Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.scielo.br/bjce

Vol. 34, No. 03, pp. 691 – 700, July – September, 2017
dx.doi.org/10.1590/0104-6632.20170343s20150822

USE OF ELEPHANT GRASS (*Pennisetum purpureum*) AS SUBSTRATE FOR CELLULASE AND XYLANASE PRODUCTION IN SOLID-STATE CULTIVATION BY *Penicillium echinulatum*

D. Menegol[1,*], A.L. Scholl[1], A.J.P. Dillon[1] and M. Camassola[1]

[1]University of Caxias do Sul, Institute of Biotechnology
Rua Francisco Getúlio Vargas 1130, 95070-560 Caxias do Sul, RS, Brazil
Phone/fax: 55 54 3218 2149

*Corresponding author: Email: dmenegol@ucs.br

(Submitted: December 26, 2015; Revised: February 29, 2016; Accepted: April 7, 2016)

Abstract - The high cost of the enzymes used for the hydrolysis of lignocellulosic biomass is one of the bottlenecks in the production of second-generation ethanol and the use of local biomass, such elephant grass, can help to reduce this cost. In this investigation elephant grass biomass was evaluated as a carbon and inducer source of production of cellulases and xylanases by the fungus *Penicillium echinulatum* strain 9A02S1 in solid-state cultivation. The highest filter paper activity (13.26 $U.g^{-1}$ dry matter) and β-glucosidase activity (138.34 $U.g^{-1}$ dry matter) were obtained on the fifth day of cultivation, in medium containing biomass pre-treated with sulfuric acid and untreated, respectively. The highest endoglucanase activity was 158.44 $U.g^{-1}$ dry matter in the presence of elephant grass pre-treated with sulfuric acid. The xylanase activity was highest with medium that was formulated with 75% untreated elephant grass and 25% wheat bran (372.62 $U.g^{-1}$ dry matter). In conclusion, the results showed that it is possible to obtain large amounts of cellulases and xylanases using a cosmopolitan and very low cost substrate such as elephant grass.

Keywords: cellulases; xylanases; elephant grass; pre-treatment; solid-state cultivation.

INTRODUCTION

Ethanol is mostly produced from sugar and starch feedstocks; however, the interest in using biomass and bioenergy has been initiated due to the recognition that the global crude oil reserve is finite, and its depletion is occurring much faster than previously predicted (Bai et al., 2008; Macedo et al., 2013; Scholl et al., 2015a). Lignocellulosic materials have a high potential for ethanol production, do not compete with food production, could lead to the use of large quantities of agroindustrial wastes and could use marginal or degraded agricultural lands for growing energy crops (Eliana et al., 2014; Idrees et al., 2014).

In this context, the species *Pennisetum purpureum*, known as elephant grass, has been considered a new alternative for energy crops, and this species is expected to provide abundant and sustainable resources for biofuel production (Scholl et al., 2015a; Xie et al., 2011). Another interesting aspect of elephant grass is its productivity, which results in the production of 45 tons of dry mass per hectare per year. The production of sugar cane and corn is approximately 22 tons (sugar and bagasse) and 13 tons (grain and stover), respectively (Scholl et al., 2015a; Somerville et al., 2010).

* To whom correspondence should be addressed

For ethanol production, an enzymatic hydrolysis step is required; moreover, the crucial step in the lignocellulosic bioconversion to biofuels is the cost-effective maximisation of cellulose and hemicellulose saccharification to fermentable sugars. A continuing challenge is the high cost of the enzymes involved in saccharification of the cellulosic components (Ogeda & Petri, 2010). Hemicellulose components are known to exert a significant influence on the effectiveness of the enzymatic hydrolysis of the cellulosic components (Várnai et al., 2010). In this context, several studies have suggested cocktails for the effective enzymatic degradation of lignocellulosics (Banerjee et al., 2010). Enzymes are produced by various microorganisms and mixtures. However the enzymes that are required in higher amount are cellulase and xylanase, since cellulose, the principal constituent of plant biomass, can be hydrolyzed by the action of endogluconases, cellobiohydrolases and β-glucosidases. Composed mainly of D-xylose, xylan is the most common hemicellulose and it is the second most abundant biopolymer found in nature. Xylanase is the major component of a group of enzymes, and acts by depolymerizing the xylan molecules into monomers (Padilha et al., 2015; Reis et al., 2015). Because of the complex composition of elephant grass, several enzymes are required for the enzymatic hydrolysis of the biomass to allow the release of reducing sugars.

Among the many microorganisms that produce both cellulases and xylanases are the mutant strains of *Penicillium echinulatum* (Dillon et al., 2006). These mutants produce a cellulolytic enzyme complex that is stable at 50°C, which is the relevant condition for the application of these enzymes for enzymatic hydrolysis (Camassola et al., 2004). These mutants also contain β-glucosidase activity that is present in a greater proportion than in *Trichoderma reesei* (Martins et al., 2008).

Moreover, the conduction of enzyme production is important. Solid-state cultivation is a process in which an insoluble substrate is fermented with sufficient moisture but without free water (Camassola & Dillon, 2007). The use of solid-state cultivation as an enzyme production method may offer economic and engineering advantages over the classical submerged cultivation method. These advantages include a high concentration of the product, the use of simple cultivation equipment, low effluent generation and limited requirements for aeration and agitation during enzyme production (Camassola & Dillon, 2010; Viniegra-González et al., 2003). This method also employs agricultural residues *in natura*, which helps to prevent environmental impacts that are caused by the accumulation of these residues (Camassola & Dillon, 2010).

Pre-treatments aim to disrupt the lignocellulosic matrix, reducing the amount of or modifying the structure of lignin and hemicellulose, and changing the crystalline structure of cellulose to make it more susceptible to enzymatic attack (Guilherme et al., 2015). These modifications also contribute to the development of microorganisms favoring the production of enzymes (Scholl et al., 2015b). Pre-treatments with acids, such as sulfuric, nitric or hydrochloric can solubilize hemicelluloses, exposing the cellulose to enzymatic attack, while alkaline pretreatments remove lignin and reduce the degree of cellulose crystallinity (Guilherme et al., 2015).

