Original Article

PRODUCTION OF XYLANASE BY A NEW STRAIN OF *Thermoascus aurantiacus*: OBTAINMENT OF ENZYMATIC EXTRACT WITH REDUCED CELLULOLYTIC ACTIVITY FOR APPLICATION IN PULP AND PAPER INDUSTRIES

PRODUÇÃO DE XILANASE POR NOVA LINHAGEM DE Thermoascus aurantiacus: *OBTENÇÃO DE EXTRATO ENZIMÁTICO COM REDUZIDA ATIVIDADE CELULOLÍTICA PARA APLICAÇÃO EM INDÚSTRIA DE PAPEL E CELULOSE*

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ABSTRACT: Xylanases are useful in several industrial segments, including pulp and paper bleaching, animal feed, and bread-making processes. However, the industrial use of these enzymes is closely related to its production cost and its catalytic properties. The process of solid state fermentation enables the use of agro-industrial residues as substrates for microbial cultivation and enzymes production, reducing costs. In the present study, different cultivation parameters were evaluated for the xylanase production by the thermophilic fungus *Thermoascus aurantiacus*, by solid state fermentation, using agro-industrial residues as substrates. High production of xylanase (1701.9 U g^{-1} of dry substrate) was obtained using wheat bran containing 65% of initial moisture, at 120 h of cultivation, and 45°C. The xylanase showed optimal activity at pH 5.0 and 75°C; its stability was maintained at pH 3.0–11.0. The enzyme retained its catalytic potential after 1 h, at 75°C. The enzymatic extract produced under optimized conditions showed reduced activities of endoglucanase and FPase. Our results, including the xylanase production by *T. aurantiacus* in low-cost cultivation medium, high structural stability of the enzyme, and reduced cellulolytic activity, encourage the application of this enzymatic extract in pulp and paper bleaching processes.

KEYWORDS: Solid State Fermentation. Agro-industrial residues. Phisico-chemical characterization.

INTRODUCTION

Hemicellulose is the second-most abundant polysaccharide in nature, as it is a structural constituent of plant cell wall, disposed between cellulose and lignin. This heteropolysaccharide is composed of several sugars, including glucose, arabinose, mannose, galactose, and galacturonic acid, branched in the main chain of xylan, composed of xylose monomers linked through β-1,4-glycosidic bonds (ALVES-PRADO et al., 2010; HADDAR et al., 2012).

Xylanases are enzymes responsible for hydrolyzing the β-1,4-xylan bonds to release xylose oligosaccharides, thus reducing the degree of polymerization of the main hemicellulose chain and disrupting the plant cell wall (HADDAR et al., 2012; GOWDHAMAN et al., 2014). This catalytic property enables these enzymes to be used in the hydrolysis of vegetal biomass for the production of biofuels, the formulation of animal feed, or even in bread-making processes to improve the quality of dough, hydrolyzing xylooligosaccharides that are disadvantageous to the formation of gluten and impairs the crumb structure (BECKER et al., 2009; BRIENZO et al., 2012; OLIVEIRA et al., 2014; PIROTA et al., 2015; TERRASAN and CARMONA, 2015). However, the biobleaching of kraft pulp during the processing of cellulose for paper production is the more widespread and desired use of xylanolytic enzymes, due of the economic importance of this industrial segment (MASUI et al., 2012; MITTAL et al., 2013).

The use of enzymes in industrial scale remains limited because of their high production costs (OLIVEIRA et al., 2015). An alternative for reducing the final cost of these biomolecules involves the use of agro-industrial residues in solid-state fermentation to produce microbial enzymes (BRIENZO et al., 2012; MASUI et al., 2012; SILVA et al., 2013; TERRASAN and CARMONA, 2015). Approximately 40% of the final cost of an

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enzyme is derived from the medium used for microorganism cultivation (ROMERO et al., 2007). The complex composition of agro-industrial residues enables the use of these by-products to replace traditionally employed formulations for microbiological cultures, particularly in countries with intense agricultural activity, and can sustain the large-scale economic application of the enzyme of interest, mainly to production of enzymes associated with plant cell wall degradation (BOCCHINI-MARTINS et al., 2011; SANTOS; ISHII, 2011).

The industrial environment is hostile to biocatalysts; generally, these enzymes are exposed to extremes of pH and temperature, which alter their structural conformations and reduce their catalytic efficiencies. Thus, thermophilic microorganisms capable of producing enzymes with high structural stability have been explored to withstand industrial conditions (GOMES et al., 2007).

