

SUBSTRATE AND TEMPERATURE EFFECT ON XYLANASE PRODUCTION BY *ASPERGILLUS FUMIGATUS* USING LOW COST AGRICULTURAL WASTES

EFEITO DA TEMPERATURA E DO SUBSTRATO NA PRODUÇÃO DE XILANASE POR *ASPERGILLUS FUMIGATUS* UTILIZANDO RESÍDUOS AGROINDUSTRIAIS DE BAIXO CUSTO

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ABSTRACT: This study reports the optimization of xylanase production under solid state fermentation (SSF) by a thermotolerant *Aspergillus fumigatus* strain (SCB4) isolated from sugarcane bagasse piles of Brazilian Cerrado. Different combinations of low-cost agricultural byproducts in SSF were evaluated: sugarcane bagasse and wheat bran (1:1), sugarcane bagasse and corn straw (1:1) and only sugarcane bagasse. The enzyme biosynthesis by SSF was carried out at different temperatures (40, 45, 50 and 55 °C). The maximum levels of xylanase activity were obtained after 24 h at 45 °C using a culture medium containing sugarcane bagasse and wheat bran (1:1). Under optimal conditions, the fungal culture produced 574 U g⁻¹ of xylanase (units/g of dry substrate). The crude enzyme showed optimal activity at 60 °C and pH 4.5. It exhibited thermostability up to 55 °C, wide range of pH stability and tolerance to ethanol, xylose and glucose. The physicochemical properties shown by this enzyme are appropriate for its application in hydrolysis of lignocellulosic residues for ethanol production and other bioproducts.

KEYWORDS: Sugarcane bagasse. Wheat bran. Corn straw. Xylanase, *Aspergillus fumigatus*.

INTRODUCTION

Xylan is the major polysaccharide of hemicelluloses and is the second most abundant polymer in plant cell wall. It consists of a homopolymeric backbone of β -1,4 linked D-xylopyranosyl units with short chain branches of O-acetyl, α -L-arabinofuranosyl, and α -D-glucuronid residues (SÁNCHEZ, 2009). Xylanases (β -1,4-D-xylan-xylanohydrolase, EC 3.2.1.8) are the key enzymes responsible for the hydrolysis of xylan, providing the availability of cellulose for the action of cellulases and contributing for an increase in the efficiency of the process of biomass degradation (POLIZELI et al., 2005). These enzymes have high commercial applications. For instance, xylanases can be used in paper industry, in the improvement of the quality of animal feed, textile and food processing industries and also in the transformation of lignocellulosic materials in fermentable sugars for the production of second generation ethanol (NAIR et al., 2010; BAJAJ; ABBASS, 2011; SOUZA et al., 2012).

Filamentous fungi are known to be good producers of extracellular xylanases by solid-state fermentation (SSF) using lignocellulosic agricultural

by-products as components of microbial growth media, with several economic advantages: low water output, cheap substrates, low risk of contamination and low operating costs (MORETTI et al., 2012; TERRASAN; CARMONA, 2015). Among these residues, sugarcane bagasse is one of the most important in Brazil and is composed by approximately 50% cellulose, 25% hemicellulose and 20% lignin (PANDEY et al., 2000). Wheat bran is also a mostly available waste which comprises about 46% of polysaccharides (glucuronarabinoxylans, cellulose, mixed linked (1–3)(1–4)- β -D-glucans and starch), proteins (15–22%) and lignin (4–8%) (SOUZA et al., 2012). These substrates have been tested as carbon sources for the production of cellulases and hemicellulases by fungal strains (LIU et al., 2011; DELABONA et al., 2013).

In industrial processes, thermotolerant xylanases are desirable since high temperatures are required to increase solubility of substrates, to reduce viscosity and to reduce the risk of contamination (MORETTI et al., 2012). As a result, the search for thermophilic microorganisms with potential for the synthesis of such enzymes has increased considerably in the last years, mainly

filamentous fungi, which have been the most chiefly employed (JOSHI; KHARE, 2012; MORETTI et al., 2012).