In this context, the objective of this study was to evaluate the production of cellulases and xylanases by *P. echinulatum* through solid-state cultivation using low cost biomass, untreated or pre-treated elephant grass. These enzymatic complexes are required for the hydrolysis of lignocellulosic materials with the aim of ethanol production.

MATERIALS AND METHODS

Microorganism

The cellulolytic mutant *P. echinulatum* 9A02S1 (DMS 18942) was used in this study. This strain was obtained by exposing the wild type *P. echinulatum* 2HH to ultraviolet light (UV) and hydrogen peroxide (H_2O_2) (Dillon et al., 2006). The strain is stored in the culture collection of the Enzymes and Biomass Laboratory at the Institute of Biotechnology in Caxias do Sul, Rio Grande do Sul, Brazil. The strain was grown on C-agar slants (Dillon et al., 2006) for up to 7 days at 28°C until conidia formed and were then stored at 4°C until use.

Substrate and Pre-treatments

The elephant grass samples were collected in the city of Nova Petrópolis, Rio Grande do Sul, Brazil. Plant cutting was carried out six months after planting. This material was initially dried at 60°C for 3 days and then triturated with a forage chopper obtaining a particle size between 200 and 4 mesh.

Pre-treatments were then carried out according to Menegol et al. (2014). Physical pre-treatment occurred in an autoclave at 120 ºC for 15 min at a 1:4 solid/liquid ratio. For the chemical pre-treatment, samples were pre-treated with sodium hydroxide solutions (2%, 3%, 4% and 6% w/v), sulfuric acid (5% and 10% v/v) and ammonium hydroxide (10% and 20% v/v) at a 1:4 solid/liquid ratio. These mixtures were then autoclaved at 120 ºC for 15 min. After pre-treatments, the biomass was washed with tap water until a neutral pH. Then, a fraction of the substrate was dried to determine the moisture, and the material was used in the assays of enzymatic production.

Enzyme production

Elephant grass biomass and wheat bran were used as the support and main carbon sources. The culture media

consisted of mixtures of wheat bran and elephant grass. The enzyme production was initially tested with a 50% substitution of wheat bran with elephant grass that was untreated or pre-treated by different methods, and then 75% and 100% substitution of wheat bran for elephant grass. Control samples were composed of 100% wheat bran. The experiments were conducted in flasks with a 12×3 cm concave base; the flasks were closed with a gauze-covered cotton wool plug that contained 1 g of dry mass of production media and 1 mL of basal salt solution (Mandels & Reese, 1957) that contained ($g.L^{-1}$): KH_2PO_4, 20; $(NH_4)_2SO_4$, 13; $CO(NH_2)_2$, 3; $MgSO_4 \cdot 7H_2O$, 3; $CaCl_2$, 3; $FeSO_4 \cdot 7H_2O$, 0.05; $MnSO_4 \cdot H_2O$, 0.0156; $ZnSO_4 \cdot 7H_2O$, 0.014; $CoCl_2$, 0.002.

All of the flasks were autoclaved at 1 atm for 20 min. Each flask was then inoculated with a sufficient conidial suspension to give a final concentration of 1×10^6 conidia per gram of dry mass of production media. The moisture of the media was adjusted to 67% by the addition of sterile distilled water. The flasks were incubated in a chamber at 28°C with 90% of air relative humidity during five days. Experiments were carried out with three replicates for the same strains for each incubation time. To extract the enzymes after incubation, the contents of each flask were separately added to a 125 mL-Erlenmeyer flask that contained 10 mL of distilled water. The pH was measured, and 17 mL of 0.05 $mol.L^{-1}$ citrate buffer (pH 4.8) was added, mixed, incubated under agitation for 30 min at 4°C and filtered. The filtrate was assayed for enzymes as described below.

Enzyme assays

Enzyme activities were analysed on filter paper (Filter Paper Activity, FPA) according to Camassola & Dillon (2010) and Ghose (1987). β-glucosidase activity was determined using 4-nitrophenyl β-D-glucopyranoside as the substrate according to (Daroit et al., 2008). Endoglucanase activity was determined according to Ghose (1987) using 2% (w/v) carboxymethylcellulose solution in citrate buffer. Xylanase activity was determined using 1% (w/v) xylan from oat spelts solution according to Bailey et al. (1992). The reducing sugar was estimated as either xylose or glucose equivalents by using the 3,5-dinitrosalicylic acid (DNS) method according to Miller (1959).

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar from the appropriate substrates per min under the assay conditions. The enzymatic activities are expressed as units per gram ($U.g^{-1}$) of dry medium.

Determination of mycelial mass, total soluble protein and pH determination

Mycelial mass was estimated with N-acetylglucosamine according to Novello et al. (2014). The total soluble protein was determinaed according to Bradford (1976). The pH was determined with an Orion 420A pH meter.

RESULTS AND DISCUSSION

Solid-state cultivations were performed using different proportions of untreated or pre-treated elephant grass and wheat bran to monitor the secretion of cellulases and xylanases by *P. echinulatum* 9A02S1. The control culture was formulated with 100% wheat bran. The wheat bran was used in the production of these enzymes as a nutrient-rich substrate to provide the appropriate conditions for cellulases and xylanases production. According to Archana & Satyanarayana (1997), hydrolysed wheat bran contains significant amounts of soluble sugars that are required for the growth of the microorganisms, including 42.5% glucose, 15.4% xylose, 3.1% arabinose and 2.7% galactose. Untreated elephant grass composition in sugars is 35.97% glucose, 15.15% xylose and 5.36% arabinose (Menegol et al., 2014).

In this work, enzyme production was initially tested with a 50% substitution of wheat bran with elephant grass that was untreated or pre-treated by different methods (Figures 1–3), and then 75% and 100% substitution of wheat bran for elephant grass (Table 1).

Table 1. Comparisons of enzyme activities in media formulated with different proportions of untreated or pre-treated elephant grass and wheat bran in solid-state cultivation.

