-His-Asn-Gly-Tyr-Phe-Tyr-,

or a partial amino acid sequence with a homology thereto of at least 80%, preferably at least 90%.

Surprisingly, it has been found that it is possible to produce the xylanase according to the invention as part of a xylanase preparation, which contains enzymatic activities, e.g. cellulases besides xylanase in very small concentrations. Especially, it has to be noted that the xylanase according to the invention is a special xylanase selected among the several xylanases produced inherently from *Humicola insolens*, DSM 1800, which is excellently suited both as an agent for addition to

paper pulp, as a baking agent and as an additive to animal fodder. Also, it has been found that the xylanase according to the invention exhibits a specific activity which is larger than the specific activity of any of the prior art xylanases. It is most surprising that the xylanase according to the invention exhibits superior properties in relation to all these technical areas, which are otherwise unrelated to each other.

The xylanase according to this invention exhibits a significant effect in removal of lignin from pulp at pH 8, as will be demonstrated later. The lignin removing effect is significantly higher than prior art (*B. pumilus*), as will be shown later.

The high performance in regard to lignin removal is surprisingly obtained with xylanase according to the invention alone, even though it is one out of several xylanase components in *H. insolens*. The process for lignin removal is more economical as only one xylanase component is needed, a component which by genetic engineering can be produced in high yields.

Surprisingly it is possible to produce the xylanase according to the invention without significant cellulase activity, thereby avoiding yield loss caused by cellulolytic attack on the cellulose fibres in the pulp during the use of the xylanase for lignin removal.

As will be shown later the xylanase according to the invention surprisingly can remove more lignin from softwood than the *B. pumilus* xylanase even though the two xylanases have very similar pH profiles.

Xylanase (the designation pentosanase is commonly used in the baking industry) is used as a baking agent for wheat bread for several purposes:

- dough development
- improving dough elasticity and stability
- increasing bread volume
- improving crumb structure
- anti-staling

It is believed that some xylanases degrade the pentosans (arabinoxylans) in such a way that the thereby modified pentosans improve dough elasticity, stability and development. Surprisingly the xylanase according to the invention can modify the pentosans in such a way. The xylanase according to the invention can be produced without other xylanases and xylan modifying enzymes and thereby makes it possible to obtain a controlled modification of the pentosans.

The pH in dough is 6.0 to 5.5 which makes this xylanase ideal for the use as a baking agent, as the pH optimum of the xylanase according to the invention is 5.5 to 7.5.

The xylanase according to the invention can also be used for modification of animal feeds or for addition to animal feeds for *in vivo* breakdown of the hemicellulose fraction. The xylanase according to the invention can be used as a preparation with practically no side activities, whereas the prior art xylanase, represented by an *H. insolens* xylanase product, would contain several side activities, including other xylanases. It was found that the xylanase according to the invention and the prior art xylanase gave rise to similar effects in regard to weight gain in a chicken feeding trial. This shows that the xylanase according to the invention is the active or one of the active *H. insolens* xylanases in relation to the feed additive application. Furthermore, the xylanase according to the invention exhibits the significant advantage that it contains a very pure xylanase, whereby a reproducible weight gain can be obtained, in contradistinction to the prior art xylanase, which contains many different side activities with varying proportions from batch to batch. Also, use of the xylanase according to the invention, which is a single component xylanase, opens up the possibility of a more economic feed additive.

A preferred embodiment of the xylanase according to the invention is characterized by the fact that the xylanase is immunoreactive with an antibody raised against a purified xylanase derived from *Humicola insolens*, DSM 1800.

A preferred embodiment of the xylanase according to the invention is characterized by the fact that the xylanase has an isoelectric point of 7.5 - 9.5, preferably 8.0 - 8.5.

A preferred embodiment of the xylanase according to the invention is characterized by the fact that the xylanase exhibits a specific activity above 330 EXU/mg of protein, preferably above 400 EXU/mg of protein. The EXU xylanase activity unit is defined in AF 293.9/1. This AF publication and other AF publications indicated in this specification are available on request from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

Also, the invention comprises a recombinant DNA sequence, which is characterized by encoding for the xylanase according to the invention.

A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises the following partial DNA sequence

```
1                   5                   10                  15
ATG GTC TCG CTC AAG TCT GTC CTC GCG GCC GCC ACG GCT GTG AGC

                    20                  25                  30
TCT GCC ATT GCT GCC CCT TTT GAC TTC GTT CCT CGG GAC AAC TCG

                    35                  40                  45
ACG GCC CTT CAG GCT CGC CAG GTG ACC CCC AAC GCC GAG GGC TGG

                    50                  55                  60
CAC AAC GGC TAC TTC TAC TCG TGG TGG TCC GAC GGC GGA GGC CAG

                    65                  70                  75
GTT CAG TAC ACC AAC CTC GAG GGC AGC CGC TAC CAG GTC AGA TGG

                    80                  85                  90
NNN AAC ACC GGC AAC TTC GTC GGT GGT AAG GGT TGG AAC CCG GGA

                    95                  100                 105
ACC GGC CCC ACG ATC AAC TAC GGC GGC TAC TTC AAC CCC CAG GGC

                    110                 115                 120
AAC GGC TAC CTG GCC GTC TAC GGC TGG ACC NNN AAC CCG CTC GTC

                    125                 130                 135
GAG TAC TAT GTC ATC GAG TCG TAC GGC ACG TAC AAT CCC GGC AGC

                    140                 145                 150
CAG GCT CAG TAC AAG GGC ACA TTC TAT ACC GAC GGC GAT CAG TAT

                    155                 160                 165
GAC ATC TTT GTG AGC ACC CGT NNN AAC CAG CCC AGC ATC ACG GCA

                    170
CCC GGA CGT CCA GCT AGT ACT
```

A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises a DNA sequence selected from

a) the *Humicola insolens* xylanase DNA insert in pHD 450

b) a DNA sequence which hybridizes to the coding region for the mature xylanase DNA comprised by the DNA insert of a) and which comprises a structural gene for a polypeptide with xylanase activity, and optionally a promoter, a coding region for a signal or leader peptide and/or transcriptional terminator

c) a DNA sequence with a homology sufficient to hybridize to the sequence indicated in Claim 6 under relative stringent conditions (1.0 x SSC, 0.1% SDS, 65°C), reference being made to T. Maniatis, A laboratory Manual (CSH)

d) a derivative of a DNA sequence defined in a), b) or c), or

e) a DNA sequence which codes for a mature xylanase or a signal peptide or a leader peptide thereof and which is degenerate within the meaning of the genetic code with respect to a DNA sequence of a), b) or c).

Also, the invention comprises a vector, which is characterized by the fact that it comprises the recombinant DNA sequence according to the invention.

A preferred embodiment of the vector according to the invention is characterized by the fact that the promoter is the *Aspergillus oryzae* takaamylase promoter and/or the xylanase gene is isolated from EC 3-2, and/or the terminator is the *Aspergillus oryzae* AMG terminator, preferably pHD450.

Also, the invention comprises a transformed host, which is characterized by the fact that it contains the vector according to the invention.

A preferred embodiment of the transformed host according to the invention is characterized by the fact that it is an *Aspergillus* strain.

A preferred embodiment of the transformed host according to the invention is characterized by the fact that it is a strain belonging to the species *Aspergillus aculeatus*, *Aspergillus niger*, *Aspergillus oryzae* or *Aspergillus awamori*.

