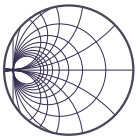
Guimarães et al., J Biocatal Biotransformation 2013, 2:1
http://dx.doi.org/10.4172/2324-9099.1000105



Journal of Biocatalysis & Biotransformation

Research Article

A SCITECHNOL JOURNAL

Xylanase Production from *Aspergillus japonicus* var *aculeatus*: Production using Agroindustrial Residues and Biobleaching Effect on Pulp

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Abstract

This study describes a xylanase produced by *A. japonicus* var *aculeatus,* and evaluated the suitability of this protein for cellulose pulp biobleaching process. The best production of xylanase was achieved with wheat bran (AE=11 U/mg of protein). We observed that the xylanase was induced similarly, when the *A. japonicus* was grown on xylan 0.5% (10.3 U/ml), and on a mixture of wheat bran and xylan (10 U/ml). The optimum xylanase pH and temperature corresponded to 5.0 and 55°C, respectively. The xylanase remained stable at 45 and 50°C, retaining 64% of the initial activity during 1 hour of incubation at 45°C, and had its half-life corresponded to 1 hour at 50°C. The xylanase activity increased 22% with glycerol, when compared to the control (without additives), incubated during 1 hour at 50°C. The best result of biobleaching was obtained with 10 U/g dry pulp for 3 hours of treatment, that decreased 3.9 points of the kappa number, in comparison to the control (kappa efficiency-25.2%). Using the xylanase, the brightness improved 2.8, 2.2 and 3.1 points. The results showed that the xylanase produced by *A. japonicus* has promising characteristics, to be industrially applied on the biobleaching of cellulose pulp.

Keywords

Xylanase; *Aspergillus japonicus* var *aculeatus*; Agroindustrial residues; Biobleaching

Introduction

Due to the ever-increasing demand for paper, the paper pulp industry is rapidly growing and becoming one of the worst offenders in environmental terms [1]. The use of eco-friendly technology is actually preferred in any industrial process; therefore, due to severe concern over the environmental hazards of toxic chemicals used in pulp and paper industries, alternative methods for bleaching of pulp have been suggested [2].

At present, the use of xylanases for biobleaching of pulp is recognized as an economically feasible biotechnology-based process in the pulp and paper industries. Pre-bleaching with xylanases does exactly this function of reducing the need for toxic bleaching chemicals, and is thus environmentally and economically advantageous [3].

An alternative approach in eliminating chlorine in bleaching, reducing chlorinated organic compounds bleach plant effluents, reducing the kappa number (residual lignin content in the pulp) and increasing the brightness of the pulp, is the use of xylanases in prebleaching of cellulose pulp [4]. It is based on the unique specificity of hemicellulases, particularly xylanolytic enzymes, in attacking the hemicellulose component in pulp [3].

Xylanases and other hemicellulases are used to modify the structure of xylan and glucomannan in pulp fibers, in order to enhance chemical delignification efficiency. Endo-β-xylanases cause hydrolysis of the main chain of xylan in pulp, reducing the degree of polymerization of substrate, and are used primarily for the removal of the lignin-carbohydrate complex (LCC), generated in the kraft process, acting as a physical barrier against the entry of bleaching chemicals. LCC removal, thus, facilitates the efficient extraction of high molecular mass lignin from pulp [5].

For biobleaching applications, the candidate xylanase should be thermostable, alkali tolerant and stable on kraft pulping, and its various properties, such as low molecular weight and specific action pattern, must suit the pulping process requirements. Moreover, to avoid damage to cellulose pulp, enzyme preparations should be free from cellulase activity [6].

The aim of the present study was to describe the extracellular xylanase produced by *Aspergillus japonicus* var *aculeatus,* and test its suitability for cellulose pulp biobleaching.