	Endoglucanase ($U.g^{-1}$)	FPA ($U.g^{-1}$)	β-glucosidase ($U.g^{-1}$)	Xylanase ($U.g^{-1}$)
(7.5) untreated EG: (2.5) WB	84.89±3.84	7.91±0.73	87.86±0.73	372.62±37.62
(7.5) H_2O 121ºC: (2.5) WB	52.36±0.67	4.10±0.05	23.53±3.85	243.40±21.94
(10) H_2O 121ºC	23.65±2.98	2.32±0.40	*	128.05±0.46
(7.5) 2% NaOH EG: (2.5) WB	33.34±5.00	3.79±0.39	77.44±0.40	140.20±4.31
(10) 2% NaOH EG	23.04±1.20	1.71±0.22	41.80±0.58	84.02±5.09
(7.5) 20% NH_4OH EG: (2.5) WB	68.59±1.30	8.60±1.39	79.97±0.56	264.48±19.70
(10) 20% NH_4OH EG	72.20±4.06	5.61±0.29	75.16±2.37	146.22±4.00
(7.5) 5% H_2SO_4 EG: (2.5) WB	58.81±0.93	8.46±0.66	86.33±0.20	243.75±17.46
(10) 5% H_2SO_4 EG	26.75±1.01	1.80±0.46	35.07±3.92	97.73±13.17
(10) untreated EG	72.20±4.07	8.13±0.09	87.66±1.07	349.67±23.97
(10) WB	79.28±3.09	4.14±0.23	81.14±1.80	261.02±9.26

* Value less than 5 U.g-1.

EG: elephant grass; WB: wheat bran. The numbers that are shown inside the brackets in the legend indicate the proportion of each component that was used. The percentages indicate the concentrations of the solutions that were used in the pre-treatment.

Endoglucanase

The cultures formulated with 50% untreated elephant grass and 50% wheat bran ((5) untreated EG:(5) WB) showed a peak endoglucanase activity of 135.55 ± 9.07 $U.g^{-1}$. Endoglucanase values decreased during the remaining days of cultivation. The cultures with 50% elephant grass pre-treated with sulfuric acid displayed activities that increased and reached a maximum value of 142.32±3.89 $U.g^{-1}$ for elephant grass pre-treated with 5% (w/v) H_2SO_4 and 158.44±9.79 $U.g^{-1}$ for elephant grass pre-treated with 10% (w/v) H_2SO_4 after five days of cultivation. Elephant grass pre-treated with sulfuric acid has induced the greater production of these enzymes. This may be explained by the fact that the cultures formulated with elephant grass pre-treated with sulfuric acid showed a more significant reduction in pH. The control samples with 100% wheat bran showed two peaks of activity, which were on the second and fourth days of cultivation with enzyme activities of 72.42±2.48 $U.g^{-1}$ and 79.28±3.09 $U.g^{-1}$, respectively. Deschamps et al. (1985) observed similar activities (198 $U.g^{-1}$) using *Trichoderma harzianum* that had been grown on straw and wheat bran. When 75% and 100% substitutions were tested, endoglucanase activity decreased in relation to 50% substitution. However, the media with 75% untreated elephant grass and 25% WB provided enzyme activity superior to the control with 100% wheat bran (84.89±3.84 $U.g^{-1}$).

Filter Paper Activity

FPA assays (Figure 1 D to F) indicated that most of the pre-treatments favoured the production of enzymes in relation to the control cultivation with only wheat bran.

