

Full Length Research Paper

Optimization of xylanase biosynthesis by *Aspergillus japonicus* isolated from a “Caatinga” area in the Brazilian state of Bahia

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The objective of this research was to investigate the potential of xylanase production by *Aspergillus japonicus* and to determine the effects of cultivation conditions in the process, aiming toward optimization of enzyme production. The best temperature, as well as the best carbon source, for biomass production was determined through an automated turbidimetric method (Bioscreen-C). The enzyme activity of this fungus was separately evaluated in two solid substrates (wheat and soybean bran) and in Vogel medium, adding other carbon sources. Temperature effects, cultivation time, and spore concentrations were also tested. The best temperature for enzyme and biomass production was 25°C; however, the best carbon source for growth (determined by the Bioscreen C) did not turn out to be a good inducer of xylanase production. Maximum xylanase activity was achieved when the fungus was cultivated in wheat bran (without the addition of any other carbon source) using a spore concentration of 1×10^7 spores/mL (25°C, pH 5.0, 120 h). *A. japonicus* is a good xylanase producer under the conditions presented in these assays.

Key words: *Aspergillus japonicus*, xylanase activity, enzyme optimization.

INTRODUCTION

Xylanase is one of the microbial enzymes that has aroused great interest recently due to its biotechnological potential in many industrial processes, e.g. in xylitol and ethanol production (Beg et al., 2001), in the cellulose and paper industry (Wong et al., 1988), in the production of oligosaccharides (Pellerin et al., 1991), to obtain cellular proteins, liquid fuels, and other chemical substances (Biely, 1985), in the food industry (Haltrich et al., 1996), and in poultry, pork, and caprine feeding (Pucci et al., 2003). Xylanases are extracellular enzymes produced by microorganisms such as bacteria (saprophytic and

phytopathogenous), mycorrhizic fungi, and some yeasts. The enzyme is also found in protozoa, insects, crustaceans, snails, seaweed, and also seeds of plants during the germination phase in the soil (Wong et al., 1988).

Xylanases catalyze xylan hydrolysis, the major hemicellulose component in plant cell walls. The xylan structure, however, can differ greatly depending on its origin (Huisman et al., 2000). Basically, a xylan structure consists of D-xylopyranose units, linked by β -1,4 bonds, which can be exhibited either in linear or branched form (Bastawde et al., 1992). A complete and efficient enzymatic hydrolysis of this complex polymer depends mainly on two types of enzymes: endo-1,4- β -xylanases (1,4- β -D-xylohydrolase; EC 3.2.1.8), which hydrolyze the xylanopyranose of the central chain, and the β -xylosidases (1,4- β -D-xylano xylohydrolase; EC 3.2.1.37), which hydrolyze xylobiose and other xylooligossa-

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charides resulting from the action of endoxylanases. Other enzymes (e.g., acetylxylo esterases, α -glucuronidase, and α -L-arabinofuranosidase) act in synergism with xylanases to degrade specific groups (Beg et al., 2001).

Many environmental factors affect microbial metabolic activity (which can induce or repress enzyme biosynthesis) such as the substrate used (Lenartovicz et al., 2002), pH, temperature, and cultivation time (Haltrich et al., 1996) as well as spore concentration (Kuhad et al., 1998; Fadel, 2000). The objective of this research was to evaluate the best cultivation conditions for optimizing the production of xylanase by *Aspergillus japonicus*.

MATERIALS AND METHODS

Microorganism

A strain of *Aspergillus japonicus*, isolated from a depth of 10-15 cm in the soil of a "Caatinga" area, in the Contendas do Sincorá National Forest, which is located in the micro-area of the homogeneous Chapada Diamantina, in Ituaçu, Bahia, Brazil, was used. Stock cultures were kept in malt extract Agar, performing constant replications and maintained at 4°C.

Effect of carbon source on *A. japonicus* growth

The selection of the best carbon source for *A. japonicus* was performed in a LabSystem Bioscreen C automated growth system. The culture medium containing 1% liquid malt extract was distributed in penicillin flasks and supplemented with a single carbon source (1%, w/v): lactose, sucrose, maltose, glucose, glycerol, sorbitol, fructose, xylose, or galactose. The flasks were sealed, labeled, and autoclaved at 121°C for 20 min. Afterwards 40 μ L of a suspension of 1×10^7 spores/mL of *A. japonicus* in 360 μ L of the culture medium were placed in each culture, in quadruplicates. In the control cells, 400 μ L of each culture medium was used. The plates were placed in the Bioscreen C, using the following settings: 25°C, absorbance at 540 nm, reading intervals every 4 h, cell agitation 10 s before each reading, and a total experiment time of 60 h. The automatic readings were sent to a computer, where the growth curves were determined after the 60-h experiment for each carbon source. The same procedure was performed at 35°C.