Previous studies have described different strains of the filamentous fungi *Thermoascus aurantiacus* as excellent producers of xylanases and cellulases, particularly when grown in agro-industrial residues (KALOGERIS et al., 2003; DA-SILVA et al., 2005), precluding the application of these enzymatic extracts in cellulose pulp bleaching processes due to the high concentration of cellulolytic enzymes. Recently, our research group isolated a new strain of *T. aurantiacus* with considerable potential for the production of xylanase and reduced cellulolytic activity. In this study, we optimized the xylanase production by the selected strain, using agro-industrial residues in solid-state fermentation. The enzyme produced under optimized conditions was characterized biochemically to evaluate its structural stability for future application in the pulp and paper industry.

MATERIAL AND METHODS

Microorganism

The filamentous fungus used in this study was isolated from leaf litter of a fragment of the Semideciduous Atlantic Forest (Mata do Azulão) located in Dourados, Mato Grosso do Sul State, Brazil. The thermophilic fungus was identified as *T. aurantiacus* by the Laboratory of Fungal Ecology and Systematics in the Biosciences Institute of the São Paulo State University, IB/UNESP Rio Claro, São Paulo State, Brazil. The microorganism was kept on Sabouraud Dextrose Agar medium at 4°C.

Inoculum

The inoculum was prepared in 250 mL Erlenmeyer flasks containing 40 mL of inclined Sabouraud Dextrose Agar and incubated for 72 h at 45°C. The microbial suspension was obtained by adding 50 mL of nutrient solution containing 0.1% ammonium sulfate, 0.1% magnesium sulfate heptahydrate, and 0.1% ammonium nitrate (m/v), followed by gentle scraping of the medium surface. Inoculation of the microorganism was performed by transferring 5 mL of the microbial suspension to the flasks containing the agro-industrial residues (PEREIRA et al., 2015).

Solid-state fermentation for xylanase production

Several agro-industrial residues were tested for the production of xylanase, such as wheat bran, soy bran, corn cob, corn straw, rice peel, or sugarcane bagasse. All substrates were washed with distilled water and dried at 50°C for 48h. The fungus was cultivated in 250 mL Erlenmeyer flasks containing 5 g of substrate (agro-industrial residue) moistened with nutrient solution (described above). Initially, the moisture content was adjusted to 70% (w/v), the cultivation time was fixed at 96 h, and the temperature was maintained at 45°C. The best substrate for production of the enzyme was selected to evaluate other culture parameters, such as temperature (35-55°C), initial moisture (50-70%), and cultivation time (24-168 h). The parameter optimized in each step was adopted in subsequent cultivation. All assays were performed in duplicate and the values described represent the respective averages.

Enzyme extraction

The enzymatic extract was obtained by adding 50 mL of distilled water in Erlenmeyer flasks containing the fermented residue. The flasks were agitated for 1 h at 150 rpm, and then the samples were filtered through nylon cloth, and subsequently centrifuged at 3000×*g* for 5 min. The supernatant was used in subsequent assays.

Determination of enzymatic activities

The activities of endoglucanase (or CMCase) and xylanase were quantified using 3% carboxymethylcellulose (C5678; Sigma) and 1% xylan (Birch-Wood; Sigma). The filter paper activity (FPase) was quantified according to Ghose (1987), using a strip of n. 1 Whatman filter paper (1.0 × 6.0 cm) as substrate. The reducing sugars released were quantified using the DNS method (MILLER, 1959). The activities of β-glucosidase and β-xylosidase were measured with the respective synthetic substrates, including 4 mM p-NP-β-D-glucopyranoside and 4 mM p-NP-β-D-xylopyranoside (Sigma) as described by Pereira et

al. (2015). All assays were performed at 50°C in 0.1 M sodium acetate buffer, pH 5.0. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of product per minute of reaction.

Effect of pH and temperature on the xylanolytic activity

The xylanase produced under optimized conditions was characterized biochemically. The optimal pH was determined by quantifying the enzymatic activity, at 50ºC, at different pH values (3.0–8.0) using 0.1 M citrate-phosphate buffer. The optimum temperature was determined by measuring the enzymatic activity from 65 to 85°C, at the optimal pH of the enzyme activity. pH stability was determined by incubating the enzyme for 24 h, at different pH values, at 25°C using 0.1 M citrate-phosphate (3.0–8.0), 0.1 M Tris-HCl (pH 8.0–8.5), and 0.1 M glycine-NaOH (8.5–11) buffers. Thermostability was evaluated by incubating the enzyme, for 1 h, at different temperatures from 45 to 85°C. Residual activities were measured under optimal enzyme conditions, adopting as 100% the highest value of residual activity obtained after the samples treatment.