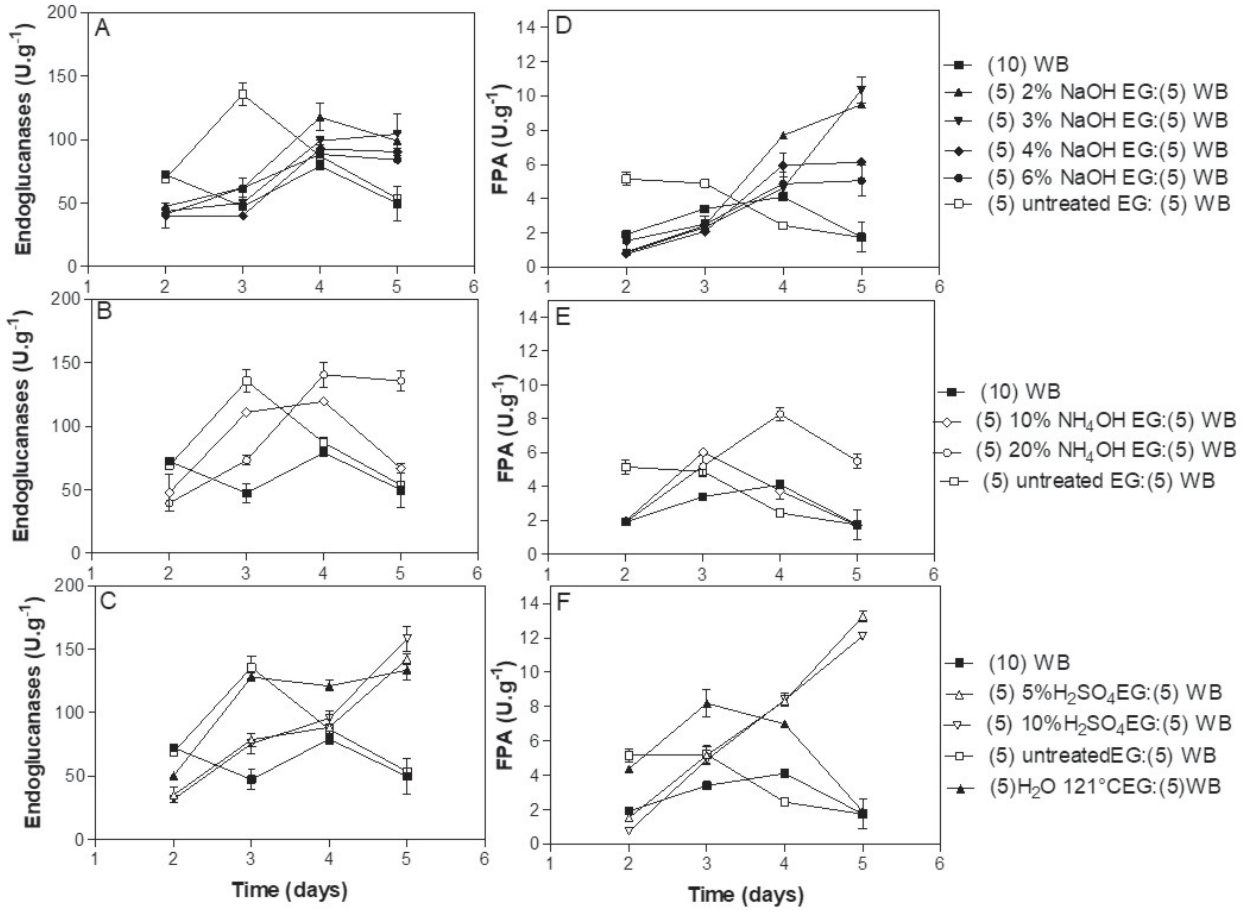

Figure 1. Variations in the endoglucanase activities (A, B and C) and filter paper activities (D, E and F) in media that was formulated with different proportions of untreated or pre-treated elephant grass and wheat bran in solid-state fermentation using the *Penicillium echinulatum* strain 9A02S1. EG: elephant grass; WB: wheat bran. The numbers that are shown inside the brackets in the legend indicate the proportion of each component that was used. The percentages indicate the concentrations of the solutions that were used in the pre-treatment.

The highest activities were obtained when elephant grass pre-treated with sulfuric acid was used as the carbon source. According to Alvira et al. (2010) dilute acid pre-treatment at elevated temperature (>120 ºC) increased biomass porosity by hemicellulose removal, improving enzyme accessibility to cellulose.

The peak activity for the control cultivation (wheat bran) was 4.11±0.24 $U.g^{-1}$ on the 4th day of cultivation. The cultivations that were performed with elephant grass pre-treated with sulfuric acid at concentrations of 5% and 10% displayed activities of 13.26±0.33 $U.g^{-1}$ and 12.11± 0.21 $U.g^{-1}$, respectively, on the fifth day of cultivation. These data are presented in Figures 1 D-F.

The cultivation that was performed with untreated elephant grass showed higher activities than the control containing only wheat bran on the first day of cultivation, and these values decreased on the following days. This result may be related to sugars, proteins and vitamins that are easily assimilated and present in the biomass of the elephant grass. The higher enzymatic activity that was observed with the cultivations with elephant grass that were pre-treated only in hot water in relation to cultivations with untreated biomass may be due to the removal of growth inhibitory substances. Rezaei et al. (2011) described the inhibitory effects of phenolic compounds that are present in switchgrass on the growth of *Acidothermus cellulolyticus,* but these compounds lost their effectiveness at temperatures above 50°C. Replacing 75% of wheat bran by elephant grass, FPA activity was mostly favoured in relation to control with 100% WB, but once again with lower activity when compared to the substitution of 50%.

β-glucosidase

Analysis of β-glucosidase activity (Figure 2 A to C) showed that there was no need to pre-treat elephant grass. The cultures that were formulated with either 50% untreated grass and 50% wheat bran or with 100% wheat bran showed similar activity profiles on the second to fifth days of cultivation. On the fifth day of cultivation, the

