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# β-Xylanase produced by *Aureobasidium pullulans* CBS 58475

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**Summary.** *Aureobasidium pullulans* CBS 58475 produced β-xylanase with an activity of 5 units/ml culture filtrate. Xylose, xylan and complex substrates containing xylose served as strong inducers. Purification of the enzyme was achieved by two sets of gel permeation chromatography. The enzyme has an pH optimum at 4.25 and a temperature optimum at 60° C. At slightly acid pH and temperatures up to 60° C β-xylanase showed good stability. The analysis of cleavage products classified the β-xylanase as an endoenzyme. Together with an endopolygalacturonase, the β-xylanase enhanced the maceration of carrots compared to endopolygalacturonase alone.

## Introduction

Hemicelluloses, particularly β-1.4-xylan, make up the major part of monocotyledonous primary cell walls, up to 30% of dicotyledonous secondary cell walls and up to 10% of gymnosperm walls. The backbone of β-1.4-xylan is, depending on the source and method of preparation, slightly to highly branched and has several substituents such as acetyl, arabinosyl and glucuronosyl residues (Aspinall 1980). The hydrolysis of β-1.4-xylan plays an important role in the conversion of renewable resources such as straw and wood into easily fermentable products. As opposed to chemical degradation, enzymatic hydrolysis shows specific reactions and milder conditions are required for the reaction (Thomson 1983).

Several species of bacteria and fungi have been reported to produce xylanolytic enzymes (Dekker and Richards 1976). In most cases these enzymes show high multiplicity and specificity of reaction. β-1.4-Xylanase appears in all systems as the dominant enzyme and is often accompanied by β-xylosidase, arabinofuranosidase, acetylesterase and α-glucuronidase, achieving high rates of hydrolysis (Biely et al. 1986; Poutanen et al. 1987). In combination with other polysaccharidases β-1.4-xylanases appear to open up new possibilities in the food industry and for bioconversion of agricultural wastes (Wong et al. 1988; Biely 1985).

Because of cultivation advantages several strains of the yeast-like fungus *Aureobasidium pullulans* have been examined for the production of β-xylanase. The present study was undertaken in order to find out the best conditions for the production and purification of this enzyme. The characteristics of purified β-xylanase from *A. pullulans* are compared to those of other fungal β-1.4-xylanases.

## Materials and methods

*Enzyme preparation.* The *A. pullulans* strain CBS 58475 was cultivated in a medium containing 1% xylose, 1% Yeast Carbon Base (YCB) (Difco, Detroit, Mich, USA) and 1% $(NH_4)_2SO_4$ in a 0.1 *M* citrate-phosphate buffer, pH 4. The cultivation was carried out in a Fernbach flask for 72 h at 28° C.

*Induction of β-xylanase.* Production of the enzyme was investigated on media containing 1% different carbon sources, 1% YNB (Difco) in 0.1 *M* citrate-phosphate buffer, pH 4. The carbon sources used were monosaccharides (glucose, galactose, arabinose, xylose, galacturonic acid), polysaccharides (starch, β-1.4-glucan, carboxymethylcellulose (CMC), pectic acid, galactan, araban, xylan) and complex substrates (barley straw, beet rasps, draff, spruce wood pulp). The concentration of complex substrates amounted to 5%.

*Enzyme assays.* Xylanase activity was measured by incubating 0.1 ml enzyme and 0.9 ml of 1% xylan solution (larchwood, Sigma, St. Louis, Mo, USA). The protein content of the enzyme samples was 330 μg/ml culture filtrate and 130 μg/ml P6

concentrate (Table 2), which was used for chemical characterization. Xylan was resuspended in 0.1 *M* citrate-phosphate buffer, pH 4. The incubation lasted 10 min at 37° C. Reducing sugars were quantified using the method of Nelson (1944) and Somogyi (1952) modified by Spiro (1966). The unit of activity is expressed as micromoles of monosaccharide equivalents released per minute and per millilitre of enzyme. The activity of endopolygalacturonase was estimated by analogy with β-xylanase.

Protein was measured as described by Lowry et al. (1951).

*Purification.* The culture filtrate was lyophilized and desalted on a P6-column (Bio-Rad Richmond, Calif., USA). After a second lyophilisation the enzyme was chromatographed on an HR200 column (Pharmacia, Uppsala, Sweden). Xylanase-active fractions were concentrated and again chromatographed on a G50 column (Pharmacia). After another desalting step β-xylanase was lyophilized for storage at 4° C.

