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ORIGINAL PAPER

Production of xylanase by Aspergilli using alternative carbon sources: application of the crude extract on cellulose pulp biobleaching

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Abstract The ability of xylanolytic enzymes produced by *Aspergillus fumigatus* RP04 and *Aspergillus niveus* RP05 to promote the biobleaching of cellulose pulp was investigated. Both fungi grew for 4–5 days in liquid medium at 40°C, under static conditions. Xylanase production was tested using different carbon sources, including some types of xylans. *A. fumigatus* produced high levels of xylanase on agricultural residues (corncob or wheat bran), whereas *A. niveus* produced more xylanase on birchwood xylan. The optimum temperature of the xylanases from *A. fumigatus* and *A. niveus* was around 60–70°C. The enzymes were stable for 30 min at 60°C, maintaining 95–98% of the initial activity. After 1 h at this temperature, the xylanase from *A. niveus* still retained 85% of initial activity, while the xylanase from *A. fumigatus* was only 40% active. The pH optimum of the xylanases was acidic (4.5–5.5). The pH stability for the xylanase from *A. fumigatus* was higher at pH 6.0–8.0, while the enzyme from *A. niveus* was more stable at pH 4.5–6.5. Crude enzymatic extracts were used to clarify cellulose pulp and the best result was obtained with the *A. niveus* preparation, showing kappa efficiency around 39.6% as compared to only 11.7% for that of *A. fumigatus*.

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Introduction

In the last decades, an increasing number of studies aimed to develop environmentally clean and non-toxic methods for industrial processes. For instance, an enzymatic step in the process of cellulose pulp bleaching would contribute to reduce the use of chlorine-containing reagents. According to Valcheva [1], the utilization of enzymes, particularly xylanases, results in an easier bleaching in subsequent stages and a better pulp brightness. Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) catalyzes the hydrolysis of glycosidic bonds in the xylan backbone, reducing the degree of polymerization of the substrate [2]. This improves the access of bleaching reagents into the cellulose fibers facilitating the elimination of lignin in subsequent alkaline extraction [3].

The industrial application of xylanases takes place mainly in Scandinavia, North America and China [1, 3]. In the last decades, an increased number of studies were devoted to the biobleaching of cellulose pulp [1–9]. In order to induce xylanase synthesis from microbial sources, agricultural residues, such as rye flakes, wheat bran, oat flakes, corn flakes, crushed corncob, rice straw, sugar cane bagasse and others can be used [4, 8]. The use of agricultural residues as alternative carbon sources reduces the production costs and the price of the final product. Among the filamentous fungi employed to produce xylanase, the *Aspergillus* genus is one of the most explored. For example, production of xylanase and assays of biobleaching of cellulose pulp have been reported for *Aspergillus caespitosus*

[8], *A. nidulans* and *A. awamori* [4], *A. niger* An76 and *A. aculeatus* [2].

The aim of the present study was to describe xylanases from two fungi isolated from Brazilian soil, which were identified as *Aspergillus fumigatus* and *Aspergillus niveus*, and to test the adequacy of these enzymes for cellulose pulp biobleaching. These fungi produced high levels of xylanases [10] with special characteristics, such as high stability and high optimum temperature, in comparison to others reported in the literature [11–13].

Materials and methods

Organisms and culture conditions

The *Aspergillus* strains were isolated from soil and decomposing leaves from a reforestation area of the Campus of São Paulo University at Ribeirão Preto, SP, Brazil. These strains were identified as *A. fumigatus* RP04 and *A. niveus* RP05, and are deposited at the Pernambuco Federal University, PE, Brazil. Stock cultures of both strains were propagated at 35°C on slants of solid oatmeal medium [14]. Both microorganisms were grown in different liquid media, such as SR [15], CP [16], Khanna [17], Czapeck [18], Vogel [19] and M-5 [20], inoculated with 4×10^5 spores/ml (final concentration) in 25 ml of medium. Most cultures were supplemented with 1% birchwood xylan, but other carbon sources were also tested. Cultivation was either static or agitated at 100 rpm; the incubation temperatures and times varied according with the experiment.

Extraction of xylanases and protein determination

Mycelia were harvested by filtration, rinsed with distilled water, blotted on filter paper and stored at −15°C until use. The mycelial mass was homogenized by grinding in a mortar with glass beads at 4°C. After addition of 10 ml of 100 mM McIlvaine buffer (citrate-phosphate buffer) [21] pH 5.0–5.5 for *A. fumigatus* or 4.0–4.5 for *A. niveus*, cell disruption was continued and the slurry was centrifuged at 12,100*g*, 15 min. The supernatant was used to estimate growth as total protein, according to Lowry et al. [22], using bovine serum albumin as standard.