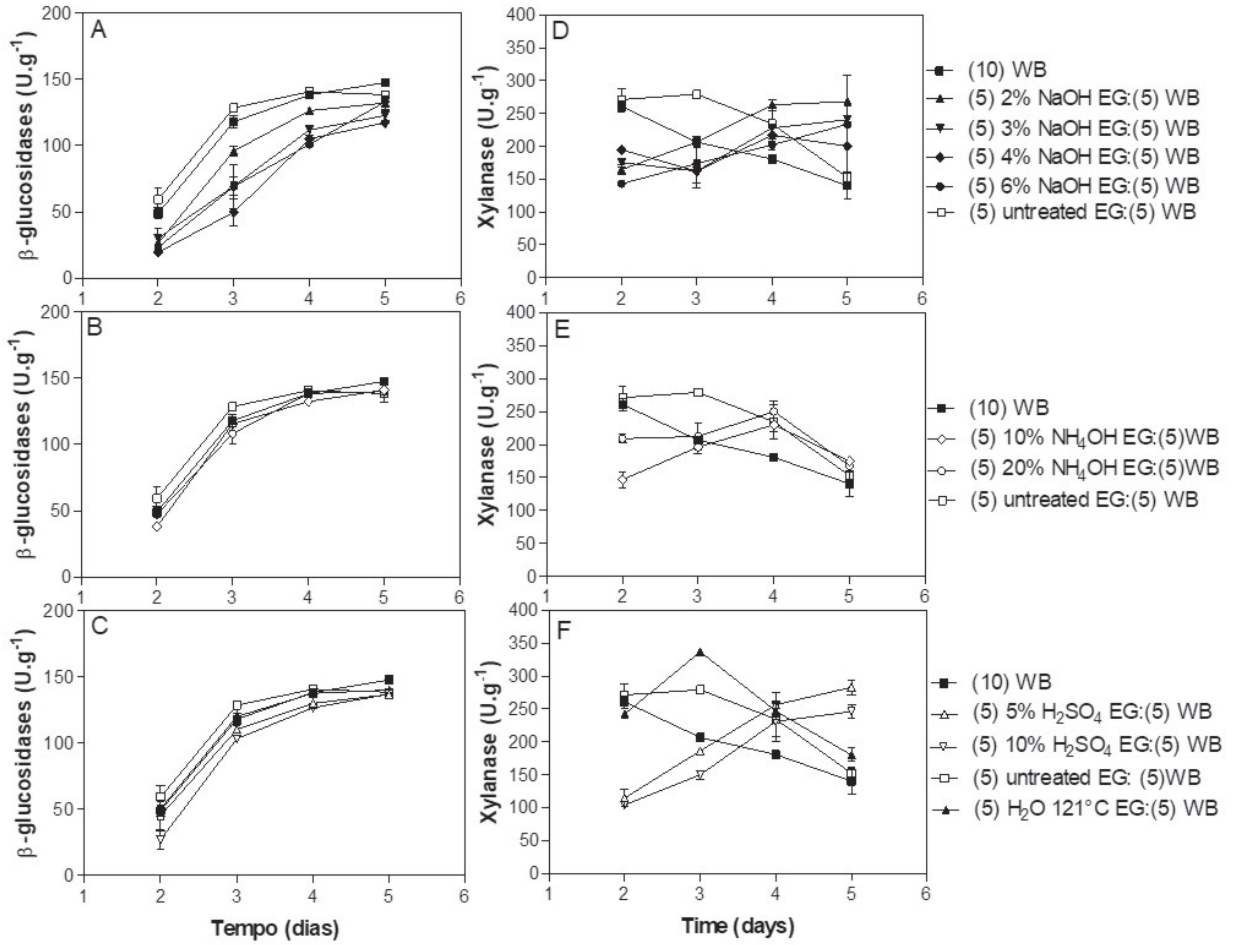

Figure 2. Variations in the β-glucosidases (A, B and C) and xylanases (D, E and F) in media that was formulated with different proportions of untreated or pre-treated elephant grass and wheat bran in solid-state fermentation using the *Penicillium echinulatum* strain 9A02S1. EG: elephant grass; WB: wheat bran. The numbers that are shown inside the brackets in the legend indicate the proportion of each component that was used. The percentages indicate the concentrations of the solutions that were used in the pre-treatment.

control sample with 100% wheat bran had an activity of 147.80 ± 1.79 $U.g^{-1}$, and the cultures that were formulated with 50% untreated elephant grass and 50% wheat bran had an activity of 138.34 ± 0.95 $U.g^{-1}$. Replacing 75% and 100% wheat bran by elephant grass gave the same profile, meaning that there was no need to pre-treat the biomass for the induction of these enzymes, since the media formulated with 75% untreated elephant grass: 25 % wheat bran and media formulated with 100% elephant grass showed higher values than the control with 100% wheat bran.

Xylanases

Analyses of xylanases activity (Figures 2 D to F) showed that the cultivation that was performed with 50% untreated elephant grass and 50% wheat bran displayed an activity that was higher than the control on the second to the fifth days of cultivation. However, the cultivation that was performed with 50% elephant grass pre-treated with water displayed an activity of 337.31±4.59 $U.g^{-1}$ on the third day of cultivation. Greater activity was obtained with 75% untreated elephant grass and 25% wheat bran (372.62±37.62 $U.g^{-1}$). Basso et al. (2014) verified that for xylanase production, the highest yields were independent of the different accession cellulose concentrations. Camassola and Dillon (2010) obtained a xylanases activity that was approximately 10 $U.g^{-1}$, which was obtained using wheat bran and alkaline pre-treated sugar cane bagasse with *P. echinulatum* 9A02S1.

In most cultivation conditions, replacement of wheat bran with 50% elephant grass favoured the production of cellulases and the replacement of wheat bran with 75% elephant grass favoured xylanase production. To increase the activity of the β-glucosidases and xylanases, supplementation with untreated elephant grass has been suggested (50%:50% and 75%:25%, respectively). This supplementation helps to reduce the production cost of these enzymes. As for the FPA and endoglucanases, the acid treatment (sulfuric acid) of the fibre helped to promote a higher activity.

Comparing the data from the production of the enzymes (Figures 1 and 2) with the concentrations of cellulose, hemicellulose and lignin (Menegol et al., 2014) leads to the conclusion that the results cannot be directly related to the production of the studied enzymes. Samples with higher concentrations of cellulose (pre-treated with NaOH) and lower lignin concentrations did not have the highest FPA (data not shown). In contrast, in the samples pre-treated with H_2SO_4, the lignin concentrations were higher than in those samples that were not pre-treated and had higher enzyme titres. These data indicate that a change in biomass structure is more important than the removal of lignin in the production of cellulolytic and hemicellulolytic enzymes, as observed by Rollin et al. (2011). According to Xiao et al. (2012) some pre-treatments can increase the cellulose crystallinity, because of solubilization of hemicellulose, lignin and amorphous cellulose, which can hinder the enzyme accessibility.

Comparing the data that was obtained in this work with other data in the literature shows that elephant grass has a high potential for enzyme production. Many of the activities that were obtained are higher than those that were presented in the literature, as shown in Table 2.

Determination of the mycelial mass, total soluble proteins and pH determination in the enzyme broth

Figure 3 (A to C) presents data on the mycelial growth. The control culture that was formulated with only wheat bran and the cultivations that were pre-treated with NaOH showed higher mycelial masses. These data indicate that the form of cellulose that results from the alkaline pre-treatment is more easily assimilable for the fungus. According to the results obtained, higher mycelial growth levels are not directly related to higher enzymatic activity, as described by Scholl et al. (2015c).

Regarding the protein content that was detected, cultivations that were formulated with 50% untreated elephant grass showed high protein concentration (343.49±6.81 $mg.g^{-1}$), but these proteins may have been from the elephant grass and not the enzymes. The protein content of the elephant grass pre-treated with sulfuric acid was found to display a peak on the fifth day of cultivation, which corresponded to the increased FPA on the last day of cultivation.

The control sample formulated with wheat bran alone showed a higher concentration of protein on the fifth day of cultivation with an average value of 415.21**±20.71** $mg.g^{-1}$. The incremental changes in protein content on the fifth day may be associated with the degradation of biomass, which resulted in the highest concentration of soluble protein. This result may be associated with reduced FPA, endoglucanase and xylanase activities and increased **β**-glucosidase activity. Based on Vaheri et al. (1979), **β**-glucosidases are only detected at the end of the enzyme production process when cell lysis occurs.