*Isoelectric focusing (IEF).* This was carried out with polyacrylamide gels, pH 3–10 (Serva, Heidelberg, FRG), or polyacrylamide gels prepared with Ampholine (Serva) of a pH range from 5–8 (Görg et al. 1978); IEF was carried out as described by Höfelmann et al. (1983). The samples were desalted and resuspended in distilled water. The gels were either stained with Coomassie R250 or $AgNO_3$ (Morrissey 1981).

Specific detection of β-xylanase was carried out with polyacrylamide replicas, prepared in a similar way to the gels for IEF, except that 1% dyed xylan from birch wood (Roth, Karlsruhe, FRG) was added (Biely et al. 1985a). β-Xylanase could be visualized by layering the focused gel over the substrate gel and incubating the gels in a wet chamber at 50° C for 2 h (Biely et al. 1985b). The arrangement of protein bands was compared to bands of IEF marker proteins, pH 3–10 (Pharmacia).

*Sodium dodecyl sulphate (SDS)-PAGE.* This was carried out according to Lämmli (1970) with concentration of total monomers (T) 12.5% and proportion of crosslinker to total monomer (C) 2.5%. The gels were stained with $AgNO_3$ (Morrissey 1981). Protein bands were compared to low molecular marker proteins of 14400–94000 daltons (Pharmacia).

*Optimum pH and temperature.* The purified enzyme was incubated with 1% xylan dissolved in 0.1 *M* acetate buffers of different pH values. For determination of the temperature optimum, the standard assay was performed at different temperatures.

*Temperature and pH stability.* The enzyme was dissolved in 0.1 *M* buffers with pH values ranging from 2 to 12 and incubated for 4 days at 8° C. The test of temperature stability was carried out with the enzyme in standard buffer solution. The remaining β-xylanase activity was assessed as described above.

*Analysis of cleavage products.* The purified enzyme was incubated with a 50-fold volume of a 2% xylan solution for different periods. After heat inactivation the samples were analysed by gel permeation chromatography on a P2 column (Bio-Rad) as described by Lloberas et al. (1988).

*Maceration assay.* The maceration assay contained 9 g carrot rasps and different quantities of enzyme, suspended to a final volume of 30 ml and a final buffer concentration of 0.1 *M* citrate-phosphate, pH 4. The samples were incubated for 2 h at 45° C on a reciprocal shaker. The amount of tissue released was assayed by sedimentation. The results were compared to maceration results obtained with Rohament P (Röhm, Darmstadt, FRG), which contained 300 mg of enzyme.

## Results and discussion

Table 1 shows the varying yields of β-xylanase, which depend upon the carbon source used. The number of cells was $3–5 \times 10^8$ cells/ml culture broth, except on CMC, β-1.4-glucan, straw and wood pulp ($10^8$/ml). *Aureobasidium pullulans* produced 5 units of β-1.4-xylanase/ml culture filtrate, which is an average value compared to the yields of other moulds. In several similar studies the release of β-xylanase lay between 1 and 30 units/ml culture filtrate (Poutanen et al. 1987; Biswas et al. 1987), although there have been maximum yields of 575 units/ml (Yu et al. 1987) and 1244 units/ml (Steiner et al. 1987).

The yields of different moulds are difficult to compare, because there is no standard test available for the estimation of β-xylanase activity. Increases in enzyme activity from tenfold to 100-fold are possible by dilution of one and the same enzyme sample (Khan et al. 1986; Cauchon and Le Duy 1983). Based on the data obtained β-xylanase was mainly produced on xylan, xylose and substrates containing high amounts of xylose olig-

**Table 1.** Xylanase activity (units/ml) in culture filtrates after cultivation for 3 or 4 days on different carbon sources

| Monosaccharides (4 days) | | Polysaccharides (3 days) | | Complex substrates (4 days) | |
|---|---|---|---|---|---|
| Glucose | 1.2 | Starch | 0.4 | Straw | 2.7 |
| Galactose | 0.4 | Pectic acid | 0.3 | Draff | 4.5 |
| Arabinose | 0.9 | CMC[a] | 0.6 | Beet rasps | 0.95 |
| Xylose | 3.75 | β-1.4-Glucan | 1.2 | Wood pulp | 0.5 |
| Galacturonic acid | 0.3 | Araban | 0.7 | | |
| | | Galactan | 0.3 | | |
| | | Xylan | 5.0 | | |