Materials and Methods

Microorganism

Aspergillus japonicus var *aculeatus* strain was isolated by us from soil samples, identified in the Federal University of Pernambuco–UFPE (PE, Brazil), and deposited in our laboratory fungi collection. Stock cultures were propagated at 30°C on slants of solid potato dextrose agar (PDA) medium, stored at 4°C.

Xylanase production and enzyme extraction

Spores were inoculated into 125 ml Erlenmeyer flasks, containing 25 ml medium [7], using 1% (w/v) of the desired carbon sources (1% of xylan or agroindustrial residues, such as rice straw, sugarcane bagasse, wheat bran or corncob). The cultures were incubated under orbital agitation (100 rpm), or stationary conditions, at 30°C, during different periods (24-168 hours). The medium was subsequently vacuum-filtered using filter paper (Whatman nº1), and the crude filtrate was used for the study of the extracellular enzyme.

Enzymatic assays and protein determination

The reaction mixture consisted in 500 µl of citrate-phosphate buffer [8], pH 5.0, containing 1.0% (w/v) of xylan, and 500 µl of enzymatic extract, appropriately diluted. The samples were incubated at 55°C to determine xylanase activity. The amount of reducing sugar released was determined using the 3,5-dinitrosalicylic acid (DNS)

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Received: January 18, 2013 **Accepted:** February 15, 2013 **Published:** February 22, 2013

method, employing xylose (Sigma) as the standard. One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol of reducing sugar per minute, under assay condition. Specific activities were expressed as U/mg of protein. Protein concentrations were determined by the Lowry method [9], using bovine serum albumin (BSA), as the standard.

Effects of pH and temperature

The effect of pH and temperature on xylanase activity was analyzed using crude filtrate from *A. japonicus*. The suitable pH value for xylanase activity was assayed using McIlvaine buffer, in the pH range 3.5-8.0, at 55°C. The assays of temperature were performed in the same buffer pH 5.0, incubated at different temperatures (35-60°C). To determine thermal stability, the enzyme was incubated between 45 and 60°C for different durations (5 to 60 min), and the assays were performed using McIvaine buffer pH 5.0 at 55°C.

Biobleaching

The amounts of enzyme used for this treatment was 10, 20 or 40 units of enzyme per gram of dried cellulose pulp from *Eucalyptus grandis*. The treatments were performed during different periods (1-24 hours). All calculations and procedures were determined, according to the standard methods of Technical Association of the Pulp and Paper Industry [10]. The consistency was determined on a percent dry weight basis. The volume of enzyme or distilled water was added, until it reached a 10% pulp consistency. Crude xylanase extract from *A. japonicus* was added to the treated pulp, and the control was prepared by adding distilled water, instead of enzyme. The samples were incubated inside sealed polyethylene bags at 50°C for 24 hours, 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 brightness parameters. The liberation of aromatic compounds was monitored by absorbance values at 237 nm.

Reproducibility of results

All results are expressed as the means of at least three independent experiments.

Results and Discussion

Time course for xylanase production

Nutritional and environmental factors, such as the type of carbon source, time course, use of agitation (or not), temperature and pH, may affect enzyme synthesis and production by fungi [11]. To evaluate the growth time and the effect of stationary and agitated conditions in the production of extracellular xylanase by the fungus *A. japonicus*, it was grown in SR medium [7], with 1% wheat bran for 168 hours. It was observed that the production of xylanase was better in medium under stationary condition than in the medium under agitation (Figure 1). Under stationary conditions, there was a progressive increase of xylanase production in the period, 24-96 hours of cultivation (the period in which there was maximum production), followed by a reduction (45%), and stabilization in the production of the enzyme. Probably, the reduction in xylanase yield was due to the depletion of available nutrients to microorganism, or due to the proteolysis [12]. Under agitated condition, there was a xylanolytic production between 24 and 96 hours, with maximum peak at 96 hours, with a subsequent decrease, but these values are much lower than those produced under stationary condition.