Enzymatic assays

Xylanase activity was determined in the crude filtrate by measuring the reducing groups released from birchwood xylan [23]. The reaction mixture consisted of 200 µl of 1% (w/v) xylan in McIlvaine buffer at the ideal pH for each enzyme, and 200 µl of enzymatic extract appropriately diluted. The reaction mixture was incubated at 70°C for the extract of *A. fumigatus* and 65°C for that of *A. niveus*. One unit was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute determined according to Miller [23]. Total activity (total U) was defined as units/ml multiplied by the total sample volume. Identical conditions of assay were employed for cellulase, amylase and pectinase determination, using as substrate 1% (w/v) carboxymethyl-cellulose, avicel or filter paper for cellulase, polygalacturonic acid for polygalacturonase and starch for amylase activities. The assay temperature was 60°C for these other enzymes. One unit was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute, using monogalacturonic acid as standard for polygalacturonase, or glucose for cellulase and amylase.

Effects of temperature and pH

The effect of temperature on xylanase activity was analyzed using crude filtrate from *A. fumigatus* and *A. niveus* cultures. The assays were performed in McIlvaine buffer at the ideal pH for each enzyme, incubated at 25–80°C, with intervals of 5°C. The effect of pH was assayed using the same buffer in the pH range 2.5–8.0, at the ideal temperature for each enzyme. Thermal inactivation was analyzed with enzymes incubated at 70°C for 1 h and the assays were performed at the optima of temperature and pH for each enzyme. At different time intervals, aliquots were withdrawn and residual activities were measured as described above. The pH stability was analyzed incubating the enzymes at 25°C with McIlvaine buffer in different pH values (range 2.5–8.0) for 1 h, and after that the assays were carried out at the optima of temperature and pH for each enzyme.

Biobleaching

The amount of enzyme used for this treatment was 10 U/g of dried cellulose pulp extracted from *Eucalyptus grandis*. All calculations and procedures were carried out according to TAPPI, Atlanta GA [24] methodology. The volume of enzyme or distilled water added corresponded to 10% (dry weight) of the pulp mixture. The samples were incubated in polyethylene bags at the adequate temperature for 1 h, after that the cellulose pulp was filtered on a Büchner funnel, rinsed with 200 ml of distilled water and used for determination of kappa, viscosity and brightness parameters.

Reproducibility of results

All results are the mean of at least three independent experiments.

Results and discussion

Optimization of xylanase production

In order to find the best culture conditions, both *Aspergilli* were grown on six different liquid media. The best enzymatic yields were obtained in Vogel or Czapeck media for *A. fumigatus* RP04 and *A. niveus* RP05, respectively, (Table 1).

The time-course of growth and xylanase production for *A. fumigatus* and *A. niveus* was followed in Vogel and Czapeck media, respectively, supplemented with 1% birchwood xylan, for 196 h. *A. fumigatus* grew along all the experimental time (Fig. 1a), reaching the maximum of xylanase production at 96 h (Fig. 1c). By the end of the experiment, a decrease in xylanase activity was observed, probably due to inhibition of the enzyme by end products. For *A. niveus*, the peak of growth was observed at 72 h (Fig. 1a), but the maximal enzyme production occurred at 120 h, suggesting partial autolysis of the mycelium at the end of cultivation. Comparing xylanase production (total units) of both fungi (Fig. 1a, c) it can be seen that *A. fumigatus* produced 2.1-fold more xylanase than *A. niveus*, but in contrast, due to lesser growth, the specific activity (U/mg protein) of *A. niveus* xylanase was 25.5-fold higher than that of *A. fumigatus*. The time required for production of xylanase for *A. fumigatus* was 96 h and it is in agreement with that reported for *A. awamori* and *A. oryzae* [4]. *A. tamarii* required 120 h, similar to *A. niveus* RP05 [4].