The fungus *Aspergillus fumigatus* is a widespread species commonly found in agricultural residues with numerous biotechnological applications. Previous studies reported its ability to produce secondary metabolites with antimicrobial activity (ARAÇARI et al., 2002), and many enzymes such as proteases, cellulases and xylanases (OYELEKE et al. 2010; MORETTI et al., 2012; SOUZA et al., 2012). In this work, a thermotolerant *A. fumigatus* SCB4 strain isolated from sugarcane bagasse piles from Brazilian Cerrado was evaluated for the production of extracellular xylanase. Cultivation conditions of *A. fumigatus* SCB4 in SSF for enzyme biosynthesis were optimized. The physicochemical characteristics of the crude enzyme were also investigated.

MATERIAL AND METHODS

The filamentous fungus *A. fumigatus* SCB4 was obtained from the microbial collection of Laboratory of Environmental Microbiology, Uberlandia Federal University (LAMIC, UFU). This strain was previously isolated from internal portions of sugarcane piles (Araporã, Minas Gerais, Brazil, 18° 26' 13" S and 49° 11' 13" W), identified by conventional morphological examination and by gene sequencing of ITS region from ribosomal DNA (dos SANTOS et al., 2015).

Aspergillus fumigatus SCB4 was cultivated under solid state fermentation (SSF) using three

types of substrates: a mixture of sugar cane bagasse and wheat bran (SCB/WB, 1:1 w/w); sugar cane bagasse and corn straw (SCB/CS, 1:1 w/w) and only sugar cane bagasse (SCB) (Table 1). Sugar cane bagasse was obtained from a Brazilian ethanol industrial plant (Usina Alvorada, Araporã, MG). Before SSF, it was washed with distilled water, dried at 50 °C for 48 h and grinded to obtain particles of 2.0 mm (dos SANTOS et al., 2015). Wheat bran was brought from a local retailer and was submitted to the same process. Corn straw was obtained from a local farm. SSF was carried out using 2.5 g of each substrate (5 g in total) in erlenmeyer flasks of 250 mL, containing 20 mL of sterile nutrient solution. The flasks were inoculated with a volume equivalent to 10⁷ spores g⁻¹ of substrate suspended in a nutrient solution constituted by (g L⁻¹): (NH₄)₂SO₄, 3.5; KH₂PO₄, 3.0; MgSO₄ 7H₂O, 0.5; CaCl₂, 0.5 and Tween 80 (1.0% v/v). SSF was performed during seven days at 45 °C. To ascertain the time of peak of enzyme synthesis, protein extraction was carried out at intervals of 24 h with the addition of 100 mL of distilled water, followed by shaking at 100 rpm for 1 h. The samples were filtered and centrifuged at 15,000 rpm for 20 min at 5 °C. The supernatant was used as crude enzymatic extract and stored at -20 °C. In order to determine the optimal temperature of fungal growth and enzyme production, SSF fermentations were executed at the same conditions but at different temperatures: 40, 50 and 55 °C (Table 1).

Table 1. Substrates and temperatures evaluated in SSF for optimization of xylanase production by *A. fumigatus* SCB4 strain.

Substrate	Temperature
SCB/WB (1:1 w/w) ^a	40 °C
SCB/WB (1:1 w/w) ^a	45 °C
SCB/WB (1:1 w/w) ^a	50 °C
SCB/WB (1:1 w/w) ^a	55 °C
SCB/CS (1:1 w/w) ^b	45 °C
SCB ^c	45 °C

^a Sugar cane bagasse + wheat bran (1:1); ^b Sugar cane bagasse + corn straw (1:1); ^c Sugar cane bagasse

Xylanase activity was determined by 3,5-dinitrosalicylic acid (DNS) method (MILLER, 1959). Reaction mixtures containing 10 µL of crude enzymatic extract and 90 µL of sodium acetate buffer 0.1 M, pH 5.0 with xylan (1%) as substrate were incubated at 60 °C for 10 min. The released reducing sugars were quantified at 540 nm using a xylose standard curve. One unit of enzyme activity (U) was defined as the amount of enzyme required

to release one µmol of xylose per minute under the assay conditions. Control assays using heat inactivated enzymatic extract were carried out for each sample.

