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Cellulases and Hemicellulases from Endophytic *Acremonium* Species and Its Application on Sugarcane Bagasse Hydrolysis

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Abstract The aim of this work was to have cellulase activity and hemicellulase activity screenings of endophyte *Acremonium* species (*Acremonium zeae* EA0802 and *Acremonium* sp. EA0810). Both fungi were cultivated in submerged culture (SC) containing L-arabinose, D-xylose, oat spelt xylan, sugarcane bagasse, or corn straw as carbon source. In solid-state fermentation, it was tested as carbon source sugarcane bagasse or corn straw. The highest FPase, endoglucanase, and xylanase activities were produced by *Acremonium* sp. EA0810 cultivated in SC containing sugarcane bagasse as a carbon source. The highest β-glucosidase activity was produced by *Acremonium* sp. EA0810 cultivated in SC using D-xylose as carbon source. *A. zeae* EA0802 has highest α-arabinofuranosidase and α-galactosidase activities in SC using xylan as a carbon source. FPase, endoglucanase, β-glucosidase, and xylanase from *Acremonium* sp. EA0810 has optimum pH and temperatures of 6.0, 55 °C; 5.0, 70 °C; 4.5, 60 °C; and 6.5, 50 °C, respectively. α-Arabinofuranosidase and α-galactosidase from *A. zeae* EA0802 has optimum pH and temperatures of 5.0, 60 °C and 4.5, 45 °C, respectively. It was analyzed the application of *Acremonium* sp. EA0810 to hydrolyze sugarcane bagasse, and it was achieved 63% of conversion into reducing sugar and 42% of conversion into glucose.

Keywords Cellulase · Hemicellulase · *Acremonium* · Endophyte · Ethanol · Agroindustrial residue

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Introduction

Lignocellulose is one of the most common biopolymers in nature and is composed mainly of cellulose, hemicellulose, and lignin. Cellulose is a linear polymer of glucose units which can be hydrolyzed by the action of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), exoglucohydrolases (EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21; 1). Hemicellulose is a heterogeneous and branched polymer of pentoses, hexoses, and uronic acids. Complete enzymatic hydrolysis of xylan, the major polymer founded in hemicelluloses, requires endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and several accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), α-galactosidase (EC 3.2.1.22), acetylxylan esterase (3.1.1.72), and ferulic acid esterase (EC 3.1.1.73; 2–4).

Degradation of lignocellulosic material has great importance for many industrial processes, and enzymatic hydrolysis has received attention due to its potential as an environmentally friendly process besides its enormous hydrolysis specificity [5]. Cellulases can be used in the textile industry for bio-stoning and bio-finishing of cellulosic fibers, and hemicellulases can be applied for the bio-bleaching of kraft pulps. Furthermore, in the food industry, cellulases and hemicellulases can be used for extraction and clarification of fruit and vegetable juices, and in the animal feed industry, these enzymes can promote an increase in the nutritive quality of feed [6–9].

In recent decades, the interest in cellulases and hemicellulases has increased due to the ethanol production from lignocellulosic residues. Lignocellulosic ethanol is being explored as potential low-cost gasoline and diesel substitutes and is seen as an interesting alternative because it can contribute to sustainable development as well as offset fossil fuel greenhouse emissions [10, 11]. However, this technology is not completely developed and is still expensive, mainly because of the high cost of these enzymes which are essential for the hydrolysis of raw material.

There is a general interest in obtaining new, more specific, stable enzymes and using cheap inducer sources, such as sugarcane bagasse [12, 13]. Enzymes for the mentioned process are produced by microorganisms, and the majority of research has been on the *Trichoderma* and *Aspergillus* genera [3, 14, 15]. However, little is understood about enzymes from the *Acremonium* genus [16, 17].

The genus *Acremonium* Link comprises anamorphic Hypocreales, consisting of about 100 species with a worldwide distribution [18]. The genus is reported from a variety of soil-borne, human pathogens, entomopathogenic and endophytic ecological niches [19]. *Acremonium zeae*, also reported as *Acremonium strictum*, is the most prevalent colonist in preharvest maize (*Zea maydis*), typically producing symptomless kernel infections. *A. zeae* is an extensively studied endophyte due to its production of the cephalosporin C antibiotic [20–22]. It was reported that this fungus can produce another type of antibiotic called pyrrocidines A and B that have antagonist effect against *Aspergillus flavus* and *Fusarium verticillioides* in cultural tests and interferes with *A. flavus* infection and aflatoxin contamination in preharvest maize kernels [23]. Endophytic microorganisms are a promising source of enzymes since it seems that hydrolytic enzymes are involved in the penetration and development of fungi in plants [24–26]. There are success studies about *Acremonium cellulolyticus*, a hyper-cellulase producer [16, 27, 28], and recently, *A. zeae* was studied in relation to its hemicellulases production and its capacity to hydrolyze corn arabinoxylan [29].

The purpose of this study was to investigate the ability of two species of *Acremonium* (*A. zeae* EA0802 and *Acremonium* sp. EA0810) to produce cellulases and hemicellulases in submerged culture (SC) and in solid-state fermentation (SSF), using low-cost materials as carbon source.

Materials and Methods

Materials

The substrates ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal), ρ-nitrophenyl-β-D-glucopyranoside (ρNPGlc), ρ-nitrophenyl-α-L-arabinofuranoside (ρNPAra), and ρ-nitrophenyl-β-D-xylopyranoside (ρNPXyl) were obtained from Sigma Chemical Co. (St. Louis, MO). Xylan from birch wood and D (+) xylose were purchased from Sigma Chemical Co. (Germany and USA, respectively). The sugarcane bagasse and corn straw were acquired from the local market. All other chemicals used were of analytical grade.

Microorganism

Two fungal strains, *A. zeae* EA0802 and *Acremonium* sp. EA0810, were used in the study. These fungi were isolated from commercial corn seeds by Blotter method [30] and direct culture isolation in the Laboratory of Seed Pathology and post-Harvest at UFV, Brazil. The fungi were routinely propagated in agar solid medium supplemented with 21% (*v*/*v*) tomato sauce and 0.3% (*m*/*v*) calcium carbonate. The strains were kept on Laboratory of Biochemical Technology at UFV, Brazil.

Submerged Culture

For microorganism cultivation, 50 mycelial agar disks (8 mm diameter) were transferred to 250 mL of production media in a 500-mL Erlenmeyer flask. The production media for *Acremonium* species contained per liter 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 0.01 g $ZnSO_4$, and 10 g of a carbon source: oat spelt xylan, L-arabinose, D-xylose, milled sugarcane bagasse, and milled corn straw. Both microorganisms were grown at 30 °C with agitation (180 rpm) for 23 days. Flasks were sampled daily (4 mL). Samples were centrifuged for 30 min at 3,500×*g*, and the supernatants were used as the crude enzyme.

