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# Application of xylanolytic enzymes in alkyl-β-xyloside synthesis and purification of β-xylosidase from *Sclerotinia sclerotiorum*

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***ABSTRACT*:** β-Xylosidase and endo-xylanase belong to an enzyme battery that phytopathogenic fungi use for the degradation of the cell walls. An enzyme preparation from *Sclerotinia sclerotiorum* was applied in synthesis of biosurfactants: hexyl-β-xyloside and hexyl-β-xylobioside from xylan and hexanol. Also, in order to characterize the enzyme activity, the β-xylosidase obtained in the culture filtrate was purified by the successive use of anion exchange chromatography DEAE-sepharose, anion exchange HPLC TSK-DEAE, gel filtration on TSK-200 SW HPLC followed by preparative native-PAGE. β-Xylosidase seems to be a monomeric protein with 70 kDa estimated by gel filtration and 72 kDa determined by SDS-PAGE. With pNPX as substrate, β-xylosidase has Michaelis-Menten kinetics: $K_M$ = 1.61 mM and $V_M$ = 0.14 µmol $min^{-1}$ mg protein. The enzyme showed an optimal activity at 60 °C and pH 4. It also showed stability over a wide pH range (from 2 to 9) and retained up to 50% of its activity at 50 °C. The enzyme activity was further characterized by testing several divalent ions and reagents.

**Key words**: Alkyl-xyloside, endoxylanase, Sclerotinia sclerotiorum, synthesis reaction, xylan, β-xylosidase.

## Introduction

Xylans are among the most abundant organic compounds in the biosphere. These heterogeneous hemicelluloses have basic backbone of poly β-1.4-xylose with additional side links to arabinose, glucuronic acid and arabino glucuronic acid *(1-3)*. As other lignocelluloses, they occur in agricultural, forestry, and fruit and vegetable processing wastes. This biomass has an immense potential as a renewable raw material. Thus, suitable and economic technology can be developed in order to allow its use in novel and non-classical fields for edible or for non-edible products. The valorization of xylans using the biotechnology approach can lead to added value natural products.

Industrial and fundamental researches have shown that many carbohydrate-hydrolyzing enzymes can be used for the production of added-value compounds *(3-4)*. Pathogenic fungi has a strong enzyme battery that allows the degradation of the plant cell walls. The degradation of xylan is performed by several enzyme activities, especially the xylanases and the b-xylosidase. These enzymes have found applications mainly in synthesis reactions. One of these applications is the synthesis of alkyl-xylosides from xylose derivatives or xylose polymers and alcohols by reversed hydrolysis or transglycosylation in organic-aqueous two-phase media *(5-8)*. The importance of alkyl-glycosides, in general, has been demonstrated for many industrial applications as non-ionic surfactants *(9)*. They are one of the most promising candidates of new surfactants because they are made from naturally occurring renewable resources of carbohydrates and fatty alcohols. However, the application of the enzymatic processes in industry remains limited especially by the production cost and enzyme purification.

In this study we attempted the production of xylanolytic enzymes from the fungus: *Sclerotinia*

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*sclerotiorum*, in order to use them in synthesis reaction of alkyl-b-xylosides in a biphasic medium. The purification of a b-xylosidase as well as its biochemical and catalytic properties were studied. A strong hydrolytic enzyme battery in *Sclerotinia sclerotoirum* was first described by Saidane et al *(10)* and Smaali et al. *(11-12)*. In previous work, we evaluated different supports for immobilization of b-xylosidase and b-glucosidase. Highest yields of alkyl-glycoside synthesis have been obtained when enzymes were immobilized by adsorption *(24)*.

## Experimental

### *Microorganisms and culture conditions*

*Sclerotinia sclerotiorum* was isolated from sunflower (local isolate from Tunisia) with a screening program for xylanolytic activity-producing microorganisms. The fungus was obtained from the national fungi collection of the "Laboratoire de Cryptogamie INRAT in Tunis". It was maintained on potato dextrose agar.

