



Biomass of unconventional plants from Brazilian semiarid as substrate for hydrolytic enzymes production by *Aspergillus niger* under solid and submerged fermentation

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ABSTRACT. *Aspergillus niger* KIJH was grown in solid and submerged fermentation using leaves and roots (with and without bark) of plants typically from Brazilian semiarid as substrate to produce a multienzymatic extract, which was characterised for its potential biotechnological applications. Solid-state fermentation (SSF) was applied to select the most promising plants biomass as induction substrates for the production of hydrolytic enzymes by fungus. The best biomasses were used as substrate in submerged fermentation (SmF) assays at two scales. Samples of up scale fermented culture were partially purified by ultrafiltration and activity and pH and temperature stability of CMCase and xylanase were evaluated. *A. niger* KIJH produced hydrolytic enzymes under SSF containing unconventional plants biomass from Brazilian semiarid. In SmF conditions, maximum CMCase (0.264 U mL⁻¹) and xylanase (1.163 U mL⁻¹) activities were induced by *Jacaratia corumbensis*. Scaling up the SmF to 500 mL of medium was able to maintain constant the production of CMCase (0.346 U mL⁻¹) and xylanase (1.273 U mL⁻¹) on the fermented culture. Ultrafiltered and concentrated extract presented CMCase activities practically constant in all temperature ranges (30–80°C) and pH (3.0–9.0), while xylanase optimum activity temperature was 50°C and pH in the range of 3.0 to 5.0. CMCase activity remained stable for 24 hours at 50°C and xylanase was reduced in 53% after two hours incubation at the same temperature. CMCase and xylanase obtained by *A. niger* KIJH cultivated in submerged culture containing *J. corumbensis* as carbon source may have application in biotechnology processes that require enzymes that remain active under routine extreme conditions.

Keywords: Bioprocess; endoglucanases; *Jacaratia corumbensis*; xylanase.

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Introduction

Hydrolytic enzymes are valuable commercial enzymes been widely applied in the textile (amylase, cellulase, pectinase), leather (lipase, protease), pulp and paper (lipase, xylanase) and food industries (cellulase, lactase, lipase, pectinase, protease) (Kirk, Borchert, & Fuglsang, 2002).

Although hydrolytic enzymes can be derived from several sources, including plants, animals, and microorganisms, microbial enzymes generally meet industrial demands (Corrêa, Moutinho, Martins, & Martins, 2011). Fungal strains belonging to the genus *Aspergillus* are among the most significant producers of hydrolytic enzymes (Sohail et al., 2009). For example, for *A. niger* it was reported the production of diverse enzymes such as pectinase (Patil & Dayanand, 2006); cellulase (Bhat, 2000); amylase (Gupta, Gupta, Modi, & Yadava, 2008) and lactase (Kazemi, Khayati, & Faezi-Ghasemi, 2016).

The enzymes produced by different species of *Aspergillus* vary not only in type but also in optimum pH and temperature (Coral, Arikan, Ünalı, & Güvenmez, 2002; Subramaniyan & Prema, 2002; Romanowska, Polak, & Bielecki, 2006). Enzymes that maintain its high activity even under extreme conditions of temperatures and pH are attractive for several industrial segments (Mitidieri, Martinelli, Schranka, & Vainstein, 2006).

Solid and submerged (liquid) fermentation have been exhaustively applied for optimization of hydrolytic enzymes production (Couri, Terzi, Pinto, Freitas, & Costa, 2000; Cunha, Esperança, Zangirolami, Badino, & Farinas, 2012; Mrudula & Murugammal, 2011). Aiming a cost effective, readily procurable, abundant and

environmental correct sources method for obtaining enzymes, a diversity of alternative biomass has been studied. Generally, these substrates are divided into three groups according to the main source of carbon present: those containing starch, such as rice, potatoes, corn, among others; those that present cellulose or lignocellulose such as wood and straw; and those with soluble sugars such as fodder, fruit pulp and beet, among others.