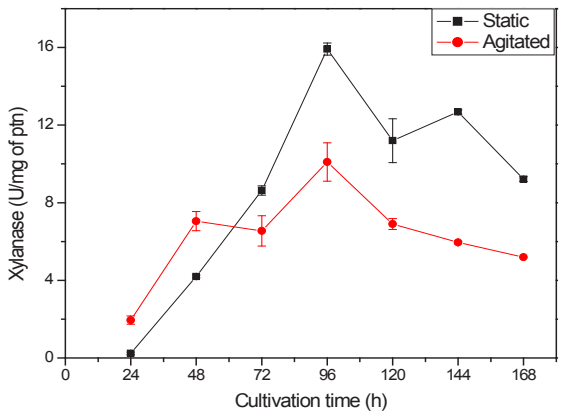

Figure 1: Growth time of extracellular xylanase production by *A. japonicus* (24-168 hours), under stationary and agitated conditions (100 rpm).

Aspergillus foetidus MTCC 4898 grown on solid fermentation using wheat bran as carbon source, also had maximal xylanase production between 24 and 96 hours of cultivation [13]. A similar result was observed at work using the fungus *A. casielus,* with brewery industrial residue as carbon source [14]. *A. fumigatus* RP04 [5] and *A. niger* [15], also had maximal xylanase production with 96 hours of cultivation.

Xylanase production using different agroindustrial residues and substrates

In order to induce xylanase synthesis from microbial sources, agricultural residues such as wheat bran, oat flakes, corn flakes, corncob, rice straw, sugarcane bagasse and others, can be used. The use of agricultural residues as alternative carbon sources reduces the production costs and the price of the final product [16].

The xylanase production by *A. japonicus* was evaluated using different alternative carbon sources. The best production of xylanase was achieved with wheat bran (AE=11 U/mg of protein), followed by soybean bran (AE=7 U/mg of protein), and sugarcane bagasse (AE=5.6 U/mg of protein) (Table 1).

In literature, we found several studies using industrial and agroindustrial wastes as inducing sources for xylanase production [11,12,17]. Among the fungi producers of xylanase from agroindustrial wastes, has *Aspergillus terreus* [11], *Aspergillus terricola* Marchal [17], *Aspergillus foetidus* MTCC 4898 [13] and *Aspergillus ficuum* AF-98 [18], producing xylanase from wheat bran. And in work with *Aspergillus japonicus* C03, the xylanase production was better induced with soybean bran [19].

After finding that the wheat bran was the best substrate inducer of xylanase production by *A. japonicus*, the influence of mixtures of substrates was evaluated (Table 1). We can note that the xylanase was similarly induced, when the *A. japonicus* was grown in xylan, and in the medium containing a mixture of wheat bran and xylan. The enzymatic activity produced in the presence of 0.5% birchwood xylan or oat spelt xylan (10.3 and 9 U/ml, respectively), was almost the same of the mixtures of xylans with wheat bran (10 and 10.6 U/ml, respectively), and of the test only with 0.5% of wheat bran (8.7 U/ml), demonstrating that the use of alternative carbon sources shown viable, as well as favoring the cheaper of the cost of enzyme

production, since the commercial xylan is extremely expensive for widespread use.

There are several studies using different combinations of industrial and agroindustrial wastes as carbon sources for xylanase production in literature [3,19]. Facchini et al. [19] found that a mixture of soybean meal and crushed corncob increased xylanolytic activity by 8.5%. In work using the fungus *A. niger*, *A. niveus* and *A. ochraceus,* and some agroindustrial residues, wheat bran mixed with corncob increased production by about 20% for *A. ochraceus* and *A. niger*, however, not improved the production of xylanase by *A. niveus* [16]. In studies with *A. fumigatus* RP04 and *A. niveus* RP05 was possible to prove that the production of xylanase by *A. fumigatus* was between 5 and 6% higher with corncob meal and wheat meal, as compared with media containing birchwood xylan as carbon source [5].