The enzyme production was achieved on media containing KCl (1 g $L^{-1}$), $KH_2PO_4$ (1 g $L^{-1}$), $MgSO_4$ (0.5 g $L^{-1}$), $(NH_4)_2SO_4$ (1.4 g $L^{-1}$), $NaNO_3$ (4.33 g $L^{-1}$), yeast extract (2 g $L^{-1}$), phtalate potassium buffer (5 g $L^{-1}$) with starting pH 5.5 *(13)*, in presence of 1% (w/v) xylan from birch wood (Sigma, France). b-Xylosidase and endoxylanase activities were measured after 9 days of culture at 25 °C respectively using pNPX and DNS (Sigma, France).

The incubation was carried out on shaker at 25 °C and 150 rpm for 9 days. The mycelia were then removed by filtration on GF/D paper and centrifugation 8000 rpm during 30 min at 4 °C. The supernatant containing the enzyme activity was then recovered.

### *Enzyme assay*

The β-xylosidase activity was measured in presence of 0.5 mM p-nitrophenyl-b-D-xylopyranoside (pNPX, Sigma, France) in 50 mM sodium acetate buffer containing 200 mM NaCl. An available quantity of the enzyme preparation was added to the medium and the 500 µl reaction mixture was incubated for 30 min at 60 °C. The reaction was stopped by adding 600 ml 0.4 M glycine buffer pH 10.8 and the liberated p-nitrophenol (pNP) quantity was measured at 410 nm using a computer-controlled spectrophotometer (Beckman DU 530, France). 1U activity was defined as the amount of enzyme producing 1 mmol pNP per min. The kinetic behavior of the enzyme was studied in the absence or the presence of several inhibitors and chemicals.

The β-glucosidase activity was measured using the same method and conditions described below, with p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate and without addition of NaCl.

Endo-xylanase activity was assayed on the basis of reducing sugars cleaved from xylan. 1% substrate was incubated with an appropriate enzyme solution at 55°C for 30 min. The concentration of reducing sugars was measured by the DNS method *(14)* and expressed as xylose equivalent. One unit of endo-xylanase activity corresponded to 1 mmole equivalent xylose liberated per minute at these conditions.

### *Synthesis reaction*

The preparation of alkyl-β-xylosides was achieved by reverse-hydrolysis reaction or transxylosylation in biphasic medium (aqueous/alcohol, phase ratio: ¼) at 50 °C and 250 rpm. 4 mL alcohol were mixed with aqueous phase containing 50 mg xylan or xylose in 50 mM acetate buffer pH 4. The reaction started when adding 50 ml enzyme preparation (5U $mL^{-1}$). The final aqueous phase volume is 1 mL.

The synthesis of alkyl-xyloside or alkyl-xylobioside was qualitatively detected by thin layer chromatography (TLC) using silica gel and elution phase composed of 1-propanol and water (85/15, v/v). The visualization was achieved with 20 % sulfuric acid in methanol followed by heating 10 min at 150 °C.

RP-HPLC (HP 1100, France) was used for the measurement of the product synthesis with C18 reversed phase column Ultrasep 6 mm (Bischoff, France, 4 x 250 mm), heated to 50 °C, followed by refractometer and spectrophotometer UV 210 nm and 280 nm. The mobile phase (0.5 mL $min^{-1}$) is a mixture of methanol and ultra pure water. The percentage used for each phase depends on the carbon chain length of the alcohol; varying from 40/60 (methanol/water) with butanol as substrate, to 60/40 (methanol/water) with octanol as substrate, containing 0.2% acetic acid. Samples are homogenized with equal volumes of acetonitrile. After the centrifugation, 20mL of the liquid is injected. Octyl-β-D-glucopyranoside, heptyl-β-D-glucopyranoside, hexyl-β-D-glucopyranoside, pentyl-β-D-glucopyranoside, butyl-b-D-glucopyranoside and butyl-β-D-glucopyranosyl-galactopyranoside (Sigma, France) were used as standards diluted in their corresponding alcohols mixed with 1 v acetonitrile.

