Full Length Research Paper

Production of cellulase-free xylanase by *Aspergillus flavus*: Effect of polyols on the thermostability and its application on cellulose pulp biobleaching

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The production of xylanase without cellulase is required for prebleaching of pulp in pulp and paper industry. *Aspergillus flavus* produced high levels of xylanase on agricultural residues with wheat bran and sugarcane bagasse (4.17 U/mg), and wheat bran and corncob (2.97 U/mg). Xylanase was found to be stable at 45°C with 100% of its original activity remaining after 2 h incubation. At 50°C, xylanase was stable for the first twenty minutes, and had half-life of 50 min. The pH stability for the xylanase from *A. flavus* was most stable in the range of pH 3.0-8.0 retaining more that 100% activity after 1 h. The addition of 5% glycerol, mannitol or xylitol protected the xylanase from thermal inactivation at 50°C. The protective effect by glycerol, xylitol and mannitol resulted in increases of 162, 262.5 and 150% when compared with the control at 120 min, approximately. Increasing the polyols concentration up to 20% (w/v) further improved the thermostability of xylanase after 120 min at 50°C by 300% when compared with the control (no additive). The kappa number reduced 2.56 points, which corresponds to 18.34 kappa efficiency. This xylanase is an attractive enzyme for potential future application in the pulp and paper industries, since industrial application requires a cellulase-free activity, maintenance of high temperature and enzyme stability are desirable.

Key words: *Aspergillus flavus*, polyols, xylanase, biobleaching cellulose pulp.

INTRODUCTION

After cellulose, hemicellulose is the second most abundant renewable biomaterial available in nature and, among the hemicelluloses constituents, xylans are the major portion of the hemicellulose. Xylan is a hetero-polymer consisting principally of xylose and arabinose (Abdel-Sater and El-Said, 2001). A complex of enzymes are responsible for the hydrolysis of xylan, but the main enzymes involved are endo-1,4-β-xylanase and β-xylosidase. These enzymes can be produced by bacteria, yeast or insect, but the principal commercial source is filamentous fungi (Biswas et al., 2010; Guimaraes et al., 2013a). Among the microbial sources, filamentous fungi

are especially interesting as they secrete these enzymes into the medium and their xylanase levels are very much higher than those found in bacteria (Michelin et al., 2010; Guimaraes et al., 2013b). A large number of different *Aspergillus* species have been reported as good producers of xylanases (Abdel-Sater and El-Said, 2001; Sandrim et al., 2005; Betini et al., 2009; Guimarães et al., 2013b).

In recent years, there has been increasing interest in the use of xylanases, particularly in the bleaching process of pulp and paper industry (Comlekcloglu et al., 2014; Guimaraes et al., 2013b; Michelin et al., 2010; Peixoto-Nogueira et al., 2009; Abdel-Sater and El-Said, 2001). Xylanases are used in the pretreatment of pulp to increase the liberation of lignin through the hydrolysis of hemicellulose (Guimaraes et al., 2013b; Khonzue et al., 2011). In fact, treating cellulosic pulps with xylanases selectively removes residual xylan and hence reduces the usage of chlorine during the bleaching process (Woolridge, 2014; Bankeeree et al., 2014; Nawel et al., 2011; Abdel-Sater and El-Said, 2001). Chlorine is the base of bleaching process of pulp and paper industry and present serious environmental effects such as the production of toxic and mutagenic residues (Dedhia et al., 2014; Goluguri et al., 2012; Yeasmin et al., 2011); therefore, environmental demands have necessitated that the pulp and paper industry find various alternatives to chlorine-based chemical bleaching processes for the production of bleached kraft pulp. A xylanase pretreatment, always used as a mixture, can deink pulp of waste paper, lower bleaching chemical use by 10-20% and usually results in greater final brightness (Goluguri et al., 2012). In paper manufacturing, xylanases are efficient in biobleaching and so are regularly used for process efficiency, improving enhancement of products quality (Paes et al., 2012; Kenealy and Jeffries, 2003). For such biotechnological applications, xylanases are required to be stable at elevated temperatures, to be active at alkaline pH, to be devoid of cellulose activity, to avoid deterioration of strength properties and to minimize yield loss. Accordingly, lignocellulosic extract has been employed for xylanase production in the present study. The purpose of this work was to characterize the production of xylanase, and application of the crude extract on cellulose pulp biobleaching.

MATERIALS AND METHODS

Microorganism

Aspergillus flavus was isolated from soil samples in Campo Grande region, Mato Grosso of Sul, Brazil, and was deposited in the laboratory fungal herbarium. The *A. flavus* was grown on potato dextrose agar (PDA) slants at 30°C for 5 days and subsequently stored at 4°C.