A higher xylanase production using wheat bran may possibly be due to its low lignin content and higher protein content, compared to other substrates (14.87% protein) [13]. So to optimize the xylanolytic activity, tests were performed with different concentrations of wheat bran, as shown in table 2. The results showed that with increasing of wheat bran concentrations, there was a gradual reduction of xylanolytic activity. At a concentration of 0.5 to 1.5%, there was a high xylanase production as compared to higher values of wheat bran, showing that a small amount of wheat bran is sufficient for a good production of xylanase, consequently reducing production costs. Presumably, 4% inoculum level was so high, that the nutrients were consumed faster, and it overall resulted in a lower enzyme yield [12]. There are few studies of this test with fungi, but in work with *Bacillus mojavensis*, maximum xylanase activity was observed in the presence of 2% oat bran as substrate inducer of the enzyme, and this also had

Table 1: Effect of carbon source on xylanase production by *A. japonicas*.

Carbon Source	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)
Glucose	1	1	1 (± 0.25)
Rice straw	1	1	1 (± 0.21)
Corncob	3	1	3 (± 0.25)
Sugarcane bagasse	9	2	5 (± 0.21)
Soybean bran	7	1	7 (± 0.26)
Avicel 1%	0.3	0.5	0.6 (± 0.3)
Avicel 0.5%	0.5	0.5	1 (± 0.26)
Wheat bran 1%	11	1.2	9 (± 0.25)
Wheat bran 0.5%	8.7	0.8	11 (± 0.2)
Xylan (oat spelt) 1%	10.3	0.7	15 (± 0.2)
Xylan (oat spelt) 0.5%	9	0.5	18 (± 0.3)
Xylan (birchwood) 1%	10.8	0.7	15 (± 0.2)
Xylan (birchwood) 0.5%	10.3	0.6	17 (± 0.3)
Xylan (oat spelt) 0.5%+Wheat bran 0.5%	10.6	0.9	12 (± 0.3)
Xylan (birchwood) 0.5%+Wheat bran 0.5%	10	1	10 (± 0.2)
Avicel 0.5%+Wheat bran 0.5%	6.2	0.9	7 (± 0.27)

The fungus *A. japonicus* was grown in Erlenmeyer of 125 ml containing SR liquid medium [7], and different carbon sources, which were incubated for 96 h at 30°C in stationary condition. The temperature used for enzyme assay was 55°C.

Table 2: Effect of different concentrations of wheat bran on xylanase production by *A. japonicas*.

[Wheat bran]	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg of protein)
0.5%	8.7	0.8	10.9 (± 0.2)
1.0%	11	1.2	9.2 (± 0.25)
1.5%	6.8	1.0	6.8 (± 0.29)
2.5%	5.6	1.1	5.1 (± 0.31)
4.0%	3.5	0.9	3.9 (± 0.30)
8.0%	3.6	1.2	3.0 (± 0.25)

The fungus *A. japonicus* was grown in Erlenmeyer of 125 ml containing SR liquid medium [7], and different concentrations of wheat bran (0-8.0%), which were incubated for 96 h at 30°C in stationary condition. The temperature used for enzyme assay was 55°C.

a decreased production in higher concentrations [12]. In studies with *Pseudomonas* sp. WLUN024, the yield of xylanase increased greatly with increasing concentrations of wheat bran [20], as well as work with *Streptomyces cyaneus* SN32 [21].

Effect of pH on xylanase activity

The extracellular xylanolytic activity produced by *A. japonicus* showed stable enzymatic activity in the pH range between 4.0 and 6.5, reaching maximum activity at pH 5.0 (Figure 2). These results were similar to other studies, where most of the fungal species, *Aspergillus* sp. showed maximum xylanolytic activity at pH ranging from 4.0 to 6.0, as *A. japonicus* (5.0) [22], *A. ochraceus* (5.0) [17]; *A. fumigatus* RP04 (5.0-5.5), and *A. niveus* RP05 (4.5-5.0) [5]; *A. ochraceus* (5.0), *A. niger* (5.5-6.0) and *A. niveus* (5.0-5.5) [16].