An interesting effect of temperature was observed (Fig. 1 b, d). Despite of the maximal xylanase levels being attained with incubation at 40°C (Fig. 1d), the optimum temperature for growth was distinct, being 40°C for *A. fumigatus* and 30°C for *A. niveus* (Fig. 1b). This result suggested a thermotolerant character for *A. fumigatus*, as previously reported for *A. phoenicis* [2, 6] and *A. caespitosus* [8]. For other *Aspergillus* species, such as *A. awamori*, *A. niger*, *A. nidulans*, *A. oryzae* and *A. tamari*, the best xylanase production occurs when the fungi are grown at lower temperatures [4].

Still aiming to improve xylanase production, cultures from both fungi were carried out under the optimized conditions, with agitation (100 rpm), or under static conditions. For both strains, the best growth was obtained with agitated cultures (Fig. 2a), but in contrast, static conditions (Fig. 2b) improved xylanase production and the enzyme levels were 2.3-fold higher for *A. fumigatus* and 1.46-fold for *A. niveus*, as compared to agitated cultures.

Aspergillus fumigatus produced approximately 3–4-fold more xylanase than *A. niveus* (Fig. 1c). Comparing with the literature, *A. fumigatus* produced 16–55% more xylanase than other fungi, such as *A. caespitosus* [8], *A. nidulans* and *A. awamori* [2, 4], while *A. niveus* produced 25 and 39% more xylanase than *A. awamori* and *A. nidulans*, respectively [2, 4], and 64% more xylanase in comparison with other strains of *A. niveus* used for prebleaching tests [13]. It is important to emphasize that the best enzymatic production under static conditions may lower the production cost.

Effect of alternative carbon sources on xylanase production

Aspergillus fumigatus RP04 and *A. niveus* RP05 were cultured in the optimized media, supplemented with different carbon sources (Table 2). *A. fumigatus* produced 5.0–6.0% more xylanase on powdered corncob, wheat bran and crushed corncob as compared to birchwood xylan. Rice straw and rye flakes were as favorable to production of xylanase as was birchwood xylan, and could be good alternative carbon sources. Xylanase from *A. niveus* was produced preferentially on wheat bran and powdered corncob, but birchwood xylan, xylose, *E. grandis* xylan and oat spelt xylan also were good substrates for the production of this

Table 1 Production xylanase by *Aspergillus fumigatus* and *Aspergillus niveus* in different culture media

Fungus	Culture medium	Protein (total mg)	Activity (total U)	Activity (U/mg protein)
Aspergillus fumigatus	SR	3.9 (± 0.3)	140.7 (± 0.1)	36.1 (± 0.2)
	CP	2.2 (± 0.1)	62.0 (± 0.3)	28.2 (± 0.12)
	Khanna	2.7 (± 0.2)	116.3 (± 0.1)	43.1 (± 0.1)
	Czapeck	3.6 (± 0.12)	93.0 (± 0.1)	25.8 (± 0.1)
	Vogel	3.2 (± 0.3)	348.0 (± 0.1)	108.7 (± 0.13)
	M-5	1.3 (± 0.1)	46.5 (± 0.24)	35.7 (± 0.18)
Aspergillus niveus	SR	1.5 (± 0.2)	24.6 (± 0.17)	16.4 (± 0.1)
	CP	1.4 (± 0.1)	20.8 (± 0.3)	14.8 (± 0.3)
	Khanna	1.4 (± 0.1)	28.7 (± 0.1)	20.5 (± 0.1)
	Czapeck	2.5 (± 0.15)	100.0 (± 0.2)	40.0 (± 0.1)
	Vogel	1.4 (± 0.1)	26.8 (± 0.1)	19.2 (± 0.4)
	M-5	1.3 (± 0.32)	11.8 (± 0.1)	9.1 (± 0.24)

A fumigatus and *A. niveus* were grown under static conditions, at 40°C for 96 h, according to Sect. "Materials and methods"