[a] Carboxymethylcellulose

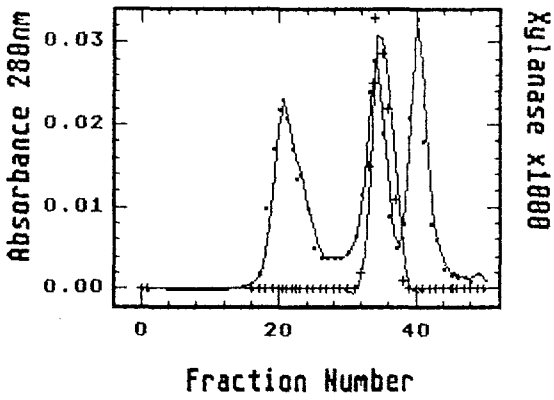


Fig. 1. Sephacryl HR200 chromatography of β-xylanase (P6 concentrate). The column (95 × 2.6 cm) was equilibrated and eluted with 0.1 *M* acetate buffer. The flow rate was 150 ml/h and the fraction volume was 10 ml: ·, absorbance at 280 nm; +, activity of β-xylanase

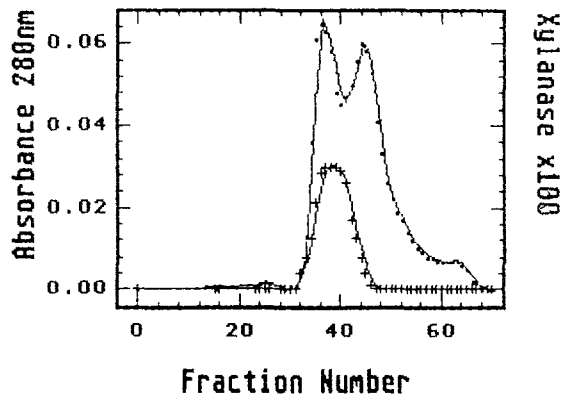


Fig. 2. Sephadex G50 chromatography of β-xylanase (HR200 concentrate). The column (55 × 1.5 cm) was equilibrated and eluted with 20 m*M* acetate buffer, pH 4. The flow rate was 9 ml/h and the fraction volume was 3 ml: ·, absorbance at 280 nm; +, activity of β-xylanase

omers. Cultivation on glucose and β-1.4-glucan resulted in slightly higher production than the base level.

Production of β-xylanase is strongly inducible; xylose and its oligomers are the strongest inducers. These results correspond to the results of Pou-Llinas and Driguez (1987) and Leathers et al. (1986). In the absence of xylose the production of β-xylanase was tenfold lower.

Purification of β-xylanase was achieved in two steps. In the first step, i.e. gel filtration using an HR200 column, β-xylanase was separated from other enzymes such as polygalacturonases, α-1.4-glucoamylase, β-xylosidase and β-glucosidase. These enzymes have a higher molecular mass and were eluted at an earlier stage (Fig. 1). In the second step, i.e. gel filtration over a G50 column, β-xylanase was purified from arabinofuranosidase (Fig. 2). The purification is easy and can be rapidly performed to electorphoretical homogeneity.

Purification by ion-exchange chromatography failed because α-1.4-glucoamylase, endopolygalacturonase, exopolygalacturonase and arabinofuranosidase show several isoenzymes with isoelectrical points near to the ones of β-xylanase. In contrast, purification succeeded by gel permeation chromatography because β-xylanase exhibits the smallest molecular mass of the polysaccharidases. The purification data are summarized in Table 2.

The purification factor represents only a standard value since increasing dilution of the samples would cause increasing activities. The specific activity is similar to the ones of the culture strains of *A. pullulans* screened by Leathers (1986). Contrary to his results, no mutants or wild strains of *A. pullulans* with extremely high β-xylanase yields could be isolated.

In IEF-PAGE (Fig. 3) β-xylanase exhibited three bands of protein and substrate stain. The released Remazol Brillant Blue of the dyed substrate resulted in blue bands in lane 2, while the blue substrate gel was decolourised in these zones. The main band had a pI at pH 8.0 and the two weaker bands were focussed at pH 7.4 and pH 6.75. The pIs of fungal xylanases are in a wide pH range (4–9.5) (Dekker and Richards 1975; Tan et al.1985).

