Acta Scientiarum

http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v40i1.41512

BIOTECHNOLOGY

Filamentous fungi producing enzymes under fermentation in cassava liquid waste

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ABSTRACT. The conversion of agroindustrial residues by microorganisms has been explored from fermentative processes to obtain several bioactive molecules. The objective of this work was to isolate and select filamentous fungi present in cassava liquid waste for the production of amylase, carboxymethylcellulose (CMCase), pectinase and xylanase using the same residue as induction substrate in fermentative processes. A total of 65 filamentous fungi were isolated and qualitative tests indicated that approximately 86% of these strains were able to produce at least one of the enzymes and 32% capable of producing the four enzymes. Fermentation assays in cassava liquid residue-containing medium showed 6 fungal lines as potential enzyme producers. The maximum activities of pectinase, xylanase, amylase and CMCase were respectively observed at 96 hours of fermentation by the strain by the strain *Aspergillus* sp. B5C; at 120 hours (163.6 ± 0.13 nKat mL^{-1}), by *Aspergillus* sp. B4I; at 144 hours (99.8 ± 0.24 nKat mL^{-1}), by *Penicillium* sp. B3A; and at 48 hours (55.5 ± 0.21 nKat mL^{-1}), by *Aspergillus* sp. B4O. These results suggest that cassava liquid waste was source of filamentous fungi producing amylase, CMCase, pectinase and xylanase, as well as a promising alternative substrate for bioprocesses aiming the production of enzymes.

Keywords: enzymatic activity; bioprocess; agroindustrial residues.

Fungos filamentosos produtores de enzimas sob fermentação em manipueira

RESUMO. A conversão de resíduos agroindustriais por micro-organismos tem sido explorada a partir de processos fermentativos para obtenção de diversas moléculas bioativas. O objetivo deste trabalho foi isolar e selecionar fungos filamentosos presentes em manipueira para produção de amilase, carboximetilcelulase (CMCase), pectinase e xilanase utilizando o próprio resíduo como substrato indutor. Um total de 65 fungos filamentosos foi isolado e testes qualitativos indicaram que, aproximadamente, 86% dessas linhagens foram hábeis em produzir pelo menos uma das enzimas e 32% capazes de produzir as quatro enzimas. Ensaios fermentativos em meio contendo manipueira apontaram 6 linhagens fúngicas como potenciais produtoras de enzimas. As atividades máximas de pectinase, xilanase, amilase e CMCase foram observadas, respectivamente, às 96 horas de fermentação (67.4 ± 0,6 nKat mL^{-1}), pela linhagem *Aspergillus* sp. B5C; às 120 horas (163.6 ± 0,13 nKat mL^{-1}), por A*spergillus* sp. B4I; às 144 horas (99.8 ± 0,24 nKat mL^{-1}), por *Penicillium* sp. B3A; e às 48 horas (55.5 ± 0,21 nKat mL^{-1}), por *Aspergillus* sp. B4O. Estes resultados sugerem a manipueira como fonte de fungos filamentosos produtores de amilase, CMCase, pectinase e xilanase, além de um promissor substrato alternativo para bioprocessos visando a produção dessas enzimas.

Palavras-chave: atividade enzimática; bioprocesso; resíduos agroindustriais.

Introduction

Agroindustrial residues are secondary products of industrial and economical activities and stand out as potential raw materials for the generation of high value-added products such as microbial proteins, organic acids, ethanol, enzymes and biologically active secondary metabolites (Alexandrino, de Faria, de Souza, & Peralta, 2007; Ferreira-Leitão et al., 2010; Oliveira, Vendruscolo, Costa, & Araújo, 2016). Advances in industrial biotechnology have offered opportunities for the economic use of agroindustrial residues, and prevents its accumulation, which is of great environmental concern due to its potential for contamination of soils, rivers and underground water (Stroparo, Beitel, de Resende, & Knob, 2012).