Solid-State Fermentation

Three mycelial agar disks (8 mm diameter) of *Acremonium* sp. EA0801 and *A. zeae* EA0802 were transferred to 125-mL Erlenmeyer flasks containing 7.0 g of humidified carbon source (sugarcane bagasse or corn straw). The moisture content was adjusted to 74% with mineral salt solution containing per liter 1.5 g NaH_2PO_4, 0.5 g $MgSO_4$, and 1.0 g $(NH_4)_2SO_4$. The flasks were incubated under static conditions at 25–28 °C. The culture flasks were removed at 3-day intervals over a period of 39 days. In each culture was added 50 mL of 0.05 M sodium acetate buffer pH 5.0 under agitation at room temperature for 30 min. The extract was filtered through a nylon filter, and the filtrate was used as crude enzyme for further analysis. The experiment was carried out in duplicate, and the results represent the mean of values.

Enzyme Assay

Endoglucanase activity was assayed on 0.5% carboxymethylcellulose at 50 °C for 30 min. Xylanase activity was assayed on 1% birchwood xylan at 40 °C for 15 min. Filter paper activity (FPase) was assayed on Whatman no. 1 filter paper (1×5 cm, 40 mg) at 50 °C for

60 min. Assays were carried out in 0.05 M sodium acetate buffer pH 5.0 in a final volume of 0.5 mL. The release of reducing sugars for these assays was determined using the 3,5-dinitrosalicylic acid (DNS) method [31] and was calculated according to the standard curve (0.11–1.11 μmol of glucose). The activities of α-L-arabinofuranosidase, α-galactosidase, β-xylosidase, and β-glucosidase were measured using 0.5 mM ρNPAra, ρNPGal, ρNPXyl, and ρNPGlc, respectively. These four enzymes assays were carried out at 40 °C for 15 min in 0.05 M sodium acetate buffer, pH 5.0 in a final volume of 0.5 mL. The absorbance of the mixture was then measured at 410 nm. The amount of pNP released was calculated according to the standard curve (0.0017–0.0625 μmol of pNP). One enzyme unit was defined as the amount of enzyme that released 1 μmol of product per minute under the assay conditions.

Biochemical Characterization of Enzymes

The effect of the temperature on the enzyme activities was determined in 0.05 M sodium acetate buffer, pH 5.0 at a temperature range of 10–80 °C.

For determination of thermostability, the enzymatic extracts were preincubated with 0.05 M sodium acetate buffer, pH 5.0, during 150 min at various temperatures: β-glucosidase, 60 °C; endoglucanase, 70 °C; FPase, 55 °C; α-galactosidase, 45 °C; α-arabinofuranosidase, 60 °C; and xylanase, 50 °C.

The pH effect on α-galactosidase, α-arabinofuranosidase, xylanase, endoglucanase, FPase, and β-glucosidase activities were investigated at different pH values (from 3.6 to 8.0) using McIlvaine buffer (citric acid/sodium phosphate; 32) at optimum temperature for each enzyme. Results of the analyses are presented as mean±SD for three measurements.

Biomass Pretreatment

Sugarcane bagasse was dried in an oven at 70 °C until the weight was constant. The dry sugarcane bagasse was milled in rotary mill (model MA-580, Marconi Co. Piracicaba, SP) and passed through a 1.0-mm screen. Sodium hydroxide at concentration of 1% was used to pre-treat 25 g of milled sugarcane bagasse samples at a solid loading of 10% (*w*/*v*). Treatments were performed in duplicate in an autoclave at 121 °C for 60 min. The pretreated materials were separated into solid and liquid fractions using a Buchner funnel fitted with filter paper. The solid fraction was washed thoroughly with distilled water, sealed in hermetic vessel to retain moisture, and then stored at −20 °C.

The chemical composition of the untreated and alkaline pretreated sugarcane bagasse was determined using a modified Klason lignin method derived from the TAPPI Standard Method T222 om-98 [33].

Biomass Saccharification

Enzymatic saccharification of the pretreated sugarcane bagasse at a solid loading of 2% dry matter (*w*/*v*) was performed in a orbital shaker, at 40 °C and 250 rpm for up to 72 h in flasks containing 25 mL of appropriately diluted enzymes in 50 mM phosphate buffer, pH 6.0, plus sodium azide (10 mM) and tetracycline (0.04 $g mL^{-1}$) to inhibit microbial contamination. Crude enzymatic extract produced by *Acremonium* sp. under submerged fermentation using corn straw as carbon source were used in the enzymatic hydrolysis. The enzymatic extract was concentrated tenfold before being employed in the experiment using an Amicon Ultrafiltration system (Millipore Co., Billerica, MA, USA) using a YM-10 (cut-

off Mr 10,000 Da) membrane filter. The enzyme loading for all experiments was 10 FPase units per gram of dry matter. Samples (0.5 mL) were taken from the reaction mixture at different time intervals. These samples were centrifuged for 10 min at 13,000 rpm, immediately incubated in boiling bath to denature the enzymes, and then stored at −20 °C. The supernatant was used for the determination of total reducing sugar and glucose released during the reaction. The total reducing sugar concentration was quantified by DNS, while glucose concentration was estimated using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil). The products released were calculated by averaging values for samples triplicates and subtracting average values for the respective controls which were carried out parallel, replacing the active enzymatic extract by enzymatic samples boiled for 30 min. The substrate conversion was calculated using the following equation [34]:

$$\text{Conversion}(\%) = \frac{\text{reducing sugar}(\text{mg/mL}) \times 0.9 \times 100}{\text{initial substrate}\,(\text{mg/mL})}$$

Results and Discussion

Enzymes Production

Filter Paper Activity—FPase

The complex and simple carbon sources in the culture medium induced FPase activity when the fungi were cultivated in SC and in SSF (Tables 1 and 2). Total cellulase activity over filter paper was present in the cultures during practically all the period of time of analysis (Tables 1 and 2). Although reported before for *Thermoascus aurantiacus* and *Aspergillus fumigatus,* it is not common that an organism produces cellulolytic enzymes on so wide a substrate range [35, 36]. The high FPase production is an indicative of endophytic *Acremonium* ability to digest cell wall components, an important characteristic to fungal survival in its environment. The secretion of cellulases implies that the fungus is well equipped for both penetration of living cells and decomposition of dead tissues [37].