A preferred embodiment of the transformed host according to the invention is characterized by the fact that it is a microorganism, which in its non-transformed condition does not produce xylanase or only produces xylanase in insignificant amounts, preferably *Bacillus sp.*, *E. coli* or *S. cerevisiae*.

Also, the invention comprises a method for production of xylanase, which is characterized by the fact that the method utilizes a transformed host according to the invention.

Also, the invention comprises the xylanase, produced by means of the method according to the invention.

Also, the invention comprises an agent containing the xylanase according to the invention, preferably in the form of a non-dusting granulate, a stabilized liquid or a protected enzyme, whereby the xylanase comprises at least 10%, preferably at least 30% of the total enzyme protein.

A preferred embodiment of the agent according to the invention is characterized by the fact that the proportion between the xylanase activity and the cellulase activity, as expressed by the ratio between the xylanase activity in EXU/g and the cellulase activity in ECU/g has a value above 10, preferably above 30, most preferably above 100. The cellulase activity unit ECU is defined in AF 302-1.

A preferred embodiment of the agent according to the invention is characterized by the fact that it contains a xylanase activity of at least 10 EXU/mg of enzyme protein, preferably at least 100 EXU/mg of enzyme protein, more preferably at least 300 EXU/mg of enzyme protein.

Also, the invention comprises a use of the agent according to the invention, the use being characterized by the fact that the agent is used for xylan degradation.

A preferred embodiment of the use according to the invention is characterized by the fact that the use is related to chemical pulp or recycle paper pulp before or as part of bleaching, preferably at a pH value above 7.

A preferred embodiment of the use according to the invention is characterized by the fact that the use is related to production of bread, which contains wheat flour.

A preferred embodiment of the use according to the invention is characterized by the fact that the use is related to animal feed.

The xylanase according to the invention can be produced in the following manner.

Xylanase was produced by cultivating *Humicola insolens* DSM 1800, as described in US 4,435,307, Example 6 from the beginning thereof to column 11, line 29. The freeze dried powder was diluted with water to a dry matter content of 10%, and pH was lowered to 2.3 with 10% HCl. The mixture was left alone at 22°C for 50 minutes, and subsequently pH was increased to 8.0 with NaOH. Then a salt precipitation with 250 g of Na_2SO_4 per kg of liquid at pH 5.0 was carried out. The salt cake was redissolved and then concentrated and washed by ultrafiltration. Finally the preparation was frozen down.

In a 12% dry matter solution the xylanase preparation exhibited the following enzyme activities: 444 CSU/ml and 144 EXU/ml. CSU is the cellulase activity unit, vide AF 267.

This preparation was further purified by batch ion exchange with DEAE Sephadex A-50 (Pharmacia), concentration by AMICON ultrafiltration, gel filtration on Sephacryl S-200 (Pharmacia), and cation exchange with high load S-Sepharose (Pharmacia).

It was found that the xylanase did not exhibit any detectable cellulase side activity. Thus, the purified xylanase product showed a cellulase activity of less than 0.1 CSU/mg protein and a xylanase activity higher than 350 EXU/mg protein. The purified xylanase product shows only one SDS-PAGE band with molecular weight 22 kD. The pI was determined to 8.5 by isoelectric focusing.

The antibody reactive with this purified xylanase product was produced in the following manner. Antiserum against the purified xylanase was raised by immunizing rabbits according to the procedure described by N. Axelsen et al.: A

Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23. A purified immunoglobulin was obtained from the antiserum by salt precipitation ($(NH_4)_2SO_4$), followed by dialysis and ion exchange chromatography on DEAE-Sephadex. Immunochemical characterization of the purified xylanase was conducted by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2,). With the antibody indicated above the purified xylanase showed a single arch moving against the cathode in rocket immunoelectrophoresis.

In order to characterize the xylanase chemically the total amino acid composition was determined by acid hydrolysis according to Moore and Stein (1963), Methods Enzymol. 6, 819-831, and by qualitative and quantitative determination of the amino acids in the hydrolysis mixture according to Heinrikson and Meredith (1984), Anal. Biochem. 136, 65-74 (derivatisation) and Edelhoch (1967), Biochemistry 6, 1948-1954 (tryptophan determination). The last indicated literature reference describes the spectrophotometric determination of the tryptophan content. The following amino acid composition was found.

	Composition (mole per cent)	Standard deviation	Approximated composition according to molecular weight	Maximum number of amino acid residues	Minimum number of amino acid residues
Asx (B)	13.16	0.75	25	27	24
Glx (Z)	12.94	0.37	25	25	24
Ser (S)	6.07	0.16	12	12	11
Gly (G)	14.01	0.48	27	28	26
His (H)	2.49	0.10	5	5	5
Arg (R)	4.91	0.33	9	10	9
Thr (T)	7.19	0.09	14	14	14
Ala (A)	2.94	0.23	6	6	5
Pro (P)	4.16	0.13	8	8	8
Tyr (Y)	9.77	0.21	19	19	18

Val (V)	6.10	0.38	12	12	11
Met (M)	0.93	0.07	2	2	2
Cys (C)	0.00	0.00	0	0	0
Ile (I)	2.68	0.12	5	5	5
Leu (L)	2.15	0.05	4	4	4
Phe (F)	3.50	0.07	7	7	7
Lys (K)	2.09	0.05	4	4	4
Trp (W)	4.89	0.10	9	10	9
Total	99.98	193	198	186	

The N-terminal amino acid sequence of the purified xylanase was found to be

```
1               5                  10                  15
Gln-Val-Thr-Pro-Asn-Ala-Glu-Gly-Trp-His-Asn-Gly-Tyr-Phe-Tyr-
```
(SEQ ID NO. 6)

Also, after enzymatic degradation with chymotrypsin three peptides are sequenced, whereby one of these is determined as a side sequence (ss) during the sequencing of another peptide:

Chymo-22: Thr-Asn-Thr-Gly-Asn-Phe-Val-Gly-Gly-Lys-Trp-Asn-Pro-Gly-Thr-Gly-Arg-Thr-Lys-Asn-Tyr- (SEQ ID NO. 1)

Chymo-22: Thr-Ala-Asn-Pro-Leu-Val-Glu-Tyr-Tyr- (ss) (SEQ ID NO. 2)

Chymo-24: Ser-Trp-Trp-Ser-Asp-Gly-Gly-Gly-Gln-Val-Gln-Tyr- (SEQ ID NO. 3)

After chemical degradation with CNBr followed by enzymatic degradation with pepsin two peptides are sequenced.

CNBr/Pep-16: Val-Ser-Thr-Arg-Tyr-Asn-Gln-Pro-Ser-Ile-Asp-Gly-Thr-Arg-Thr-Phe-Gln-Gln-Tyr-Trp-Ser-Ile-Arg-Lys- (SEQ ID NO. 4)

CNBr/Pep-23: Tyr-Val-Ile-Glu-Ser-Tyr-Gly-Thr-Tyr-Asn-Pro-Gly-Ser-Gln-Ala-Gln-Tyr-Lys-Gly-Thr-Phe-Tyr-Thr-Asp-Gly-Asp-Gln-Tyr-Asp- (SEQ ID NO. 5)

It has been found that the xylanase according to the invention exhibits a weak homology to prior art xylanases, e.g. xylanases producible from *Schizophyllum communue*, *Bacillus pumilus* and *Bacillus subtilis*.