*β-Xylosidase purification protocol*

After enzyme production with xylan as unique carbon source and recovery of extracellular proteins, the culture filtrate was applied to an anion exchange chromatography on DEAE-sepharose CL6B column (15x3 cm, Pharmacia) equilibrated with 25 mM Tris-HCl buffer, pH 7.25. The elution was achieved with a linear NaCl gradient (0-0.5 M) in the same buffer with 30 mL $min^{-1}$ flow rate. The fractions were collected and screened for b-xylosidase activity. The b-xylosidase active fractions were pooled and diluted with 50 mM Tris-HCl buffer pH 7.25, then concentrated by ultrafiltration on amicon membrane (PM 10).

The whole fraction was injected onto an anion exchange HPLC column (TSK-DEAE 7.5 x 75 mm), equilibrated with 50 mM Tris-HCl buffer (pH 7.25). A linear NaCl gradient (0-0.3 M) was used in the same buffer at a flow rate of 0.5 mL $min^{-1}$. The active fractions were then collected and concentrated by ultrafiltration.

The obtained fraction was chromatographied by gel filtration on TSK-200 SW HPLC column (7.5 x 60 mm, Supelco, France). The column was equilibrated and eluted with 50 mM Tris-HCl buffer pH 7.25 containing 100 mM NaCl at a flow rate of 0.5 mL $min^{-1}$.

Finally preparative polyacrylamide gel electrophoresis (native-PAGE) was carried out using a discontinuous buffer system: stacking gel (6%) at pH 6.8 and resolving gel (10%) at pH 8.8 under 4 °C overnight with 40 V voltage *(23)*. After running, the enzyme was recovered by electroelution and it served for subsequent studies.

This enzyme preparation was analyzed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). SDS-PAGE was carried out according to Laemmli *(15)* and was performed using a discontinuous buffer system: stacking gel (6%) at pH 6.8 and resolving gel (10%) at pH 8.8. Gels were stained by silver stain. The high molecular weight calibration mixtures (Sigma, France) were used as standards.

Protein concentrations were determined with Bradford's method (595 nm) using 1 mg $mL^{-1}$ bovine serum albumin solution.

*pH and temperature optimization*

The β-xylosidase activity was measured at several pH values ranging from 2 to 9 (50 mM buffer: glycine-HCl pH 2, 3, sodium acetate pH 4, 5, 6 or Tris-HCl pH 7, 8, 9) at 60 °C for 30 min. The purified enzyme was incubated with 0.5 mM pNPX.

For the determination of the optimum temperature of β-xylosidase activity, the standard assay was performed at different temperatures (4 to 80 °C) at pH 4.

*Stability as function of pH and temperature*

β-Xylosidase was dissolved in 50 mM buffer at pH values ranging from 2 to 9 as cited above. The incubation took 24 h at 4 °C. In a second step, the residual activity was measured in standard conditions.

The enzyme stability as function of temperature was studied by measuring the remaining of the b-xylosidase activity in standard conditions after incubation 30 min in 50 mM sodium acetate buffer pH 4 under different temperatures.

*Effect of different metal ions and chemicals on β-xylosidase*

The β-xylosidase activity was tested in the presence of different divalent cations. The enzyme was added to 0.5 mM pNPX in 50 mM sodium acetate buffer pH 4 in presence of 5 mM cation.

The effect of EDTA or DTT were studied after incubation of the enzyme in presence of 1.5 or 10 mM of the chemical specie in 50 mM sodium acetate buffer pH 4 containing 200 mM NaCl, 1 h at 4 °C. 0.5 mM pNPX was then added and the residual activity was tested.

## Results and discussion

*Enzyme production*

The production of enzyme activities by *Sclerotinia sclerotiorum* was induced by xylan from birch wood which is a strong inducer for both β-xylosidase activity, and endoxylanase activity (*24*). In other work, xylan was shown to be the best inducer of the β-xylosidase activity from the pathogenic fungus *C. carbonum (16)*. Otherwise, b-xylosidase production decreases in presence of glucose as carbon source *(24)* because of carbon catabolic repression by the protein CreA *(17)*.