It is known that different carbon source can affect the classes and maximum activity of enzymes produced by a single strain (Solís-Pereira, Favela-Torres, Viniegra-González, & Gutiérrez-Rojas, 1993; Gautam, Bundela, Pandey, Awasthi, & Sarsaiya, 2010; Mojsov, 2010). Consequently, studies are focusing on optimizing enzymes production by using unusual biomass and agro-industrial waste as substrate (Silva, Lago, Merheb, Macchione, Yong, & Gomes, 2005; Díaz, Ory, & Blandino, 2012; Salihu, Abbas, Sallau, & Alam, 2015; Saini, Aggarwal, & Yadav, 2017).

Brazilian semiarid, also known as caatinga, is a unique ecosystem exclusive from Brazil. The semi-arid climate of this region is characterized by elevate temperature and evaporation, and long irregular dry season (Araújo, Castro, & Albuquerque, 2007). The flora in this region is diverse, with native xerophilous vegetation but also with introduced ('alien') plants (Almeida, Lopes, Tabarelli, & Leal, 2014). Adaptive structures such as high root/shoot ratio, high ability to accumulate reserves and water in stem and roots, presence of small and thick leaves allow these plants to be very adapted to the drought (Ferreira, Lacerda, Costa, & Filho, 2015). Plants such as forage cactus (*Opuntia ficus-indica* (L.) Mill), agave (*Agave attenuata*), mamaozinho (little papaya) (*Jacaratia corumbensis* O. kuntze), cassava (*Manihot esculenta* Crantz) and bonina (*Mirabilis jalapa*) show these characteristics and are targets for extractive and agricultural activities, for human and animal feeding and for medicinal use, such as *M. jalapa*.

Higher economic value could be added to these plants by using them as substrates in fermentative processes for enzymes production, since these plants have high biomass accumulated in their physical structures and are still under-exploited. Also, unconventional carbon source for hydrolytic enzymes production have been advised due to its potential to induce enzymes production (Jun, Kieselbach, & Jönsson, 2011).

The objective of this study was to evaluate and optimize the hydrolytic enzymes production by *A. niger* KIJH under solid and submerged fermentation using leaves and roots (with and without bark) of plants typically from Brazilian semiarid as substrate.

Material and methods

Microorganism and inducer substrate

Aspergillus niger KIJH was previously isolated from decomposing cactacea collected in Vitória da Conquista, Bahia, Brazil (14°51'58"S; 40°50'22"W). The fungal was cultivated for 7 days in BDA plates and stored at 4°C. All biomass used as substrate: forage cactus (*Opuntia ficus-indica* (L.) Mill), agave (*Agave attenuata*), papaya (*Jacaratia corumbensis* O. kuntze), cassava (*Manihot esculenta* Crantz) and bonina (*Mirabilis jalapa*) were collected in rural area of Vitória da Conquista and stored at 4°C for posterior use.

Solid-state fermentation (SSF)

Experiments were conducted in Erlenmeyer flasks (125 mL) containing 50 g of grinded biomass, in a total of seven different treatments: T1) *O. ficus-indica* (leaves); T2) *M. jalapa* (roots with husk); T3) *M. jalapa* (roots without husk); T4) *J. corumbensi* (roots with husk); T5) *J. corumbensi* (roots without husk); T6) *M. esculenta* (roots without husk); T7) *A. attenuata* (leaves). All experiment was conducted on triplicate, and the results were averaged and obtained the standard error of each data.

The moisture of the biomass varies between 70 to 80%. The flasks were autoclaved for 20 minutes at 121°C and 1 atm, and cooled at room temperature. Inoculum concentrations were adjusted to 10⁶ spores per mL and fermentations were carried out in static conditions at 28°C for 7 days.

Enzyme assay

After fermentation period, 50 mL of sodium acetate buffer were added to each Erlenmeyer and centrifuged at 10.000 rpm for 5 minutes. Then filtration was performed using Whatman paper number 1. The crude extracts (filtered) were collected in separate tubes after addition of 0.1% of sodium azide solution, it was stored at 4°C.