The invention will be illustrated by the following examples.

Example 1 illustrates the selection of the xylanase producing gene and production of the xylanse by means of a genetic modified host organism. Example 2 illustrates the production in pilot plant of the xylanase and purification of the xylanase. Example 3 illustrates the use of the xylanase as a bleach booster during paper pulp production and Example 4 illustrates the use of the xylanase as a baking agent.

EXAMPLE 1

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. Autoclaved, 90 ml 20% glucose (sterile filtered) added.

10 x Basal salt: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20 % casamino acids, 9 ml 1% tryptophane, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose added.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan and 20 g/l agar (Bacto). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

YNB-1 agar: 3.3 g/l KH_2PO_4, 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 min. at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

Oat spelt xylan overlayer gel: 1% agarose, 1% oat spelt xylan (Sigma Chemical Company) in Tris-maleic buffer, pH 7. The gel was boiled and then cooled to 55°C before the overlayer is poured onto agar plates.

FG-4-Agar: 35 g/l agar, 30 g/l Soy bean meal, 15 g/l maltodextrin (Glucidex 6), 5 g/l Bacto pepton, pH 7. Autoclaved 40 min at 121°C

FG-4 medium: 30 g/l Soy bean meal, 15 g/l maltodextrin (Glucidex 6), 5 g/l Bacto pepton. Autoclaved 40 min at 121°C.

Construction of a yeast expression plasmid

The commercially available plasmid pYES II (Invitrogen) was cut with SpeI, filled in with Klenow DNA polymerase + dNTP and cut with ClaI. The DNA was size fractionated on an agarose gel, and a fragment of about 2000 bp was purified by electroelution. The same plasmid was cut with ClaI/PvuII, and a fragment of about 3400 bp was purified by electroelution. The two fragments were ligated to a blunt-ended SphI/EcoRI fragment containing the yeast TPI promoter. This fragment was

isolated from a plasmid in which the TPI promoter from *S. cerevisiae* (cf. T. Albers and G. Kawasaki, J. Mol. Appl. Genet. 1, 1982, pp. 419-434) was slightly modified: an internal SphI site was removed by deleting the four bp constituting the core of this site. Furthermore, redundant sequences upstream of the promoter were removed by Bal1 exonuclease treatment followed by addition of a SphI linker. Finally, an EcoRI linker was added at position -10. After these modifications, the promoter is included in a SphI-EcoRI fragment. Its effeciency compared to the original promoter appears to be unaffected by the modifications. The resulting plasmid pYHD17 is shown in Fig. 1.

Donor organism

H. insolens, DSM 1800, grown in a cellulose-rich fermentation medium with agitation to ensure sufficient aeration.

Isolation of mRNA

Total RNA was isolated from approximately 7 g of mycelium. The mycelium was frozen in liquid nitrogen and ground in a mortar with 1 g of quartz sand to a consistency of flour. The RNA was extracted with guanidinium thiocyanate and centrifuged through CsCl essentially as described in Sambrook et al., 1989, op. cit.. Poly A RNA was isolated from total RNA by chromatrography on oligo dT cellulose.

cDNA synthesis

cDNA synthesis was carried out by means of a cDNA synthesis kit from Invitrogen according to the manufacturer's specifications. The DNA was adapted to the expression vectors by addition of a BstxI linker (Invitrogen) and size fractionated on an agarose gel. Only DNA larger than 5-600 bp was used in the library construction. The adapted cDNA was ligated into an appropriate yeast expression vector cut with BstxI. Following test ligations (in order to determine the size of the library) the library was plated onto 50 agar plates amounting to approximately 5000

transformants per plate. To each plate containing approximately 5000 individual clones was added 3 ml of medium. The bacteria were scraped off, 1 ml glycerol was added, and stored at -80°C as 50 pools. The remaining 2 ml were used for DNA isolation. If the amount of DNA was insufficient to give the required number of yeast transformants (see below), large scale DNA was prepared from 500 ml medium (TB) inoculated with 50 µl -80°C bacterial stock propagated over night. DNA was isolated from 20 individual clones from the library and subjected to analysis for cDNA insertion. The insertion frequency was found to be >90% and the average insert size was approximately 1400bp.

Transformation of yeast

The yeast strain used was yNG231. (MAT alpha, leu2, ura3-52, his4-539, pep4-delta 1, cir+). One colony was grown at 30°C overnight in 10 ml of YPD. 10, 30, and 60 µl of this culture were added to 3 shaker flasks containing 100 ml YPD, and incubated with shaking overnight at 30°C. The culture with an OD_{600} closest to 0.3-0.4 was selected. The cells were harvested in 50 ml tubes in a Beckman centrifuge (speed 6, 10 minutes), the cells were resuspended in 2 x 5 ml H_2O, centrifuged as described above, resuspended in 5 ml buffer containing 0.1 M LiAc, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5, and centrifuged again. The cells were resuspended in 500 µl of the above buffer and incubated for 60 minutes at 30°C. 250 µg carrier DNA (sterile salmon-sperm DNA 10 mg/ml, see below) was added and aliquots of 100 µl were prepared. The DNA to be transformed (approx. 5 µg) was added to the 100 µl aliquot, mixed gently, and incubated for 30 minutes at 30°C. 700 µl 40% PEG 4000, 0.1 M LiAc, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5 was added, and incubation was continued for 60 minutes at 30°C. The transformation mixture was subjected to heat shock for 5 minutes at 42°C, spun briefly in a micro centrifuge, resuspended in 100-200 µl H_2O, and plated on SC plates without uracil, followed by incubation for three days at 30°C.

Preparation of carrier DNA

100 mg salmon-sperm DNA was weighed out and dissolved overnight in 10 ml 10 mM Tris-Cl, 1 mM EDTA, pH 7,5 (TE). The solution was then sonicated in a plastic container in ice water until it was no longer viscous. The solution was then phenole extracted and EtOH precipitated, and the pellet was washed and resuspended in 5 ml TE. The suspension was EtOH precipitated, and the pellet was washed and resuspend in 5 ml TE. The OD_{260} was measured, and the suspension was diluted with TE to 10 mg/ml.

Screening of yeast

As described above DNA from the *Humicola* library, pools 1-10, was transformed into yeast, and plates containing 20-25,000 colonies were obtained from each pool. The colonies were scraped off and stored in glycerol at -80°C.

Yeast cells from the library were spread onto YNB agar to a total of about 400,000 colonies. The number of colonies per plate varied from 50 to 500. After 4 or 5 days of growth, the agar plates were replica plated onto a set of SC-H agar plates. These plates were then incubated for 2-4 days at 30°C before the agar plates were overlayered with a oat spelt xylan overlayer gel for the detection of xylanase. After incubation overnight at 40°C, enzyme reactions were visualised with Congo Red. 10-15 ml of a 0.1% solution of Congo Red was poured onto the overlayer and removed after 10-20 min. The plates were then washed once or twice by pouring 10-15 ml of 2M NaCl onto the plates. The NaCl solution was removed after 15-25 min. Xylanase-positive colonies identified on the plates overlayers as colourless or pale red clearing zones on a red background.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the xylanase-producing colonies identified.