The production of enzymes from the microbial culture using lignocellulosic agroindustrial residues (sugarcane bagasse, rice straw, wheat straw, oat bark

and wood chips) as substrate is well documented in the literature. Approximately 70% of the dry matter of these wastes consist of carbohydrates (cellulose, hemicellulose and lignin) that can be converted into sugars by the action of microbial enzymes. Among the bacteria, the species of the genus *Bacillus* are one of the most investigated groups regarding the production of enzymes, especially amylase, under cultivation in agroindustrial residues as substrate (Corrêa, Moutinho, Martins, & Martins, 2011; Barros, Simiqueli, de Andrade, & Pastore, 2013). However, the main biological sources of enzymes are filamentous fungi, especially the genus *Aspergillus* for which was reported the production of cellulase, pectinase, amylase, lipase and others enzymes under culture in many different agroindustrial residues, such as wheat bran, paddy straw, sugarcane bagasse, manioc peel and coffee husk (Cruz et al., 2011; Gusmão, Ferraz, Rêgo, Assis, & Leal, 2014; Kanimozhi & Nagalakshmi, 2014).

However, peculiarities of the composition of some agroindustrial residues can compromise the microbial activity and consequently the conversion of these materials by the enzymes action. In this way, the cassava liquid residue (manipueira), generated by the processing of cassava for the production of flour, presents in its composition a glycoside (linamarin) potentially hydrolyzable to hydrocyanic acid (Barana & Cereda, 2000). In such cases, there is a need to employ appropriate and metabolically adapted microbial strains to cover the residue in the product of interest, involving the isolation and selection of microorganism from the most diverse environments, including agroindustrial residues (Celestino et al., 2014).

In Brazil, the northeast region is the main producer of cassava, and have hundreds of flour houses dedicated to the production of cassava flour low volumes (*Serviço Brasileiro de Apoio às Micro e Pequenas Empresas* [Sebrae], 2012). Considering that, in the production of cassava flour, an average of 300 liters of manipueira per ton of processed root is generated, with about 50 g L^{-1} of COD (Chemical Oxygen Demand) and 140 ppm of hydrocyanic acid (Chuzel, 2001), the region Northeast is also largest generator of this waste, in Brazil. In order to minimize the environmental impacts resulting from the inappropriate disposal of cassava liquid waste in soils and water sources, studies have been developed to reuse this residue, including the use of this residue as a carbon source for microbial cultivation and obtention of biotechnological products of interest: Leonel and Cereda (1995) verified the biosynthesis of citric acid by *Aspergillus niger*; Barros, de Quadros, and Pastore (2008) reported biosurfactant production by *Bacillus subtilis* LB5a; Oliveira et al. (2013) used cassava liquid waste as source of carbon for the production of 2-phenylethanol by *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Geotrichum fragrans*.

The high organic load found in manipueira also makes it interesting as source of carbon for the cultivation of microorganisms producing enzymes of industrial interest (Teixeira et al., 2017). Amylase, carboxymethylcellulose (CMCase), pectinase and xylanase are examples of microbial hydrolytic enzymes utilized in several applications, such as food, textile, paper, pulp and industrial detergents, that can be obtained in the saccharification of agricultural residues (El-Shishtawy et al., 2014).

In this sense, and knowing that microorganisms used in enzyme production tests can be obtained from natural sources, in which, the desired enzymes must act, the objective of this work was to isolate and select filamentous fungi from cassava liquid waste for the production of hemicellulases and amylases using the same residue as induction substrate, and adding value to the secondary products of agroindustry.

Material and methods

Samples of fresh cassava wastewaterfrom the first cassava pressing were collected in flour production houses located in the municipality of Vitória da Conquista/BA (14º51'58"S; 40º50'22"W). The sample used as substrate for fermentation, composed of eight sub-samples of cassava liquid residue, was characterized for pH, phosphorus, potassium, calcium, magnesium, sulfur, copper, iron, zinc, sodium, aluminum, nitrogen and free cyanide (*Empresa Brasileira de Pesquisa Agropecuária* [EMBRAPA] 2009), as shown in Table 1.