Xylanase, mannanase, pectinase, endoglycanase (carboxymethyl cellulase), total cellulases (FPase), exoglycosidase (avicellase) and amylase assay was carried out according to the DNSA (3, 5 dinitro salicylic acid) method (Miller, 1959; Siqueira, Siqueira, & Siqueira, 2010). For determination of xylanase, pectinase, mannanase and carboxymethylcellulose (CMCase) activities, were used 50 µL of crude extracts and 100 µL of

1% solution of xylan, pectin, mannan and CMCase, respectively. The solution was incubated at 50°C for 30 min. The reaction was stopped by adding 300 µL of DNS and kept in boiling water bath for 10 min. Then, 1.5 mL of water was added, and the absorbance was read at 540 nm.

Activity of FPase was accessed by incubation of 150 µL of crude extract with 16 discs (6 mm) of Whatman paper number 1 and 1.50 µg of substrate, approximately, at 50°C for 60 minutes. Avicellase activity was determined using 100 µL of 1% microcrystalline cellulose solution (avicel) in 50 mM sodium acetate buffer (pH 5) and 100 µL of crude extract followed by incubation at 50°C for 2 hours. The addition of DNS and absorbance reading follows the same procedure as described above.

The enzyme activities were calculated by standard curve prepared with each standard compound: glucose monomers for CMCase, FPase, avicellase and amylase activities, xylose for xylanase, galacturonic acid for pectinase and mannose for mannanase activity.

Submerged fermentation (SmF)

Biomass from *J. corumbensis*, *M. esculenta* and *M. jalapa* were selected by the results obtained on SSF. The culture was conducted in 125 mL Erlenmeyer flasks by adding a volume of spore suspension, calculated to give a concentration of 10^6 spores per mL, to 50 mL of 1% of dry substrate medium enriched with basic medium (KH_2PO_4 - 7 g L⁻¹; K_2HPO_4 - 2 g L⁻¹; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.1 g L⁻¹; $(\text{NH}_4)_2\text{SO}_4$ - 1.6 g L⁻¹). The incubation was carried out for 7 days in an orbital shaker incubator, at 28°C and 150 rpm. The determination of enzymatic activities was as described before. All experiment was conducted on triplicate, and the results were averaged and obtained the standard error of each data.

Large-scale enzyme production under SmF

Jacaratia corumbensis biomass was selected for scale up fermentation due to its ability of induction of CMCase and xylanase. SmF was conducted on 500 mL of substrate prepared as previously described (2.4) on 1 L Erlenmeyer. The experiment was conducted on triplicate, and the results were averaged and obtained the standard error of each data.

Partial purification by ultrafiltration

The scale up fermented culture was ultrafiltered using the Amicon System with PM10 membrane (10 kDa cut-off) in order to obtain an extract concentrated 10 times. After filtration it was obtained 450 mL of ultrafiltered extract and 50 mL of concentrated extract. Samples of fermented culture, ultrafiltered and concentrated extract were used for determination of CMCase and xylanase activity as described previously.

Effects of temperature and pH on enzymatic activity

The concentrated extract was used to determine the effects of temperature and pH variation on the CMCase and xylanase activities.

Optimum temperature for the enzymes were assessed by varying the incubation temperature of the assay from 30 to 80°C.

Optimum pH assessed by performing the assay with buffers ranging from pH 3 to 9 (50 mM sodium acetate buffer for pH 3.0-6.0; 50 mM sodium phosphate buffer for pH 6.0-7.5; 50 mM Tris-HCl buffer for pH 7.0-9.0). The assays were conducted by incubation of 75 µL of buffer (pH 3 to 9); 25 µL concentrated extract; 50 µL of carboxymethylcellulose and xylan (1%; m:v) at 50°C for 30 minutes.

Thermostability of enzymes

To investigate the thermostability, CMCase and xylanase were incubated at 50°C for 1, 2, 4, 8 and 24 hours, measuring its activities in each interval.

Results and discussion

Profiles of xylanase, mannanase, pectinase, endoglycanase (CMCase), total cellulases (FPase), exoglycosidase (avicellase) and amylase activities obtained by *A. niger* KIJH grown in solid cultures containing plants from Brazilian semiarid biomass as carbon source are in Table 1. It was observed that *J. corumbensis* was the optimum inducer for xylanase (3.403 U. mL⁻¹), mannanases (1.258 U mL⁻¹), FPase (0.377 U mL⁻¹) and

amylase (2.725 U mL^{-1}) production. On the other hand, maximum activities of pectinase (4.845 U mL^{-1}) e CMCase (1.017 U mL^{-1}) were registered when *M. esculenta* was applied as carbon source. *Mirabilis jalapa* (roots with bark) shows satisfactory results, especially for pectinase activity (3.425 U mL^{-1}). No plant biomass proved to be able to induce avicellase production by *A. niger* KIJH under solid or submerged fermentation.