Characterization of xylanase positive clones

Each of the 147 of the xylanase-producing colonies were isolated. Some of these colonies were inoculated into 20 ml YNB-1 broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

The cells were resuspended in 1 ml 0.9 M sorbitol, 0.1 M EDTA, pH 7.5. The pellet was transferred to an Eppendorf tube, and spun for 30 seconds at full speed. The cells were resuspended in 0.4 ml 0.9 M sorbitol, 0.1 M EDTA, 14 mM β-mercaptoethanol. 100 µl 2 mg/ml Zymolase was added, and the suspension was incubated at 37°C for 30 minutes and spun for 30 seconds. The pellet (spheroplasts) was resuspended in 0.4 ml TE. 90 µl of (1.5 ml 0.5 M EDTA pH 8.0, 0.6 ml 2 M Tris-Cl pH 8.0, 0.6 ml 10% SDS) was added, and the suspension was incubated at 65°C for 30 minutes. 80 µl 5 M KOAc was added, and the suspension was incubated on ice for at least 60 minutes and spun for 15 minutes at full speed. The supernatant was transferred to a fresh tube which was filled with EtOH (room temperature) followed by thorough but gentle mixing and spinning for 30 seconds. The pellet was washed with cold 70% ETOH, spun for 30 seconds and dried at room temperature. The pellet was resuspended in 50 µl TE and spun for 15 minutes. The supernatant was transferred to a fresh tube. 2.5 µl 10 mg/ml RNase was added, followed by incubation at 37°C for 30 minutes and addition of 500 µl isopropanol with gentle mixing. The mixture was spun for 30 seconds, and the supernatant was removed. The pellet was rinsed with cold 96% EtOH and dried at room temperature. The DNA was dissolved in 50 µl water to a final concentration of approximately 100 µl/ml.

The DNA was transformed into *E. coli* by standard procedures. Two *E. coli* colonies were isolated from each of the transformations and analysed with the restriction enzymes HindIII and XbaI which excised the DNA insert. DNA from one of these clones was retransformed into yeast and rescreened for enzyme activity.

The DNA sequences of several of the positive clones were partially determined. Based on the DNA sequence, 15 clones were classified as the same family, the sequence of this xylanase family showed full homology with the amino

acid sequence of the purified xylanase according to the invention. A partial sequence appears in claim 5.

A strain of *E. coli* containing the xylanase HindIII/XbaI cDNA fragment in pYES2 was deposited in DSM on March 18, 1992, as DSM 6995. The xylanase cDNA fragment was isolated from one of the clones by cleavage with HindIII/XbaI. The HindIII/XbaI fragment was purified by agarose gel electrophoresis electroeluted and made ready for ligation reactions. The cDNA fragment is ligated to HindIII/XbaI digested pHD414 (see below) to generate pHD 450 in which the cDNA is under transcriptional control of the TAKA promotor from *Aspergillus oryzae* and the AMG terminator from *Aspergillus niger*.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described as follows.

Construction of an *Aspergillus* expression vector

The vector pHD414 (Fig. 2) is a derivative of the plasmid p775 (described in EP 238 023). In contrast to this plasmid, pHD 414 has a string of unique restriction sites between the promoter and the terminator. The plasmid was constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3'end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5'end of the promoter, also containing undesirable sites. The 200 bp region was removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase +dNTP, purification of the vector fragment on gel and religation of the vector fragment. This plasmid was called pHD413. pHD413 was cut with StuI (positioned in the 5'end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated, resulting in pHD414. Fig. 11 is a map of plasmid pHD414, wherein "AMG Terminator" indicates the *A. niger* glucoamylase terminator, and "TAKA Promoter" indicates the *A. oryzae* TAKA amylase promoter.

Transformation of *Aspergillus oryzae*

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of *A. oryzae* or and incubated with shaking at 37°C for about 2 days. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$. 10 mM NaH_2PO_4, pH = 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234 is added. After 5 minutes 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayered with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation is performed for 15 minutes at 100 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5. 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifugated for 5 minutes at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576). 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on the appropriate plates. Protoplasts are spread on minimal plates (Cove, see Biochem.Biophys.Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is

repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Expression of the xylanase in *Aspergillus*

11 transformants were obtained and inoculated and maintained on YPG-agar. Each of the 8 selected transformants were inoculated from YPG-agar slants on 500 ml shake flask with 150 ml FG-4 media. After 4 days of fermentation with sufficient agitation to ensure good aeration, the culture broths were centrifuged for 10 minutes at 2000 g and the supernatants were analyzed. The best yielded 72 EXU/ml. There is no background from the untransformed host strain. This strain is designated Axy40/8.

EXAMPLE 2

The strain Axy40/8 was fermented in a pilot plant scale in the following way.

An agar substrate with the following composition was prepared in a Fernbach flask:

Sucrose	30 g/
KH_2PO_4	1 g/l
$NaNO_3$	3 g/l
$MgSO_4$, $7H_2O$	0,5 g/l
$FeSO_4$, $7H_2O$	0,01 g/l
KCl	0,5 g/l
Agar	25 g/l

pH was adjusted to between 6.4 - 6.5, autoclaved for 20 minutes at 121°C.

The Fernback flask was inoculated with a spore suspension and cultivated 5 days at 30°C.

A substrate with the following composition was prepared in a 500 litre seed fermenter:

Yeast extract, 50%	6 kg
Potato starch	9 kg
$CaCO_3$	0,15 kg
Pluronic L61	150 ml
Termamyl® 60 (L)*)	9 g

*) Termamyl is an alpha-amylase obtainable from Novo Nordisk A/S

Tap water was added to a total volume of around 225 litres. pH was adjusted to around 6.5 with H_3PO_4.

The temperature was raised from 60 to 90°C in 30 minutes, held for 30 minutes at 90°C, before the substrate was sterilized in the seed fermenter for 1.5 hour at 121°C. Final volume before inoculation was around 300 litres.

The Fernbach flask spore suspension was transferred to the seed fermenter. Seed fermentation conditions were:

Fermenter type: Conventional aerated and agitated fermenter with a height/diameter ratio of around 2.3.

Agitation:	250 rpm
Aeration:	300 normal litre air per minute
Temperature:	34°C
Time:	around 24 hours

Around 35 hours after inoculation 150 litres was transferred from the seed fermenter to the main fermenter.

A substrate with the following composition was prepared in a 2500 litre main fermenter:

$MgSO_4$, $7H_2O$	2.6 kg
KH_2PO_4	2.6 kg
K_2SO_4	3.9 kg
Trace metal solution	650 ml
Potato starch	39.0 kg
Yeast extract, 50%	28.6 kg
Urea	5.2 kg
Citric acid	1.053 kg
Pluronic L61	650 ml
Termamyl® 60 L	39 g

The trace metal solution has the following composition:

$ZnSO_4$, $7H_2O$	14.3 g/l
$CuSO_4$, $7H_2O$	2.5 g/l
$NiCl_2$, $6H_2O$	0.5 g/l
$FeSO_4$, $7H_2O$	13.5 g/l
$MnSO_4$, H_2O	8.5 g/l
Citric acid, monohydrate	3.0 g/l

Tap water was added to a total volume of around 900 litres. pH was adjusted to 6.5 with H_3PO_4. The temperature was raised from 60 to 90°C in 30 minutes, held for 30 minutes at 90°C. Then pH was adjusted to 4.5 before the substrate was sterilized in the main fermenter for 1.5 hours at 123°C. Final volume before inoculation was around 1300 litres.