**Table 2.** Purification of β-xylanase

| Purification step | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Culture filtrate | 132 | 930 | 7.0 | 100 | 1 |
| P6 concentrate I | 23.65 | 848 | 35.6 | 91.2 | 5 |
| HR200 concentrate | 11.3 | 435 | 38.4 | 46.8 | 5.5 |
| G50 concentrate | 5 | 340 | 68.0 | 36.6 | 9.6 |
| P6 concentrate II | 5 | 340 | 68.0 | 36.6 | 9.6 |

Each concentrate represents the β-xylanase active fractions after the respective chromatography step

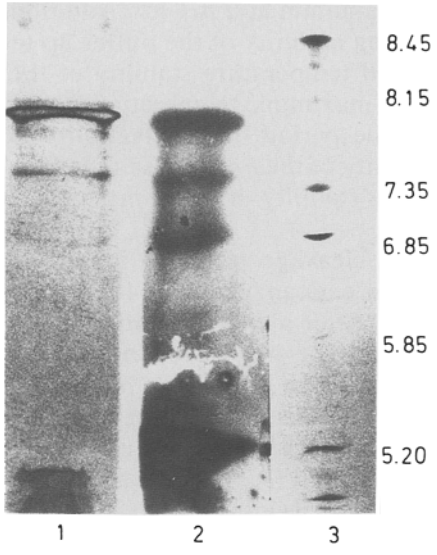


**Fig. 3.** Isoelectric focusing of β-xylanase by polyacrylamide gel electrophoresis (PAGE) with a pH gradient of 5–8: *lane 1,* β-xylanase stained with $AgNO_3$; *lane 2,* β-xylanase after incubation with the substrate gel; *lane 3,* standard proteins stained with Coomassie R250. The isoelectric points of standard proteins are indicated by the *numbers* on the right

The SDS-PAGE of β-xylanase (Fig. 4) showed a small protein with an apparent molecular mass of 24000 daltons, which is typical for fungal xylanase. Similar to the results of Leathers (1986) the β-xylanase sometimes exhibited a second protein band with a slightly higher molecular mass. This protein band seemed to be an artefact of the 24000 dalton band, which appeared depending upon the increasing age and preparation procedure of the enzyme for SDS-PAGE.

In gel filtration on Superose 12 HR10/30 (Pharmacia) connected to a Pharmacia FPLC System the purified enzyme showed a single peak. In contrast to SDS-PAGE the apparent resulting molecular mass was 14000 daltons, compared to the partition coefficient $(K_{av} = V_c - V_o)/(V_t - V_o)$ of cytochrome C, ovalbumin and bovine serum albumin. The molecular mass differs widely depending on the method of estimation, and the varying conformation of the respective protein markers compared to β-xylanase.

The pH optimum of β-xylanase was 4.25 and the temperature optimum was 60° C in a 10 min incubation assay. The data of the respective experiments are shown in Figs. 5 and 6. These optima do not differ from other fungal optima, which are generally at a slightly acid pH and a temperature between 50° C and 70° C. The pH

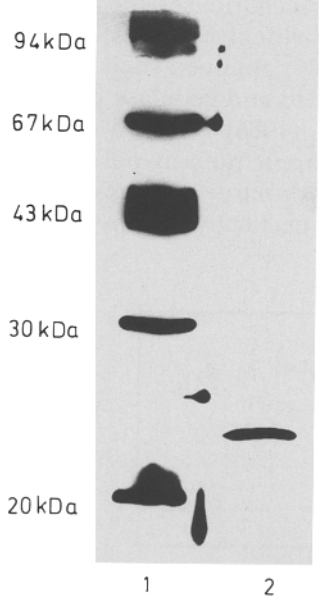


**Fig. 4.** Sodium dodecyl sulphate (SDS)-PAGE of β-xylanase, stained with $AgNO_3$: *lane 1,* standard proteins; *lane 2,* β-xylanase. The *numbers* on the left are the molecular masses of the standard proteins