Culture conditions and xylanase production

Spores were inoculated in 25 ml of liquid medium SR (Rizzatti et al., 2001) supplemented with 1.0% (v/w) carbon source (sugarcane bagasse or wheat bran or rice straw, etc.) contained in 125 ml Erlenmeyer flasks. The cultures were incubated at 30°C, subjected to 110 rpm agitation for 96 h. After this period, the medium was filtered using vacuum and the filtrate used as a source of crude extracellular enzymes.

Enzymatic assay and protein determination

The xylanolytic activity was assayed using 3´,5´-dinitrosalicylic acid (DNS) as described by Miller (1959), using 1% (v/w) Birchwood xylan as substrate, at 50°C. The reaction mixture consisted of 500 µl of McIlvaine buffer (McIlvaine, 1921) pH 5.0 containing the substrate diluted and 100 µl of enzymatic extract appropriately diluted. One unit (U) was defined as the amount of enzyme that releases 1 µmol of xylose (Sigma) per minute under the assay conditions. Protein content was estimated by the Lowry et al. (1951) method, using bovine serum albumin as standard. Specific activity corresponded to U/mg protein.

Effect of temperature and pH on enzyme stability

To determine the thermostability of the enzyme, it was incubated from 45-60°C, in the absence of substrate, and residual activity was determined for different periods (10 to 120 min). The pH stability was analyzed using McIlvaine buffer in the pH range of 2.0-8.0 for 1 h, and after that, assays were carried out at the optimal temperature and pH of enzyme.

Effect of polyols on xylanase thermostability

In order to improve the thermal stability of the xylanase, polyols including polyethylene glycol, mannitol, xylitol and glycerol were added to separate enzyme solutions at 5% (w/v) final concentration prior to incubation at 50°C. Aliquots were withdrawn and then the residual xylanase activity was assayed under the optimal conditions. The stability of the enzyme was expressed as a percentage of residual activity (%) compared with activity of the initial enzyme (before incubation and no polyols). The polyols that most improved the thermostability was selected for further study over a range of concentration on the optimal [2.5 – 20% (w/v)] at 50°C.

Biobleaching

The amount of enzyme used from *A. flavus* for this biobleaching treatment was 10 units of enzyme per gram of dried cellulose pulp from *Eucalyptus grandis*. All calculations and procedures were determined according to the standard methods of Technical Association of the Pulp and Paper Industry (TAPPI test methods, 1996). The consistency was determined on a percent dry weight basis. The volume of enzyme or distilled water was added until it

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Table 1. Effect of different carbon sources on extracellular xylanase production.

Carbon source	Total xylanase activity (units)	Total protein (mg)	Specific xylanase activity (U/mg[a])
Wheat bran 0.5% + Sugarcane bagasse 0.:	129.8	31.1	4.17 (±0.0141)
Wheat bran 0.5% + Corncob 0.5%	94.0	31.7	2.97 (±0.0141)
Corncob 0.5% + Sugarcane bagasse 0.5%	21.4	17.4	1.23 (±0.0141)
Soybean 1%	4.4	47.2	0.09 (±0.7142)
Triturated rice straw 1%	12.6	14.9	0.85 (±0.1768)
Corncob 1%	36.1	15.8	2.28 (±0.0566)
Rice bran 1%	4.1	44.9	0.09 (±0.8556)
Wheat bran 1%	47.9	32.1	1.49 (±0.0212)
Rice straw 1%	25.9	14.7	1.76 (±0.4313)
Sugarcane bagasse 1%	49.8	19.5	2.55 (±0.8061)
Avicel 1%	1.3	18.2	0.07 (±0.6576)
Glucose 1%	1.3	5.4	0.24 (±0.0354)
Carboxymethiyl-cellulose (CMC) 1%	4.1	9.3	0.44 (±0.3182)

[a]Values are means ±SD of three experiments.

reached a 10% pulp consistency. Crude xylanase extract from *A. flavus* was added for the treated pulp and the control was prepared by adding distilled water instead of enzyme. The samples were incubated inside sealed polyethylene bags at 55°C for 2 h and pH 6.5 and after that, the treated cellulose pulps were filtered on a Büchner funnel, rinsed with 200 ml of distilled water and used for determination of kappa number and viscosity. Xylan is degraded by the xylanase, in addition to xylose, it also results in the release of lignin and phenolic compounds from the pulp fibres, which ultimately causes an enhancement in the absorbance at 237 nm of pulp free samples. The correlation between the release of chromophores optical density (at 237nm) and hydrophobic compounds (at 465 nm) coupled to the release of reducing sugars suggested the dissociation of lignin-carbohydrate complex. So, the filtrate was used to analyze the liberation of aromatic and hydrophobic compounds monitored using the Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, USA).