Then 150 litres of seed culture was added.

Fermentation conditions were:

Fermenter type: Conventional aerated and agitated fermenter with a height/diameter ratio of around 2.7.

Agitation:	250 rpm (two turbine impellers)
Aeration:	1500 normal litre air per minute, rising to 1700 Nl/min at 130 hours.
Temperature:	34°C
Time:	around 130 hours

After 25 hours into the fermentation a maltodextrin solution was added aseptically to the main fermenter at a rate increasing from 1 l/hour to 4 l/hour in 12 hours. The dextrin solution was prepared with the following composition in a 550 litre feed tank:

Potato starch	184 kg
Biotin	0.04 g
Thiamin	0.4 g
Citric acid	0.2 kg
Pluronic L61	200 ml
Termamyl® 60L	360 ml

Tap water was added to a total volume of around 300 litres. The temperature was held at 90°C for 60 minutes before the substrate was sterilized in the dosing tank for 1.5 hours at 123°C. Final volume before start of dosage was around 400 litres.

After 25 hours of fermentation pH was controlled at 7.3-7.4 by NH_3.

After around 130 fermentation hours the fermentation process was stopped. The around 1850 litres of culture broth were cooled to around 5°C and the enzymes were recovered according to the following method.

The culture broth was centrifuged at pH 7 and the centrifugate was filtered on a Seitz filter sheet (type Supra EKS Neu) using Hyflo Super-Cell

diatomaceous earth as a filter aid. The pH in the filtrate was adjusted to 4.7 and the filtrate was concentrated to a dry matter content of 5% using ultrafiltration. Further concentration to a dry matter content of 30% was carried out by evaporation. The pH in the concentrate was adjusted to 6.5 and the concentrate was filtered on a frame filter using Hyflo Super-Cell as filter aid and germ filtered on a Seitz filter sheet (type Supra EKS Neu).

This concentrate is identified later in this specification as preparation 1. Preparation 1 exhibits a xylanase activity of 533 EXU/g.

Preparation 1 was purified as follows.

Totally 200,000 EXU was precipitated with 25% ammonium sulfate. The precipitate was solubilized in 500 ml of water and washed on an Amicon ultrafiltration cell with a membrane GR90PP from Dow Danmark A/S. The conductivity was reduced to 1.7 mS. The yield was 150,000 EXU. The pH was adjusted to 5.0 and the sample filtered through a 0.45 micron filter; yield 100,000 EXU. The sample was treated by cation exchange chromatography by means of 200 ml S-Sepharose fast flow column chromatography and a buffer containing 20 mM sodium acetate with pH 5.0. The xylanase bound to the column at this pH. The xylanase was eluted using a linear gradient containing 1 M sodium chloride in the same buffer. Totally 75,000 EXU was recovered in 160 ml with E_{280} 4.1 which corresponds to 114 EXU per E_{280}.

For peptide mapping and measurement of the specific activity and characterization the xylanase is obtained in high purity by a final purification step. By means of size chromatography Superdex 75 a small portion of the xylanase has been highly purified. The purified xylanase has a single band in SDS-PAGE with a molecular weight of 22 kD, and a single band in isoelectric focusing by means of Pharmacia IEF gels with a pI of 8.2.

The native and the cloned xylanase according to the invention purified as described above, react equally with rabbit serum raised against purified native xylanase. By use of rocket immunoelectroforesis both enzymes on an equal EXU basis give rise to the same immune precipitate.

By use of the EXU method the activity of the purified xylanase on colored xylan is 130 EXU per E_{280} or 450 EXU per mg protein. The extinction coefficient is 3.5 E_{280}.

EXAMPLE 3

In this example the prior art *B. pumilus* xylanase has been compared with the xylanase according to the invention. Preparation 1 described in Example 2 was used for the comparison. On a softwood pulp, it was seen that the xylanase according to the invention gave a significantly better bleach boosting effect.

Xylanase according to the invention:	*H. insolens* xylanase, liquid preparation, 533 EXU/g
Prior art xylanase:	*B. pumilus* xylanase liquid preparation, 1070 EXU/g

Pulp

Unbleached softwood from a Swedish mill. The pulp has been washed, air dried, and stored at < 5°C in a cool room.

The pulp was soaked for > 12 hours in water whereafter it was pulped in a laboratory pulper at 1% DS, 10,000 reversions.

Table 1

Data for the pulp measured after storage

Pulp data measured	Softwood
Kappa No.	25.8
pH	7.35
Dry matter (%)	97.0
ISO brightness (%)	28.3

Two bleaching trials were performed, each consisting of the following two stages:

1) Enzyme treatment
2) (D50C50)E delignification

Laboratory bleaching conditions

Enzyme

50°C, pH 8.0 (Britton-Robinson buffer). Treatment time and dosage to be described later under the heading "Enzyme treatment".

(D50C50)

45 minutes, 40°C, 5% DS, final pH: 1.9 - 2.9.

aCL-multiples: 0.15, 0.19, 0.23, 0.27

E

60 minutes, 60°C, 12% DS, final pH: 10.6 - 12.2.

The NaOH dosage is calculated from the chlorine chemicals applied in the delignification stage as

$[0.5 \cdot (\text{kg } Cl_2/\text{ton} + ClO_2/\text{ton}) + 3]$ kg/ton

The bleaching stages were carried out in plastic bags heated in water baths. The bags were kneaded by hand at regular intervals.

Enzyme treatment

Two samples of pulp were treated in the same enzyme stage: one with the xylanase according to the invention, and the other with the prior art xylanase. The treatment time was 3 hours and the dosage was 1000 EXU/kg. Afterwards, the pulp was washed thoroughly with cold water. A third sample - the control - was given the same treatment, but without enzyme addition.

A significant difference between the two enzymes was seen in this experiment.

The Kappa numbers and absorbances measured after enzyme treatment are presented in Table 2.

Table 2

Data measured after the enzyme stage

	Control	Xylanase according to the invention	Prior art xylanase
Kappa no.	24.3	23.0	23.7
Abs. 280 nm	0.31	1.36	1.32

(D50C50)E delignification

From each of the three samples, four portions each of about 17 g of pulp were delignified with different dosages of active chlorine. Afterwards, the Kappa nos. and the ISO brightness values were measured. The results are shown in Table 3.

From Table 3 it appears that the *H. insolens* xylanase according to the invention provides a markedly lower Kappa no. and a better brightness than the prior art xylanase. Even if it appears from Table 3 that the Kappa no. in relation to the xylanase according to the invention is only slightly smaller than the Kappa no. in relation to the prior art xylanase, this slight difference corresponds to a much larger and significant saving of used active chlorine.

Table 3

Kappa nos. and brightness after enz(D50C50)E delignification of the pulp

aCl multiple*		0.15	0.19	0.23	0.27
kg of aCl/ton		36.5	46.2	56.0	65.7
Control	Kappa No.	8.48	6.33	4.55	3.89
	% ISO	36.1	40.8	45.0	47.5
Xylanase according to the invention	Kappa No.	7.22	4.92	3.88	3.03
	% ISO	39.1	44.0	47.9	51.5
Prior art xylanase	Kappa No.	7.54	5.66	4.07	3.28
	% ISO	38.7	42.0	46.5	51.1

* Multiple on Kappa no. of control

For the pulp used in this experiment it can be concluded that the xylanase according to the invention will generate a higher bleach boosting effect than the prior art xylanase.