Table 1. Hydrolytic enzyme activities (U mL^{-1}) produced by *Aspergillus niger* KIJH grown in solid cultures containing plant biomasses from Brazilian semiarid as the only nutrient source.

Plant biomass	Hydrolytic enzyme activities (U mL^{-1}) ^a						
	Xylanase	Mannanase	Pectinase	CMCase	FPase	Avicellase	Amylase
<i>O. ficus-indica</i> (leaves)	1.218	0.858	0.279	0.426	0.001	0.001	0.590
<i>M. jalapa</i> (roots with husk)	1.327	0.268	3.425	0.425	0.140	0.014	2.315
<i>M. jalapa</i> (roots without husk)	0.743	0.099	3.070	0.165	0.211	0.029	1.236
<i>J. corumbensi</i> (roots with husk)	3.403	1.258	2.565	0.800	0.377	0.012	2.725
<i>J. corumbensi</i> (roots without husk)	2.508	0.492	1.387	0.424	0.462	0.002	1.177
<i>M. esculenta</i> (roots without husk)	2.101	0.186	4.845	1.017	0.285	0.000	1.419
<i>A. attenuata</i> (leaves)	1.132	1.030	1.567	0.339	0.299	0.014	0.472

^aValues are given as means of three replicate cultures. Standard deviations were less than 10% of the means.

Different plants biomass and agro-industrial waste have been tested as carbon source for optimization of hydrolytic enzymes production by *Aspergillus* genus. Enzyme production using grape pomace by *A. awamori* presented high xylanase ($40.4 \text{ IU gd s}^{-1}$) and cellulase (9.6 IU gd s^{-1}) high production in only 24 hours of culturing; however increasing the fermentation time to 7 and 5 days for xylanase and cellulase production, respectively, there was a significant reduction of these enzymes activity (Botella, Ory, Webb, & Blandino, 2005). However, using different substrate, wheat bran, and strain, *A. niger*, the optimum cultivation time was 6 days obtaining xylanase activity of $9,87 \text{ U mL}^{-1}$ (Kavya & Padmavathi, 2009).

For other hydrolytic enzymes also is observed variation of activity depending on the substrate used even if it is maintained the fungal species. For example using banana peel as substrate, the pectinase activity achieved in 24 hours was 6.6 U mL^{-1} by *A. niger* (Barman, Badwaik, & Deka, 2015). Mannanase activity (918.68 U g^{-1}) was observed in culture of *A. niger* using palm kernel cake as substrate (Ab Rashid, Ibrahim, & Omar, 2012). The same fungi species shows endoglucanase activity of 35.7 U g^{-1} on wheat bran in 72 hours (Pirota, Miotto, Delabona, & Farinas, 2013). On wheat bran, *A. niger* presented 74 U mg^{-1} of amylase activity, in 96 hours (Varalakshmi et al., 2009). And CMCase and FPase activities was recorded as 8.89 and 3.56 U g^{-1} , respectively, when applied 96 hours of SSF by *A. niger* using coir waste as substrate (Mrudula & Murugammal, 2011). Thus, it is possible to assume that several factors such as fermentation time, fungi specie, carbon source, cultivation condition (temperature, pH, inoculum size) are crucial for hydrolytic enzymes production. Even though, the hydrolytic enzyme produced on Brazilian semiarid plants biomass is low compared with that found in other substrates, these lignocellulosic biomasses have the potential to induce the production of several important commercial enzyme in a single fungi strain.