RESULTS AND DISCUSSION

Production of xylanase by solid-state fermentation

The fungus *T. aurantiacus* showed growth and xylanase production in all evaluated agro-industrial residues (Figure 1A). However, the highest enzyme production was obtained using wheat bran as substrate, producing 1301.1 U g^{-1} of dry substrate (or 130.11 U mL^{-1}).

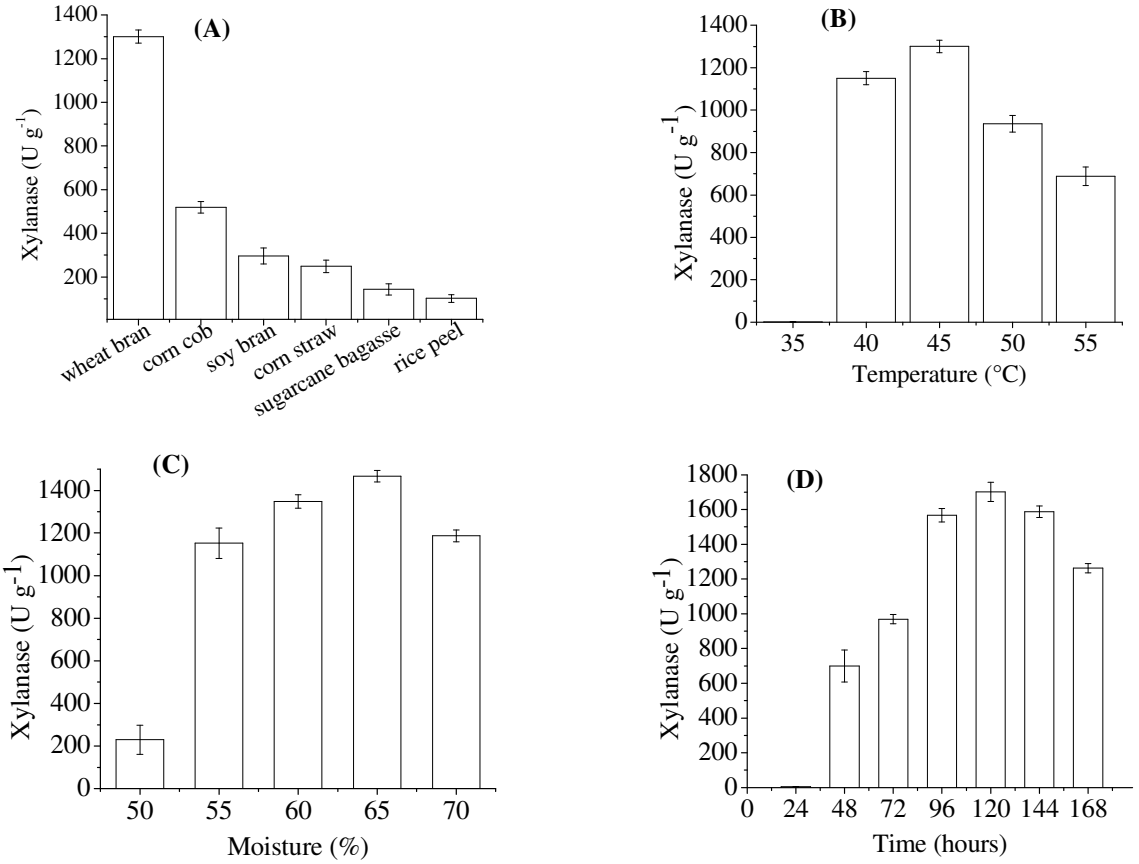

Figure 1. Influence of fermentation parameters on xylanase production by *T. aurantiacus* in solid-state fermentation. (A) Production of xylanase in several agro-industrial residues; (B) Influence of cultivation temperature; (C) Influence of initial substrate moisture; (D) Influence of cultivation time.