In the condition 50 % WB and 50% elephant grass pre-treated with H_2SO_4 10% (w/v), the specific activity was 12.8 $UI.g^{-1}$ and for the condition of 25 % WB: 75 % elephant grass pre-treated with NH_4OH 20% (w/v), the activity was 80 $UI.g^{-1}$. The highest specific activity detected by replacing 75 % of wheat bran by pre-treated elephant grass with NH_4OH indicates a greater amount of enzymes of interest in relation to the total proteins secreted.

The pH values were decreased on the second day of cultivation for all of the conditions that were tested. This drop in pH indicates an increase in fungal metabolism that was associated with the release of H^+ into the medium. The cultures that were formulated with the elephant grass that was pre-treated with sulfuric acid showed a more significant reduction in pH (between 3.7 and 3.9) (data not shown).

Table 2. Comparisons of enzymes productions from different fungi grown on lignocellulosic materials.

Organism	Substrate	Enzyme activities (U g^{-1}) Substrate used to enzymatic assay				Reference
		FPA*	β-glucosidase	Endoglucanase**	Xylanase	
P. echinulatum 9A02S1	Elephant grass	13.26	138.34	158.44	372.62 Oat spelts xylan	This work
P. echinulatum 9A02S1	Wheat bran and untreated sugar cane bagasse	45.82	40.13 Salicine	290.47	37.87 Oat spelts xylan	Camassola & Dillon (2010)
P. echinulatum 9A02S1	Wheat bran and pre-treated sugar cane bagasse	32.89	58.95 Salicine	282.36	10.0 Oat spelts xylan	Camassola & Dillon (2010)
P. decumbens	Wheat straw and wheat bran	17.7	52.8 Cellobiose	–	–	Mo et al. (2004)
T. reesei and *A. phoenicis*	Sugar cane bagasse	13.4	18.1 pNPG	73.8	2,842 Xylan	Gutierrez-Correa & Tengerdy (1997, 1998)
T. reesei	Sugar cane bagasse	5.3	7.7 pNPG	18.8	1,968 Xylan	Gutierrez-Correa & Tengerdy (1997, 1998)
Myceliophthora sp.	Rice straw	2.44	7.48 pNPG	32,9	900 Birch wood xylan	Badhan et al. (2007)
Myceliophthora sp.	Bagasse	0.7	2.01 pNPG	6.62	620.1 Birch wood xylan	Badhan et al. (2007)
A. niger	Wheat straw and wheat bran	–	-	14.8	–	Jecu (2000)
T. aurantiacus	Untreated wheat straw	–	105 pNPG	1235	–	Kalogeris et al. (2003)
T. aurantiacus	Alkali treated wheat straw	–	0.4 pNPG	66	–	Kalogeris et al. (2003)
T. aurantiacus	Untreated corn cobs	–	ND	18	–	Kalogeris et al. (2003)
Myceliophthora sp.	Wheat bran	0.74	3.83 pNPG	26.6	128.9 Birch wood xylan	Badhan et al. (2007)
A. awamori	Grape pomace	–	–	–	38 Birch wood xylan	Botella et al. (2009)
A. niger	Wheat bran	–	–	35.7	–	Pirota et al. (2013)

* Measured using filter paper (Whatman No. 1)