Jacaratia corumbensis and *Mirabilis jalapa* (roots with bark) and *M. esculenta* were selected as substrate for submerged fermentation once these plant biomasses induced higher production of the majority hydrolytic enzymes evaluated in SSF. These assays showed that *J. corumbensis* was the source of carbon that presented the higher enzyme induction by *A. niger* KIJH in FmS (Table 2): 1.163 U mL^{-1} (xylanase activity); 0.133 U mL^{-1} (mannanase activity); 0.404 U mL^{-1} (pectinase activity); 0.264 U mL^{-1} (CMCase activity) and 0.192 U mL^{-1} (FPase activity).

Highlight is given to xylanase (1.163 U mL^{-1}) and CMCase (0.264 U mL^{-1}) activities since they have the greater activities detected in the SmF assay. These results can be considered satisfactory when compared to other studies that conducted *Aspergillus* strains in submerged culture containing alternative substrates as carbon source: Reddy et al. (2015) observed that *A. niger* under submerged culture containing saw dust and corn cobs provided CMCase activity approximately equal 0.2 and 0.3 U mL^{-1} , respectively, after 7 days. Okafor, Okochi, Onyegeme-okereanta and Nwodo-Chinedu (2007) verified maximum activity of xylanase (0.95 U mL^{-1}) by *A. niger* ANL 301 under submerged culture in media containing sugarcane pulp at 96 hours. Also *A. niger* ANL 301 cultivated in media containing sawdust and oat spelt xylan promoted, respectively, enzyme activities peak equal to 0.65 and 0.80 U mL^{-1} , at 120 hours fermentation.

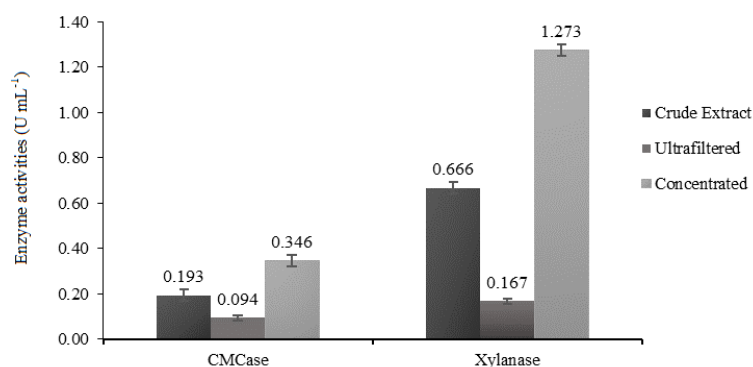
Table 2. Hydrolytic enzyme activities (U mL⁻¹) obtained by *Aspergillus niger* KIJH grown in submerged cultures containing plants biomass as the only nutrient source (carbon and other nutrients).

Plant biomass	Hydrolytic enzyme activities (U mL ⁻¹) ^a						
	Xylanase	Mannanase	Pectinase	CMCase	FPase	Avicellase	Amylase
<i>M. jalapa</i> (roots with husk)	0.943	0.074	0.114	0.164	0.063	0.016	0.041
<i>J. corumbensis</i> (roots with husk)	1.163	0.133	0.404	0.264	0.192	0.009	0.040
<i>M. esculenta</i> (roots without husk)	0.433	0.027	0.285	0.203	0.025	0.035	0.045

^aValues are given as means of three replicate cultures. Standard deviations were less than 10% of the means.

Fermentation scale up with *A. niger* KIJH in submerged culture were conducted using *J. corumbensis* as a carbon source, which was the better inducer for CMCase and xylanase inducer in SSF experiment. Fermented culture, ultrafiltered and concentrated ultrafiltered extracts were obtained for evaluate of xylanase and CMCase, which presented the highest activities. Considering the fermented culture as 100% of activity, it was possible to determine a reduction of CMCase activity equal 51% in the ultrafiltrated extract and an increase equal 79% in the concentrated extract, showing that a largest fraction of the enzymes was retained by membrane (10 kDa cut-off) (Figure 1).