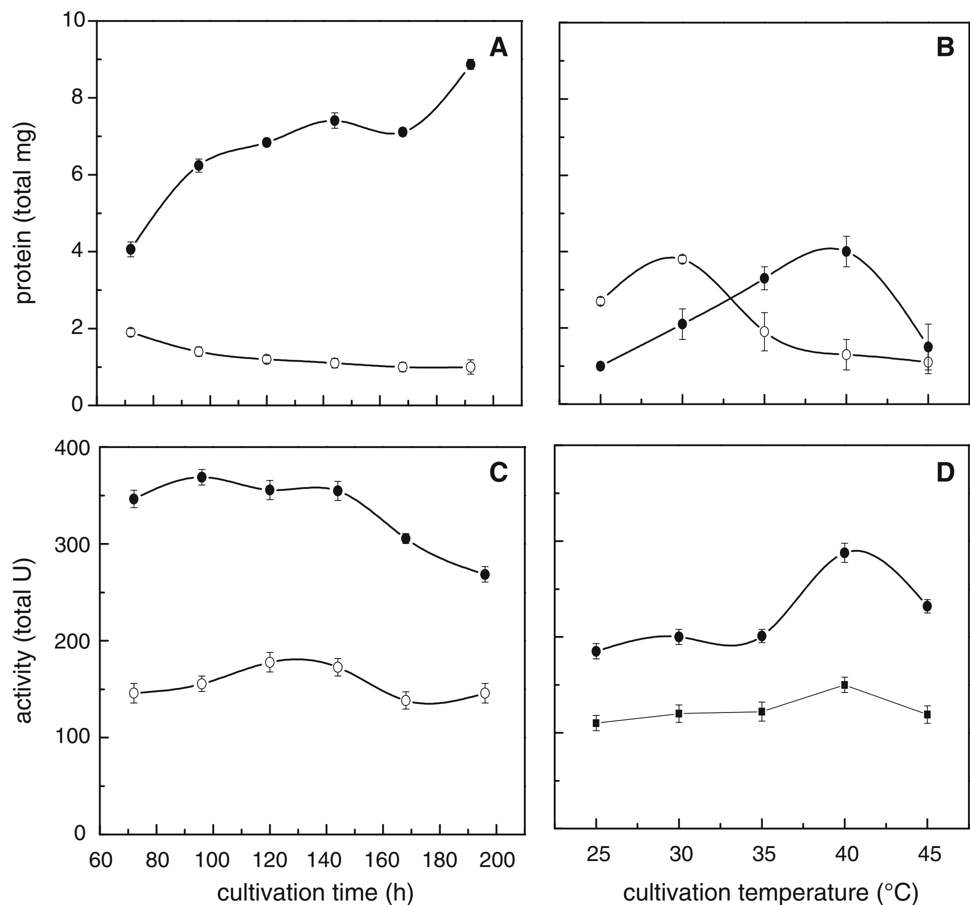

Fig. 1 Time-course and culture temperature from *Aspergillus fumigatus* and *Aspergillus niveus* for xylanase production. Total protein (**a**, **b**) and total activity (**c**, **d**) during different cultivation periods at 40°C (**a**, **c**) and on different temperatures during 96 h for *A. fumigatus* or 120 h for *A. niveus* (**b**, **d**). Microorganisms were cultivated in Czapeck (*A. niveus)* or Vogel (*A. fumigatus*) media supplemented with 1% birchwood xylan. Symbols: *Filled circle with dash*, *Aspergillus fumigatus*; *Empty circle with dash*, *Aspergillus niveus*

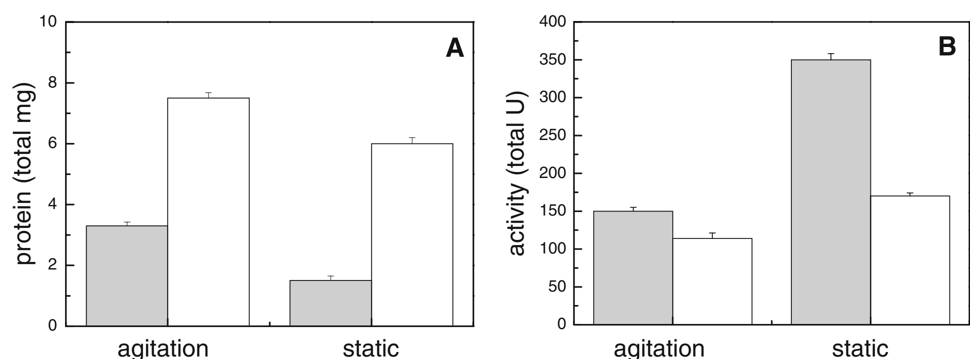

Fig. 2 Influence of aeration in the cultivation of *Aspergillus fumigatus* and *Aspergillus niveus* for xylanase production. Total protein (**a**) and total activity (**b**) under agitation (100 rpm) or on static conditions. Microorganisms were cultivated in Czapeck (*A. niveus)* or Vogel (*A. fumigatus*) media supplemented with 1% birchwood xylan. *White column Aspergillus fumigatus*, *gray column Aspergillus niveus*

enzyme. The possibility of using agricultural residues to produce enzymes may lower the production costs resulting in a cheaper product [25]. But it is known that agro-industrial residues are complex and when they are used as carbon source may induce the fungi to produce other enzymes in the crude extract. Because of that, it was decided to use xylan as the only carbon source to continue this study. In order to assure that cellulase activities were absent, we assayed the activity of the crude extract with three different substrates (carboxymethyl-cellulose, avicel or filter paper) as described in Sect. "Materials and methods", and no activity was detected. Other enzyme activities, such as amylase and pectinase were found in diminished levels, suggesting constitutive activities for both fungi.