Table 1. Chemical characterization of the composite sample of cassava wastewater obtained from sub samples collected in flour houses, in the micro-region of Vitória da Conquista – BA.

pH	P	K	Ca	Mg	S	Cu	Fe	Zn	Na	Al	N	CN
	mg L^{-1}											
4.7	198.1	302.7	9.27	30.17	22.7	1.2	3.6	3.5	44.25	81.64	370	186.82

The isolation of filamentous fungi from the cassava liquid waste was carried out from serial dilutions (10 -1 to 10 -6) using saline solution 0.85%, followed by the surface plating method, in triplicate, using a Dextrose Potato Agar culture medium (pH 5.6 ± 0.2) plus Tetracycline (1%). Plates were maintained at 30°C, for four days, and the isolates obtained were transferred to tubes containing Dextrose Potato Agar culture medium (pH 5,6 ± 0,2) plus Tetracycline (1%), in order to obtain pure cultures. The Castellani method was used for the preservation and maintenance of fungal cultures.

Pure cultures of fungal strains were submitted to qualitative tests in triplicate to determine the enzymatic potential, using solid culture media in Petri plates (0.5 g L^{-1} of $MgSO_4$; 3.0 g L^{-1} of $NaNO_3$; 1.0 g L^{-1} of KH_2PO_4; 0.5 g L^{-1} of KCl; 0.01 g L^{-1} of Fe_2SO_4; 15 g L^{-1} of Agar) supplemented with starch, carboxymethyl cellulose, pectin or xylan for induction of the bioproduction of amylases, CMCases, pectinases and xylanases enzymes, respectively (Griebeler et al., 2015). After four days of incubation at 25°C, the formation of translucent halo around the microbial colony was evaluated, indicating the degradation of the specific substrates contained in the culture media. Halos with diameter above 3 mm were considered positive for enzymatic activity.

From the qualitative tests, the selected strains were cultivated on Petri plates containing Dextrose Potato Agar culture medium (pH 5.6 ± 0.2) plus Tetracycline (1%), at 30°C, for seven days. 5 mm diameter discs containing fungal mycelium were removed from each plate and transferred individually into Erlenmeyers flasks of 250 mL, containing 100 mL of autoclaved cassava wastewater sample, then maintained at incubator, with orbital agitation of 130 rpm, at 38°C, for six days.

Aliquots of 1 mL of crude enzyme extract from the fermentation assays were removed at 24 hours culture intervals and added to 9 mL of distilled water. The mixture was filtered and centrifuged at 5,000 rpm for 15 minutes to obtain a clear crude extract. The obtained supernatants were used as crude extract of the enzymes to determine the enzymatic activities.

The activities of amylase, CMCase, pectinase and xylanase were determined by mixing 100 µL of the enzymatic substrate to 1% p/v (starch, carboxymethylcellulose, pectin or xylan, respectively) with 50 µL of the crude extract and incubated at 50°C in a water bath for 30 minutes (Siqueira, de Siqueira, Jaramillo, & Filho, 2010). After incubation, 300 µL of dinitrosalicylic acid solution (DNS) was added for reaction for 10 minutes in a water bath at 98°C. The reaction was stopped by the addition of 1.5 mL distilled water and the absorbance reading was done in a spectrophotometer at 540 nm (Miller, 1959).

The activity assays of all the enzymes were conducted in triplicate, with the preparation of each reaction control: 1) Replacement of crude enzymatic extract with distilled water; 2) Replacement of the substrate with distilled water. The enzyme activities unit (nKat mL^{-1}) was defined as the amount of enzyme capable of releasing 1 µmol of product (reducing sugars) per second per gram of sample (polysaccharides) under the reaction conditions using as the standard curve the glucose monomers corresponding to the enzymes.

The averages of enzymatic activities obtained at the end of the fermentation process for each fungal line were submitted to analysis of variance (ANOVA), and differences between the averages were analyzed by the Scott-Knott test, at 1% probability, using the software Assistat. Correlation between the fermentation time and enzyme activity were evaluated using the Pearson coefficient.

Fungal lines with higher yields for enzyme production were identified at the genus level, based on the macroscopic morphological analysis of the colonies and on the study of reproductive structures of the lines using optical microscopy (Seifert, Morgan-Jones, Gams, Kendrick, 2011).