** Measured using carboxy methyl cellulose (CMC)

rNPG: 4-nitrophenyl β-D-glucopyranoside

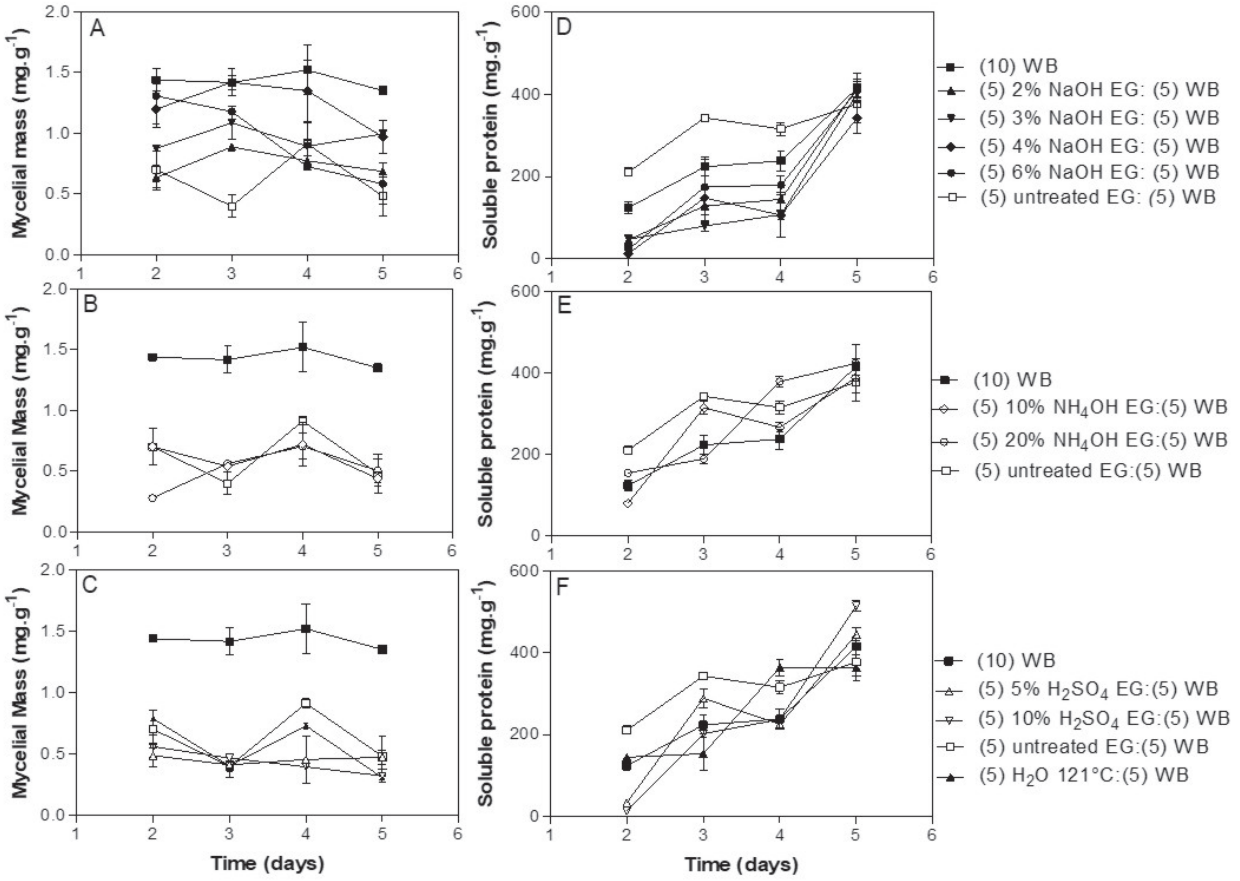

Figure 3. Variations in the mycelial mass (A, B and C) and soluble protein (D, E and F) in media that was formulated with different proportions of untreated or pre-treated elephant grass and wheat bran in solid-state fermentation using the *Penicillium echinulatum* strain 9A02S1. EG: elephant grass; WB: wheat bran. The numbers that are shown inside the brackets in the legend indicate the proportion of each component that was used. The percentages indicate the concentrations of the solutions that were used in the pre-treatment.

CONCLUSIONS

The results obtained in this work showed that the solid-state growth of the fungus *P. echinulatum* on a simple medium that consisted of elephant grass and a low cost mineral source proved to be a promising alternative for the simultaneous production of cellulases and xylanases. These data also indicate that changes in biomass structure are more important than the removal of lignin in the production of cellulolytic enzymes and that untreated elephant grass can be used for the induction of **β-**glucosidase and hemicellulolytic enzymes.

ACKNOWLEDGEMENTS

The authors are grateful for the financial and technical support from UCS, CNPq (310590/2009-4), and FAPERGS (10/1972-5).

REFERENCES

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology, 101, 4851-4861 (2010).

Archana, A., Satyanarayana, T., Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. Enzyme and Microbial Technology, 21(1), 12-17 (1997).

Badhan, A.K., Chadha, B.S., Kaur, J., Saini, H.S., Bhat, M.K., Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099. Bioresource Technology, 98, 504-510 (2007).

Bai, Z.G., Dent, D.L., Olsson, L., Schaepman, M.E., Proxy global assessment of land degradation. Soil Use and Management, 24, 223-234 (2008).

Bailey, M.J., Biely, P., Poutanen, K., Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology, 23, 257-270 (1992).

Banerjee, G., Car, S., Scott-Craig, J.S., Borrusch, M.S., Bongers, M., Walton, J.D., Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass. Bioresource Technology, 101, 9097-9105 (2010).

Basso, V., Machado, J.C., da Silva Lédo, F.J., da Costa Carneiro, J., Fontana, R.C., Dillon, A.J.P., Camassola, M., Different elephant grass (*Pennisetum purpureum*) accessions as substrates for enzyme production for the hydrolysis of lignocellulosic materials. Biomass and Bioenergy, 71, 155-161 (2014).

Botella, C., Diaz, A.B., Wang, R., Koutinas, A., Webb, C., Particulate bioprocessing: A novel process strategy for biorefineries. Process Biochemistry, 44, 546-555 (2009).

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254 (1976).

Camassola, M., De Bittencourt, L.R., Shenem, N.T., Andreaus, J., Dillon, A.J.P., Characterization of the Cellulase Complex of *Penicillium echinulatum.* Biocatalysis and Biotransformation, 22, 391-396 (2004).

Camassola, M., Dillon, A.J., Cellulases and xylanases production by *Penicillium echinulatum* grown on sugar cane bagasse in solid-state fermentation. Applied and Biochemistry Biotechnology, 162(7), 1889-1900 (2010).

Camassola, M., Dillon, A.J., Effect of methylxanthines on production of cellulases by *Penicillium echinulatum*. Journal of Applied Microbiology, 102, 478-485 (2007).

Daroit, D.J., Simonetti, A., Hertz, P.F., Brandelli, A., Purification and characterization of an extracellular beta-glucosidase from *Monascus purpureus.* Journal Microbiology and Biotechnology, 18, 933-941 (2008).

Deschamps, F., Giuliano, C., Asther, M., Huet, M.C., Roussos, S., Cellulase production by *Trichoderma harzianum* in static and mixed solid-state fermentation reactors under nonaseptic conditions. Biotechnology and Bioengineering, 27, 1385-1388 (1985).

Dillon, A.J., Zorgi, C., Camassola, M., Henriques, J.A., Use of 2-deoxyglucose in liquid media for the selection of mutant strains of *Penicillium echinulatum* producing increased cellulase and beta-glucosidase activities. Applied Microbiology and Biotechnology, 70, 740-746 (2006).

Eliana, C., Jorge, R., Juan, P., Luis, R., Effects of the pretreatment method on enzymatic hydrolysis and ethanol fermentability of the cellulosic fraction from elephant grass. Fuel, 118, 41-47 (2014).

Ghose, T.K., Measurement of cellulase activities. Pure and Applied Chemistry, 592, 257-268 (1987).

Guilherme, A.A., Dantas, P.V.F., Santos, E.S., Fernandes, F.A.N., Macedo, G.R., Evaluation of composition, characterization and enzymatic hydrolysis of pretreated sugar cane bagasse. Brazilian Journal of Chemical Engineering, 32, 23-33 (2015).

Gutierrez-Correa, M., Tengerdy, R., Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnology Letters, 19, 665-667 (1997).

Gutierrez-Correa, M., Tengerdy, R., Xylanase production by fungal mixed culture solid substrate fermentation on sugar cane bagasse. Biotechnology Letters, 20, 45-47. (1998).