Reproducibility of results

All the results are the mean of at least three independent experiments [±standard deviation (SD)].

RESULTS AND DISCUSSION

Effect of different carbon sources on xylanase production by *A. flavus*

Xylanase production by *A. flavus* was investigated using several carbon sources including agroindustrial residues. These substrates with high hemicelluloses contents include wheat bran, corn cob and rice straw. Table 1 shows the activity of xylanase produced by *A. flavus* when growing on different carbon sources, including agroindustrial residues. Maximum xylanase production was observed in wheat bran and sugarcane bagasse

(4.17 U/mg), followed by wheat bran and corncob (2.97 U/mg), as carbon source. Other materials assayed, such as soybean, triturated rice straw, rice bran and avicel were poor substrates for xylanase production.

In literature, the xylanase production using different combinations of agroindustrial residues as carbon sources, has been reported for *Aspergillus* species. *A. niger* had its highest xylanase production in media containing a mixture of wheat bran and corncob (Guimaraes et al., 2013b) or wheat bran and soybean as described by Pal and Khanum (2010), for *Aspergillus japonicas,* the combination of soybean and crushed corncob was described by Facchini et al. (2011). The possibility of using agricultural residues to produce enzymes may reduce the production costs resulting in a cheaper product. Wheat bran is the agricultural waste most often included in nutrient media for microbial xylanase production (Techapun et al., 2003), and furthermore the substrate content lower lignin content and increase protein concentration, as compared to other substrates which promotes a higher xylanase production.

Studies on pH stability indicated that *A. flavus* xylanase was most stable in the range of pH 3.0-8.0 retaining more than 100% activity after 1 h (Figure 1). In literature, the xylanases of *A. niveus* and *A. ochraceus*, were stable between pH 3.5-7.0, retaining more than 75% activity, approximately (Betini et al., 2009). *Aspergillus fumigatus* (Peixoto-Nogueira et al., 2009), *Aspergillus oryzae* (Polizeli et al., 2005) and *Aspergillus fischeri* (Techapun et al., 2003) showed a considerable stability on ranges of pH from 5.0 to 8.0. Shah and Madamwar reported that *Aspergillus foetidus* xylanase activity has a favorable pH range of 4.6-.56. Studies on pH stability indicated that *A. niger* xylanase was most stable in the range of pH 2-7 retaining more that 75% activity (Betini et al., 2009).
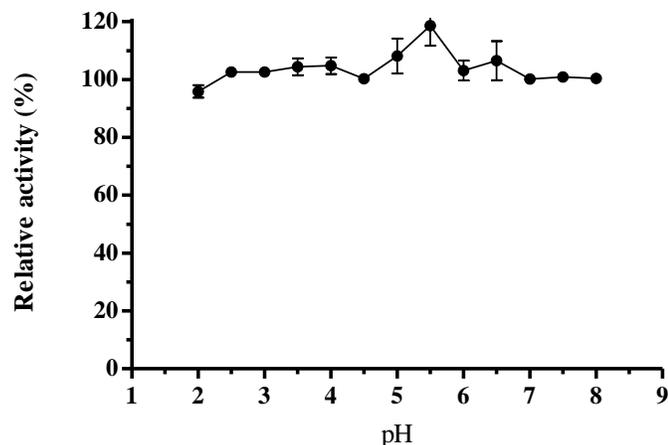
Figure 1. The pH stability of xylanase by *A. flavus*. The influence of pH on xylanase was verified using McIlvaine buffer 2.0-8.0. Residual activity was assayed after 1 h.
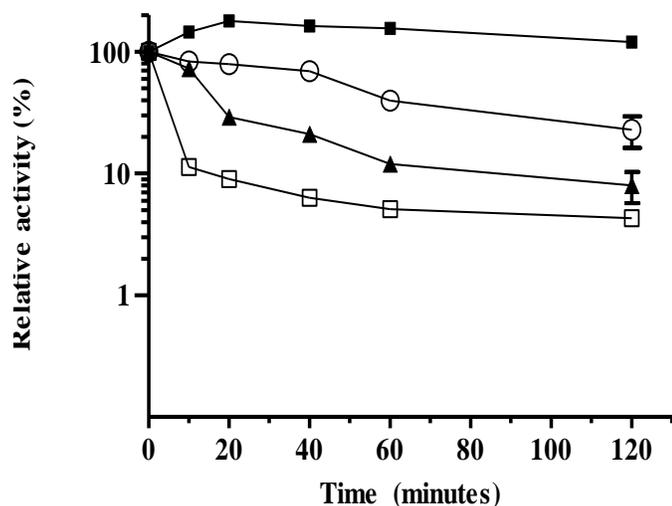