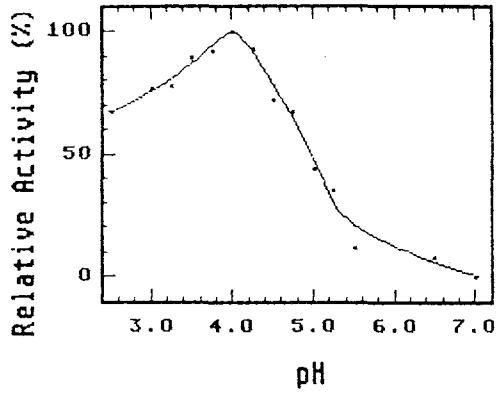


**Fig. 5.** Effect of pH on the activity of purified β-xylanase

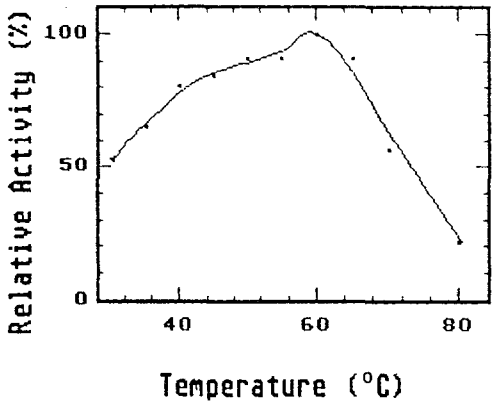


**Fig. 6.** Effect of temperature on the activity of purified β-xylanase

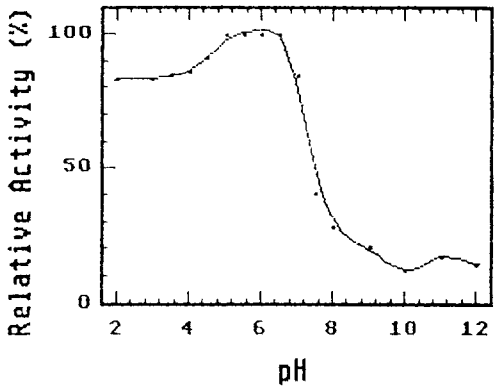


**Fig. 7.** Effect of pH on the stability of purified β-xylanase

and temperature stability were also maximal in these ranges. The enzyme was stable at pH values between 2 and 6.5 for 4 days at 4° C and showed a loss of 20% activity at 40° C, or a loss of 65% activity in the 60 min incubation assay. Figures 7 and 8 demonstrate these data.

The activity of β-xylanase was influenced in a positive way when the buffer contained phosphate ions. With citrate-phosphate buffers for determination of the pH optimum, the enzyme showed a second maximum at a pH 5.5. Addition of NaCl or increasing molarity of the buffer up to 1 *M* led to increased temperature stability of the enzyme. While the maximum temperature could not be increased, the period of inactivation was extended. The stability of the β-xylanase therefore corresponded to the stability of other mesophilic fungal xylanases.

The analysis of cleavage products identified the β-xylanase as an endoenzyme (β-1.4-xylanhydrolase EC 3.2.1.8). After a short period of incubation the proportion of oligosaccharides (Decamer of Xylose Dp10 to Pentamer of Xylose Dp5) increased while after incubation for 24 h only xylotetrose, xylotriose, xylobiose and small amounts of xylose resulted (Fig. 9). For an exoenzyme the proportion of xylose would rise soon after the start of incubation and would be the dominant cleavage product. The endomechanism was confirmed by viscosimetric data.

β-Xylanase alone showed no maceration activity on carrots. Together with the endopolygalacturonase of *Candida macedoniensis* maceration was increased compared to maceration using endopolygalacturonase alone, as presented in Fig. 10. While the amount of β-xylanase was constant at 9 units per probe the amount of endopolygalacturonase increased. This result showed the synergistic effect of β-xylanase and endopolygalacturonase in the maceration of carrots, exhibiting 84% of the maceration obtained by Rohament P. Several reports describe similar synergistic effects, where combined action of polysaccharidases shows better yields than from individual enzymes. While Sreenath and Radola (1986) obtained such results with polyendogalacturonase and cellulase on carrot rasps, Voragen et al. (1986) received almost complete liquefaction of apple pulp by polygalacturonase and cellobiohydrolase and enhanced cloud stability of apricot nectar by the snynergis-