Idrees, M., Adnan, A., Bokhari, S.A., Qureshi, F.A., Production of fermentable sugars by combined chemo-enzymatic hydrolysis of cellulosic material for bioethanol production. Brazilian Journal of Chemical Engineering, 31, 355-363 (2014).

Jecu, L., Solid state fermentation of agricultural wastes for endoglucanase production. Industrial Crops and Products, 11, 1-5 (2000).

Kalogeris, E., Christakopoulos, P., Katapodis, P., Alexiou, A., Vlachou, S., Kekos, D., Macris, B.J., Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes. Process Biochemistry, 38, 1099-1104 (2003).

Macedo, E.P., Cerqueira, C.L.O., Souza, D.A.J., Bispo, A.S.R., Coelho, R.R.R., Nascimento, R.P., Production of cellulose-degrading enzyme on sisal and other agro-industrial residues using a new Brazilian actinobacteria strain *Streptomyces* sp. SLBA-08. Brazilian Journal of Chemical Engineering, 30, 729-735 (2013).

Mandels, M., Reese, E.T., Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. Journal of Bacteriology, 73, 269-278 (1957).

Martins, L.F., Kolling, D., Camassola, M., Dillon, A.J., Ramos, L.P., Comparison of *Penicillium echinulatum* and *Trichoderma reesei* cellulases in relation to their activity against various cellulosic substrates. Bioresource Technology, 99, 1417-1424 (2008).

Menegol, D., Scholl, A.L., Fontana, R.C., Dillon, A.J.P., Camassola, M., Increased release of fermentable sugars from elephant grass by enzymatic hydrolysis in the presence of surfactants. Energy Conversion and Management, 88, 1252-1256. (2014).

Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426-428 (1959).

Mo, H., Zhang, X., Li, Z., Control of gas phase for enhanced cellulase production by *Penicillium decumbens* in solid-state culture. Process Biochemistry, 39, 1293-1297 (2004).

Novello, M., Vilasboa, J., Schneider, W.D.H., Reis, L.d., Fontana, R.C., Camassola, M., Enzymes for second generation ethanol: exploring new strategies for the use of xylose. RSC Advances, 4, 21361-21368 (2014).

Ogeda, T.L., Petri, D.F.S., Biomass Enzymatic Hydrolysis. Quimica Nova, 33, 1549-1558 (2010).

Padilha, I.Q.M., Carvalho, L.C.T., Dias, P.V.S., Grisi, T.C.S.L., Silva, F.L.H., Santos, S.F.M., Araújo, D.A.M., Production and characterization of thermophilic carboxymethyl cellulase synthesized by *Bacillus* sp. growing on sugarcane bagasse in submerged fermentation. Brazilian Journal of Chemical Engineering, 32, 35-42 (2015).

Pirota, R.D.P.B., Miotto, L.S., Delabona, P.S., Farinas, C.S., Improving the extraction conditions of endoglucanase produced by *Aspergillus niger* under solid-state fermentation. Brazilian Journal of Chemical Engineering, 30, 117-123 (2013).

Reis, L.d., Ritter, C.E.T., Fontana, R.C., Camassola, M., Dillon, A.J.P., Statistical optimization of mineral salt and urea concentration for cellulase and xylanase production

by *Penicillium echinulatum* in submerged fermentation. Brazilian Journal of Chemical Engineering, 32, 13-22 (2015).

Rezaei, F., Joh, L.D., Berry, A.M., VanderGheynst, J.S., Xylanase and cellulase production by *Acidothermus cellulolyticus* grown on switchgrass in solid-state fermentation. Biofuels, 21-32 (2011).

Rollin, J.A., Zhu, Z., Sathitsuksanoh, N., Zhang, Y.H., Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. Biotechnology and Bioengineering, 108, 22-30 (2011).

Scholl, A.L., Menegol, D., Pitarelo, A.P., Fontana, R.C., Filho, A.Z., Ramos, L.P., Dillon, A.J.P., Camassola, M., Ethanol production from sugars obtained during enzymatic hydrolysis of elephant grass (*Pennisetum purpureum*, Schum.) pretreated by steam explosion. Bioresource Technology, 192, 228-237 (2015a).

Scholl, A.L., Menegol, D., Pitarelo, A.P., Fontana, R.C., Filho, A.Z., Ramos, L.P., Dillon, A.J.P., Camassola, M., Elephant grass (*Pennisetum purpureum* Schum.) pretreated via steam explosion as a carbon source for cellulases and xylanases in submerged cultivation. Industrial Crops and Products, 70(0), 280-291 (2015b).

Scholl, A.L., Menegol, D., Pitarelo, A.P., Fontana, R.C., Filho, A.Z., Ramos, L.P., Dillon, A.J.P., Camassola, M., Elephant grass pretreated by steam explosion for inducing secretion of cellulases and xylanases by Penicillium echinulatum S1M29 solid-state cultivation. Industrial Crops and Products, 77, 97-107 (2015c).

Somerville, C., Youngs, H., Taylor, C., Davis, S.C., Long, S.P., Feedstocks for Lignocellulosic Biofuels. Science, 329, 790-792 (2010).

Vaheri, M., Leisola, M., Kauppinen, V., Transglycosylation products of cellulase system ofTrichoderma reesei. Biotechnology Letters, 1, 41-46 (1979).

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Rómero-Gomez, S.d.J., Díaz-Godínez, G., Augur, C., Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochemical Engineering Journal, 13, 157-167 (2003).

Várnai, A., Siika-aho, M., Viikari, L., Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose. Enzyme and Microbial Technology, 46, 185-193 (2010).

Xiao, W., Yin, W., Xia, S., Ma, P., The study of factors affecting the enzymatic hydrolysis of cellulose after ionic liquid pretreatment. Carbohydrate Polymers, 87, 2019-2023 (2012).

Xie, X.-M., Zhang, X.-Q., Dong, Z.-X., Guo, H.-R., Dynamic changes of lignin contents of MT-1 elephant grass and its closely related cultivars. Biomass and Bioenergy, 35, 1732-1738 (2011).