It is interesting that oat spelt xylan or D-xylose were reasonable inducers of FPase activity by *Acremonium* species cultivated in SC. FPase activities, about 0.18 U mL^{-1} were detected in the second day of growing using oat spelt xylan. In the case of D-xylose, the maximum FPase activity, about 0.12 $U.mL^{-1}$, was detected in the range of 8–14 days of cultivation (Tables 1 and 2). Similarly, this monosaccharide was also an inducer of FPase activity in *A. fumigatus* culture [36]. The *Acremonium* species studied show very low or no FPase activity when were cultivated in SC with L-arabinose as carbon source (Tables 1 and 2). This effect was also observed in *Aspergillus niger* cultivated with arabinose [38].

As expected, highest FPase activity was detected when sugarcane bagasse or corn straw were the carbon sources (Tables 1 and 2), probably as function of the high cellulose content present in these materials [39, 40]. In general, cellulosic materials act as inducers and readily metabolized carbon compounds as repressors [36]. In addition, the *Acremonium* species studied exhibited maxima FPase activities when they were cultivated in SC using sugarcane bagasse or corn straw, compared with SSF using the same carbon sources (Tables 1 and 2). The highest FPase activities, 0.55 and 0.51 $U.mL^{-1}$, were achieved for *Acremonium* sp. EA0810 and *A. zeae* EA0802 on fourth and eighth cultivation day,

Table 1 *A. zeae* EA0802 enzyme activity (units per milliliter) in solid-state fermentation (SSF) and in submerged culture (SC) with different carbon sources

Carbon source	ABF	α-Gal	β-Xyl	β-Glc	Endo	FPase	Xyl
SSF-Corn straw	0.01^{32}	0.01^{6}	0.01^{39}	0.01^{39}	0.03^{39}	0.06^{99}	0.26^{12}
SSF-Sugarcane bagasse	0.02^{36}	< 0.01	0.01^{30}	0.08^{36}	0.08^{33}	0.14^{33}	0.78^{15}
SC-Corn straw	0.01^{23}	0.03^{5}	<0.01	0.02^{6}	0.09^{6}	0.14^{18}	0.76^{9}
SC-Sugarcane bagasse	<0.01	0.02^{7}	<0.01	0.03^{7}	0.09^{3}	0.51^{8}	2.16^{14}
SC-L-Arabinose	0.02^{4}	0	0	0.02^{6}	0	0	0.08^{15}
SC-Oat spelt xylan	0.04^{18}	0.09^{4}	<0.01	0.03^{9}	<0.01	0.17^{2}	1.56^{15}
SC-D-Xylose	<0.01	0	<0.01	0.01^{4}	<0.01	0.11^{8}	0.32^{9}

All standard deviation were less than 10%. Superscript numbers stand for the day of growth, referring to the day of greatest activity

ABF α-L-arabinofuranosidase activity, *α-Gal* α-galactosidase activity, *β-Xyl* β-xylosidase activity, *β-Glc* β-glucosidase activity, *Endo* endoglucanase activity, *FPase* FPase activity, *Xyl* xylanase activity

respectively (Tables 1 and 2). These results suggest that, for these fungi, SC is more efficient for FPase induction than SSF.

Neurospora crassa produced 0.23 UmL^{-1} of FPase when it was cultivated in liquid medium with 1% wheat straw as carbon source [41]. On the other hand, higher FPase production, 5 $U.mL^{-1}$, was reported by Yamanobe [27] using *A. cellulolyticus* strain grown in a liquid medium with 4% of cellulose powder as carbon source. A hyper-producer mutant of this original strain produced 15 FPU mL^{-1} after 8 days growing in an optimized medium [28]. In an optimized culture medium for the production of cellulase by a mutant strain of *Trichoderma reesei* using 4% corn cobs as carbon source, maximum FPase activity at 5.2 $U.mL^{-1}$ was obtained after cultivation for 168 h, with a yield of 213.4 Ug^{-1} and productivity of 31.3 UL^{-1} h^{-1} [42].

In the case of *Acremonium* sp. EA0810 cultivated in non-optimized conditions, the FPase activity reached 0.55 UmL^{-1} (Table 2), showing yield of 54.6 Ug^{-1} (units of enzyme for gram of dry carbon source) and productivity of 11.45 UL^{-1} h, after 48 h of growing using only 1% sugarcane bagasse, a low-cost material, as carbon source. These results suggest

Table 2 *Acremonium* sp. EA0810 enzyme activity (units per milliliter) in solid-state fermentation (SSF) and in submerged culture (SC) with different carbons source

Carbon source	ABF	α-Gal	β-Xyl	β-Glc	Endo	FPase	Xyl
SSF-Corn straw	<0.01	<0.01	<0.01	0.02^{39}	0.07^{6}	0.06^{21}	0.2^{15}
SSF-Sugarcane bagasse	0.01^{25}	<0.01	<0.01	0.02^{15}	0	0.02^{30}	0.07^{3}
SC-Corn straw	<0.01	<0.01	<0.01	0.02^{7}	0.18^{9}	0.18^{3}	2.08^{16}
SC-Sugarcane bagasse	<0.01	<0.01	0	0.03^{7}	0.15^{4}	0.55^{4}	2.46^{10}
SC-L-Arabinose	0	<0.01	<0.01	0.09^{20}	0.02^{3}	0.03^{20}	0.14^{17}
SC-Oat spelt xylan	0.01^{9}	0	<0.01	0.06^{9}	0.06^{5}	0.18^{2}	2.02^{3}
SC-D-Xylose	0	<0.01	<0.01	0.17^{20}	0.07^{9}	0.12^{14}	0.15^{7}

ABF α-L-arabinofuranosidase activity, *α-Gal* α-galactosidase activity, *β-Xyl* β-xylosidase activity, *β-Glc* β-glucosidase activity, *Endo* endoglucanase activity, *FPase* FPase activity, *Xyl* xylanase activity

All standard deviation were less than 10%. Superscript numbers stand for the day of growth referring to the day of greatest activity

that *Acremonium* sp. EA0810 is a promising fungus to produce FPase, since its culture medium was not optimized and the carbon sources were tested in a low concentration. Besides, low-cost agro-industrial residues as sugarcane bagasse and corn straw were shown to be good carbon sources for cellulases production by *Acremonium* species, contributing, in this way, for an appropriate utilization of this agro-industrial residue.

Endoglucanase

Acremonium sp. EA0810 showed high endoglucanase activities, 0.18 and 0.15 $U.mL^{-1}$, when grown in the SC with corn straw or sugarcane bagasse as carbon source, respectively (Table 2). On the other hand, when this fungus was cultivated in SSF with sugarcane bagasse, no endoglucanase activity was detected (Table 2). Arabinose, oat spelt xylan, or xylose induced low endoglucanase activities at 0.02, 0.06, and 0.07 $U.mL^{-1}$, respectively, in SC *Acremonium* sp. EA0810 cultures (Table 2).