Characterization of xylanase activity in the crude extract

It is important for practical applications that industrial enzymes display some adequate properties, such as resistance to temperature and pH. The temperature optimum for *A. fumigatus* and *A. niveus* was 70 and 60–65°C, respectively (Fig. 3a). The temperature optimum observed for *A. fumigatus* was the same as that of the thermophilic fungus *Thermomyces lanuginosus* [26], but it was different of those

Table 2 Effect of the carbon source on the production of xylanase by *A. fumigatus* and *A. niveus*

Carbon Source	Activity (total U)	
	A. fumigatus	*A. niveus*
Without addition	28.9 (± 0.2)	6.9 (± 0.12)
Glucose	78.9 (± 0. 14)	43.9 (± 0. 11)
Xylose	161.4 (± 0.13)	99.5 (± 0.13)
Xylan (birchwood)	347.0 (± 0.11)	100.3 (± 0.12)
Xylan (oat spelt)	223.2 (± 0.1)	93.5 (± 0.2)
Xylan (*Eucalyptus grandis*)	136.8 (± 0.2)	97.8 (± 0.1)
β-Methylxyloside	155.3 (± 0.21)	9.2 (± 0.1)
Arabinose	60.3 (± 0.3)	64.8 (± 0.1)
Avicel microcrystalline cellulose	44.7 (± 0.14)	0.0 (± 0.3)
Cellobiose	17.2 (± 0.17)	6.9 (± 0.2)
Xylitol	91.2 (± 0.1)	25.4 (± 0.12)
Rye flakes	343.1 (± 0.16)	60.2 (± 0.11)
Wheat bran	368.8 (± 0.19)	114.3 (± 0.11)
Oat flakes	103.8 (± 0.3)	37.1 (± 0.2)
Barley flakes	121.7 (± 0.14)	69.4 (± 0.3)
Soy flakes	170.5 (± 0.2)	37.1 (± 0.11)
Corn flakes	142.8 (± 0.3)	11.6 (± 0.12)
Crushed corncob	366.8 (± 0.2)	55.6 (± 0.13)
Powdered corncob	371.1 (± 0.17)	111.8 (± 0.12)
Rice straw	349.1 (± 0.19)	32.4 (± 0.14)
Sugar cane bagasse	164.1 (± 0.15)	20.8 (± 0.1)

A fumigatus and *A. niveus* were grown on Vogel (96 h) and Czapeck (120 h) liquid media, respectively, at 40°C under static conditions, in according to Sect. "Materials and methods"

reported for most *Aspergilli* [2, 6, 8], including other strains of *A. fumigatus* [12]. *A. niveus* exhibited an optimum temperature similar to those of *A. fischeri*, *A. kawachii*, *A. oryzae*, *A. sojae* and *A. sydowii* [2, 5].

For *A. fumigatus* the pH optimum was 5.0–5.5 and for *A. niveus*, 4.5–5.0 (Fig. 3b). In comparison with other fungi, these values of pH optimum are close to those of *A. niger*, *A. fischeri*, *A. sojae* and *A. nidulans* [2, 5], and to one of the xylanases (xyl II) produced by *A. caespitosus* [8]. Others fungal xylanases are alkalophilic, as those from *Thermomyces lanuginosus* [26], *A. nidulans* KK-99, *A. terreus* [2], and one of the xylanases (xyl I) from *A. caespitosus* [8].

Even though *A. fumigatus* produced approximately 3–4-fold more xylanase than *A. niveus*, the thermostability of both enzymes was similar at 60°C for 30 min. After this period, the residual activity of *A. fumigatus* xylanase diminished considerably and, after 120 min the xylanase from *A. fumigatus* retained only 10% of activity, although the xylanase from *A. niveus* still maintained 30% of the initial activity (Fig. 3c). Although the thermostability of the xylanase from *A. niveus* was higher, the temperature optimum of the xylanase from *A. fumigatus* was 70°C, while the xylanase from *A. niveus* corresponded to 60–65°C (Fig. 3a), suggesting that the substrate xylan may protect the enzyme.