Studies have indicated that molecular weight of CMCase produced by *Aspergillus* strains is greater than 10 kDa: Coral et al. (2002) estimated molecular weights around 83 and 50 kDa for CMCase by *A. niger* Z10 grown in a liquid Czapek Dox medium, containing oat spelts xylan as the sole carbon source. Onsori, Zamani, Motallebi and Zarghami (2005) verified molecular weights equals 18.5; 23 e 28 kDa for CMCase obtained by *Aspergillus* sp. cultured in modified liquid medium containing CMC. Begum, Absar and Alam (2009) identified the presence of extracellular cellulase with 41 kDa molecular weight produced by *A. oryzae* ITCC-4857.01 grown in Czapek liquid medium with CMC (1%). All of the cited studies determined the CMCase molecular weight by SDS-PAGE.

**Figure 1.** CMCase and xylanase activities from crude extract and ultrafiltered extract (ultrafiltered and concentrated) by membrane 10 kDa obtained by *Aspergillus niger* KIJH grown submerged culture containing *Jacaratia curubensis* as carbon source.

For xylanase, was verified an activity reduction of 75% in ultrafiltrated extract in relation to the crude extract and an increase of 91% in the concentrated extract indicating that a largest fraction of the enzymes has molecular weights greater than 10 KDa (Figure 1). This corroborates the studies of Lemos and Pereira Jr. (2013) that reported molecular weights of 31, 51.4 and 55 KDa for three isoenzymes with xylanolytic activities obtained by *A. awamori* grown in sugarcane bagasse. The low xylanolytic activity (0,167 U mL⁻¹) detected in the ultrafiltered extract is possibly due to the presence of endo-1,4- β -xylanases (EC 3.2.1.8) belonging to the Family G/11 having low molecular weight and are able to permeate through the membrane (Yi et al., 2010).

Generally, microorganisms are able to produce more than one type of xylanase as result of differential mRNA processing, partial proteolysis or differences in the degree of amidation and glycosylation (Kocabas, Kocabas, & Bolukbasi, 2011). According to Yi et al. (2010), *Aspergillus niger* produces a broad spectrum of enzymes that degrade xylan, most of them belonging to the Family G/11.

CMCase and xylanase are known to be active in a wide range of temperature and pH. In this study, CMCase in the concentrated extract demonstrate a constant activity practically in all temperature ranges (30-80°C) and pH (3.0-9.0), having a small peak at 65°C and pH 6.0 (Figures 2 and 3). These results differ from those

reported by Coral et al. (2002) for CMCase obtained from *A. niger* Z10 whose optimum temperature was 40°C at pH 7.5. Thus, CMCase from *A. niger* KIJH produced on *J. corubensis* biomass is a potential enzyme that could be used on process which required thermostable components.

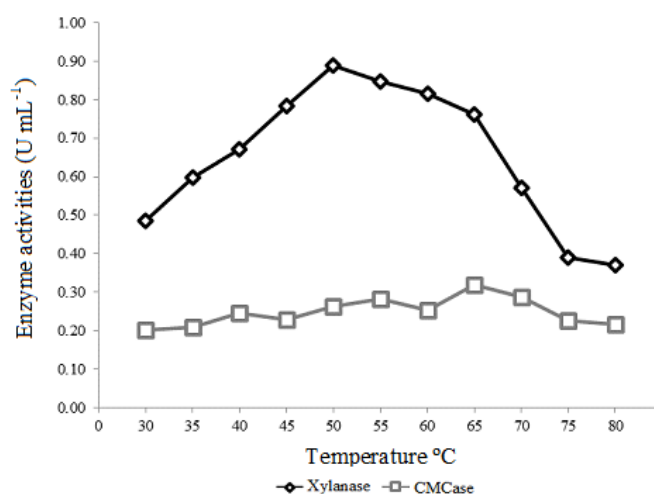


Figure 2. Effect of temperature variation on CMCase and xylanase activities obtained by *Aspergillus niger* KIJH grown submerged culture containing *Jacaratia curubensis* as carbon source.

