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# Immobilization and biochemical properties of a β-xylosidase activated by glucose/xylose from *Aspergillus niger* USP-67 with transxylosylation activity

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ABSTRACT

β-Xylosidases have important applications in many biotechnological processes. In this context, the aim of this work was the purification, immobilization and characterization of a β-xylosidase produced by a new isolate of *Aspergillus niger* USP-67. β-Xylosidase was produced on static conditions in liquid Benassi medium supplemented with xylan birchwood, initial pH 3.0, for 6 days, at 30 °C. The enzyme was purified on DEAE-Sepharose followed of Superdex™ 200, and the molecular mass of the β-xylosidase was estimated to be 100 kDa, with 90% similarly to the β-xylosidase xlnD from *A. niger* (gi 146230215 accession), using MS sequencing. The enzyme was immobilized on DEAE-Sepharose, Polyethyleneimine (PEI)-Sepharose, Q-Sepharose, CM-Sepharose, Sulphopropil-Sepharose and MANAE-agarose, but the best result was obtained with PEI-Sepharose, which presented 94% of immobilization yield. Moreover, this derivative was more thermal stable than the soluble enzyme and other supports, which presented a half-life of about 50 min, at 65 °C. The enzyme immobilized on PEI-Sepharose had an optimum pH more acidic (around 4.5) than the purified enzyme (pH 5.5). Metal ions inhibited the soluble enzyme activity more than the immobilized form; however, $Zn^{2+}$ increased the activity of the immobilized enzyme in 29%. The specific activity of the immobilized enzyme corresponded to 98.15 U/mg, but the soluble enzyme was 77.96 U/mg. Furthermore, the $K_M$ and $K_{cat}$ values for the purified enzyme with *p*-nitrophenyl-xylopyranoside as substrate were 0.654 mM and 58.87 $s^{-1}$ and for the immobilized enzyme the values were 0.587 mM and 88.95 $s^{-1}$, respectively. The purified enzyme efficiently hydrolyzed xylooligosaccharides until xylose, but other xylooligosaccharides (X2–X6) were formed, suggesting transxylosylation action. The immobilized β-xylosidase of *A. niger* was not inhibited by xylose (100 mM) and glucose (200 mM), what confers to this enzyme a potential application in biotechnological processes.


## 1. Introduction

Xylan represents up to 30–35% of the total dry weight of hemicelluloses, and is a complex heterogeneous polymer composed by a backbone xylopyranose residues linked by β-1,4-bounds modified by a variety of substituents, such as acetyl, L-arabinofuranosyl, galactosyl, glucuronyl and 4-methyl-*O*-glucuronyl groups side chain [1]. The enzymatic degradation to its monomer is a complex process involving the synergistic action of several enzymes with different functions, mainly endo-β-1,4-xylanases (EC 3.2.1.8), which cleave the β-1,4-glycosidic bond between xylose residues to produce xylooligosaccharides (XOS) of weak polymerization degree, and β-xylosidase (EC 3.2.1.37), which hydrolyzes short xylooligomers and xylobiose from the non-reducing end releasing xylose [2].

The β-xylosidases hydrolyze the glycosidic bonds by one of two major mechanisms, giving rise to either an overall retention or an overall inversion of the configuration of the anomeric substrate carbon [3]. In both mechanisms, the hydrolysis usually requires two carboxylic acids, which are conserved within each glycoside hydrolase family, and proceeds through oxocarbonium ion-like transition states. According to carbohydrate active enzymes database (CAZy), the β-xylosidases are divided into families 3, 30, 39, 43, 52 and 54

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of glycoside hydrolases (GHs) [4]. However, on the literature have been described only for families 3, 43 and 54 for the filamentous fungal β-xylosidases [5].

The xylooligosaccharides naturally occur in fruit and vegetables and they are commercially produced by enzymatic and/or chemical hydrolysis of xylan, for example from barley hulls, corn cobs, corn straw, rice hulls, wheat straw and bamboo. The XOS can act as prebiotics and have important applications in food industry as functional ingredients promoting growth of probiotic bacteria in the human gut [6]. One characteristic of fungal β-xylosidase is its transxylosylation activity, i.e., the formation of larger xylooligosaccharides from small olygosaccharides. The transfer reactions can result in a complex structure of xylans. The hydrolytic activity would be restrained by xylan side chains, whereas the presence of high amounts of small sugars in the reaction mixture would encourage the transglycosylation activity, and makes it widely employed in biotechnology [7].

β-Xylosidases are attracting much attention for their potential application in a number of biotechnological processes. On the other hand, some problems such as: (1) the poor stability, (2) the high production cost, and (3) the difficulty in recovery, should be overcome in order to industrially apply them in lignocellulose saccharification process [8].

Using immobilized enzymes in industrial processes provide many important advantages as to reduce the cost because they can be reused in continuous operation, the product formation is controlled and the process efficiency is enhanced. Reversible soluble-insoluble polymers have been reported as carriers for the immobilization of enzymes, and the advantage of using such polymers is that the bound enzyme can be used in a soluble form for mediating the desired reaction, thus overcoming the steric hindrances and mass transfer limitations presented by insoluble matrices [9,10]. Although some bacterial and fungal β-xylosidases have been purified and characterized, only few enzymes synthetized from thermotolerant fungi have been reported in the literature [11].

