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Cellulases and Xylanases Production by *Penicillium echinulatum* Grown on Sugar Cane Bagasse in Solid-State Fermentation

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Abstract To investigate the production of cellulases and xylanases from *Penicillium echinulatum* 9A02S1, solid-state fermentation (SSF) was performed by using different ratios of sugar cane bagasse (SCB) and wheat bran (WB). The greatest filter paper activity obtained was 45.82±1.88 U gdm^{-1} in a culture containing 6SCB/4WB on the third day. The greatest β-glucosidase activities were 40.13±5.10 U gdm^{-1} obtained on the third day for the 0SCB/10WB culture and 29.17±1.06 U gdm^{-1} for the 2SCB/8WB culture. For endoglucanase, the greatest activities were 290.47±43.57 and 276.84±15.47 U gdm^{-1}, for the culture 6SCB/4WB on the fourth and fifth days of cultivation, respectively. The greatest xylanase activities were found on the third day for the cultures 6SCB/4WB (36.38± 5.38 U gdm^{-1}) and 4SCB/6WB (37.87±2.26 U gdm^{-1}). In conclusion, the results presented in this article showed that it was possible to obtain large amounts of cellulases and xylanases enzymes using low-cost substrates, such as SCB and WB.

Keywords Solid-state fermentation · Cellulases · Xylanases · Sugar cane bagasse · Second-generation ethanol

Introduction

Solid-state fermentation (SSF) is a process whereby an insoluble substrate is fermented with sufficient moisture, but without free water [1, 2]. The use of SSF as a production method of enzymes could offer some apparent economic and engineering advantages over the classical submerged fermentation (SmF). These include high concentration of the product and simple fermentation equipment, as well as low-effluent generation and low requirements for aeration and agitation during enzyme production [3]. Furthermore, this method employs agricultural residues in their natural form, thus helping to prevent the environmental impact caused by their accumulation. Sugar cane bagasse (SCB) is one of the largest lignocellulosic agroindustrial byproducts in Brazil. It consists approximately of cellulose

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(50%), hemicellulose (25%), and lignin (25%) [4], which can be used for production of cellulases and xylanases by microorganisms.

In recent years, the interest in cellulases and hemicellulases has increased due to the numerous potential applications for these enzymes. Xylanases have a variety of applications, such as biobleaching of Kraft pulp, clarification of juice and wine, starch separation, and production of functional food ingredients, improving the quality of bakery products and in animal feed biotechnology [5]. Cellulases can be used in the formulation of washing powders, in the textile industry [6, 7], in processes that include supplementation of animal feeds [8], in the extraction of fruit and vegetable juices, as well as in pulp and paper manufacturing and starch processing [9, 10]. Moreover, the growing concerns about the potential consequences of a worldwide shortage of fossil fuels, the emission of green house gasses, and the air pollution (by incomplete combustion of fossil fuel) have also resulted in an increased focus on the production of bioethanol from lignocellulosics [11], especially considering the possibility of using cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic material [12]. However, in order to make the production of bioethanol an economically feasible process, the costs of the enzymes to be used for hydrolysis of the raw material need to be reduced and their efficiency increased [11, 12]. Many countries are investing heavily to competitively produce ethanol from cellulosic material, particularly the USA and members of the Europen Union. However, the result has been rather disappointing, as costs have remained high and will probably remain so for years to come [13].

The use of a low-cost source of substrate and inducer, such as SCB, can reduce significantly the cost of enzyme production. High cellulase productivities can potentially be achieved by the use of chemically pretreated biomass as a carbon source [14, 15]. However, pretreatment has been viewed as one of the most expensive processing steps when transforming cellulosic biomass into fermentable sugar [16], and it can generate toxic effluent.

This study evaluates the SSF production of cellulases and xylanases by *Penicillium echinulatum* 9A02S1, in experiments carried out with different ratios of SCB and wheat bran (WB). We used untreated SCB to avoid the production of effluent during the pretreatment process and to reduce the cost of enzyme production, given that we have not costs with reagents and/or energy during pretreatment.

Materials and Methods

Microorganism

The cellulolytic mutant *P. echinulatum* strain 9A02S1 (DSM 18942) was used in this study. This strain was obtained by exposing wild type *P. echinulatum* strain 2HH to ultraviolet (UV) light and hydrogen peroxide (H_2O_2) [17]. These strains are stored in the culture collection of the Enzymes and Biomass Laboratory, Institute of Biotechnology, Caxias do Sul, Rio Grande do Sul, Brazil. The strain was grown on C-agar slants [17] for up to 7 days at 28 °C until conidia were formed, and then stored at 4 °C until use.