A. zeae EA0802 did not show ability to produce endoglucanase activity on so wide a substrate range like *Acremonium* sp. EA0810. The highest endoglucanase activity, 0.09 U mL^{-1}, was measured after 6 or 3 days of cultivation on SC using corn straw or sugarcane bagasse as carbon source, respectively (Table 1). In SSF, sugarcane bagasse induced the endoglucanase activity of 0.08 UmL^{-1}, similar to that activity on SC, however, after 33 days of growth. The highest endoglucanase activity observed in the *Acremonium* cultures containing complex carbon sources as corn straw and sugarcane bagasse can be due to the presence of amorphous cellulose in these substrates, since endoglucanases are more active on this kind of polymer [1].

In SC using arabinose, oat spelt xylan, and xylose as carbon source, none or low activities were detected (Table 1). *Mucor circinelloides* grown on lactose exhibited endoglucanase activity from 0.17 to 0.25 UmL^{-1}, similar to the values obtained in this work [43].

β-Glucosidase

Acremonium sp. EA0810 exhibit higher β-glucosidase activities than *A. zeae* EA0802 in SC (Table 2). Highest β-glucosidase activity (0.17 UmL^{-1}) was obtained in the SC of *Acremonium* sp. EA0810 culture using D-xylose as carbon source in (Table 2). *A. fumigatus* culture induced with xylose also secreted β-glucosidase [36]. About 0.09 UmL^{-1} was detected after 20 days of growth in SC with arabinose as carbon source, and 0.06 UmL^{-1} was detected in SC with oat spelt xylan after 9 days (Table 2). The β-glucosidase induction with arabinose was also observed in *Clostridium papyrosolvens* [44]. *Acremonium persicinum* produced approximately 0.16 UmL^{-1} of β-glucosidase when grown on laminarin after 240 h [45].

Low β-glucosidase activity was achieved in *A. zeae* EA0802 cultivated on SC. Highest activity, 0.08 $U.mL^{-1}$ or 2.88 $U.g^{-1}$, was obtained by this fungus in SSF on sugarcane bagasse after 36 days of growth (Table 1). *Fusarium oxysporum*, an endophyte microorganism like *Acremonium*, cultivated on corn stover in SSF produced 0.14 Ug^{-1} of β-glucosidase [46].

Xylanase

Considerable xylanase activity was obtained for *Acremonium* cultures grown using sugarcane bagasse, corn straw, oat spelt xylan, arabinose, and xylose as carbon sources. This observation can be explained by endophyte nature of the strains, since xylan is one of the

major components of the plant cell wall [47]. The highest xylanase activities were found to be 2.46 UmL^{-1} and 2.16 UmL^{-1}in the SC of *Acremonium* sp. EA0810 and *A. zeae* EA0802, respectively, using sugarcane bagasse as carbon source (Tables 1 and 2). Corn straw and oat spelt xylan, in SC of *Acremonium* sp. EA0810 cultures, also induced higher xylanase activities of 2.08 UmL^{-1} in the 16th cultivation day and 2.02 UmL^{-1} after 3 days of growth, respectively. This result showed that the presence of oat spelt xylan in the culture of *Acremonium* sp. EA0810 led to high levels of xylanase activity in a very short time. Nevertheless, compared with these activities, *A. zeae* EA0802 culture with corn straw or oat spelt xylan produced smaller xylanase activity of 0.76 UmL^{-1}, on ninth day and 1.56 UmL^{-1} after 15 days of growth, respectively (Table 1). *Hymenoscyphus ericae*, an endophyte fungus, produced two xylanase forms, with a total activity of 5.8 UmL^{-1}, in liquid medium with oat spelt xylan [48]. *Rhizopus oryzae* produced 0.39 U mL^{-1} using 1% xylan as carbon source after 6 days, and when 2% corn cob was used, the fungi produced 2.8 UmL^{-1} after 5 days [49].

The SSF of *A. zeae* EA0802 culture with sugarcane bagasse or corn straw as carbon source achieved 0.78 UmL^{-1} or 29.4 Ug^{-1} and 0.26 UmL^{-1} or 6.5 Ug^{-1} of xylanase activity, respectively (Table 1). *Penicillium echinulatum* grown on wheat bran and pretreated sugarcane bagasse produced 10 Ug^{-1} of xylanase activity [12]. The *A. zeae* EA0802 results encourages more studies to optimize it cultivation on SSF.

α-Galactosidase

Reduced levels of α-galactosidase activity were detected in the *Acremonium* cultures. The maximum activity, 0.09 UmL^{-1}, was produced by *A. zeae* EA0802 in SC with oat spelt xylan as carbon source after 4 days of growth (Table 1). The SSF *A. zeae* EA0802 culture with corn straw produced 0.01 UmL^{-1}, which represents 0.27 Ug^{-1}. Similar results were obtained to *Aspergillus oryzae* in non-optimized SSF with bagasse, which produced 0.19 U g^{-1} [50]. *Penicillium brasilianum* grown in 2.5% of a mixture of purified cellulose, birchwood xylan, and oat spelt xylan was found to produce 1.5 UmL^{-1} of α-galactosidase activity [51].

α-Arabinofuranosidase

The highest α-arabinofuranosidase level, 0.045 UmL^{-1}, was achieved by *A. zeae* EA0802 in SC using oat spelt xylan as carbon source (Table 1). When this strain was grown in SSF with corn straw or sugarcane bagasse, it was detected at 0.01 or 0.29 and 0.02 or 0.66 Ug^{-1}, respectively. When these carbon sources were used in SC, no significant values of α-arabinofuranosidase activity were detected (Table 1). *Acremonium* sp. EA0810 produced, in SSF with sugarcane bagasse, 0.01 or 0.21 Ug^{-1} of arabinofuranosidase activity and 0.01 UmL^{-1} in SC using oat spelt xylan (Table 2). These results suggest that *A. zeae* EA0802 is a more interesting α-arabinofuranosidase producer than *Acremonium* sp. EA0810. A *Thermomyces lanuginosus* strain produced maximum of 0.11 UmL^{-1} of α-arabinofuranosidase when grown on oat spelt xylan during 7 days [52], and *T. reesei* grown on sugar beet pulp showed an α-arabinofuranosidase activity of 0.02 UmL^{-1} [5].