Figure 2. Thermostability of the xylanase of *A. flavus*. The thermostability was determined using McIlvaine buffer pH 5.0 at 55°C after incubating the enzyme on temperatures of 45 (■), 50 (○), 55 (▲) and 60°C (□).The residual xylanase activity was calculated as the percentage of initial enzyme (before incubation).

The thermal stability of the xylanase from *A. flavus* was tested at 45-60°C at pH 5.0. Xylanase was found to be stable at 45°C with 100% of its original activity remaining after 2 h incubation (Figure 2). At 50°C, xylanase was stable for the first twenty minutes, and had half-life of 50 min. At 55°C and above, the activity decreased with increasing temperature.

In studies with *A. terreus*, the xylanase was thermotolerant at 45 and 50°C, but had half-life of only 25 min at 50°C (Sorgatto et al., 2012). And the xylanase of *A. phoenicis* had a half-life of only 25 min at 50°C (Rizzatti et al., 2001).
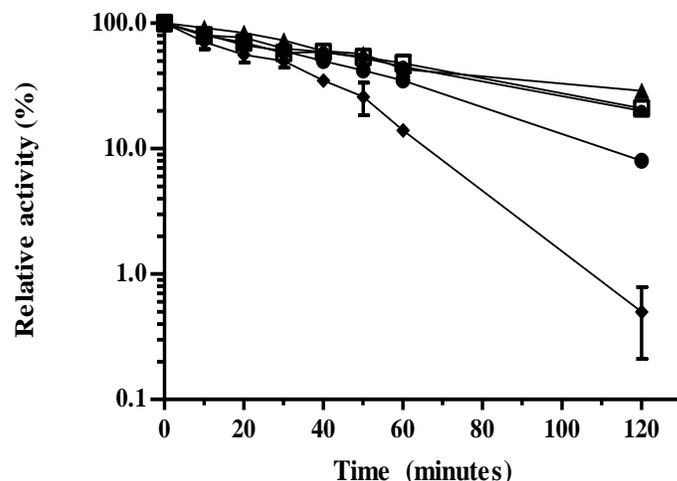

Figure 3. The effect of polyols on thermostability of the xylanase from *A. flavus*. The enzyme solutions were preincubated at 50°C without the presence of substrate for various times (0 - 120 min) in the presence of glycerol (▲), mannitol (●), xylitol (□) and polyethyleneglycol (♦) at a concentration of 5% prior to enzyme assay at the optimal condition. Control (△) without the presence of polyols. The residual xylanase activity was calculated as the percentage of initial enzyme (before incubation).

Effect of polyols on thermostability of xylanase from *A. flavus*

The addition of 5% glycerol, mannitol or xylitol somehow protected the xylanase from thermal inactivation at 50°C (Figure 3). The protective effect by glycerol, xylitol and mannitol resulted in 162, 262.5 and 150% retention of the original enzyme activity after 120 min at 50°C, approximately.

The polyethyleneglycol was not effective as protector for xylanase from *A. flavus*. The addition of polyols improves the thermostability of enzymes from fungi (Bourneow et al., 2012; Bankeeree et al., 2014), including xylanases from *Trichoderma reesei* (Cobos and Estrada, 2003), *A. niger* (Pal and Khanum, 2010) and *Aureobasidium pullulan* (Bankeeree et al., 2014).

The effect of concentration of manitol, xylitol and glycerol on the thermostability of the xylanase was also evaluated (Figure 4). Increasing the polyols concentration up to 20% (w/v) further improved the thermostability of xylanase after 120 min at 50°C by 300% as compared to the control (no additive). It has further been suggested that the protective role of polyols is due to their capability to form hydrogen bonds that support and stabilize the native conformation of the enzyme (Cobos and Estrada, 2003).