The stability of the xylanases at different pH was also tested (Fig. 3d) and the results were different for both fungi. *A. fumigatus* presented a xylanase more stable in pH from 6.0 to 8.0, while the xylanase from *A. niveus* was more stable from 4.5 to 6.0. *A. oryzae* [2], *A. fischeri* [2, 4] and *Termomyces lanuginosus* [26] also presented a considerable stability on ranges of pH from 5.0 up to 8.0.

Assays of cellulose biobleaching using xylanase

The xylanases produced by *A. fumigatus* and *A. niveus* were tested on cellulose pulp biobleaching. The amount of enzyme was 10 U/g of dry pulp, for 1 h, at the temperature optimum of each enzyme. To compare the efficiency of the crude extracts from both fungi in biobleaching, the kappa number was determined. Kappa number is an indication of the lignin content or bleach ability of wood pulp. It estimates the amount of chemicals required to obtain a pulp with a given degree of brightness. Thus, it is expected to lower the kappa number after the enzyme treatment. The best result was obtained with the crude extract from *A. niveus* (Table 3), that decreased 4.6 points the kappa number in comparison with the control. When the cellulose pulp was treated with xylanases from *A. fumigatus*, a decrease of 0.9 points was observed, corresponding to a kappa efficiency of 39.6% for *A. niveus* and 11.7% for *A. fumigatus*. Using xylanase from *A. niveus*, the brightness improved 3.4 points, against 2 points using xylanase from *A. fumigatus*. The viscosity decreased 9.2% when xylanase from *A. fumigatus* was used, but the xylanase from *A. niveus* promoted no decrease of viscosity, confirming that this crude filtrate was free of cellulase. The presence of cellulase in the xylanolytic extract may affect the cellulose pulp properties, and is a constant preoccupation of the users [4, 7, 26]. Comparing these results to those obtained with other microorganisms such as *Termomyces lanuginosus* [26] and *A. caespitosus* (xyl I and II) [8], we observed that the kappa number was reduced only 1.0, 0.2 (xyl I) or 1.5 (xyl II) and 1.1 points, respectively, comparing to their control after at least 1 h of treatment using a quantity of xylanase equivalent to 10 U/g of dry pulp. A reduction of 3.3 points in kappa number (similar to our *A. niveus* strain) was observed when xylanase from *Arthrobacter* sp. MTCC 5214 was used; but to reach this performance in the treatment, it was necessary to double the amount of enzyme and the time of treatment (20 U/g of dry pulp/2 h) and, unfortunately, using *Arthrobacter* sp. MTCC 5214 xylanase a decrease of 7.5% in the cellulose viscosity was observed [27].