β-Xylosidase

Both *Acremonium* species analyzed in this study were shown to be poor producers of β-xylosidase activity. β-Xylosidase activity from *A. zeae* EA0802 was 0.01 UmL^{-1} or 0.4 U g^{-1} when it was cultivated in SSF with corn straw and sugarcane bagasse (Table 1).

Acremonium sp. EA0810 did not produce any significant value of β-xylosidase activity in the cultures tested.

Activity Profile of the Main Enzymes Produced by Acremonium Cultures

The profile of FPase activity secreted by *Acremonium* sp. EA0810 cultivated in SC using sugarcane bagasse as carbon source revealed high FPase activity from the second cultivation day (Fig. 1). One activity peak corresponding to 0.55 UmL^{-1} was observed on fourth day, after which it slightly decreased. The second activity peak (0.53 UmL^{-1}) was detected on 12^{th} day, and this activity was maintained until 16^{th} day. Similarly, endoglucanase activity reached a peak corresponding to 0.15 UmL^{-1} on day 4, then the enzyme activity declined to zero and peaked again (0.14 UmL^{-1}) on day 9 (Fig. 1). The enzymatic preparation obtained on fourth cultivation day was chosen to analyze FPase and endoglucanase biochemical properties.

Xylanase activity production from the same fungal culture slightly increased during the first 8 days and quickly reached the maximum level of 2.46 UmL^{-1} on day 11 and maintained this level until the end of the cultivation (Fig. 1). The enzymatic preparation obtained from this last time period was utilized to biochemical characterization of the xylanase.

The β-glucosidase activity, secreted by *Acremonium* sp. EA0810 grown in SC using xylose, increased during the cultivation period and reached its maximum of 0.17 UmL^{-1} on 20^{th} day. However, from 14^{th} day, this activity was closer to maximum, and then the enzymatic preparation obtained in this time period was utilized for β-glucosidase characterization (Fig. 2).

The highest α-arabinofuranosidase and α-galactosidase activities were produced by *A. zeae* EA0802 in SC using oat spelt xylan as carbon source. α-Arabinofuranosidase activity

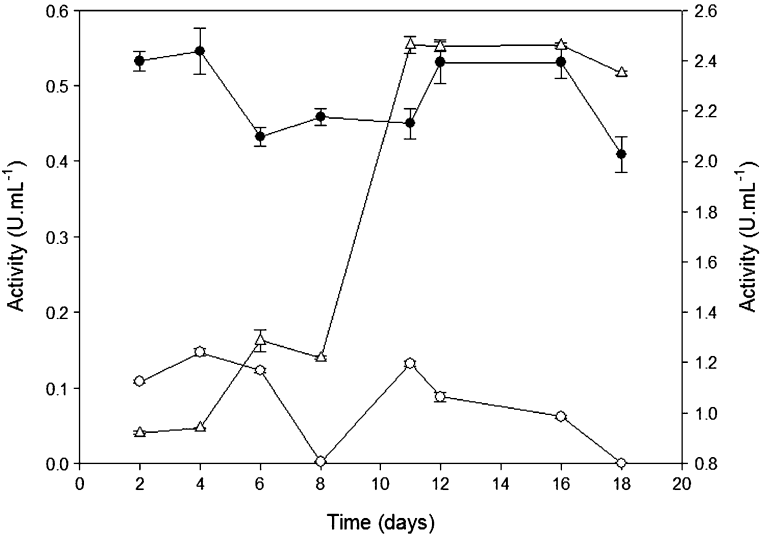

Fig. 1 (*Filled circle*) FPase, (*empty circle*) endoglucanase, and (*upright open triangle*) xylanase activities in the culture medium of *Acremonium* sp. EA0810 grown on SC with sugarcane bagasse as carbon source at 28 °C. *Left axis* corresponds to FPase and endoglucanase activities, and *right axis* correspond to xylanase activity. Units per milliliter units per milliliter of culture

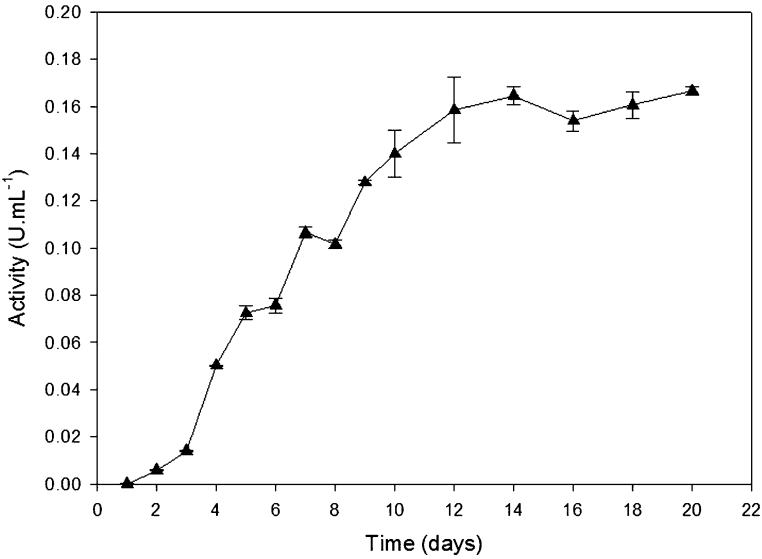

Fig. 2 (*Filled upright triangle*) β-Glucosidase activity in the culture medium of *Acremonium* sp. EA0810 grown on SC with xylose as carbon source at 28 °C. units per milliliter of culture

increased steadily during the cultivation time period and reached its peak value of 0.04 U mL^{-1} on 18th day (Fig. 3). *A. zeae* EA0802 produced, in the same liquid culture medium, α-galactosidase activity peak of 0.09 UmL^{-1} on day 4, then the level of enzyme activity declined slightly and was maintained until the end of cultivation (Fig. 3). Although 10 days