Microorganism cultivation to determine xylanolytic activity (SSF- solid state fermentation)

The culture media used were wheat bran (Yoshioka et al., 1981) and soybean bran. In a 250-mL Erlenmeyer

flask, 5 g of wheat bran and 5 mL of distilled water were added, and the pH was adjusted to 5.0 To prepare the soybean bran medium, 10 g were finely ground, 5 mL of distilled water were added, and the pH was adjusted to 5.0 The media were homogenized using a spatula. The flasks were covered and autoclaved at 121°C for 30 min, after which they were inoculated. The same procedure was performed, adding individually to each medium the best carbon source as determined by the Bioscreen C, xylan and carboxymethyl cellulose (1%, w/v).

Microorganism cultivation to determine xylanolytic activity (SmF- submerged fermentation)

Erlenmeyer flasks (250 mL) were prepared with 25 mL Vogel medium (Vogel, 1956). The flasks were covered and autoclaved at 121°C for 30 min, after which they were inoculated. The same procedure was performed, adding individually to each medium and the best carbon source as determined by the Bioscreen C (sorbitol) and xylan and carboxymethyl cellulose (all three at 1%, w/v).

Cultivation conditions

The inocula used were obtained from colonies grown in Agar malt extract for 7 days at 25°C. The spores were withdrawn and placed in a 0.1% Tween 80 solution and counted in a Neubauer chamber. Erlenmeyer flasks containing wheat bran medium were inoculated with 1 mL of the following spore concentrations: 1×10^6 , 1×10^7 , 2×10^7 and 4×10^7 spores/mL, respectively, and incubated at 20, 25, 35, and 40°C, for 24, 48, 72, 96, 120, 144, and 168 h. Erlenmeyer flasks containing soybean bran and Vogel medium were inoculated with 1 mL of 1×10^7 spores/mL and incubated at 25°C for 120 h. The flasks containing Vogel medium were kept under agitation (125 rpm). The pHs of the different culture media were 5.0.

Determination of the xylanolytic activity

After the incubation, 30 mL of sterilized distilled water were added to the flasks. The cultures were homogenized, and the flasks were maintained at 4°C for 3 h, then the cultures were filtered under vacuum. The biomass obtained in Vogel medium was also filtered using the methodology mentioned above, and the filtrates were considered as gross enzyme extract (Linko et al., 1978). The determination of xylanolytic activity was carried out at 50°C, using Xylan Birchwood (Sigma, U.S.: Bailey et al., 1992) 1% (w/v) in sodium acetate buffer 50 mmol (pH 5.0). The release of the reducing sugars was determined by the dinitrosalicylic acid method (ADNS) (Miller, 1959). One unit of enzyme activity (U) was

Table 1a. Influence of carbon source on xylanase activity produced by *Aspergillus japonicus* when cultivated in wheat bran and soybean bran media (pH 5.0) for 5 days at 25°C.

Carbon Source	Xylanase activity					
	Wheat bran			Soybean bran		
	U/mL	U/mL Total	U/mg Prot.	U/mL	U/mL Total	U/mg Prot.
None	143.9	4317.0	39.7	9.7	438.0	25.3
Xylan	104.1	3124.5	46.1	8.5	1285.3	63.6
CMC*	102.5	3075.6	40.9	N/A**	557.5	28.7
Sorbitol	43.7	2211.9	16.9	N/A	N/A	N/A

*CMC, carboxymethyl cellulose. **N/A, no activity.

Table 1b. Influence of carbon source on xylanase activity produced by *Aspergillus japonicus* when cultivated in Vogel media (pH 5.0) for 5 days at 25°C.

Carbon Source	Xylanase activity		
	U/mL	U/mL Total	U/mg Prot.
None	18.2	438.0	25.3
Xylan	20.2	1285.3	63.6
CMC*	19.4	557.5	28.7
Sorbitol	N/A**	N/A	N/A

*CMC, carboxymethyl cellulose. **N/A, no activity.

defined as the amount of enzyme necessary to produce 1 $\mu\text{mol/mL/min}$ of glucose or xylose under the assay conditions.