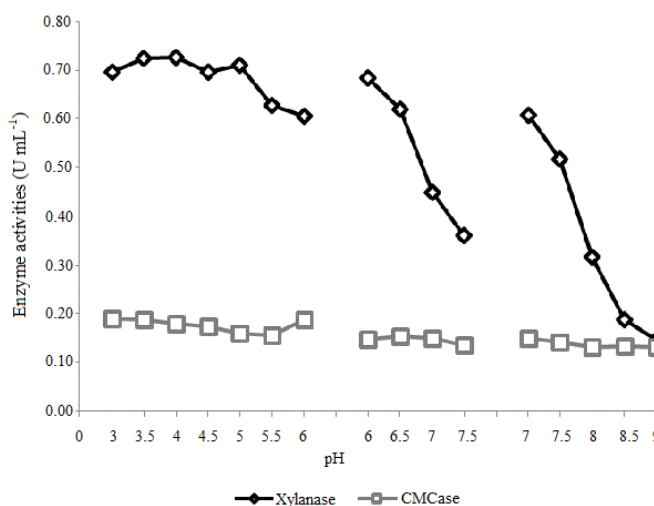


Figure 3. Effect of pH variation on CMCase and xylanase activities obtained by *Aspergillus niger* KIJH grown submerged culture containing *Jacaratia curubensis* as carbon source.

Xylanase presented optimum temperature activity of 50°C also its activity persists constant in pH range of 3.0 to 5.0 (Figures 2 and 3). In general, β -xylanases from fungal shows higher activity and stability in the temperature range of 40-55°C under acidic conditions (Taneja, Gupta, & Kuhad, 2002). This corroborates with the present study findings and those results from Krisana et al. (2005) and Romanowska et al. (2006) which verified optimal temperature of 55°C at pH 5 for CMCase produced by *A. niger* strains. According Subramaniyan and Prema (2002), xylanases produced by *Aspergillus* genus are known to be active in a range of temperature at 45-60°C.

The stability of the enzymes at 50°C for 24 hours is presented in Figure 4. The CMCase was found to be stable at 50°C for 8 hours, retaining 75% of its activity. While xylanase presented an activity reduction of 53%, after 2 hours at 50°C. At the end of the incubation period, this enzyme conserved only 16% of its original activity. These results are consistent with the findings of Coral et al. (2002) which detected CMCase from *A. niger* with 60% of its original activity after heat treatment at 50°C, and Teixeira, Siqueira, Souza, Filho and Bom (2010) that the xylanase from *A. awamori* conserved 50% of the original activity retained after heat treatment for 72 hours at 50°C.

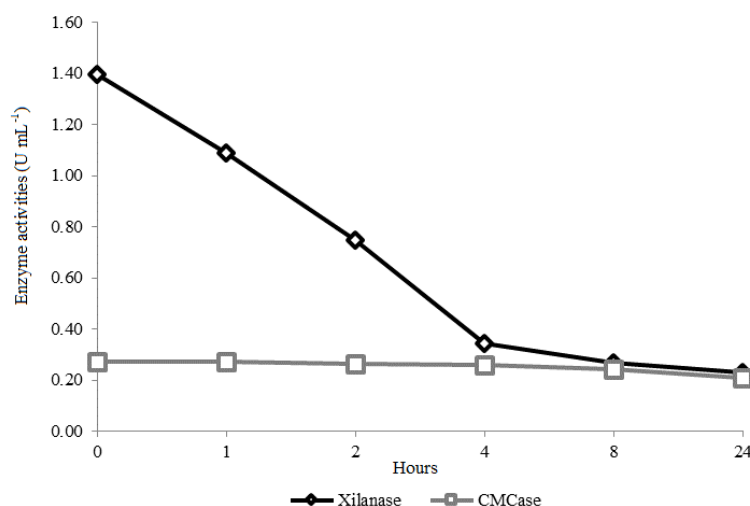


Figure 4. Thermostability at 50°C of CMCase and xylanase activities obtained by *Aspergillus niger* KIJH grown submerged culture containing *Jacaratia curubensis* as carbon source.

Conclusion

Plant biomasses commonly found in the Brazilian semiarid were able to induce the production of several enzymes by *A. niger* KIJH when grown in solid cultures. However, *J. corumbensis* was the most promising biomass for use as a substrate in solid and submerged cultures for production especially of CMCase and xylanase, by *A. niger* KIJH. The results of temperature and pH effects and thermostability of the enzymes suggest that CMCase and xylanase obtained by *A. niger* KIJH cultivated in submerged culture containing *J. corumbensis* as carbon source may have application in biotechnology processes that require enzymes that remain active under routine extreme conditions.

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