*Synthesis of alkyl-xylosides*

β-Xylosidase is an enzyme that can catalyze transfer reactions with xylosyl residues *(7,18)*. This enzyme could help an effective use of biomass of hemicellulose like xylan, which is present in large amounts in agricultural and industrial wastes. β-Xylosidase uses xylooligosaccharides as substrates for transxylosylation

reactions. This is why the hydrolysis of xylan by a xylanase activity must precede the b-xylosidase reaction in order to liberate the required oligosaccharides for the second reaction. Both of the two steps (hydrolysis and transxylosylation) were tested in the same medium, by using a mixture containing the two enzymes (endo-xylanase and b-xylosidase).

In this work, alkyl-xylosides are enzymatically produced from xylose monomers or polymers and alcohols of different sizes. These compounds are biosurfactants with surface-active properties useful in pharmacy and cosmetic industries. This application needs a simple and cheap enzyme preparation. The enzyme preparation used in this reaction is ammonium sulfate precipitate of *Sc. sclerotiorum* crude extract. The fungus was cultivated on xylan from birch wood. When using xylan and hexan-1-ol as substrates the synthesis reaction takes 3 to 4 days in the biphasic medium (aqueous/ alcohol, Figure 1). Two enzymes are involved in this reaction. After hydrolysis of xylan with endo-xylanase, the β-xylosidase is able to catalyze transxylosylation between partially hydrolyzed xylan and alcohol (Figure 1). Two products: hexyl-β-xyloside and hexyl-β-xylobioside were obtained (Figure 2) in this reaction with more than 20% conversion yield. The first one is produced with higher concentration These results were confirmed by TLC. When using xylose as substrate instead of xylan in the same conditions described above, no product was detected after 4 days. This supports the hypothesis that alkyl-xyloside and alkyl-xylobioside are produced by transxylosylation between oligosaccharides liberated

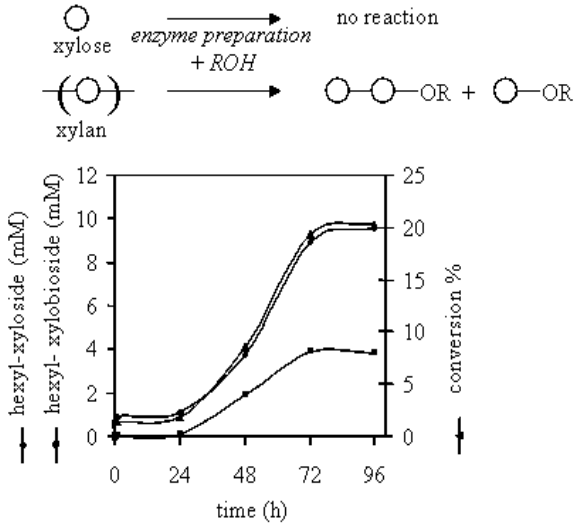


**Figure 1:** Synthesis of hexyl-xyloside and hexyl-xylobioside from xylan and hexan-1-ol in biphasic medium at 50 °C and pH 4.0 in presence of enzyme preparation from *Sc. sclerotiorum*.