Effect of temperature on xylanase activity and stability

The optimum temperature for *A. japonicus* was evaluated in the range 35-60°C, where the xylanolytic activity had a gradual increase with higher temperatures, and had maximum activity at 55°C, and after a drop of 20% at 60°C (Figure 3). This result agrees with the fact that the optimum temperature for xylanase produced by most fungi is in the range of 40-60°C [23]. In the literature, it cites other microorganisms with similar optimum temperatures such as 55°C for xylanolytic activity of *A. terreus* and 50°C to *A. aculeatus* and *A. sydowii* [24], and *A. casielus* [14]. Studies with *A. niger*, *A. niveus* and *A. ochraceus* shows optimum temperature range 55-65°C [16], and work of Khonzue et al. [3] relates to an optimum temperature range 50-60°C, showing that the temperature found in A. *japonicus* resembles other *Aspergillus* species.

The xylanase produced by *A. japonicus* remained stable at 45 and 50°C, retaining 64% activity with 1 hour of incubation at 45°C and had a half-life of 1 hour at 50°C (Figure 4). In studies with *A. terreus*, the xylanase was also thermotolerant at 45 and 50°C, but had half-life of only 36 minutes at 50°C [11]. And, *A. phoenicis* had a half-life of only 25 minutes at 50°C [15]. At 55°C, the xylanase of *A. japonicus* had a half-life of 15 minutes, while the xylanase of *A. carneus* M34 had only 7.5 minutes [25].

The addition of 5% glycerol or polyethyleneglycol somehow protected enzyme from thermal inactivation at 50°C (Figure 5). The xylanase activity increased 22% with glycerol, when compared to the control (without additives), with 1 hour of incubation. It was observed a half-life of 32 minutes to the control, and more than 60 minutes with glycerol (57% activity with 60 minutes). In studies of

Sandrim et al. [26], both xylanases were inactivated with a half-life (T_{half}) value of 2.3 min at 65°C, but the addition of 30% glycerol or polyethyleneglycol somewhat protected the enzyme from thermal inactivation.

Assays of cellulose biobleaching using xylanase

In recent years, a wide variety of studies about xylanases have been reported to have potential application in the bleaching process. Among the filamentous fungi used to produce xylanase, *Aspergillus* species are one of the most explored. For example, production of xylanase and cellulose pulp biobleaching processes have been reported for *Aspergillus niger* [3]; *A. terricola* Marchal and *A. ochraceus* [17]; *A. niger, A. niveus* and *A. ochraceus* [16]; *A. niveus* RP05 and *A. fumigatus* RP04 [5]; *A. nidulans* and *A. awamori* [27]; *A. fumigatus* [1], and *A. caespitosus* [26].

For estimation of the pulp bleaching potential of the crude xylanase from *A. japonicus* var *aculeatus*, two approaches were used: measurement of absorbance at 237 nm and estimation of kappa number. Cellulose pulp was pretreated with 10, 20 or 40 units of crude xylanase from *A. japonicus* per gram of dry cellulose pulp for 2 hours or with 10 units for different periods as 1, 2, 3 or 24 hours at 50°C, pH 5.2. Kappa number is an indication of the lignin content or bleach ability of wood pulp. Thus, it is expected to reduce the

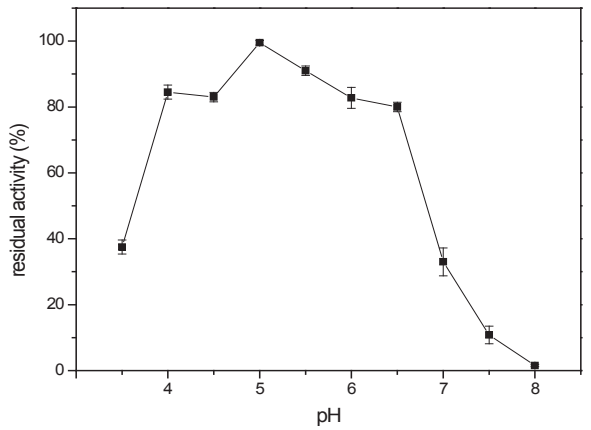

Figure 2: Xylanase pH was determined at 55°C using McIlvaine buffer on pH ranging from 3.5-8.0.