Few studies have been conducted with immobilized microbial β-xylosidase and including β-xylosidase from *Bacillus halodurans* C-125 immobilized in chitosan mediated with glutaraldehyde and deployed in packed bed reactor [12]. Commercial *Aspergillus niger* β-xylosidase was immobilized in alumina with TiCl4 and to alkylamine porous silica with glutaraldehyde [13].

Commercially available ion exchange chromatographies are used to immobilize and purify enzymes, such as DEAE-cellulose, Q Sepharose, CM-cellulose. Once, they are rapid methods for protein separation and are also highly activated with very strong ionic groups, so they can adsorb the maximum percentage of proteins from the extract [14,15]. The other support used for the enzyme immobilization was Polyethylenimine (PEI)-Sepharose [14], which has been used in some instances to stabilize proteins in solution by preventing the oxidation, aggregation, and others. PEI-Sepharose is a polymer that has a high density of ionized primary, secondary and tertiary amino groups.

On this context, the aim of this work was to determine the optimal operative conditions of purification, reversible immobilization, and characterization of the β-xylosidase produced by the thermotolerant fungi *A. niger* USP-67. This work obtained novel relevant results with respect to the immobilization of β-xylosidase in PEI-Sepharose support. Highlighting, there are not any previous studies in the literature using PEI-Sepharose support for the immobilization of β-xylosidases. In addition, the PEI-Sepharose derivative presented better characteristics compared to the purified enzyme.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

The microorganism was maintained on slants of complete medium Vogel [16], stored at 4 °C. Conidia were incubated in liquid Benassi medium constituted by 0.1 g $NH_4NO_3$, 0.065 g $KH_2PO_4$, 0.0181 g $MgSO_4{\cdot}7H_2O$, 0.0049 g KCl, 0.35 mg $ZnSO_4{\cdot}7H_2O$, 0.69 mg $MnSO_4{\cdot}H_2O$, 0.0033 g $FeCl_3{\cdot}6H_2O$, 0.031 mg $CuSO_4{\cdot}5H_2O$, 25 μL of vitamin solution (10 mM pyridoxine hydrochloride, 10 mM thiamine hydrochloride, 10 mM *p*-aminobenzoic acid, 10 mM D-pantothenic acid, 100 μM folic acid, 100 μM riboflavin), 0.025 g yeast extract, 0.25 g xylan from birchwood (Sigma–Aldrich®) and 25 mL distilled water. The pH was adjusted to pH 3.0. The incubation was carried out at 30 °C, for 6 days. After that, the cultures were filtrated in a Büchner funnel using vacuum pump and filter paper and the filtrate was used to determination of the crude extracellular β-xylosidase activity.

### 2.2. Enzymatic assay and protein determination

The protein quantification was carried out according to Bradford [17], with bovine serum albumin as standard. The values were expressed as mg of protein per mL of solution. The β-D-xylosidase activity was determined using 1% *p*-nitrophenyl-β-D-xylopyranoside (*p*NP-xyl), as substrate, in 100 mM sodium succinate buffer, pH 4.5, at 65 °C [18]. The absorbance was determined at 405 nm, and *p*-nitrophenol was used as standard. Substrate specificity was analyzed with the appropriate substrate: *p*-nitrophenyl-α-D-xylopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-galactopyranoside under the same conditions described for *p*NP-xyl, and 1% xylan from beechwood Sigma–Aldrich® and 1% xylan from birchwood Sigma–Aldrich® were measured for the amount of reducing sugar according to the Miller method [19]. Xylose was used as standard. One enzyme unit (U) was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute or 1 μmol of reducing sugar per minute, respectively. The total unit (total U) was defined as unit (U) multiplicity by volume of extracellular extract. The specific activity was expressed as U/mg protein of extracellular extract.

### 2.3. β-xylosidase purification

Approximately 250 mL of enzymatic extract was loaded on 6 g of DEAE-Sepharose equilibrated with 10 mM sodium phosphate buffer, pH 7.5. The unabsorbed proteins were removed by washing the resin with 250 mL of the same buffer. The enzyme was eluted with 700 mM of sodium chloride in the same buffer. This pool was dialyzed against 50 mM sodium succinate buffer, pH 5.0, and loaded on Superdex$^{TM}$ 200 column (1.5 × 45 cm) equilibrated with the same buffer, and 1 mL fractions were collected at a flow rate of 0.2 mL/minute. The fractions containing β-xylosidase activity were pooled and used for enzyme characterization.

### 2.4. Polyacrylamide gel electrophoresis analysis and mass spectrometry

Non-denaturing electrophoresis (PAGE) was carried out according to Davis [20], using 8% acrylamide, and the denaturing electrophoresis (SDS-PAGE) was carried out according to Laemmli [21], using 12% acrylamide. Molecular mass standards were: phosphorylase b (97 kDa); albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) from GE Healthcare UK Limited®. Protein was stained with Coomassie blue (EZ Blue$^{TM}$ Gel Staining