The optimum pH of xylanase was measured by DNS method incubating the reaction mixture at 60 °C for 10 min with buffer solutions at a pH range from 3.0 to 8.0 (dos SANTOS et al., 2015). The buffers used were: acetate buffer (pH 3.0 to 5.5);

citrate / phosphate buffer (pH 5.5 at 7.0), and Tris / HCl (pH 7.0 at 8.5). Optimum temperature was obtained by the same method but incubating the reaction mixture at temperatures from 30 to 80 °C at the optimum pH. To determine the pH stability, the extracts were diluted with acetate buffer at pH between 3.0 and 8.0 and incubated at room temperature for 24 h. After this period, the residual enzymatic activity was quantified in optimum pH and temperature. To evaluate the thermostability, the enzymatic extracts were incubated for 1 hour at temperatures ranging from 30 to 80 °C. After this period, the residual enzymatic activity was measured in optimum pH and temperature (dos SANTOS et al., 2015).

The effect of ethanol on xylanase activity was evaluated by the same method at the optimal pH and temperature, using increasing concentrations of ethanol in the reaction mixture (0 - 40%). Likewise, the enzyme activity in the presence of sugars was evaluated in concentrations from 0 to 20

mM (glucose or xylose). All assays were performed in duplicates.

RESULTS AND DISCUSSION

The xylanase production was strongly affected by the initial moisture contents of the substrates and the results were different for each evaluated carbon source (Figure 1). Maximum xylanase activity (574 U g⁻¹) was achieved using a mixture of sugarcane bagasse and wheat bran (1:1 w/w) after 24 h of SSF at 45 °C. This enzymatic biosynthesis in a short period of time is advantageous for industrial processes because it permits more repetitions of the process and higher productivity. The enzyme production also varied considerably with the temperature. At the temperatures of 40, 50 and 55 °C the enzyme production was smaller, indicating that these temperatures inhibited the growth of the microorganism and consequently its enzymatic production.

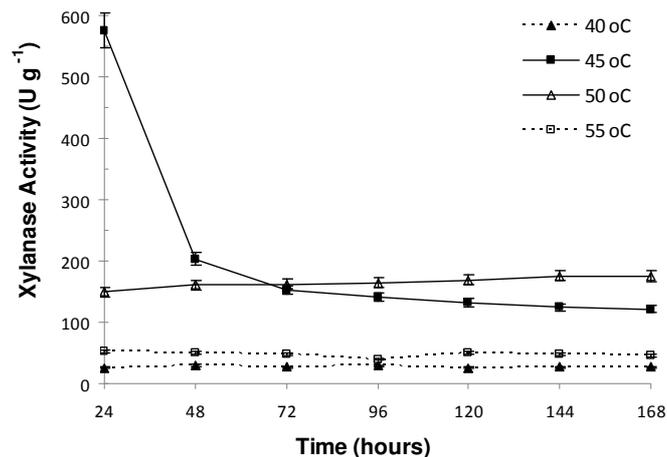


Figure 1. Xylanase production by *A. fumigatus* SCB4 using sugar cane bagasse and wheat bran (SCB/WB) at different temperatures.

The greatest biosynthesis of xylanase at 45 °C suggests that *A. fumigatus* SCB4 can be a thermotolerant fungal strain. Previous studies reported the effects of time and temperature during SSF on xylanase synthesis by other *Aspergillus* species and described higher production of xylanases at the same temperature (SALES et al., 2010, DELABONA et al., 2012). This peak of xylanase activity at 45 °C suggest that this mold can be employed in bioreactors at high temperatures, which could contribute to better conditions of operations, such as fluidity of the medium and reduction of the risk of contamination by mesophilic microorganisms (GOMES et al., 2007).

