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Research article

# Agroindustrial biomass for xylanase production by *Penicillium chrysogenum*: Purification, biochemical properties and hydrolysis of hemicelluloses

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ABSTRACT

*Background:* In this work, the xylanase production by *Penicillium chrysogenum* F-15 strain was investigated using agroindustrial biomass as substrate. The xylanase was purified, characterized and applied in hemicellulose hydrolysis.
*Results:* The highest xylanase production was obtained when cultivation was carried out with sugar cane bagasse as carbon source, at pH 6.0 and 20°C, under static condition for 8 d. The enzyme was purified by a sequence of ion exchange and size exclusion chromatography, presenting final specific activity of 834.2 $U \cdot mg \cdot prot^{-1}$. The molecular mass of the purified enzyme estimated by SDS-PAGE was 22.1 kDa. The optimum activity was at pH 6.5 and 45°C. The enzyme was stable at 40°C with half-life of 35 min, and in the pH range from 4.5 to 10.0. The activity was increased in the presence of $Mg^{+2}$ and $Mn^{+2}$ and reducing agents such as DTT and β-mercaptoethanol, but it was reduced by $Cu^{+2}$ and $Pb^{+2}$. The xylanase presented $K_m$ of 2.3 mM and $V_{max}$ of 731.8 $U \cdot mg \cdot prot^{-1}$ with birchwood xylan as substrate. This xylanase presented differences in its properties when it was compared to the xylanases from other *P. chrysogenum* strains.
*Conclusion:* The xylanase from *P. chrysogenum* F-15 showed lower enzymatic activity on commercial xylan than on hemicellulose from agroindustry biomass and its biochemistry characteristics, such as stability at 40°C and pH from 4.0 to 10.0, shows the potential of this enzyme for application in food, feed, pulp and paper industries and for bioethanol production.



## 1. Introduction

Lignocellulosic materials are mainly composed by cellulose, hemicellulose and lignin, and smaller portion of pectin, waxes and mineral salts [1]. Hemicellulose is a class of structurally variable heteropolysaccharides that, in association with cellulose and lignin, aid in the adhesion and cohesion of plant fibers [2]. Xylan is the most abundant hemicellulose in lignocellulosic biomass from many agroindustrial wastes. These polysaccharides represent up to 50% of tissues from monocots, grasses and cereals [3]. It is composed by a backbone of (1–4) linked β-D-xylanopyranosyl residues, with substituents such as glucuronosyl, arabinosyl and acetyl groups, among others [4,5,6,7]. The complete and efficient xylan biodegradation requires the action of several enzymes, known as xylanolytic system. The main enzyme is endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) which hydrolyze the xylan backbone, reducing the degree of polymerization and releasing smaller oligosaccharides and xylobiose [5,7,8,9]. Xylanases have been used for many biotechnological applications such as treatment of juices, beer and wine; in bakery industries; for production of xylooligosaccharides (XOS) and xylitol; improvement of animal feed digestibility; and bleaching of cellulosic pulp. These enzymes also have been used to degrade polysaccharides from lignocellulosic materials to produce second-generation bioethanol [3,6,7,10,11,12,13]. Fungi are important xylanolytic enzyme producers, because they secrete the enzymes to the external medium, and the levels are much higher than those verified in yeasts and bacteria [14,15]. Several fungal producers such as *Aspergillus niger*, *Humicola insolens*, *Termonospora fusca*, *Trichoderma reesei*, *Trichoderma longibrachiatum* and *Trichoderma koningii* have been manipulated to produce commercial xylanases [11,16]. The production of xylanolytic enzymes by *Penicillium* has also been explored in many species [17] such as *Penicillium purpurogenum*, *Penicillium janthinellum*, *Penicillium funiculosum*, *Penicillium herquei*, and *Penicillium capsulatum* [18,19,20,21,22]. Some *Penicillium* species

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such as *Penicillium sclerotiorum* [23], *Penicillium janczewskii* [24] and *Penicillium glabrum* [25] have been cultivated in brewer's spent grains for xylanolytic enzymes production. *Penicillium chrysogenum* was previously reported as producer of xylanase and other lignocellulosic enzymes with potential application to degradation winery-derived biomass waste [26,27]. Biomass degradation studies are required due to the high demand for alternative treatments for agricultural and industrial wastes. This degradation is important because it allows the reuse of these materials and the rational use of the degradation by-products. During a screening trial, a *P. chrysogenum* strain demonstrated to be a notable xylanase producer. The aim of this work was to investigate the behavior of a *P. chrysogenum strain* in the agroindustrial wastes biodegradation for xylanases production. The study was performed with wastes from different agroindustry, an industrial sector with large waste production. In addition to the production, a xylanase purification protocol was stablished, and its physicochemical properties were determined. This knowledge can allow a targeted enzyme application in which the enzyme characteristics are compatible and adequate to be applied in specific industrial sectors.

## 2. Materials and methods

### 2.1. Microorganisms and growth

*P. chrysogenum* F-15 strain was isolated from soil of caatinga biome located in Northeast Brazil (Floresta Nacional Contendas do Sincorá, Bahia). It is available in the Culture Collection of Environmental Studies Center – CEA/UNESP, Brazil. Conidia production was carried out on solid Vogel medium [28] containing 3.0% (*w*/*v*) wheat bran and 1.5% (*w*/*v*) agar at 25°C for 7 d and inoculum corresponded to $5.10^7$ conidia·$mL^{-1}$ suspension. Xylanolytic strains from previous studies such as *Aspergillus giganteus* [29], *Aspergillus versicolor* [30], *Trichoderma inhamatum* [31], *P. janczewskii* [24] and *P. sclerotiorum* [23] were used for hydrolysis hemicelluloses comparison.