Results and discussion

A total of 65 strains of filamentous fungi were isolated from cassava liquid waste. Qualitative tests on the enzyme production potential showed that 53.84% of the lines produced CMCase, 78.46% amylase, 66.15% xylanase, 73.84% pectinase and 13.84% did not produce any of the enzymes (Table 2). The occurrence frequency of enzyme-producing fungi verified in this study was higher or similar than found by other authors using substrates diverse as source of microorganisms: Saleem and Ebrahim (2014) verified that, among the 46 fungal species isolated in legume seeds, eight isolates (17.4% of all isolates) had amylase activity; Simões, Tauk-Tornisielo, Tapia (2009) reported that 76% of total fungi isolated from Caatinga soil presented xylanase activity; Guimarães et al. (2006) verified that 40% of isolate filamentous fungi from soil and humus, plants and sugar cane bagasse presented pectinolytic activity.

Table 2. Fungal lines isolated from cassava wastewater that showed presence (+) or absence (-) of indicative halos to activity of the enzymes CMCase, amylase, xylanase and pectinase. Halos with diameter above 3 mm were considered positive for enzymatic activity

Fungal Strains	CMCase	Amylase	Xylanase	Pectinase
FUNB3B	+	+	-	+
FUNB3H	-	+	+	+
FUNB3A*	+	+	+	+
FUNB3C	-	+	+	+
FUNB40*	+	+	+	+
FUNB4D*	+	+	+	+
FUNB4I*	+	+	+	+
FUNB4O*	+	+	+	+
FUNB4P	-	-	-	-
FUNB4N	-	+	-	+
FUNB4E	-	+	+	+
FUNB6M	-	+		+
FUNB6L	+	+	+	-
FUNB6F	-	-	-	+
FUNB6I	+	+	-	+
FUNB60*	+	+	+	+
FUNB6Y		+	+	+
FUNB6C	+	+	+	-
FUNB6G	+	+	+	-
FUNB3D	-	+	+	-
FUNB2J*	+	+	+	+
FUNB5I*	+	+	+	+
FUNB5L*	+	+	+	+
FUNB5G	+	+	-	+
FUNB5H	-	-	-	-
FUNB8B	-	-	-	-
FUNB8E	-	-	-	-
FUNB8C	-	+	+	+
FUNB8G	-	-	-	-
FUNB6D	-	+	+	+
FUNB6Q	-	-	-	-
FUNB6N*	+	+	+	+
FUNB6J	-	+	+	+
FUNB2H*	+	+	+	+
FUNB2F*	+	+	+	+
FUNB2E	+		+	+
FUNB2B*	+	+	+	+
FUNB8J	-	+	-	+
FUNB8I	-	+	-	+
FUNB8H	-	-	-	-
FUNB8B	-	-	-	-
FUNB2C*	+	+	+	+
FUNB7X	-	+	+	+
FUNB7A	-	+	+	+
FUNB6P	+	+	+	-
FUNB2D	-	+	-	+
FUNB2J	+		+	+
FUNB2G	-	+	+	+
FUNB2I	-	+	-	+
FUNB2A2	+	+	-	+
FUNB2L1*	+	+	+	+
FUNB2L2	-	-	+	+
FUNB6A1*	+	+	+	+
FUNB4L*	+	+	+	+
FUNB4F*	+	+	+	+
FUNB4G	-	-	-	-
FUNB4J	+	+	+	-
FUNB5C*	+	+	+	+
FUNB5B*	+	+	+	+
FUNB5D	-	+	-	+
FUNB5F	-	+	-	+
FUNB4A	-	+	+	+
FUNB4B	+	+	+	-
FUNB4C	+	+	+	-
FUNB4H*	+	+	+	+
Total	35	51	43	48

*Fungal strains showing activity for all enzymes.

The importance of isolating fungi with a broad enzymatic spectrum is due to the possibility that a single microorganism is sufficient to hydrolyse different substrates of complex composition (Stroparo et al., 2012). In this study, approximately 32% of the fungal strains were able to produce all the enzymes evaluated and therefore, selected for the fermentative trials containing cassava liquid residue as source of carbon and nitrogen.