When wheat bran was substituted by corn straw a lower value of enzymatic activity was obtained (180 U g⁻¹) with a peak of activity after 168 h of SSF (Figure 2). This result indicates that the use of corn straw in SSF is not advantageous for xylanase production. In addition, SSF with sugarcane bagasse as single substrate obtained only 72 U g⁻¹ of xylanase after 96 h, confirming the higher efficiency of fermentation using sugarcane bagasse and wheat bran for enzyme assembly and indicating that the simultaneous reuse of these two residues is a positive combination for biomass degradation.

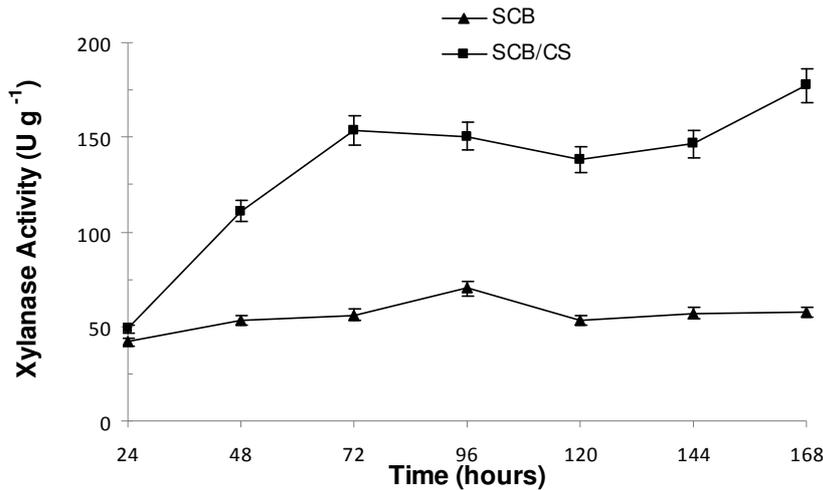


Figure 2. Xylanase production by *A. fumigatus* SCB4 using sugar cane bagasse and corn straw (SCB/CS) and only sugarcane bagasse (SCB) at 45 °C.

Maximum xylanase activity was obtained at pH 4.5 showing a typical profile of an acidophilic enzyme (Figure 3A). Moreover, 80% of the optimum activity was obtained at pH range of 3.5 to 5.0. Other studies reported an acid optimum pH for xylanase from *A. fumigatus* strains (MORETTI et al., 2012; SOUZA et al., 2012). Optimum temperature was observed at 60 °C, suggesting that this xylanase can be a thermophilic enzyme which can be useful for many biotechnological processes which operate at high temperatures (Figure 3B). This characteristic confers to this enzyme potential uses in the industry since its action represents a key step for efficient biomass degradation in saccharification processes where high temperatures are aimed (DELABONA et al., 2013). Enzymes with high optimal temperatures are also resistant to

detergent agents, to proteolytic enzymes and are stable to a wide range of pHs, increasing their application in different conditions and processes (GOMES et al., 2007).

The enzyme showed high stability in a wide pH range with a highlighted residual activity of more than 80% after an overnight exposition at pH 3.0 (Figure 3C). This stability in acid pH values shows the potential application of such enzyme in industrial bioprocesses such as the production of bioethanol (PEIXOTO-NOGUEIRA et al., 2009). The xylanase enzyme also showed high stability up to 55 °C (Figure 3D) and kept 86% of the maximum activity at 50 °C, indicating a higher stability when compared to other studies (KALOGERIS et al., 2003; KANG et al., 2004).