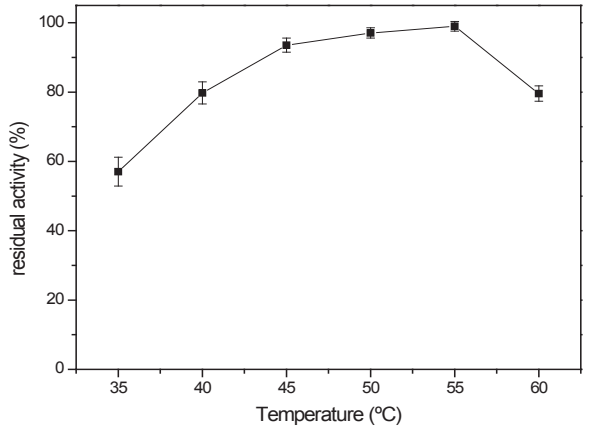

Figure 3: To test the effect of temperature, the xylanase activity was determined using McIlvaine buffer, pH 5.0, at temperatures ranging from 35-60°C.

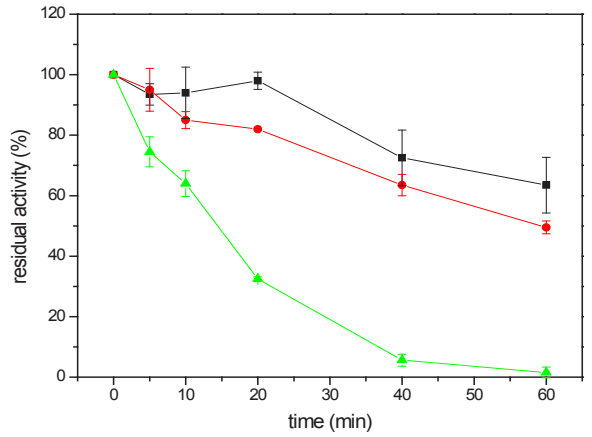

Figure 4: Thermostability of the xylanase of *A. japonicus* was determined using McIlvaine buffer pH 5.0 at 55°C after incubating the enzyme on temperatures of 45 (■), 50 (●) and 55°C (▲).

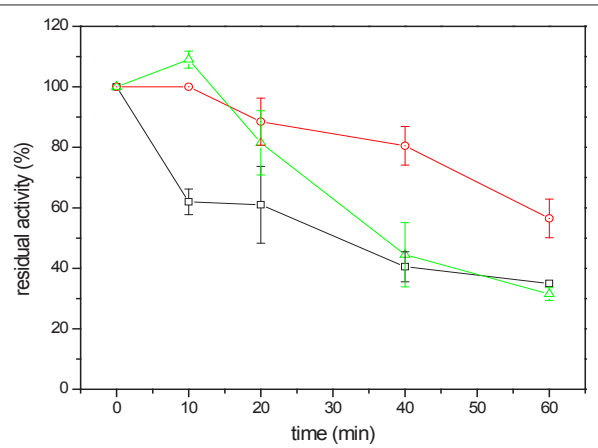

Figure 5: Thermostability of the xylanase at 50°C, without additives (□), and with 5% glycerol (○) and polyethyleneglycol (Δ).