Unlike the qualitative tests, only three of these strains (FUNB40, FUNB4I and FUNB4O) showed activity for all the enzymes under cassava wastewater fermentation (Figure 1). The pectinolytic activity at the end of 144 hours of fermentation was recorded for all strains and the averages varied between 3.8 and 26.73 nKat mL^{-1} ($p < 0.01$). The average activity of xylanases, amylases and CMCase, in medium containing cassava wastewater as substrate varied ($p < 0.01$), respectively, from 1.05 to 67.96 nKat mL^{-1}, between 13 strains; 5.91 to 32.46 nKat mL^{-1}, between 6 strains; and 2.11 to 21.41 nKat mL^{-1} between 4 strains, respectively (Figure 1).

The majority of the studies on filamentous fungi enzyme production using agroindustrial residues as substrate indicate solid state fermentation (SSF) as more advantageous compared to submerged fermentation (Hölker and Lenz, 2005; Hansen, Lübeckb, Frisvada, Lübeckb, & Andersena, 2015). However, the use of cassava wastewateras substrate in submerged fermentation to produce enzymes by filamentous fungi, provided results similar or even higher than those reported by other studies that used solid state fermentation in various types of agroindustrial residues. Stroparo et al. (2012) observed maximum activity of pectin esterase (polygalacturonase) by *P. verruculosum* and xylanase by *A. niger* J4 equal to 20.50 and 38.6 nKat g^{-1}, respectively, under SSF in wheat bran. Cunha, dos Santos, Assis, and Leal (2016) verified maximum activity of amylase and CMCase equal to 3.3 and 7.0 nKat g^{-1}, respectively, by *Penicillium* spp. LEMI A8221, under SSF in soybean crop residues.

At the end of 144 hours of cassava wastewater fermentation, the best averages of enzymatic activity in relation to the others ($p < 0.01$) were identified at the genus level, revealing that 5 of them belonged to the genus *Aspergillus* sp. (FUNB5C, FUNB40, FUNB2J; FUNBB4I, FUNB4O) and 1 to the genus *Penicillium* sp. (FUNB3A). This confirms the genera *Aspergillus* and *Penicillium* as potential producers of enzymes under fermentative processes in agroindustrial residues, as has been reported in the literature (Hansen et al., 2015).