Quantitative protein determination

The protein concentration was determined by Lowry's method (Lowry, 1951), using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Substrate effect on xylanase activity

Xylanase activity was higher in solid-state fermentation (SSF) using wheat bran as the carbon source (143.9 U/mL) when compared to that using submerged fermentation (SmF) with Vogel medium (18.3 U/mL) or that of soybean bran (9.8 U/mL, SSF) (Tables 1a and 1b).

According to Kamra et al. (2004), xylanase produced by *Humicola lanuginosa* was approximately 23-fold higher in SSF than in SmF. Production of pectinases by *Bacillus* sp. (Soares et al., 1999) in SSF was higher than that produced in SmF. These results are similar to those obtained with endoglucanases and β -glucosidases (Grajek et al., 1987), xylanases (Deschamps et al., 1985), α -amylases (Lonsane et al., 1992), and

pectinases produced by *A. niger* (Acunã-Arguelles et al., 1995).

Many authors report the advantages of using wheat bran as a substrate for xylanase production in SSF when compared to other solid wastes. De Souza et al. (2001) and Ferreira et al. (1999), cultivating *Aspergillus tamaris* in wheat bran, obtained a higher enzymatic activity than in sugar cane bagasse and corn cob. Gawande et al. (2000), using *Aspergillus terreus*, and Qinnghé et al. (2004), using *Pleurotus ostreatus*, tested the xylanase activity of these fungi in different substrates, and a highest production was obtained with wheat bran, possibly because wheat bran is rich in proteins and hemicelluloses, which are used by microorganisms as energy and carbon sources, through specific enzymes such as xylanase and α -amylases (Adolph et al., 1996).

According to de Souza et al. (2001), wheat bran, among other substrates used in solid systems, is the most efficient, because it does not perform catabolic repression in the enzymatic activity. Some authors suggest that the lack of catabolic repression is because the diffusion processes are slow due to the low water activity in the system (Ramesh et al., 1991).

Effect of the addition of other carbon sources on xylanase synthesis

Nine sugars were individually tested as sole carbon

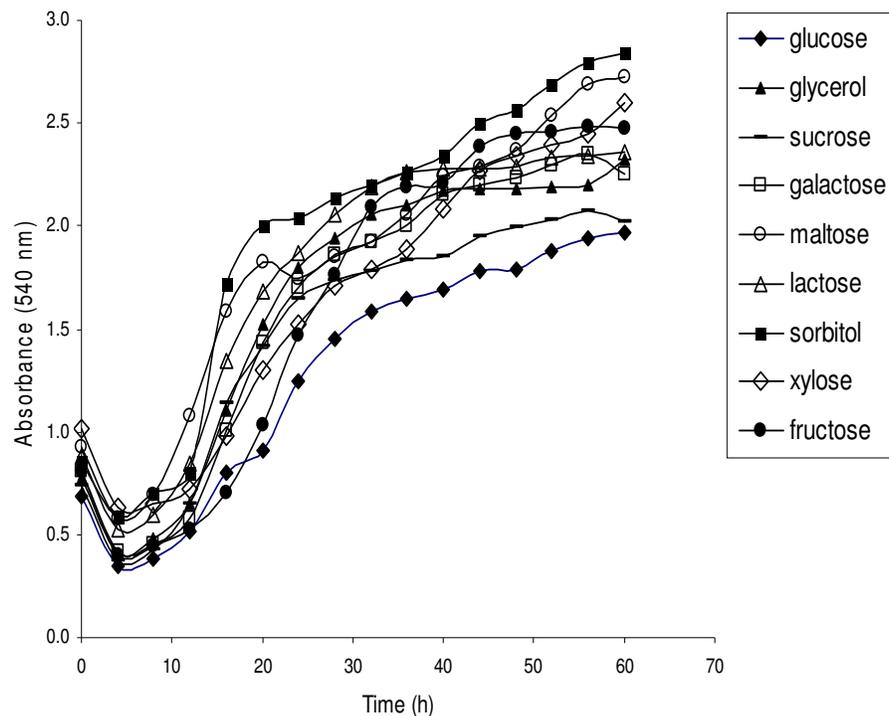


Figure 1. *Aspergillus japonicus* cultivation in different carbon sources in a Bioscreen C automated growth system at 25°C.