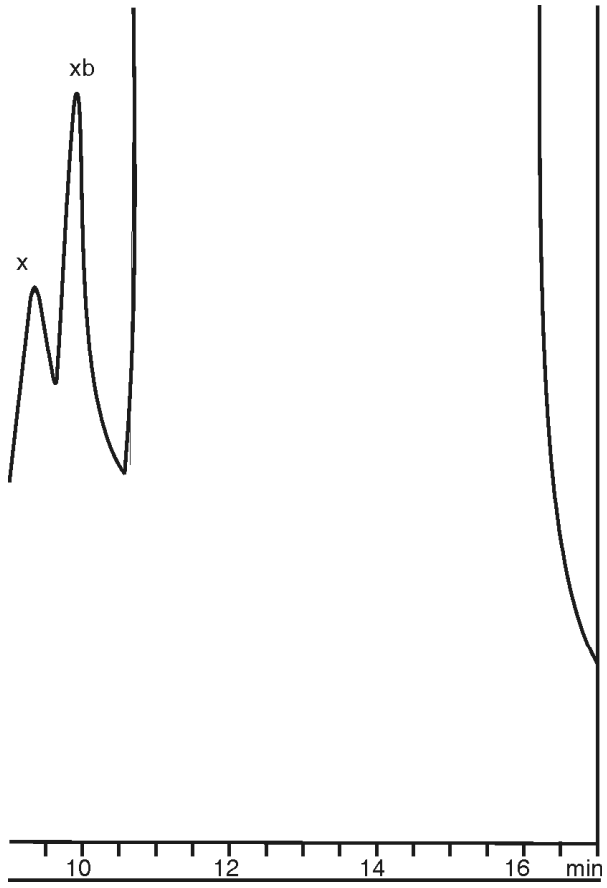


**Figure 2:** Separation of hexyl-xyloside (x) and hexyl-xylobioside (xb) by C18 reversed phase column. The product peaks are followed by the hexanol peak.

from xylan and alcohol, not by reverse-hydrolysis between xylose and alcohol. Both hexyl-β-xyloside and hexyl-β-xylobioside seem to be obtained by transxylosylation reaction catalysed by the b-xylosidase. The endo-xylanase did not catalyze synthesis reaction because no product of synthesis appeared when using xylose as substrate in presence of the same enzyme preparation.

*Enzyme purification*

In order to investigate more the xylanolytic activity produced by *Sclerotinia sclerotiorum* and its putative role in synthesis reaction, we proceeded to its purification and biochemical characterization. The purification of the β-xylosidase was achieved in four steps. In the first step the enzyme was bound to DEAE-sepharose. As shown in figure 3, elution with NaCl gradient resulted in one protein peak eluted at 0.2 M NaCl, having activity against both pNPX and p-nitrophenyl-b-glucopyranoside (pNPG, Sigma, France). Supposing that these two activities corresponded to two different enzymes: β-xylosidase and

β-glucosidase with similar properties, we tried to separate them.

The concentrate of active fraction was further subjected to HPLC anion-exchange column (TSK-DEAE 5 PW) (Figure 4) under the conditions described in the experimental section. Although the major part of β-glucosidase activity was removed, the β-xylosidase preparation exhibited activity against pNPG.

Thus, the active fractions were collected and concentrated by ultrafiltration on Centriplus PM 10 membrane, before purification by gel filtration on TSK-3000 SW HPLC column. The elution profile obtained is shown in figure 5. β-Xylosidase activity appeared as a unique major peak contaminated with very low β-glucosidase activity. The purified enzyme had no endo-xylanase activity. The test against xylan did not liberate reducing sugars. The elution volume of the peak indicated an apparent molecular mass of 70 000 Da.

An additional preparative native-gel electrophoresis was achieved in order to separate more efficiently the b-xylosidase from the small contamination. The purified enzyme was used for the determination of β-xylosidase characteristics as described in the following sections.

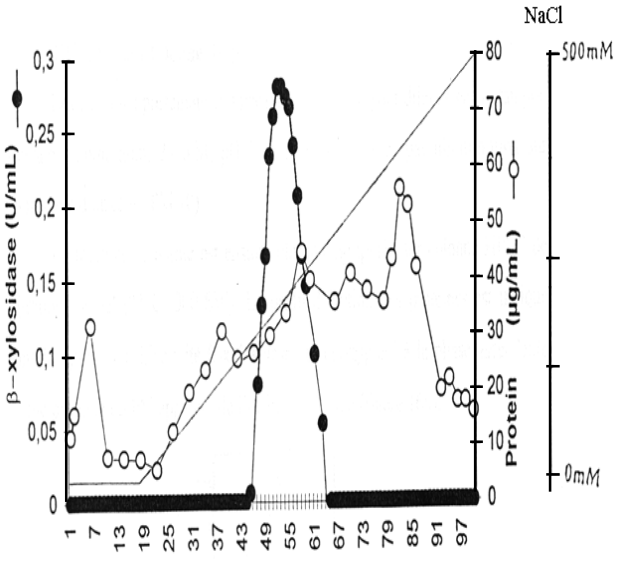


**Figure 3:** Anion exchange chromatography of enzyme concentration on DEAE-sepharose CL-6B column. The column (100 x 30 mm) was equilibrated with 25 mM Tris-HCl buffer (pH 7.25) and eluted with linear NaCl gradient from 0 to 0.5 M. The flow rate was 1 mL $min^{-1}$ and the fraction volume was 2 mL.