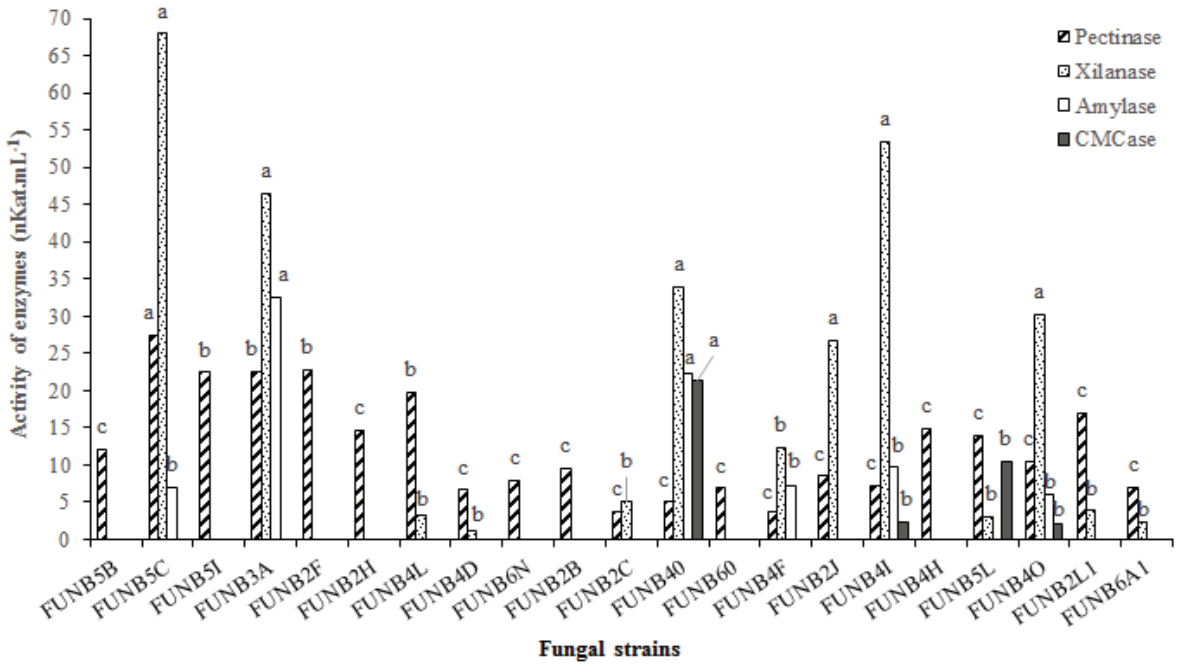

Figure 1. Average activity of the pectinase, xylanase, amylase and CMCase enzymes produced by fungal lines in fermentative trials using cassava wastewater as substrate, at the end of 144 hours of fermentation. Equal bars with different letters differ significantly by the Scott-Knott test ($p < 0.01$).

The maximum activity of pectinase, xylanase, amylase and CMCase was observed at 96 hours of fermentation by the strain *Aspergillus* sp. B5C (67.4 ± 0.6 nKat mL^{-1}); at 120 hours (163.6 ± 0.13 nKat mL^{-1}), by the strain *Aspergillus* sp. B4I; at 144 hours (998 ± 0.24 nKat mL^{-1}), by the strain *Penicillium* sp. B3A; and at 48 hours (55.5 ± 0.21 nKat mL^{-1}), by the strain *Aspergillus* sp. B4O, respectively (Figure 2).

From these results, it can be considered that the maximal enzyme activity occurred at satisfactory fermentation times and consistent with other studies. Giese, Dekker, and Barbosa (2008) verified maximum pectinolytic activity (approximately 500 nKat mL^{-1}) after 72 hours of fermentation in orange bagasse by *Botryosphaeria rhodina*. Kronbauer, Peralta, Osaku, and Kadowaki (2007) found values for maximum xylanase activity (30 nKat mg^{-1}) at 96 hours of fermentation using corn husk as substrate and *Aspergillus casielus* as a biological agent. Gusmão et al. (2014) used coffee husks as substrate for fermentation and found maximum amylase activity by *Aspergillus* spp. (178.37 nKat mL^{-1}) after 12 days of incubation. Santos, de Melo, do Bonfim, de Barros, and Santos (2015) when using banana leaves as a substrate for fermentation by *Trichoderma reesei*, it was obtained maximum CMCase activity (47.66 nKat mL^{-1}) after 96 hours.

The production of pectinase by *Aspergillus* sp. B5C, from xylanase by *Penicillium* sp. B3A and *Aspergillus* sp. B40, and CMCase by *Aspergillus* sp. B40 presented a weak positive correlation in relation to the fermentation time ($r < 0.4$) (Table 3). That is, the increase in fermentation time had little influence on the pectinolytic activity of these strains. On the other hand, the production of xylanase by *Aspergillus* sp. B5C, *Aspergillus* sp. B4I and *Aspergillus* sp. B4O, and amylase by *Penicillium* sp. B3A showed a high positive correlation with the fermentation time ($r > 0.8$), indicating that the activity of these enzymes raised as the fermentation time increased ($p<0,05$).

Finally, the production of xylanase by *Aspergillus* sp. B2J and *Aspergillus* sp. B40 showed a negative correlation ($r < 0$), that is, the activity of the enzyme decreased with increasing fermentation time (Table 3). According to Whitaker (1994), high values of enzymatic activity in the first hours of fermentation can be explained by the low availability of reducing sugars of the raw material, necessary for the development of the microorganism. This low availability stimulates the mechanism of expression of the enzymes needed to generate simple sugars. In addition, excessive fermentation time can lead to nutritional depletion of the medium, implying the reduction of microbial growth, making the conversion of biomass into products unviable and,

consequently, reducing the production of enzymes (Santos, Muruci, Damaso, Silva, & Santos, 2014).