sources to evaluate *Aspergillus japonicus* growth. Following to the culture conditions used here, sorbitol was found to be the best (Figure 1). When this sugar was added to wheat bran, soybean bran and Vogel medium, however, an inhibition of the xylanase activity was observed. Addition of xylan and carboxymethyl cellulose to wheat bran did not induce higher levels of enzyme production; the highest activity was exhibited by the pure substrate. The same occurred when these carbon sources were added to soybean wheat and Vogel medium (Tables 1a and 1b). Such results may be due to the catabolic repression processes when easily assimilated carbon sources (e.g., sorbitol) were added (Biswas et al., 1990). In earlier studies, it was found that the carboxymethyl cellulose addition was unfavorable to xylanase production, because carboxymethyl cellulose could be produced during the fermentation, which hydrolyzes the substrate in cellobiose, leading to a repressive action on xylanase production (Ahmed et al., 2003).

Xylanase production by *T. harzanium* (Seyis et al., 2005a,b) was also more advantageous without the addition of other carbon sources. However, mechanisms of synthesis control vary considerably among microorganisms (De Vries et al., 2000). Using *Streptomyces* A-151, Wang et al. (2003) report a low efficiency of xylanase production after the addition of

other carbon sources in cultures using rice bran, which also corroborates the results obtained by Chan et al. (2002), Rani et al. (2001), and Bakir et al. (2001).

Temperature effect

A. japonicus growth was evaluated in an automated cultivation system using different sugars as the sole carbon source (1%), at 25 and 35°C. The best biomass production was achieved at 25°C (results for 25°C are shown in Figure 1). Xylanase production by this fungus was tested at four different temperatures (20, 25, 35, and 40°C), with 25°C being the best temperature for xylanase production (143.9 U/mL). This was two-fold higher than the activity obtained at 20°C and 5-fold higher than that observed at 35°C. Xylanase production was extremely low at 40°C (Table 2). The results obtained are similar to those obtained by other authors who established that the best temperature range for xylanase activity is between 20°C and 30°C (Lenartovicz et al., 2003; Haq et al. 2004; Shah et al., 2005; Yuan et al., 2005). The best growth temperature for the fungus is not always the best for enzyme activity. However, Kheng et al. (2005), using *Aspergillus niger*, verified that optimal temperatures for enzyme activity and fungal growth were similar, which is also in agreement with the results obtained by Biswas et

Table 2. Influence of different cultivation temperatures on xylanase activity produced by *Aspergillus japonicus* in wheat bran (pH 5.0) for 5 days.

Temperature (°C)		Xylanase activity	
U/mL		U/mL Total	U/mg Protein
20	60.8	1825.5	6.1
25	143.9	4317.0	39.7
35	22.9	22.9	4.9
40	12.5	374.4	4.3

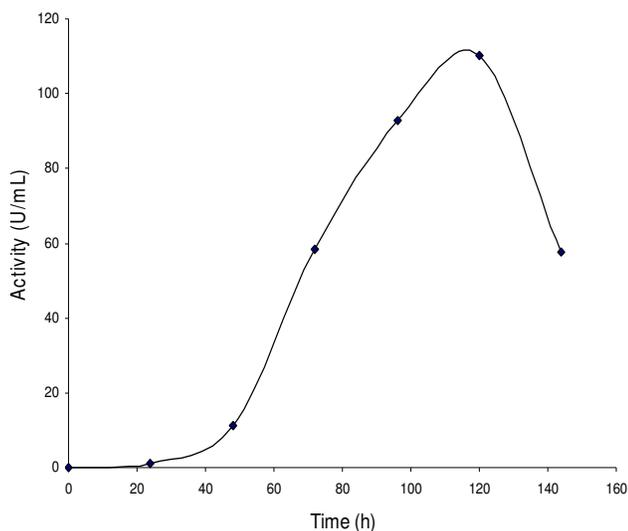


Figure 2. Xylanase activity (U/mL) at different incubation times in *Aspergillus japonicus* cultivated in wheat bran medium (pH 5.0) at 25°C for 144 h.

al. (1990). According to Yaun et al. (1999) and Rahman et al. (2003), the large decreases at very low or high temperatures are because the fungal growth is inhibited at these temperatures, leading to a decrease in enzyme synthesis.