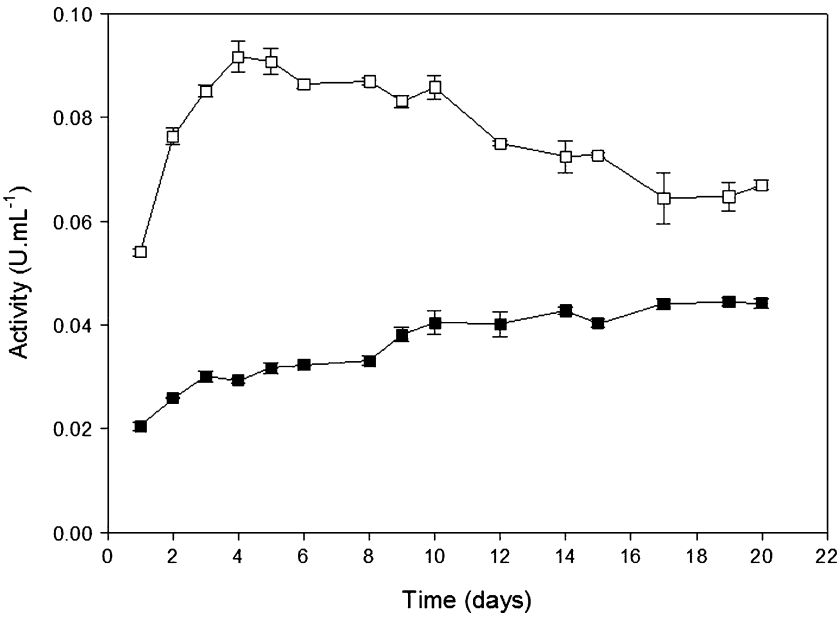

Fig. 3 (Open square) α-Galactosidase and (*closed square*) α-arabinofuranosidase activities in the culture medium of *A. zeae*. EA0802 grown on SC with oat spelt xylan as carbon source at 28 °C. Units per milliliter of culture

of growth is not the activity peak for α-arabinofuranosidase and α-galactosidase, it is a good time period to detect substantial activities of them together (Fig. 3).Then, the enzyme preparation obtained from *A. zeae* EA0802 grown for 10 days in SC using oat spelt xylan was used to characterize these enzymes.

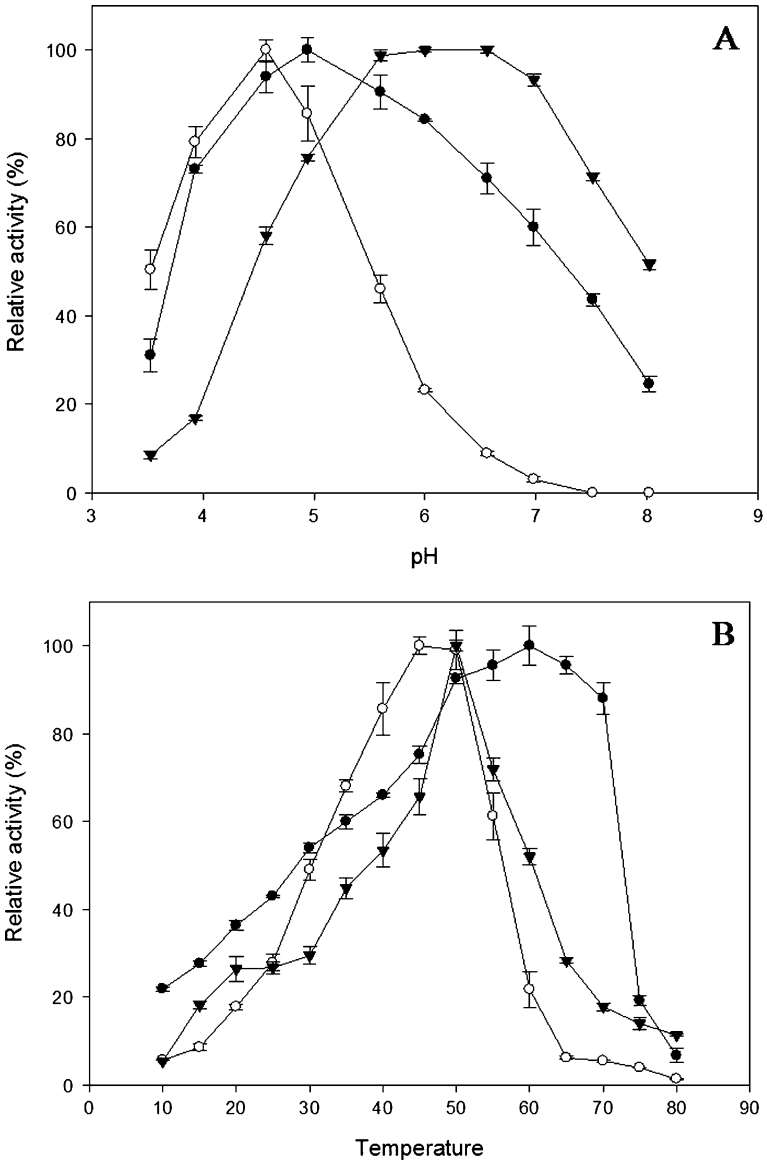

Fig. 4 Effects of pH (**a**) and temperature (**b**) on hemicellulolytic enzyme activities. (*Filled circle*) α-arabinofuranosidase activity; (*open circle*) α-galactosidase activity; (*inverted filled triangle*) xylanase activity. The highest enzyme activity value was considered 100%

Effect of pH and Temperature on Enzyme Activities

Substantial α-arabinofuranosidase activity was determined for the *A. zeae* EA0802 crude extract within the temperature range of 50–70 °C and at pH range of 4.5–6.0. Maximal substrate hydrolysis was achieved at 60 °C and pH 5.0 (Fig. 4a, b). These optimum pH and temperature values are close to those determined for α-arabinofuranosidases from *Aspergillus terreus* [53] and *Aureobasidium pullulans* [54]. Pre-incubation of the enzyme for 15 min at 60 °C promoted drastic loss in activity (Fig. 5). This result showed that α-arabinofuranosidase presented very poor thermostability at optimum temperature.

The highest α-galactosidase activity was observed at pH 4.5 and in temperature range of 45–50 °C (Fig. 4a, b). The enzyme acid character was similar to the other fungal α-galactosidases [55, 56]. The enzyme retained 99% and 36% of its original activity after pre-incubation at 45 °C for 30 and 150 min, respectively (Fig. 5). The half-life of this enzyme was 1 h 56 min at 45 °C.

Xylanase from *Acremonium sp.* EA0810 presented substantial activity at pH range of 5.0–7.5. Maximum activity was observed at pH range of 5.5–6.5 and at 50 °C (Fig. 4a, b). Similar results of pH and temperature were obtained for xylanase from *T. lanuginosus* [52] and *Aspergillus carneus* [57]. The enzyme retained more than 63% of its maximal activity after pre-incubation at optimum temperature for 30 min. The half-life of this enzyme at 50 °C was 45 min.

Substantial β-glucosidase activity was determined in the pH range of 4.0–7.0 and at 55–65 °C. Maximum activity was detected at pH 4.5 to 5.0 and 60 °C (Fig. 6a, b). After pre-incubation of the enzyme for 30 min at 60 °C, the enzyme activity was totally lost (Fig. 7). These optimum pH and temperature values are similar to those determined for the same enzyme of *Penicillium funiculosum* [58] and *A. persicinum* [45].