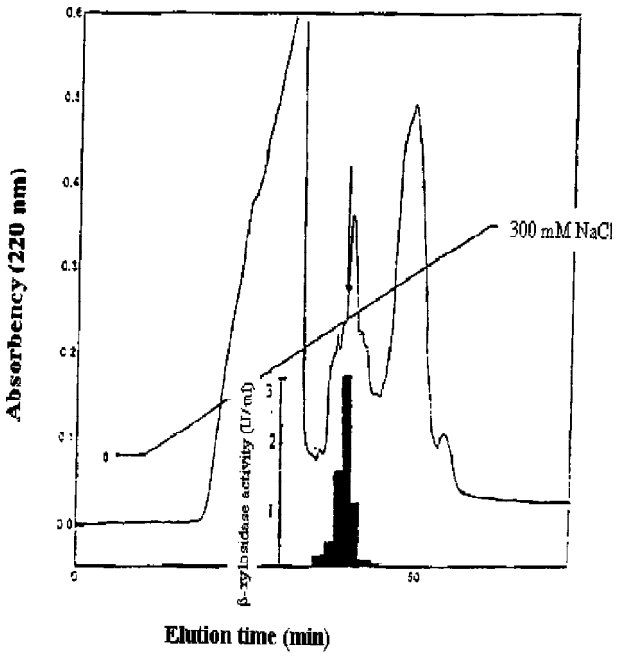


**Figure 4:** Anion exchange chromatography of the concentrated active fractions on a progel-TSK DEAE SPW HPLC column. The column (7.5 x 75 mm) was equilibrated with 50 mM ammonium acetate buffer (pH 7) and eluted with linear NaCl gradient from 0 to 0.3 M. The flow rate was 0.5 mL $min^{-1}$ and absorbency measured at 220 nm.

SDS-PAGE analysis of this preparation showed one single band corresponding to a polypeptide of molecular mass of 72 000 Da (Figure 6). These results with HPLC and electrophoresis analysis indicate that the b-xylosidase of *Sc. sclerotiorum* is a monomeric protein. Similar results were observed for other fungal b-xylosidases *(19-20)*.

*Optimal conditions for b-xylosidase*

The effect of pH and temperature on enzyme activity and stability was studied by using the purified b-xylosidase. The enzyme activity has its optimum temperature at 60 °C in the 30 min incubation assay (Figure 7 a). Besides, the b-xylosidase showed an important stability up to 60 °C. 50 % of remaining activity was observed after treatment at 65 °C (Figure 7 a). The enzyme had important activity and stability at high temperature. This property allows easily its use in industry.

According to Figure 7 b, the optimum pH for β-xylosidase activity was pH 4. The activity decreased significantly on each side of the curve. Nevertheless, the residual activity after treatment at different pH values (Figure 7 b) showed that the enzyme was stable over a wide pH range (from 2 to 9).

*β-xylosidase kinetic properties*

The maximal velocity ($V_M$) and the Michaelis constant ($K_M$) values were determined for b-xylosidase with pNPX as substrate in steady state kinetic conditions using Linweaver-Burk plots:

$K_M$ = 1.61 µM

$V_M$ = 0.14 µmol $min^{-1}$ mg protein.