### 2.2. Submerged cultivation

Cultures of *P. chrysogenum* were prepared in Vogel medium [28] containing 1% (*w*/*v*) of each substrate and the pH was adjusted for each experiment, as below. Erlenmeyer flasks (125 mL) containing 25.0 mL of medium were inoculated with 1.0 mL of the conidia suspension and incubated at different conditions, as indicated subsequently. All experiments were performed in triplicate and the results were mean values. The other strains were cultivated under conditions previously established for high xylanase production [23,24,29,30,31].

### 2.3. Enzyme preparations and assays

Cultures were harvested by filtration and the culture filtrate was used to assay extracellular enzyme activity and protein. The mycelium was washed with water, frozen and ground with sand in 0.05 M sodium phosphate buffer pH 6.0. Then, the samples were centrifuged (3.900 xg, Mach 1.6, Sorvall, Kendro, Hanau, Germany) at 4°C and the supernatants were used as intracellular protein source.

### 2.4. Xylanase activity and protein assays

Xylanase activity was determined with 1% (*w*/*v*) of substrates prepared in 0.05 M sodium phosphate buffer pH 6.0 at 50°C, according to Bailey et al. [32]. Substrates were commercial beechwood, birchwood and oat spelts xylans from Sigma–Aldrich Chemical Co (St. Louis, MO, USA) and in-house extracted hemicellulose from sugar cane bagasse, brewer's spent grain and corncobs [33]. After enzymatic reaction, reducing sugars were quantified with dinitro salicylic (DNS) acid [34] and the absorbance was read at 540 nm (spectrophotometer Ultrospec 3000, Amersham Pharmacia Biotech, Little Chalfont, UK). One unit of enzyme activity (U) was defined as the amount of enzyme which releases 1 μmol of reducing sugars per min. Specific activities were expressed as enzyme units per milligram of protein (U·mg·$prot^{-1}$). Protein was determined by the Lowry method [35] using bovine serum albumin (Sigma–Aldrich) as standard. During purification chromatography, protein in the fractions was followed by reading absorbance at 280 nm.

### 2.5. Xylanase production on different substrates and influence of particle size

Vogel medium [28] was supplemented with 1.0% (*w*/*v*) glucose, xylose, Avicel®, carboxymethyl cellulose (CMC), xylan from oat spelts, oat bran, wheat bran, sugar cane bagasse, brewer's spent grain, orange peel and corncobs. The influence of particle size on enzyme production was verified with sugar cane bagasse and brewer's spent grain particulate in the following ranges: higher than 10 mesh, between 10 and 18 mesh, and between 18 and 45 mesh.

### 2.6. Effect of culture conditions, pH and temperature on xylanase production

Cultivation was carried out under static (15 d) and shaking (9 d, 120 rpm, incubator shaker MA 830/A, Marconi, Piracicaba, SP, Brazil) conditions. The effect of initial pH on enzyme production was analyzed in the range from 2.0 to 10.0; and the influence of temperature was verified from 15 to 30°C.

### 2.7. Xylanase purification

The culture filtrate was dialyzed overnight against 0.05 M sodium acetate buffer pH 4.5 at 4°C. The dialyzed sample was applied to cationic exchange chromatography in a CM Sephadex C-50 column (17.0 × 2.2 cm) equilibrated in the same buffer. Proteins were eluted with a 0.0 to 0.7 M linear NaCl gradient. Fractions exhibiting xylanase activity were pooled, freeze-dried (Thermo Savant, Micro Modulyo, Waltham, MA, USA), suspended in 3 mL of ammonium acetate buffer 0.05 M pH 4.5 and submitted to size exclusion chromatography on a Sephadex G-100 column (64.0 × 2.6 cm) equilibrated in the same buffer. Fractions exhibiting xylanase activity were pooled and the resulting sample was submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All purification steps were carried out at 4°C, with 10% (*v*/*v*) glycerol (Merck, Darmstadt, Germany) in solution and 1 mM dithiothreitol (DTT) (Sigma-Aldrich) were added to the samples and solutions each 24 h.

### 2.8. Enzyme characterization

#### 2.8.1. Electrophoresis

SDS-PAGE was performed [36] with 8 to 18% (*w*/*v*) polyacrylamide gels. Low molecular weight proteins kit (GE Healthcare UK Limited Little Chalfont, Buckinghamshire, UK) containing phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) e α-lactalbumin (14.4 kDa) was used as standard. Proteins were stained with Coomassie brilliant blue R-250 (J.T. Baker) 0.1% (*w*/*v*) in ethanol: acetic acid: water (3:1:6, *v*/v/v).

#### 2.8.2. pH and temperature optima

Xylanase activity was measured at 50°C in different pH values using the following 0.05 M buffer systems: glycine-HCl (pH 2.0–3.5), sodium acetate (pH 4.0–5.5), imidazole (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0), glycine-NaOH (pH 9.0–10.0), at 50°C. Temperature optimum was determined by carrying out enzymatic reactions from 20 to 75°C, with 5°C intervals, at pH 6.5.



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