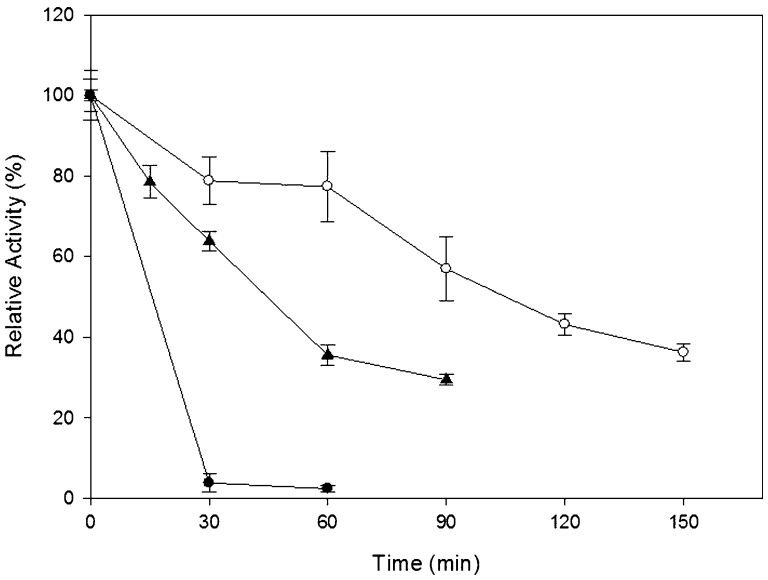

Fig. 5 Thermostability of the hemicellulolytic activities of *Acremonium* species. (*Open circle*) *A. zeae* EA0802 α-galactosidase (45 °C); (*Filled circle*) *A. zeae* EA0802 α-arabinofuranosidase (60 °C); (upright filled triangle) *Acremonium* sp. EA0810 xylanase (50 °C). The activity at 0 min of preincubation was considered to be 100%. The residual activity was determined using the standard assay

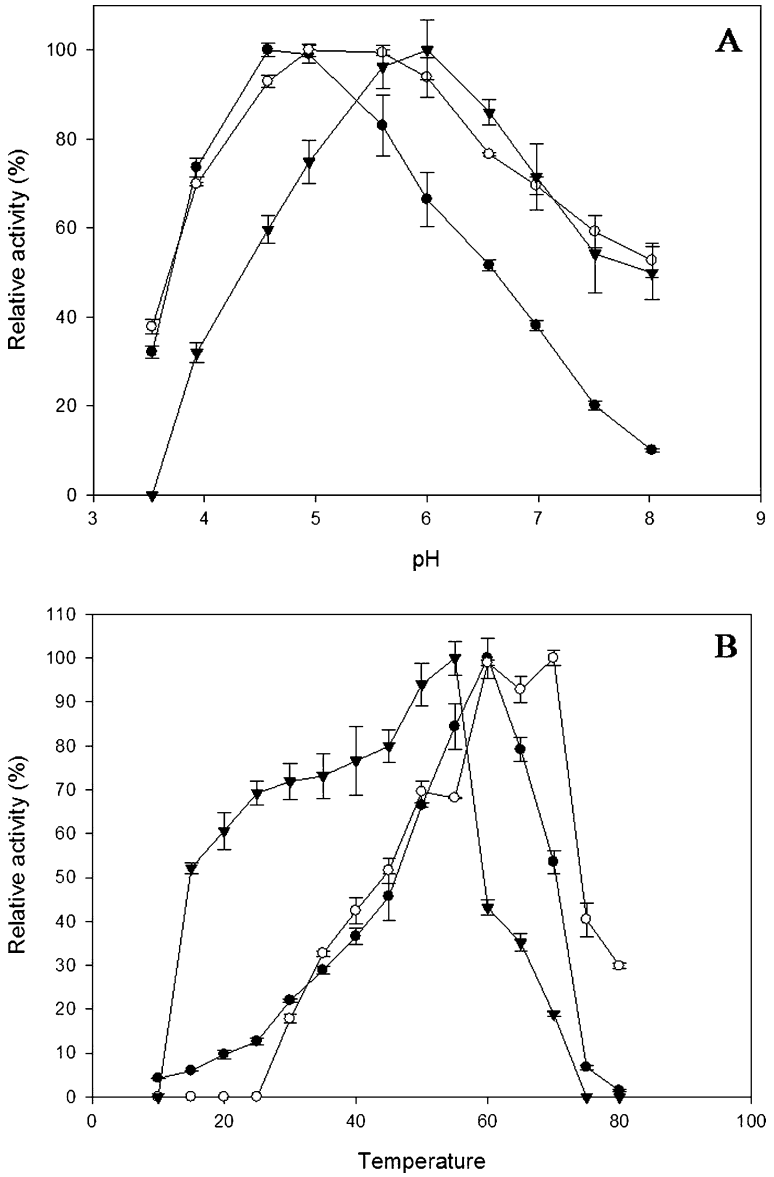

Fig. 6 Effects of pH (**a**) and temperature (**b**) on the cellulolytic activities. (*Filled circle*) β-glucosidase activity; (*open circle*) endoglucanase activity; (*inverted filled triangle*) FPase activity. The highest enzyme activity value was considered 100%

Endoglucanase displayed a wide pH range since it was highly active at pH 4.5 to 6.0 and maintained 37% and 52% of its activity at pH 3.5 and 8.0, respectively. The greatest activity was detected at pH 5.0 and at 70 °C (Fig. 6a, b). Thermostability studies of *Acremonium* sp. EA0810 endoglucanase showed that the enzyme was fairly stable at 70 °C. The enzyme retained more than 75% of its original activity after pre-incubation for 60 min at 70 °C (Fig. 7). The half-life of this enzyme at 70 °C was 1 h 43 min. Endoglucanase from