EXAMPLE 4

This example illustrates the use of the xylanase according to the invention as a baking agent.

Xylanase (the designation pentosanase is commonly used in the baking industry) is used as a baking agent for wheat bread for several purposes:

- dough development
- improving dough elasticity and stability
- increasing bread volume
- improving crumb structure
- anti-staling

Preparation 1 has been tested in wheat bread and it has very good effect on bread quality. This enzyme has sufficient dough softening effect. Addition of 43-150 FXU of preparation 1 increases the volume of rolls by 5-20% and crumb structure become more uniform and crumb is softer than bread without enzyme. It has a better effect in comparison to the commercial products. The xylanase activity unit FXU is defined in AF 293.6.

The prior art xylanase baking agents comprise several enzymatic activites, whereas the baking agent according to the invention easily can be produced with a very low content of enzymatic activities other than the xylanase activity. Thus, by use of the baking agent according to the invention bakery products with more constant characteristics from one baking operation to the next baking operation can be obtained.

Recipe and baking process

The basic recipe in this example is:

- wheat flour 1000 g
- salt 16 g
- sugar 16 g
- yeast 50 g
- water 590 g
- enzyme

The ingredients were mixed by a spiral dough-kneading machine. The dough was then kneaded for 2 mintues, at low speed and 5 minutes, at high speed. The dough temperature was approx. 26-28°C. After 10 minutes of resting, the dough was divided and formed to 30 rolls and 1 loaf. After a proofing time of 45 minutes for rolls and 40 minutes for loaf at 33°C, 80% RH, the rolls and the loaf were baked at 220°C for 15 minutes, and 30 minutes, respectively.

Results

	FXU/kg flour	Dough develop-ment	V-rolls index	V-loaf index	Crumb structure	Softness	
Reference	0	+	100	100	+	100	100
Prep. 1	43 130	++ ++	106 115	105 106	++ +	87 85	66 69
Prep. 1 without amy-lase activity	75 151	++ ++	108 116	103 104	++ +(+)	79 94	88 83
Pentopan	122 427	++ ++	108 111	101 105	++ +	82 96	94 82

In prep. 1 without amylase activity the amylase activity produced by the host organism is separated from the xylanase by means of conventional chromatographic techniques.

The roll and loaf volumes are measured using the traditional rape seeds method. The measured values are then recalculated to relative index.

The crumb softness is measured on a SMS-Texture Analyzer. A plunger with a diameter of 25 mm is pressed on the middle of a 11 mm thick slice of bread, the force needed for the plunger to depress the crumb 3 mm with a speed of 3.3 mm/s is recorded, and it is expressed as the crumb softness. The value is then recalculated to a relative index related to the reference sample which by definition has an index of 100. The lower the index for crumb softness, the softer is the crumb.

The characters for dough development and crumb structure are given according to visual evaluation: + means that the dough is normal, and that the crumb is also normal with coarse structre, and ++ means that the dough is softer than the reference and more elastic, and that the crumb structure is uniform and silky.

Pentopan™, a commercial preparation available from Novo Nordisk A/S, is a prior art baking agent which contains different xylanases from *H. insolens* and also other *H. insolens* enzyme activities. It appears from the above that the xylanase according to the invention has a better performance than the prior art baking agent as it provides larger volume and softer crumb. The most important baking advantage in relation to the xylanase according to the invention, in comparison to the prior art xylanase is the fact that the xylanase according to the invention can be used as a baking agent with practically no side activities, and thus is able to generate bread with very uniform properties from batch to batch.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: NOVO NORDISK A/S

(ii) TITLE OF INVENTION: XYLANASE, CORRESPONDING RECOMBINANT DNA SEQUENCE, XYLANASE CONTAINING AGENT, AND USE OF THE AGENT

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: Novo Allé

(C) CITY: DK-2880 Bagsvaerd

(E) COUNTRY: Denmark

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: BACH, Niels et al.

(B) REGISTRATION NUMBER: GA 24307

(C) REFERENCE/DOCKET NUMBER: 3588.204-WO

(ix) TELECOMMUNICATION INFORMATION:

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(C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Asn Thr Gly Asn Phe Val Gly Gly Lys Trp Asn Pro Gly Thr Gly
1 5 10 15

Arg Thr Lys Asn Tyr
20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Ala Asn Pro Leu Val Glu Tyr Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Trp Trp Ser Asp Gly Gly Gly Gln Val Gln Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Ser Thr Arg Tyr Asn Gln Pro Ser Ile Asp Gly Thr Arg Thr Phe
1 5 10 15

Gln Gln Tyr Trp Ser Ile Arg Lys
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Val Ile Glu Ser Tyr Gly Thr Tyr Asn Pro Gly Ser Gln Ala Gln
1 5 10 15

Tyr Lys Gly Thr Phe Tyr Thr Asp Gly Asp Gln Tyr Asp
20 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid, N-terminal

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gln	Val	Thr	Pro	Asn	Ala	Glu	Gly	Trp	His	Asn	Gly	Tyr	Phe	Tyr
1				5					10					15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 516 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: rDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola insolens

(B) STRAIN: DSM 1800

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGGTCTCGC TCAAGTCTGT CCTCGCGGCC GCCACGGCTG TGAGCTCTGC CATTGCTGCC 60

CCTTTTGACT TCGTTCCTCG GGACAACTCG ACGGCCCTTC AGGCTCGCCA GGTGACCCCC 120

AACGCCGAGG GCTGGCACAA CGGCTACTTC TACTCGTGGT GGTCCGACGG CGGAGGCCAG 180

GTTCAGTACA CCAACCTCGA GGGCAGCCGC TACCAGGTCA GATGGNNNAA CACCGGCAAC 240

TTCGTCGGTG GTAAGGGTTG GAACCCGGGA ACCGGCCCCA CGATCAACTA CGGCGGCTAC 300

TTCAACCCCC AGGGCAACGG CTACCTGGCC GTCTACGGCT GGACCNNNAA CCCGCTCGTC 360

GAGTACTATG TCATCGAGTC GTACGGCACG TACAATCCCG GCAGCCAGGC TCAGTACAAG 420

GGCACATTCT ATACCGACGG CGATCAGTAT GACATCTTTG TGAGCACCCG TNNNAACCAG 480

CCCAGCATCA CGGCACCCGG ACGTCCAGCT AGTACT 516

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 18, line 4 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
18 March 1992	DSM 6995

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Anne Rydberg
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. Xylanase wherein the xylanase has the following partial amino acid sequences

1 Thr-Asn-Thr-Gly-Asn-Phe-Val-Gly-Gly-Lys-Trp-Asn-Pro-Gly-Thr-Gly-Arg-Thr-Lys-Asn-Tyr-, (SEQ ID NO. 1)

2 Thr-Ala-Asn-Pro-Leu-Val-Glu-Tyr-Tyr-, (SEQ ID NO. 2)

3 Ser-Trp-Trp-Ser-Asp-Gly-Gly-Gly-Gln-Val-Gln-Tyr-, (SEQ ID NO. 3)

4 Val-Ser-Thr-Arg-Tyr-Asn-Gln-Pro-Ser-Ile-Asp-Gly-Thr-Arg-Thr-Phe-Gln-Gln-Tyr-Trp-Ser-Ile-Arg-Lys-, (SEQ ID NO. 4)

5 Tyr-Val-Ile-Glu-Ser-Tyr-Gly-Thr-Tyr-Asn-Pro-Gly-Ser-Gln-Ala-Gln-Tyr-Lys-Gly-Thr-Phe-Tyr-Thr-Asp-Gly-Asp-Gln-Tyr-Asp-, (SEQ ID NO. 5)

and

6 Gln-Val-Thr-Pro-Asn-Ala-Glu-Gly-Trp-His-Asn-Gly-Tyr-Phe-Tyr-, (SEQ ID NO. 6)

or a partial amino acid sequence with a homology thereto of at least 80%, preferably at least 90%.