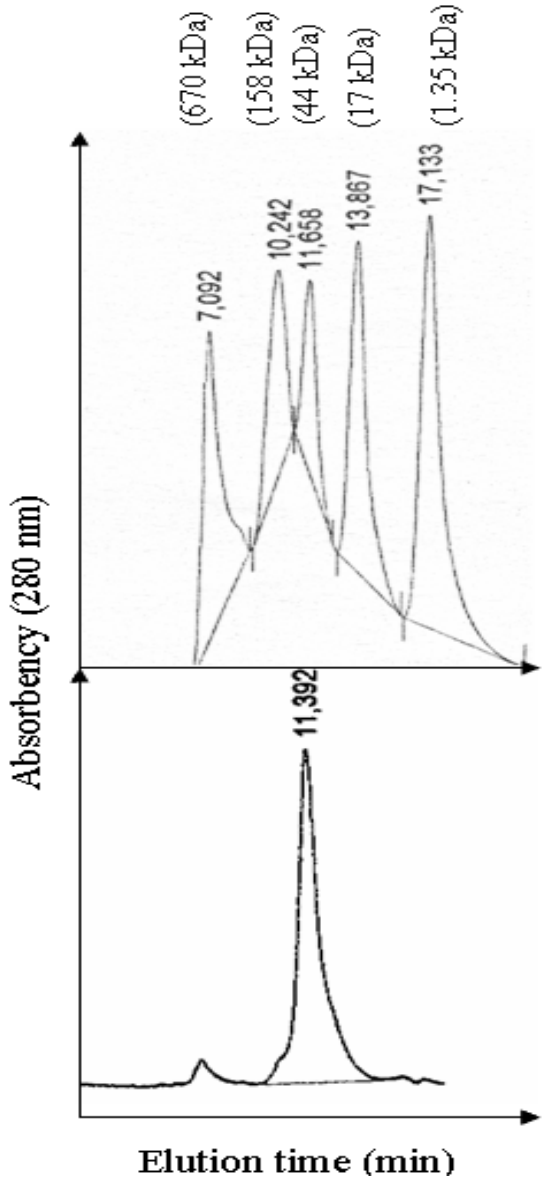


**Figure 5:** Gel filtration chromatography of enzyme concentrate compared to protein standards on TSK-3000 SPW HPLC column. The column (7.5 x 60 mm) was equilibrated and eluted with 50 mM ammonium acetate buffer (pH 7.25), 100 mM NaCl. The flow rate was 0.5 mL $min^{-1}$ and absorbency measured at 280 nm. Protein markers were thyreoglobulin: 670 kDa; bovine b-globulin: 158 kDa; ovalbumin: 44 kDa; myoglobulin: 17 kDa and B12 vitamin: 1.35 kDa.

The enzyme showed Michaelis-Menten kinetics. The Michaelis constant value we found was comparable to those described for filamentous fungi ranging between 1.0 and 6.8 mM *(21)*. Whereas these values were higher than $K_M$ determined for β-xylosidases from *A. niger*: 0.2 mM and *P. wortmanni*: 0.12 mM *(22)*. The lowest $K_M$ value described was 0.08 mM for the β-xylosidase from *T. reesei*. As described for other β-xylosidases *(22)*, we found that the reaction between *Sc. Sclerotiorum* enzyme and pNPX was inhibited by xylose. The addition of 5 mM xylose in the reaction medium led to inactivation up to 60 %. Xylose is the natural product for b-xylosidase and can play the role of substrate in synthesis reaction. This is why it is able to inhibit the reaction with pNPX. However other mono- and oligo-saccharides as glucose, galactose, maltose and cellobiose had no effect on the enzyme activity with pNPX (data not shown).

*Influence of divalent cations and other reagents on b-xylosidase*

The effect of various chemicals was investigated. As shown in Table 1, $Mg^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Zn^{2+}$, EDTA and DTT had no significant influence on the enzyme activity. Some of these divalent cations could be used as additives in industrial preparations of the enzyme without negative effect. Whereas, the enzyme activity decreased slightly in presence of $Fe^{2+}$ and $Cu^{2+}$ (Table 1).

**Table 1**
**Effect of Metallic Ions and other Reagents on the Purified β-xylosidase from Sc. sclerotiorum.**

| *reagent*[a] | *concentration (mM)* | *relative activity (%)* |
|---|---|---|
| Control | - | 100 |
| Cu $SO_4$ | 5 | 76,6 |
| Mg $SO_4$ | 5 | 106,4 |
| $CoCl_2$ | 5 | 93,6 |
| Ca $Cl_2$ | 5 | 100 |
| Mn $SO_4$ | 5 | 100 |
| Fe $SO_4$ | 5 | 61,7 |
| Zn $SO_4$ | 5 | 98 |
| EDTA | 10 | 100 |
| DTT | 10 | 100 |
| Ethanol | 5%[b] | 106 |
| NaCl | 200 | 118 |

[a] The reaction medium contains 0.15 mM pNPX, 40 µL enzyme preparation and the tested reagent in 600 µL total volume at pH 4 and 60 °C.