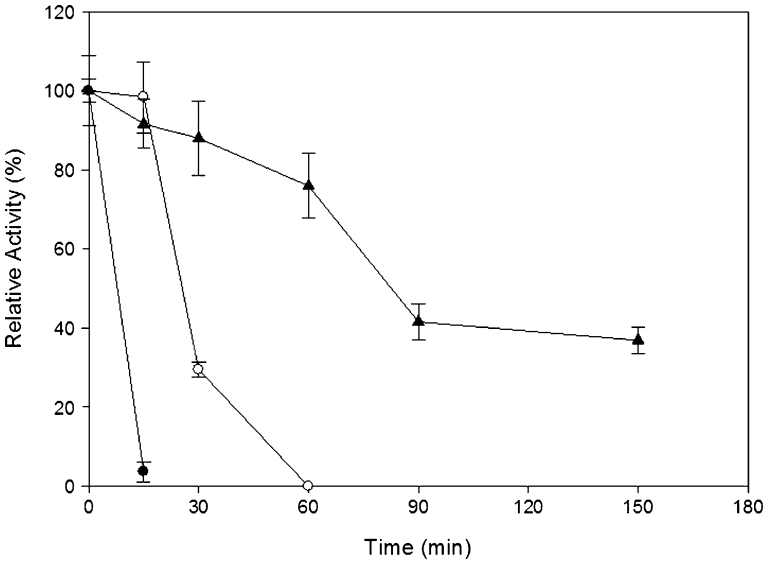

Fig. 7 Thermostability of *Acremonium* sp. EA0810 (*Filled circle*) β-glucosidase (60 °C); (Upright filled triangle) endoglucanase (70 °C); (*open square*) FPase (55 °C). The activity at 0 min of preincubation was considered to be 100%. The residual activity was determined using the standard assay

M. circinelloides and *Melanocarpus albomyces* exhibited a similar pH profile [8, 43]. Temperature optima observed is close to that determined for endoglucanase from *Daldinia eschscholzii*, however, *Acremonium* sp. EA0810 enzymes are shown to be more thermostable than endoglucanase from *D. eschscholzii* which was rapidly inactivated at 70 °C [59]. *Acremonium* sp. EA0810 endoglucanase presented a good thermostability and was active in a wide range of pHs. These results suggest that it has potential for industrial use, mainly in the biostoning process. In the textile industry, there is a need for novel cellulases that are active at neutral and alkaline pH values because they can promote low backstaining [8].

The greatest FPase activity was detected at pH 6.0 and 55 °C (Fig. 6a, b). The activity maintained relatively high at alkaline pH, retaining about 50% of its maximum activity at pH 8.0. This is in agreement with the value reported by Dutta et al. for *Penicillium citrinum* [60].

Although FPase optimum temperature was 55 °C, the enzyme exhibited more than 70% of its maximal activity between 30 °C and 40 °C. In the lignocellulose to ethanol conversion process, the use of simultaneous saccharification and fermentation process to minimize cellulase inhibition has been suggested. In simultaneous saccharification and fermentation process, biomass is hydrolyzed by cellulases and is simultaneously fermented by the microorganism. However, the optimum conditions for enzymes and fermenting microorganism are usually not the same. Cellulases have an optimum temperature around 50 °C whereas yeast have growth optima around 32–37 °C, then the operation of simultaneous saccharification and fermentation process is at suboptimal conditions for enzymatic hydrolysis [61]. In this way, an enzyme which shows higher activity at the temperature range between 32 °C and 37 °C is very interesting. Then, our result suggests that FPase from *Acremonium* sp. could be efficiently applied to simultaneous saccharification and fermentation process since it has high hydrolytic activity at lower temperatures.

Table 3 Sugar composition of sugarcane bagasse (percent dry weight)

Sample	Glucan (%)	Xilan (%)	Galactan (%)	Mannan (%)	Arabinan (%)	Soluble lignin (%)	Insoluble lignin (%)	Total lignin
Not treated	45.4	21.65	0.4	0.25	1.8	1.54	20.5	22.04
Treated	51.5	27.9	ND	ND	2.75	0.645	7.45	8.095

ND not detected

FPase showed to not be thermostable at its optimum temperature. A loss of 70% of initial activity was observed after pre-incubation at 55 °C for 30 min. The half-life of FPase was 30 min at 55 °C (Fig. 7).

Sugarcane Bagasse Saccharification

In an attempt to evaluate the performances of enzymatic complexes in biomass hydrolysis, the enzyme preparation from *Acremonium* sp. EA0810 grown in SC with sugarcane as carbon source was used to hydrolyze sugarcane bagasse. This enzymatic extract was chosen due to its high FPase and endoglucanase activities. Sugarcane bagasse was pretreated with NaOH. The sugar composition before and after the pretreatment is shown on Table 3.

The saccharification results showed that, after 15 h, a conversion of 40% of reducing sugar was already detected, and after 110 h, a biomass conversion of 63% was achieved. The release of glucose was slower than release of reducing sugar, and just after 110 h, 43% of conversion was achieved (Fig. 8). This result may be explained due to the high xylanase activity presented in the enzymatic preparation, since this enzyme acts by releasing xylose and other reducing sugars.

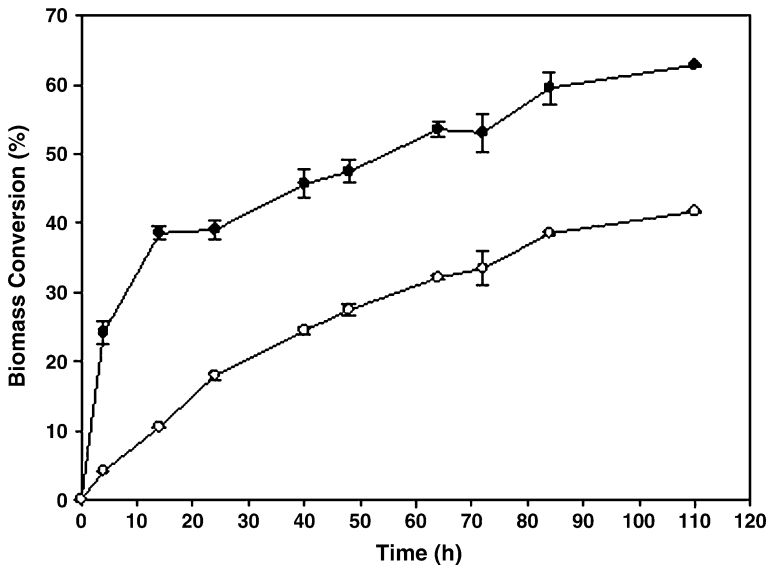

Fig. 8 Sugarcane bagasse saccharification using *Acremonium* sp. EA0810 enzymatic preparation. (*Filled circle*) Reducing sugar (*Open circle*) glucose

Conclusion

In this study, two endophytic *Acremonium* species were analyzed in relation to their ability to produce cellulases and hemicellulases. The enzyme production by endophyte microorganisms in nature could have a role in their mode of action to penetrate in plants and in their capacity to propagate [62]. Endophytes that produce both cellulases and hemicellulases should have the ability to compete with other types of fungi surviving on seeds, dead wood, and leaves [37]. *A. zeae* EA0802 and *Acremonium* sp. EA0810 showed to have efficient cellulase and hemicellulase machineries, and more specific studies are being performed to optimize their growth condition maximizing enzyme production to industrial application.

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