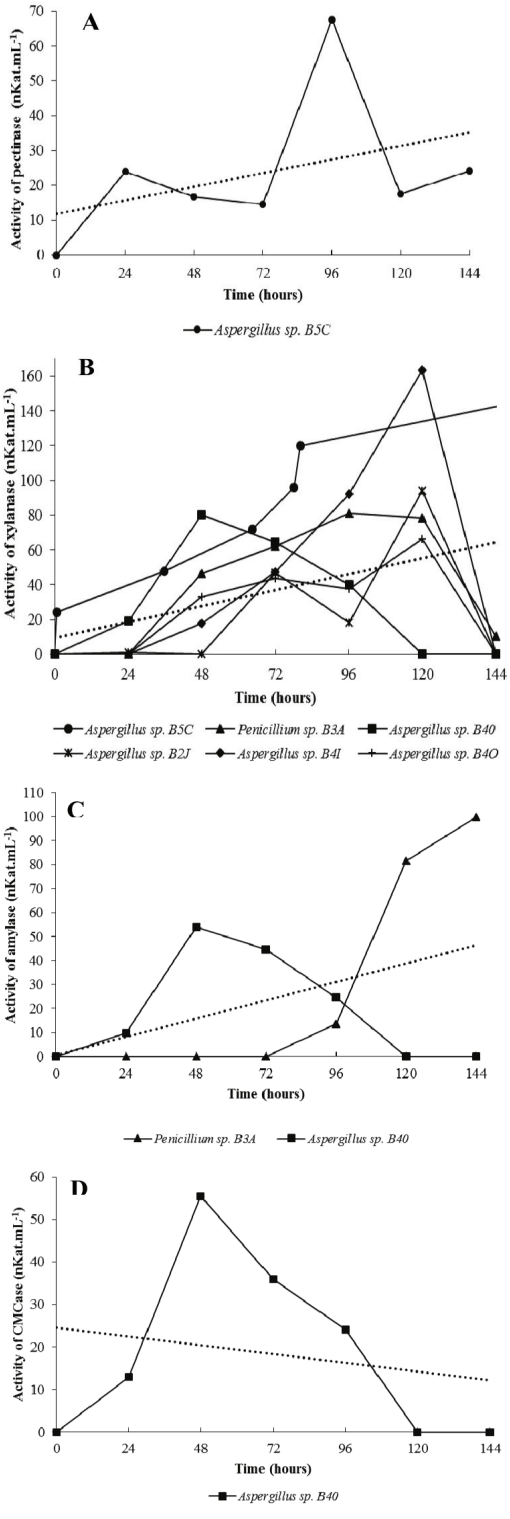

Figure 2. Activity of pectinase (A), xylanase (B), amylase (C) and CMCase (D) in function of the fermentation time (hours) in cassava wastewater by potential enzyme producing fungal strains. Dotted line represents the trend line of the enzymatic activity in relation to the fermentation time.

Table 3. Pearson correlation between fermentation time and enzymatic activity presented by fungal strains producing pectinase, xylanase, amylase and CMCase in medium containing cassava wastewater the sole source of carbon.

Fungal Strains	Pearson correlation (r)			
	Pectinase	Xylanase	Amylase	CMCase
Aspergillus sp. B5C	0.40	0.95**	SA	SA
Penicillium sp. B3A	SA*	0.32	0.84**	SA
Aspergillus sp. B40	SA	0.40	- 0.56	0.20
Aspergillus sp. B2J	SA	- 0.12	SA	SA
Aspergillus sp. B4I	SA	0.89**	SA	SA
Aspergillus sp. B4O	SA	0.88**	SA	SA

* SA = without enzymatic activity. ** $p<0,05$

Conclusion

The cassava wastewater was a source of filamentous fungi producing enzymes and appeared as a promising alternative of substrate for fermentative processes aiming to obtain pectinase, xylanase, amylase and CMCase, especially by the *Aspergillus* sp. B5C, B40 and B4I strains.

The maximum fermentation time of 144 hours was sufficient for all strains to efficiently convert substrate into product.

Acknowledgements

The authors thanks to the *Universidade Federal da Bahia* (UFBA) and *Fundação de Amparo à Pesquisa do Estado da Bahia* (FAPESB) for funding grants and fellowships.

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Received on January 30, 2018.
Accepted on June 12, 2018.