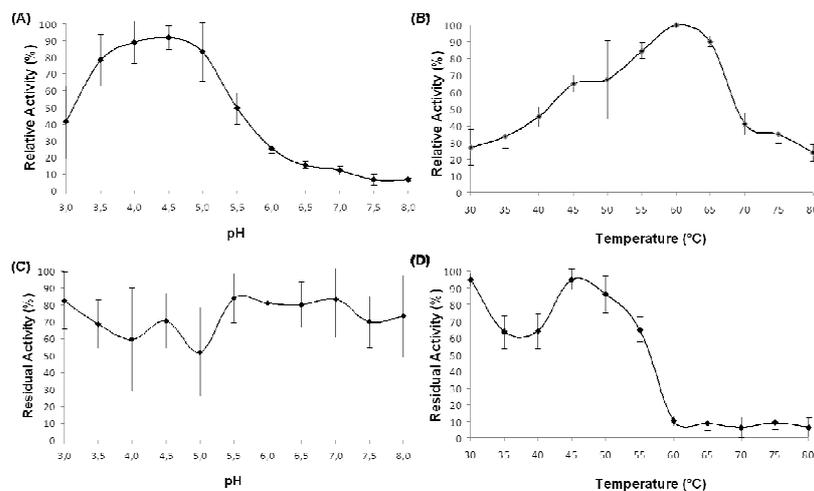


Figure 3. Effect of pH and temperature on the activity and stability of *A. fumigatus* SCB4 xylanase: (A) optimum pH; (B) optimum temperature; (C) pH stability and (D) thermostability.

The effect of sugars which are released after the hydrolysis of lignocellulosic biomass was evaluated on xylanase activity, as well as the inhibitory effect of the ethanol as the final product of the alcoholic fermentation. The enzyme was tolerant to ethanol, showing tolerance up to 40% of ethanol (Table 2). Previous studies in literature reported cellulases and hemicellulases which are inhibited by ethanol (van DYK et al., 2010; BAFFI et al., 2011). In the present study, the enzyme remained 75% of residual activity at 40% of ethanol

showing high tolerance to this compound. At the levels of ethanol assessed, high enzyme activity was still present being this xylanase also suitable for application in alcoholic fermentations. As regards the effect of sugars on enzymatic activity, the enzyme showed reasonable tolerance to glucose and xylose with nearly 70% of residual activity maintained up to 20 mM of each sugar (Table 2). Few studies in literature reported resistance to xylose by microbial xylanases (van DYK et al., 2010).

Table 2. Effects of ethanol and sugars on the activity of xylanase from *A. fumigatus* SCB4.

Substance	Xylanase tolerance
Ethanol	Up to 40%
Xylose	Up to 20mM
Glucose	Up to 20mM

CONCLUSIONS

The optimization of SSF showed high levels of xylanase production by *A. fumigatus* SCB4 strain using sugarcane bagasse and wheat bran as substrates after 24 h at 45 °C.

The enzyme was characterized as acidophilic, thermophilic and stable over a broad pH range. It also showed thermostability, high tolerance to ethanol and moderate sensitivity to reducing sugars. These properties are favorable for

application in advanced industrial processes and support data for further research involving the reuse of agro-industrial residues for the production of bioproducts from renewable sources.

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RESUMO: Este estudo descreve a otimização da produção de xilanase por fermentação em estado sólido (FES) por uma linhagem termotolerante de *Aspergillus fumigatus* isolada de pilhas de bagaço de cana-de-açúcar do Cerrado Brasileiro (linhagem SCB4). Combinações de diferentes subprodutos agrícolas de baixo custo foram avaliadas como substratos na FES: bagaço de cana-de-açúcar e farelo de trigo (1:1), bagaço de cana-de-açúcar e palha de milho (1:1) e somente bagaço de cana-de-açúcar. A produção da enzima por FES foi realizada em diferentes temperaturas (40, 45, 50 e 55 °C). Níveis máximos de xilanase (574 U g⁻¹ de substrato seco) foram obtidos após 24 h a 45 °C, utilizando bagaço de cana-de-açúcar e farelo de trigo (1:1) no meio de cultura. O extrato enzimático bruto apresentou atividades ótimas a 60 °C e pH 4,5. A enzima exibiu estabilidade térmica até 55 °C, ampla faixa de pH de estabilidade e tolerância ao etanol, xilose e glucose. Tais propriedades físico-químicas indicam que o extrato enzimático obtido é apropriado para aplicação na hidrólise de resíduos lignocelulósicos para a produção de etanol e outros bioproductos.

PALAVRAS-CHAVE: Bagaço de cana-de-açúcar. Farelo de trigo. Palha de milho. Xilanase. *Aspergillus fumigatus*.

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