The production of microbial enzymes by solid-state fermentation is directly related to the nutritional composition and accessibility of the substrate (BRIENZO et al., 2012; STROPARO et al., 2012). Wheat bran is a substrate with good water absorption capacity, and its composition favors microbial cultivation for enzymes production (OLIVEIRA et al., 2015). Previous studies have

reported the production of cellulases and hemicellulases by solid-state fermentation in wheat bran by several microorganisms (RODRIGUEZ-ZUNIGA et al., 2011; DELABONA et al., 2012; MASUI, et al., 2012; MITTAL, et al., 2013; GOWDHAMAN et al., 2014; PIROTA et al., 2015). Thus, wheat bran was used as substrate for *T. aurantiacus* cultivation in subsequent steps.

T. aurantiacus showed higher xylanase production (1301.1 U g^{-1} or 130.11 U mL^{-1}) when cultivated at 45°C (Figure 1B). However, considerable amount of enzyme and mycelial growth were obtained in cultures carried out at 40-55°C (Figure 1B), confirming the thermophilic properties of the microorganism. The capacity to grow and produce enzymes over a wide range of temperatures is extremely important for the industrial application of a microbial strain, whereas fermentation parameters may undergo significant fluctuations under industrial conditions (GARCIA et al., 2015).

Temperature influences the structure of cell constituents and therefore interferes in microbial metabolic activity. Thermophilic microorganisms present cellular adaptations that enable their survival at high temperatures. Among these adaptations, the structural stability of their proteins has gained interest in the industry. Enzymes produced by these microorganisms generally withstand greater variations in pH and temperature. This favors the application of these biocatalysts in industrial processes and validates the search for thermophilic strains to produce industrial enzymes (GOMES et al., 2007).

Wheat bran containing 50-70% moisture was used for the cultivation of *T. aurantiacus* by solid-state fermentation. Higher production of xylanase, approximately 1467 U g^{-1} (or 146.7 U mL^{-1}), was obtained in cultures containing 65% moisture (Figure 1C). Generally, filamentous fungi are better suited to grow in solid-state fermentation because of the similarity of this type of process with their natural habitat. The moisture present in the solid state fermentation should be sufficient for microorganism development without compromising substrate porosity. A reduced moisture content impairs the fungal accessibility of the medium nutrients, resulting in decreased microbial metabolic activity; in contrast, high concentrations of water favor bacterial contamination and reduce solid matrix porosity, compromising oxygen diffusion and metabolic heat dissipation (DA-SILVA et al, 2005; SADAF and KHARE, 2014). This supports the profile of enzyme production observed in this study (Figure 1C).

The optimal culture conditions established previously were adopted and the production of xylanase by the fungus *T. aurantiacus* was evaluated every 24 h for 168 h of culture. The highest xylanase production was obtained at 120 h of cultivation, showing an activity of 1701.9 U g^{-1} (or 170.19 U mL^{-1}). After this period, a significant decrease in xylanase activity was observed in the enzymatic extract (Figure 1D). The high production of xylanase over a reduced cultivation period by the fungus *T. aurantiacus* under the conditions optimized in this study became more evident when the results were compared with those from other fungal strains (Table 1).

Table 1. Xylanase production by different microorganisms by solid-state fermentation using agro-industrial residues as substrates.

Microorganism	Substrate	Cultivation Time (h)	Xylanase (U g^{-1})	Authors
Thermoascus aurantiacus	Wheat bran	120	1701	This study
Aspergillus fumigatus	Wheat bran	120	1055	Delabona et al. (2012)
Cochliobolus sativus	Wheat straw	192	1079	Bakri et al. (2008)
Lichtheimia ramosa	Wheat bran	96	28	Garcia et al. (2015)
Aureobasidium pullulans	Wheat bran	96	50	Leite et al. (2007)
Sporotrichum thermophile	*Jatropha cucas* seed cake	96	1025	Sadaf and Khare (2014)
Aspergillus japonicus	Wheat bran	72	1004	Li et al. (2015)
Penicillium janczewskii	Brewer's spent grain	168	371	Terrasan and Carmona (2015)

According to Monteiro and Silva (2009), one of the main advantages of using microbial enzymes is their reduced production period, particularly when compared to enzymes of animal and vegetal origins. Thus, the *T. aurantiacus* strain recently isolated from the region of Dourados-MS has considerable potential for the production of xylanase in low-cost culture media (agro-industrial residues). Previous studies have confirmed the potential to produce xylanase by different strains of

the same species isolated from other regions (KALOGERIS et al., 1998; DA-SILVA et al., 2005; BRIENZO et al., 2012).

Catalytic potential of the enzymatic extract

The enzymatic extract obtained at 120 hour showed high xylanase activity (170.19 U mL^{-1}) and a reduced potential to hydrolyze cellulose, containing 3.8 U mL^{-1} of endoglucanase (or CMCase) and 0.063 U mL^{-1} of FPase (Table 2). These characteristics are appreciable for application in the paper and pulp industries.