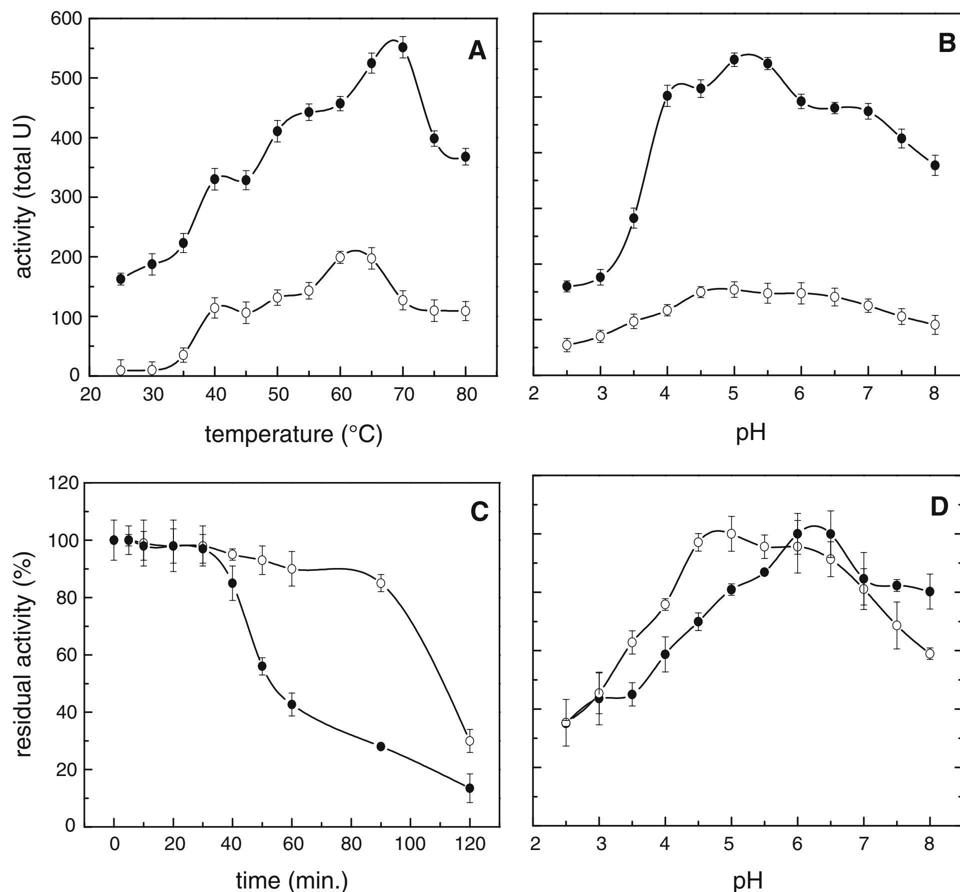

Fig. 3 Effect of temperature (**a**) and pH (**b**) in the enzyme reaction; thermostability (**c**) and pH stability (**d**) of the extracellular xylanases produced by *A. fumigatus* and *A. niveus*. The microorganisms were grown in Czapeck (*A. niveus*) or Vogel (*A. fumigatus*) supplemented with 1% birchwood xylan and the reactions were carried out as: **a** Xylanases activity were determined using McIlvine buffer, pH 5.0, at temperatures range from 25 to 80°C; **b** xylanases pH were determined at 65°C for *A. niveus* or 70°C for *A. fumigatus* using McIlvine buffer on different pHs (2.5–8.0); **c** thermal stabilities were carried out incubating both enzymes at 70°C and after the residual activities were determined at 65°C, pH 4.5–5.0 for *A. niveus* or at 70°C pH 5.0–5.5 for *A. fumigatus*; **d** pH stability were determined incubating both enzymes at different pH (2.5–8.0) at 25°C, during 1 h and after the residual activities were determined at 65°C, pH 4.5–5.0 for *A. niveus* or at 70°C pH 5.0–5.5 for *A. fumigatus*. Symbols: *Filled circle with dash*, *Aspergillus fumigatus*; *Empty circle with dash Aspergillus niveus*

Table 3 Bleaching of cellulose pulp by xylanases produced by *Aspergillus fumigatus* and *Aspergillus niveus*

	A. fumigatus		*A. niveus*	
	Control	Treated	Control	Treated
Kappa number	7.7 (± 0.1)	6.8 (± 0.12)	11.7 (± 0.2)	7.1 (± 0.3)
Kappa efficiency (%)	–	11.7 (± 0.11)	–	39.6 (± 0.3)
Viscosity (cm^3/g)	942 (± 0.16)	855 (± 0.14)	901 (± 0.1)	901 (± 0.2)
Brightness (ISO)	56.7 (± 0.18)	58.7 (± 0. 17)	54.7 (± 0.1)	58.1 (± 0.1)

The microorganisms were grown on their respectively standardized conditions. The controls corresponded to untreated samples. Treatments were carried out with extracellular xylanase (10 U/g of cellulose pulp), during 1 h at 70°C for *A. fumigatus* or 65°C for *A. niveus*

Aspergillus niveus provided a better result than *A. fumigatus*, but the performance of the xylanase obtained from the second strain was also interesting, and should receive more attention. Even when *A. fumigatus* is commonly described as a pathogenic fungus [28], it is still used to produce enzymes such as xylanase [11], applied in industry [12]. Other possibility is to carry on studies about this interesting xylanase intending to insert and express the gene in non-pathogenic yeasts, as it was done by Liu et al. [29].

Conclusions

In this work, *A. niveus* and *A. fumigatus* were described as good xylanase producers, but *A. niveus* xylanase provided the most promising characteristics to be industrially applied in the cellulose pulp biobleaching, and should receive more attention. This fungus should be studied more in order to better understand *A. niveus* xylanase synthesis system, which may enlarge the number of enzymes available in the market to be used in the paper industries.

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