Effect of pH on the culture medium

According to Subramaniyan et al. (2000), a pH of around 5.0 has, in general, been the optimum for xylanase production by fungi. The same was reported by Haq et al. (2004), Qinnqhe et al. (2004) and Shah et al. (2005). Taking into consideration these reports and the soil pH of 5.0 where the strain was isolated, all culturing were carried out at pH 5.0.

Effect of cultivation time

Xylanase activity by *A. japonicus* at 25°C (pH 5.0) increased up to 120 h, while on the sixth day there was a

large decrease (Figure 2). Several researches on xylanase production report similar data. Shah et al. (2005) obtained maximum production, using *Aspergillus foetidus*, on the fourth day (80.5 U/mL) and also observed a decrease from the sixth day on. *Aspergillus terreus* showed maximum activity (22.03 U/mL) on the fourth cultivation day; after this period, a decrease in enzyme production was observed (Ghanem et al., 2000). Other enzymes have also shown better activities at similar times, such as pectinase and cellulose produced by fungi when cultivated in solid-state fermentation (Desgranges et al., 1990).

Table 3. Effect of the different inocula concentrations on xylanase activity produced by *Aspergillus japonicus* cultivated in wheat bran medium (pH 5.0) for 120 h at 25°C.

Inocula conc. Spores/mL	Xylanase Activity		
	U/mL	U/mL Total	U/mg Prot.
1 x 10 ⁶	78.2	2347.5	19.9
1 x 10 ⁷	177.9	5336.4	38.1
2 x 10 ⁷	139.5	4186.5	36.4
4 x 10 ⁷	122.6	3677.7	28.6

Effect of the inoculum concentration

The inoculum concentration of 1 x 10⁷ spores/mL contributed to the maximum xylanase activity (177.9 U/mL) relative to the other concentrations. The lowest activity was observed when using a concentration of 1 x 10⁶ spores/mL (78.3 U/mL) (Table 3). In order to verify the enzyme activity, the spore concentration in fungi cultivation must be high enough to colonize the substrate particles (Sikyta, 1983); many studies, however, have indicated that there can be a decline in this activity over a determined spore concentration.

Kuhad et al. (1998) obtained maximum xylanase activity by *Fusarium oxysporium* using 1 x 10⁷ spores/mL; on the other hand, using 2 x 10⁷ spores/mL, they achieved the same level of activity, and the one containing higher concentrations of spores led to a decrease in activity. Shah and Madamwar (2005) observed that, during the cultivation of *Aspergillus foetidus*, maximum xylanase activity (210.0 U/mL) occurred when the inoculum used had a concentration of 1.5 x 10⁸ spores/mL, two times higher than that obtained using 1.5 x 10⁴ spores/mL. However, the increase in the inoculum concentration was not beneficial for xylanase activity, verifying that over 10⁸, a drastic decrease occurred in activity. As a general mean, the optimal spore concentration is between 10⁶ and 10⁷ spores/mL: outside this range, a decrease in xylanase activity occurred (Smith and Wood, 1991; Qinnqhe et al., 2004).

Conclusion

The best xylanase activity (177.9 U/mL) by *A. japonicus* was obtained when cultivated in wheat bran (pH 5.0, 25°C, 120 h) using an inoculum of 1×10^7 spores/mL. The results obtained indicate a significant increase in xylanase production with the use of nutrients and suitable growth conditions. As demonstrated in other studies, pure wheat bran was the best substrate for enzyme production. In the present study, soybean bran was also tested as a substrate, since it is an agricultural waste that could have good biotechnological potential due to the large area of its cultivation in Brazil. However, the results obtained were not promising. Nevertheless, as this waste was not tested extensively in previous studies, further studies are necessary, mainly with respect to particle size and the quantity of water that should be used in the preparation of the media. The new Bioscreen-C automated turbidimetric method, used to evaluate the best carbon source and temperature for biomass production of *A. japonicus*, was advantageous (due to its speed and the low cost) with sorbitol and a temperature of 25°C determined to be the best inducer for *A. japonicus* growth. This notwithstanding, the association of this sugar with wheat bran in order to evaluate its effect on xylanase production demonstrated that it is not a good inducer, because the enzymatic activity was better in pure wheat bran.

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