2. Xylanase according to Claim 1, wherein the xylanase is immunoreactive with an antibody raised against a purified xylanase derived from *Humicola insolens*, DSM 1800.

3. Xylanase according to any of Claims 1 - 2, wherein the xylanase has an isoelectric point of 7.5 - 9.5, preferably 8.0 - 8.5.

4. Xylanase according to Claims 1 - 3, wherein the xylanase exhibits a specific activity above 330 EXU/mg of protein, preferably above 400 EXU/mg of protein.

5. Recombinant DNA sequence, characterized by encoding for the xylanase according to Claims 1 - 4.

6. A recombinant DNA sequence according to Claim 5, comprising the following partial DNA sequence

```
   1                5                   10                  15
   ATG GTC TCG CTC AAG TCT GTC CTC GCG GCC GCC ACG GCT GTG AGC

10                  20                  25                  30
   TCT GCC ATT GCT GCC CCT TTT GAC TTC GTT CCT CGG GAC AAC TCG

                    35                  40                  45
   ACG GCC CTT CAG GCT CGC CAG GTG ACC CCC AAC GCC GAG GGC TGG

                    50                  55                  60
15 CAC AAC GGC TAC TTC TAC TCG TGG TGG TCC GAC GGC GGA GGC CAG

                    65                  70                  75
   GTT CAG TAC ACC AAC CTC GAG GGC AGC CGC TAC CAG GTC AGA TGG

                    80                  85                  90
   NNN AAC ACC GGC AAC TTC GTC GGT GGT AAG GGT TGG AAC CCG GGA

20                  95                  100                 105
   ACC GGC CCC ACG ATC AAC TAC GGC GGC TAC TTC AAC CCC CAG GGC

                   110                 115                 120
   AAC GGC TAC CTG GCC GTC TAC GGC TGG ACC NNN AAC CCG CTC GTC

                   125                 130                 135
25 GAG TAC TAT GTC ATC GAG TCG TAC GGC ACG TAC AAT CCC GGC AGC

                   140                 145                 150
   CAG GCT CAG TAC AAG GGC ACA TTC TAT ACC GAC GGC GAT CAG TAT

                   155                 160                 165
   GAC ATC TTT GTG AGC ACC CGT NNN AAC CAG CCC AGC ATC ACG GCA

30                 170
   CCC GGA CGT CCA GCT AGT ACT (= SEQ ID NO. 7)
```

7. A recombinant DNA sequence according to Claim 5 or 6, comprising a DNA sequence selected from

a) the *Humicola insolens* xylanase DNA insert in pHD 450

b) a DNA sequence which hybridizes to the coding region for the mature xylanase DNA comprised by the DNA insert of a) and which comprises a structural gene for a polypeptide with xylanase activity, and optionally a promoter, a coding region for a signal or leader peptide and/or transcriptional terminator

c) a DNA sequence with a homology sufficient to hybridize to the sequence indicated in Claim 6 under relative stringent conditions (1.0 x SSC, 0.1% SDS, 65°C), reference being made to T. Maniatis, A laboratory Manual (CSH)

d) a derivative of a DNA sequence defined in a), b) or c), or

e) a DNA sequence which codes for a mature xylanase or a signal peptide or a leader peptide thereof and which is degenerate within the meaning of the genetic code with respect to a DNA sequence of a), b) or c).

8. Vector comprising the recombinant DNA sequence according to Claims 5-7.

9. Vector according to Claim 8, wherein the promoter is the *Aspergillus oryzae* takaamylase promoter and/or the xylanase gene is isolated from EC 3-2, and/or the terminator is the *Aspergillus oryzae* AMG terminator, preferably pHD450.

10. Transformed host containing the vector according to Claim 8 or 9.

11. Transformed host according to Claim 10, wherein the transformed host is an *Aspergillus* strain.

12. Transformed host according to Claim 10, wherein the transformed host is a strain belonging to the species *Aspergillus aculeatus*, *Aspergillus niger*, *Aspergillus oryzae* or *Aspergillus awamori*.

13. Transformed host according to Claim 10, wherein the transformed host is a microorganism, which in its non-transformed condition does not produce xylanase or only produces xylanase in insignificant amounts, preferably *Bacillus sp.*, *E. coli* or *S. cerevisiae*.

14. Method for production of a xylanase by use of a transformed host according to Claims 10 - 13.

15. Xylanase produced by the method according to Claim 14.

16. Agent containing the xylanase according to any of claims 1 - 4 or 15, preferably in the form of a non-dusting granulate, a stabilized liquid or a protected enzyme, whereby the xylanase comprises at least 10%, preferably at least 30% of the total enzyme protein.

17. Agent according to Claim 16, wherein the proportion between the xylanase activity and the cellulase activity, as expressed by the ratio between the xylanase activity in EXU/g and the cellulase activity in ECU/g has a value above 10, preferably above 30, most preferably above 100.

18. Agent according to Claims 16 - 17 which contains a xylanase activity of at least 10 EXU/mg of enzyme protein, preferably at least 100 EXU/mg of enzyme protein, more preferably at least 300 EXU/mg of enzyme protein.

19. Use of the agent according to Claims 16 - 18, characterized by the fact that the agent is used for xylan degradation.

20. Use according to Claim 19, characterized by the fact that the use is related to chemical pulp or recycle paper pulp before or as part of bleaching, preferably at a pH value above 7.

21. Use according to Claim 19, characterized by the fact that the use is related to production of bread, which contains wheat flour.