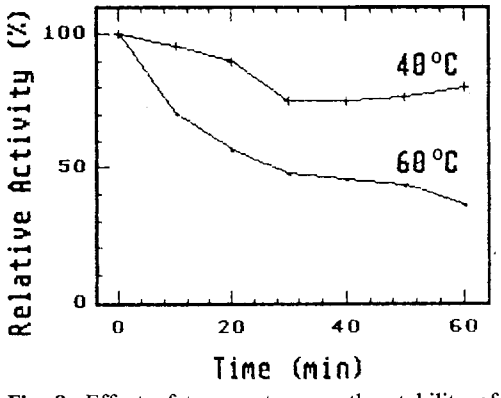


**Fig. 8.** Effect of temperature on the stability of purified β-xylanase

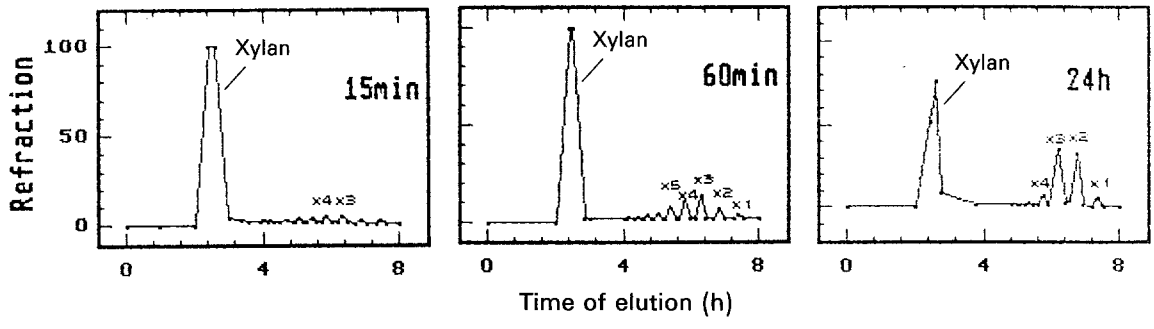


**Fig. 9.** Bio-Gel P2-chromatography of the cleavage products (xylose oligomers $X_1$ to $X_9$ released by β-xylanase after different periods of incubation (15 min, 60 min and 24 h). The column (200 × 1.5 cm) was eluted with distilled water (65° C). The flow rate was 32 ml/h

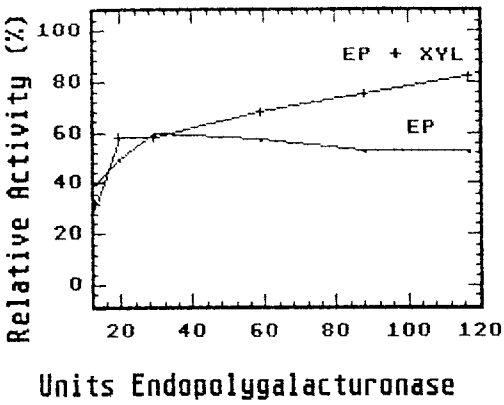


**Fig. 10.** Macerating action of endopolygalacturonase (·, EP) and endopolygalacturonase together with β-xylanase (+, EP+XYL) on carrot rasps

tic action of polygalacturonase and exoarabanase. One reason for these effects might be that endopolygalacturonase can only release protopectin, which is not linked to other polysaccharides. In combination with β-xylanase the parts of the protopectin that are linked to xylan can be hydrolysed and the quantity of liberated cells increases.

β-Xylanase seems to be an interesting enzyme for the maceration of vegetables. The synergistic effect can also be reached with native culture filtrates of *A. pullulans* and *C. macedoniensis*. Although the native culture filtrate of *A. pullulans* contains endopolygalacturonase, it is not capable of maceration. A combined application of both enzymes tends to be less problematic than the application of similar mould enzymes, which can cause allergic reactions. The separate production of culture filtrates might be less advantageous.

Another field of application might be the use of β-xylanase in the paper industry. A β-xylanase free of cellulase will achieve better results in the preparation of cellular pulps (Biely 1985). In contrast to the β-xylanases of other moulds, the β-xylanase of *A. pullulans* is produced without cellulase. As with β-xylanase, cellulytic enzymes are only produced in the presence of specific inducer, so substrates such as xylose or xylan do not lead to production of cellulase. Further, β-xylanase might improve the liberation of plant fibres from flax and hemp.

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