These compounds have been found to show similar effect on xylanases isolated from *Thermomonospora* sp. (George et al., 2001) and *Arthrobacter* sp. MTCC 5214 (Khandeparkar and Bhosle, 2006). Polyols have the capability to form hydrogen bonds that play key role in
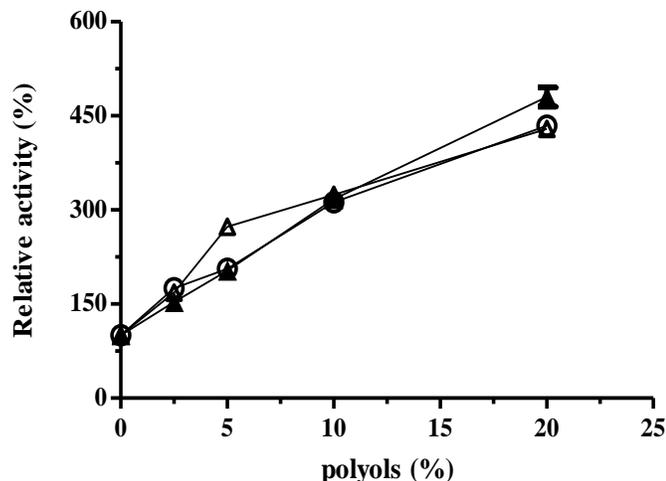
Figure 4. Thermostability of the xylanase at 50°C, with manitol (▲), xylitol (●) and glycerol (△) in different concentrations [2.5 – 20% (w/v)]. Residual activity was assayed after 120 min at 50°C.

Table 2. Properties of pulp treated with xylanase produced by *A. flavus.*

Parameters[a]	Control	*A. flavus*
Kappa number	13.96	11.40
Kappa efficiency (%)	-	18.34
CST (%)	21.70	22.10
A237 nm	-	0.100
A465 nm	-	0.043

[a]Biobleaching of bagasse pulp with 10 U xylanase g^{-1} of pulp in 0.5 M sodium citrate buffer (pH 6.5) at 55°C for 2 h.

supporting and stabilizing the native conformation of protein.

The stabilizing effect of additives is not an absolute effect valid for all enzymes, but it depends on the nature of the enzyme, on its hydrophilic and hydrophobic character and on the degree of interaction with the additive effect (George et al., 2001; Cobos and Estrada, 2003).

Assays of cellulose biobleaching using xylanase from *A. flavus*

To analyze the xylanase efficiency for cellulose pulp biobleaching assay, the cellulose pulp was clarified by *A. flavus* crude extract. The kappa number reduced 2.56 points, which corresponds to 18.34 kappa efficiency.

Alkaline-extracted pulp typically has a high brightness and low viscosity relative to xylanase-treated pulp. In fact, alkaline extraction is an aggressive treatment reducing pulp viscosity by effect of laccase-oxidized cellulose

being hydrolyzed in an alkaline medium and some glycosidic bonds between adjacent glucose molecules broken as a result. On the other hand, xylanase cleans microfibrils by hydrolyzing surface hemicellulose. Hemicellulose removal affects HexA and lignin, but does not degrade cellulose (Barneto et al., 2013). The xylanase from *A. flavus* was free of cellulase (data not shown), not changing significantly the viscosity of the pulp, meaning that the physical properties of cellulose were maintained (Table 2).

Similar results have been reported by Cheng et al. (2013) that studied the xylanase action produced by *Streptomyces griseorubens* LH-3 on eucalyptus kraft pulp, where they observed that the kappa number reduced 18,4% using 10 IU g^{-1} of xylanase, similar to our *A. flavus* xylanase kappa efficiency. Later, Cheng and colleagues (2014) studied the same xylanase of *Streptomyces griseorubens* LH-3 action, on bagasse pulp, which resulted in only 0.94 point decrease in kappa number using 30 IU g^{-1}, while *A. flavus* reduced 2.56 using 10 IU g^{-1}. But in studies of Dedhia et al. (2014) with wheat straw, a commercial xylanase reduced only 7.25% the kappa number using 6 IU g^{-1}; and *A. fumigatus* ABK9 reduced only 0.7, 1.2, 2.7, 3.3 and 4 points in kappa number, using 20, 40, 60, 80 and 100 U/g dry pulp/6 h, respectively (Das et al., 2013).

Conclusions

Xylanase has been successfully used for pre-treatment of pulp, and parameters have been optimized. The enzyme was relatively stable at 50°C, retaining more than half of its original activity after 50 min incubation. The pH stability for the xylanase was in the range of pH 3.0-8.0 retaining more that 100% activity after 1 h. The enzyme was more stable in the presence of polyols when compared with the control (no additive). The kappa number reduced with 2.56 points, which corresponds to 18.34 kappa efficiency. The results suggest the potential application of the xylanase before the pulp biobleaching process when the maintenance high temperatures, addition of polyols and enzyme stability are desirable.

Conflict of interests

The authors have not declare any conflict of interest.

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