22. Use according to Claim 19, characterized by the fact that the use is related to animal feed.

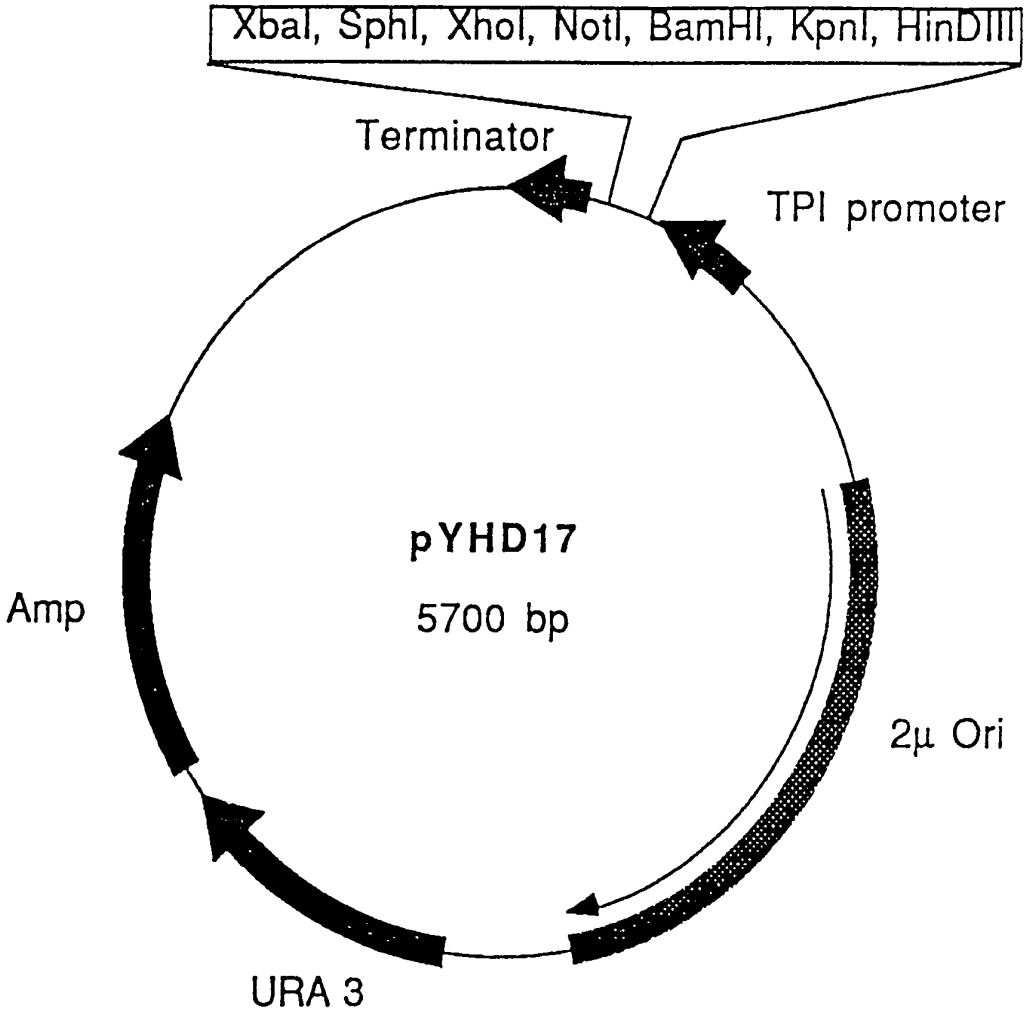
XbaI, SphI, XhoI, NotI, BamHI, KpnI, HinDIII
Terminator
TPI promoter
pYHD17
5700 bp
Amp
2μ Ori
URA 3

FIG. 1

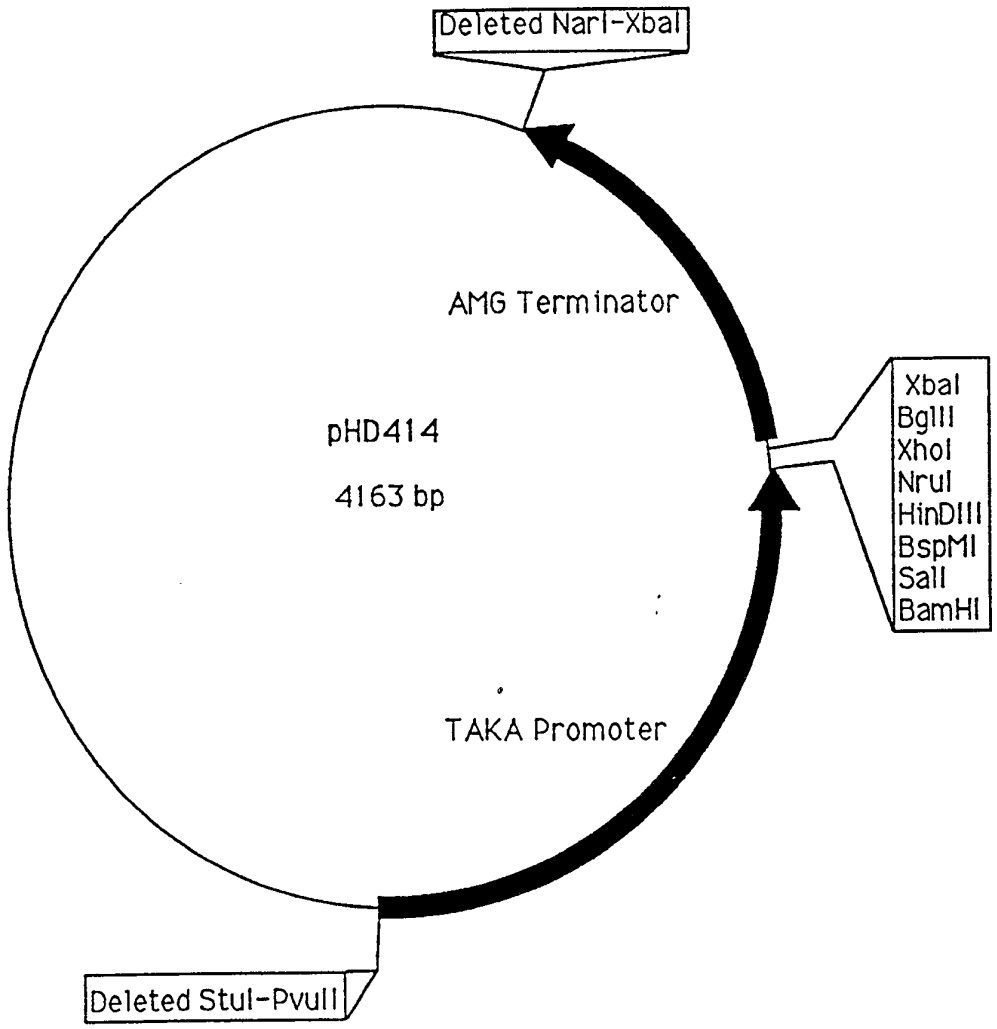
Deleted NarI-XbaI
AMG Terminator
pHD414
4163 bp
XbaI
BglII
XhoI
NruI
HinDIII
BspMI
SalI
BamHI
TAKA Promoter
Deleted StuI-PvuII

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00099

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 9/42, C 12 N 9/24, C 12 N 15/56, C 12 S 3/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; C 12 S

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCLEIC ACIDS RESEARCH, Vol. 18, No. 8, 1990 Harold Zappe et al: "Nucleotide sequence of a Clostridium acetobutylicum P262 xylanase gene (xynB)", p 2179 --	1-22
A	WO, A1, 9102839 (NOVO NORDISK A/S) 7 March 1991, see the whole document --	1-22
A	WO, A1, 9102791 (CULTOR OY) 7 March 1991, see especially the claims --	1-22
A	WO, A1, 9001060 (IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC.) 8 February 1990, see the whole document --	1-22

* Special categories of cited documents: [10]

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

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

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

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

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

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

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd July 1992	1992 -07- 07
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carolina Palmcrantz Carolina Palmcrantz

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 99, no. 23, 5 December 1983, (Columbus, Ohio, US), Isshiki, Yutaka et al: "Effect of pectinase, xylanase and cellulase supplements on the utilization of feed in chicks ", see page 652, abstract 193693d, & Nippon Kakin Gakkaishi 1983, 20(4), 237- 243 -- --------	1-22

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00099

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 29/05/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9102839	91-03-07	AU-D-	6358490	91-04-03
WO-A1- 9102791	91-03-07	NONE		
WO-A1- 9001060	90-02-08	AU-D-	2542888	90-02-19
		EP-A-	0353342	90-02-07
		JP-T-	3500366	91-01-31