[b] ethanol concentration in % of ethanol in the aqueous phase (v/v).

The chelating reagent, EDTA, does not inhibit β-xylosidase, suggesting that the enzyme is not a

metalloprotein. The non significant effect of DTT suggests that there is no sulfhydryl group involved in the catalytic active site of the enzyme as observed for β-xylosidase from *A. niger (22)*. The enzyme supports up to 5 % ethanol (v/v) in the medium (Table 1).

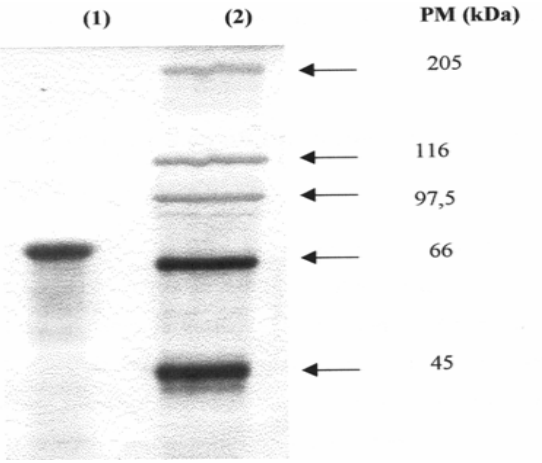


**Figure 6:** SDS-PAGE of the purified b-xylosidase. Proteins were visualized by silver stain. Lane (1): molecular weight standards (thyreoglobulin: 670 kDa; bovine b-globulin: 158 kDa; ovalbumin: 44 kDa; myoglobulin: 17 kDa and B12 vitamin: 1.35); Lane (2): purified b-xylosidase isolated by preparative gel electrophoresis.

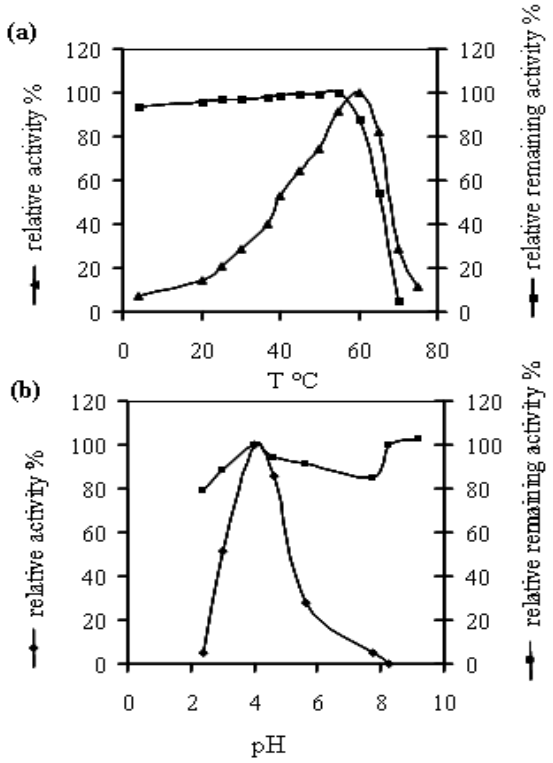


**Figure 7:** (a) Effect of temperature on activity and stability of the purified b-xylosidase in sodium acetate buffer pH 4. (b) Effect of pH on activity and stability of the purified b-xylosidase at 60 °C.

At different ionic strength b-xylosidase stills efficient. The enzyme activity improved at high concentration of sodium chloride (Table 1). The optimum activity was obtained when using 200 to 500 mM NaCl.

According to this study, b-xylosidase from *Sc. sclerotiorum* shows satisfying properties for use in some biotechnological applications.

In conclusion, the work has as main objective the identification of suitable enzyme activities for industrial use in valorization of natural polysaccharides, especially by converting these polymers as xylan to alkyl-xylosides. This is why we are studying the enzyme purification and determining the enzyme properties. Work is in progress in our laboratory for cloning and gene expression of b-xylosidase, and for purification of xylanase.

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