Table 2. Catalytic potential of enzymatic extract produced by T. aurantiacus under optimal cultivation conditions.

Enzyme	Substrate	U mL^{-1}
CMCase	Carboxymethylcellulose	3,8
β-glucosidase	Nitrophenyl β-D glucopyranoside	3,5
FPase	Filter paper	0,06
Xylanase	Xylan	170,1
β-xylosidase	Nitrophenyl β-D xylopyranoside	0,09

The main application of xylanolytic enzymes involves the bleaching of pulp to remove residual lignin still present after the Kraft process. Addition of these biocatalysts significantly reduces the amount of chemical catalysts required for oxidation of lignin. Thus, the use of xylanases in biopulping processes contributes to reduce the environmental impacts caused by effluents of this industrial segment (DURAN et al., 2008).

Enzymatic extracts used for bleaching of pulp require high concentrations of xylanase, but should have reduced cellulolytic activity so as not to decrease the quality of the pulp (DA-SILVA et al., 2005). This necessary characteristic limits the ability to obtain xylanase by microbial cultivation on complex substrates, such as agro-industrial residues, that are capable of inducing the production of several enzymes, particularly cellulases (BRIENZO et al., 2012).

Previous studies reported the fungus *T. aurantiacus* as one of the largest producers of cellulases and hemicellulases, mainly when grown in wheat derivatives (PARRY et al., 2001; KALOGERIS et al., 2003; DA-SILVA et al., 2005), preventing the application of the enzymatic extract in biopulping processes. However, the *T. aurantiacus* strain evaluated in the present study showed low potential for the production of cellulases (Table 2). Therefore, the enzymatic extract obtained can be applied in the paper and pulp industries.

Effect of pH and temperature on the xylanolytic activity

The xylanase produced by the fungus *T. aurantiacus* exhibited optimal activity at pH 5.0 and 75°C (Figure 2A and 2B). The enzyme was stable for 24 h at pH 3.0-11.0 (Figure 2C) and maintained its catalytic activity at 75°C for 1 h (Figure 2D), confirming the high enzymatic stability.

Da-Silva et al. (2005) characterized the xylanase produced by another strain of *T. aurantiacus* isolated from decayed wood, Manaus, Amazonas State, Brazil. The authors reported the optimal activity of the enzyme at values identical to those described in this study, confirming high stability at several pH levels and temperatures.

High structural stability is essential for application of an enzyme in industrial processes, where the industrial environment differs significantly from laboratory conditions with respect to the control of pH and temperature (GOMES et al., 2007). Thus, evaluating the effect of pH and temperature on enzymatic activity is essential for the industrial use of biocatalysts to ensure high catalytic efficiency throughout the process (GOWDHAMAN et al., 2014).

The removal of lignin in the production of pulp and paper is performed by cooking wood chips at high pH and temperature, which requires enzymes considerably robust (POLIZELI et al., 2005). Our results of biochemical characterization confirmed the applicability of this xylanase in the Kraft bleaching of pulp due to its high stability at pH and temperature.