kappa number after the enzyme treatment, and it is also expected the releasing of chromophores from lignin molecule (absorbing at 237nm). The filtrate extracted from the treated pulps were compared with the control pulp (not treated with xylanase), and it was observed that over the treatment time and with the increasing on enzyme concentration, there was a release of lignin (Table 3). The best result was obtained with 10 U/g dry pulp with 3 hours of treatment, which decreased 3.9 points in the kappa number comparing to the control, where the kappa efficiency corresponded to 25.2%. On the treatment of 2 hours, the xylanase (10 and 40 U/g dry pulp) reduced 2.1 points in the kappa number, which corresponded to a kappa efficiency of 18.8%. Using the xylanase, the brightness improved 2.2, 2.8 and 3.1 points in the treatment with 10, 20 and 40 U/g dry pulp, respectively, during 2 hours of treatment. The xylanase from *A. japonicus* was free of cellulase, not changing the viscosity of the pulp, meaning that the physical properties of cellulose were maintained. The delignification efficiency of *A. japonicus* in the treatment with 10 U/g dry pulp/ 1 hour (7.8%) was better than the described results with xylanases from *A. niveus* and *A. ochraceus* (6.5 and 7.5%, respectively) [16]. The xylanase of *A. caespitosus* (10 U/g dry pulp/2 hours) reduced kappa number only in 12.6% (xyl II) and 1.7% (xyl I) [26], while the

Table 3: Properties of pulp treated with xylanase produced by *A. japonicus*.

TREATMENT 1						
Xylanase (U/g dry pulp)	Time (h)	Treatment	Kappa number	Kappa efficiency (%)	Brightness (ISO)	A_{237}
10	2	Control	11.2	-	56.8	
		Treated	9.1	18.8	59.0	0.033
20	2	Control	11.2	-	56.8	
		Treated	9.4	16.1	59.6	0.066
40	2	Control	11.2	-	56.8	
		Treated	9.1	18.8	59.9	0.070
TREATMENT 2						
Xylanase (U/g dry pulp)	Time (h)	Treatment	Kappa number	Kappa efficiency (%)	Brightness (ISO)	A_{237}
10	1	Control	12.8	-	57.6	
		Treated	11.8	7.8	58.7	0.017
10	2	Control	11.2	-	56.1	
		Treated	9.1	18.8	58.3	0.027
10	3	Control	15.5	-	56.6	
		Treated	11.6	25.2	57.7	0.037
10	24	Control	12.2	-	57.0	
		Treated	10.6	13.1	57.2	0.040

The microorganism was grown on its standardized conditions. The controls corresponded to untreated samples. Cellulose pulp was pretreated with 10, 20 or 40 units of crude xylanase from *A. japonicus* per gram of dry cellulose pulp for 2 h, and with 10 units for 1, 2, 3 or 24 h at 50°C and pH 5.2.

A. japonicus xylanase kappa efficiency corresponded to 18.8%. Using the xylanase of *A. japonicus* (40 U/g dry pulp/ 2 hours), the brightness improved 3.1 points, while the xylanase of *A. ochraceus* (35 U/g dry pulp/ 2 hours) improved the brightness just 2.0 points [16].

Conclusions

The production of xylanase by *A. japonicus* using alternative carbon sources proved to be almost as efficient as when using the specific substrate, demonstrating that the use of alternative carbon sources shown viable, as well as favoring the cheaper of the cost of enzyme production, since the commercial xylan is extremely expensive for widespread use. The xylanase from *A. japonicus* showed to be relatively stable, demonstrating that it presents promising characteristics to be industrially applied on biobleaching of cellulose pulp process.

Acknowledgements

This work was supported by grants from Conselho de Desenvolvimento Científico e Tecnológico (CNPq). This work was part of Master Dissertation of NCAG (Laboratório de Bioquímica/CCBS, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil).

Declaration of Interest

This work do not presents any conflict about financial interest, and all authors are in completely agreement with the submission. The agency that financered this project, and is properly quoted in acknowledgements.

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