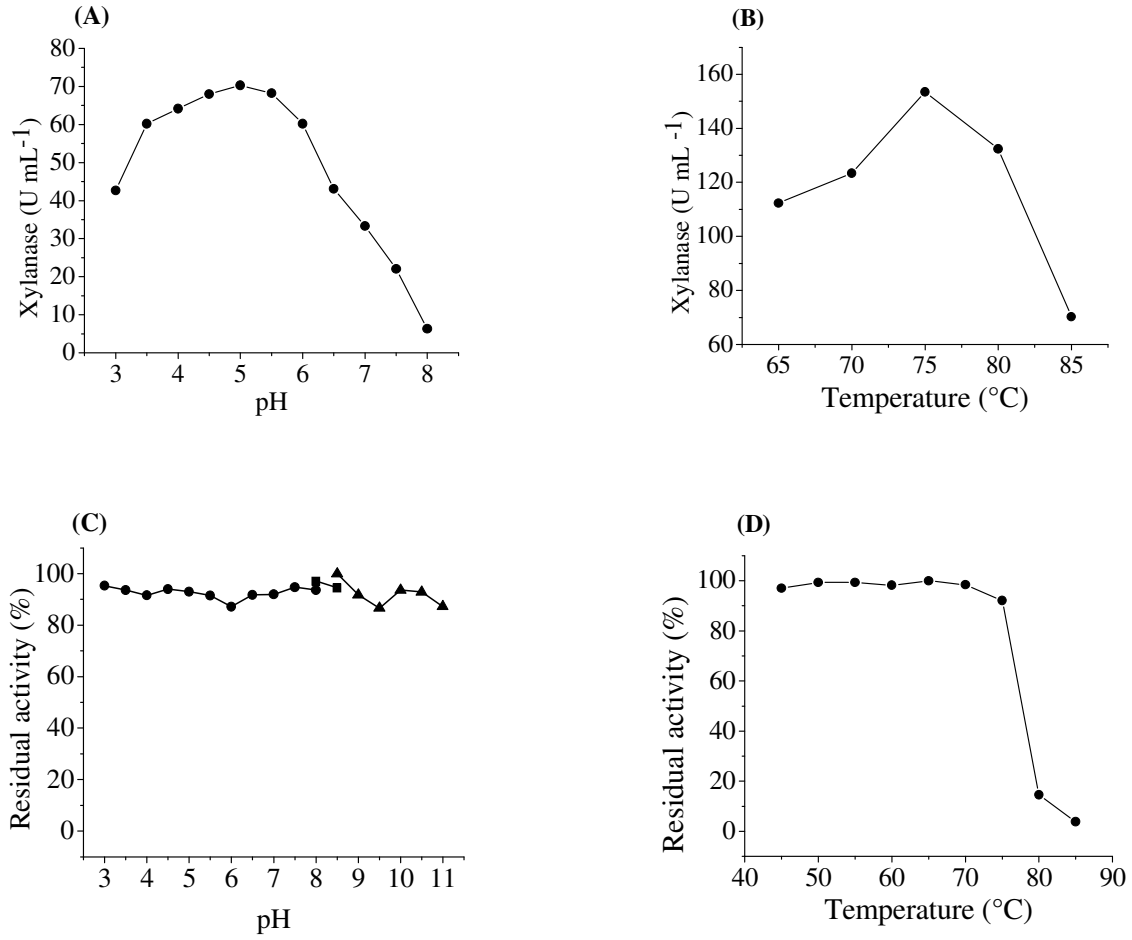

Figure 2. Biochemical characterization of xylanase produced by *T. aurantiacus* under optimal cultivation conditions. (A) Optimal pH; (B) Optimal temperature; (C) pH Stability; (D) Temperature stability.

CONCLUSIONS

T. aurantiacus strain recently isolated by our Research Group has high potential for the production of xylanase by solid-state fermentation on agro-industrial residues, resulting in the production of low-cost enzymatic extracts with negligible activity of cellulolytic enzymes.

The catalytic characteristics of the enzymatic extract, together with the high stability at pH and temperature of the xylanase produced, encourages the application of this enzyme in the bleaching of kraft pulp.

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RESUMO: As xilanases apresentam aplicabilidade em diferentes segmentos industriais, como: branqueamento de papel e celulose, ração animal e panificação. No entanto, a utilização industrial dessas enzimas está intimamente relacionada com seu custo de produção e suas propriedades catalíticas. O processo de fermentação em estado sólido possibilita o uso de resíduos agroindustriais como substratos, para o cultivo microbiano e produção de enzimas, reduzindo o custo da produção enzimática. No presente trabalho, diferentes parâmetros de cultivo foram avaliados para produção de

xilanase por cultivo em estado sólido do fungo termófilo *Thermoascus aurantiacus*, utilizando resíduos agroindustriais como substratos. A maior produção de xilanase, 1701,9 U g^{-1} de substrato seco, foi obtida no cultivo em farelo de trigo, contendo 65% de umidade inicial, em 120 horas de cultivo a 45°C. A xilanase produzida apresentou atividade ótima em pH 5,0 a 75°C, mantendo sua estabilidade em pH 3,0 a 11,0. A enzima manteve seu potencial catalítico após 1 h a 75°C. O extrato enzimático produzido nas condições otimizadas apresentou reduzida atividade de endoglucanase e FPase. Os resultados obtidos no presente trabalho (produção de xilanase pelo fungo em meios de cultivo de baixo custo, elevada estabilidade estrutural da enzima e reduzida atividade celulolítica) estimulam a aplicação desse complexo enzimático em processos de branqueamento de papel e celulose.

PALAVRAS-CHAVE: Fermentação em Estado Sólido. Resíduos agroindustriais. Caracterização físico-química.

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