Enzyme Production

WB and SCB were used as the support and main carbon sources. The culture media consisted of WB and nontreated SCB, mixed in different ratios (0SCB/10WB; 2SCB/8WB; 4SCB/6WB; 6SCB/4WB; 8SCB/2WB; 10SCB/0WB). The controls were realized with WB and SCB.

Fermentations were conducted in flasks with a 12×3-cm concave base; the flasks were closed with a gauze-covered cotton wool plug containing 2 g of dry mass of production media and 2 mL basal salt solution containing (in g L^{-1}) KH_2PO_4, 20; $(NH_4)_2SO_4$, 13; $CO(NH_2)_2$, 3; $MgSO_4.7H_2O$, 3; $CaCl_2$, 3; $FeSO_4.7H_2O$, 0.050; $MnSO_4.H_2O$, 0.0156; $ZnSO_4{\cdot}7H_2O$, 0.014; and $CoCl_2$. 0.0020. All flasks were autoclaved at 120 °C for 20 min. Each flask was then inoculated with 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 distilled water. The flasks were incubated at 28 °C and 90% humidity for 5 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 containing 10 mL of distilled water. The pH was measured and 17 mL of 0.05 M 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 Assay

The enzymatic activity was analyzed on filter paper (filter paper activity—FPA) [18]. The β-glucosidase activity was dosed using salicin as the substrate [1]. Endoglucanase activity was determined according to Ghose [18], using 2% (wt/vol) carboxymethyl cellulose solution in citrate buffer. The xylanase activity was determined in the same way as the endoglucanase activity, except that 1% of xylan from oat spelts solution was used as the substrate in place of carboxymethyl cellulose. The reducing sugar was estimated as either xylose or glucose equivalent by the dinitrosalicylic acid (DNS) method [19].

Enzyme Units

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 minute under assay conditions. The enzymatic activities are expressed as units per gram of dry medium (U gdm^{-1}).

Mycelial Mass Determination

The quantity of *N*-acetylglucosamine was determined by the method described by Reissig et al. [20] and the quantity of mycelial mass estimated according to Bittencourt et al. [21].

Statistical Tests

The results were statistically analyzed using analysis of variance with the Tukey's posttest for a $p<0.05$ using the PrismGraphPad program (GraphPad Software, Inc., USA).

Results

Production of Enzymes

The results of the enzymatic analysis are expressed as units per gram of dry medium (U gdm^{-1}) and can be found in Figs. 1, 2, 3, 4.

Filter Paper Activity

The results for FPA in this experiment are shown in Fig. 1. It can be noted that on the second day, the 2SCB/8WB culture indicated the greatest average enzymatic activity (11.75± 0.50 U gdm^{-1}); however, other cultures (0SCB/10WB; 6SCB/4WB; and 2SCB/8WB, with 10.93±0.21 U gdm^{-1}, 9.31±1.58 U gdm^{-1}, 8.72±2.14 U gdm^{-1}, respectively) showed statistically similar enzymatic activities. On the third day, the first peak of FPA activity was verified. On the same day, all cultures supplemented with WB had higher enzymatic activities than the control culture with only SCB, the 6SCB/4WB; 4SCB/6WB; and 2SCB/8WB cultures showing activities greater than the control culture formulated only with WB. In this sample collection, the greatest productivities of this experiment were also verified. Productivity values of 15.27±0.63 U gdm^{-1} day^{-1} were obtained for the 6SCB/4WB culture, 14.06±1.40 U gdm^{-1} day^{-1} for 4SCB/6WB, 13.39±0.27 U gdm^{-1} day^{-1} for 2SCB/8WB, and 11.61±3.53 U gdm^{-1} day^{-1} for the control culture 0SCB/10WB, as can be seen in Fig. 1b. On the fourth day, there was a decrease in the activities of all cultures, except for the control culture formulated with 0SCB/10WB, which showed a small increase in its enzymatic activity. On the fifth day, the cultures 8SCB/2WB and 6SCB/4WB showed the greatest enzymatic dosages. These cultures maintained the productivity observed on the fourth day of culture, whereas the other cultures presented drops (Fig. 1b).

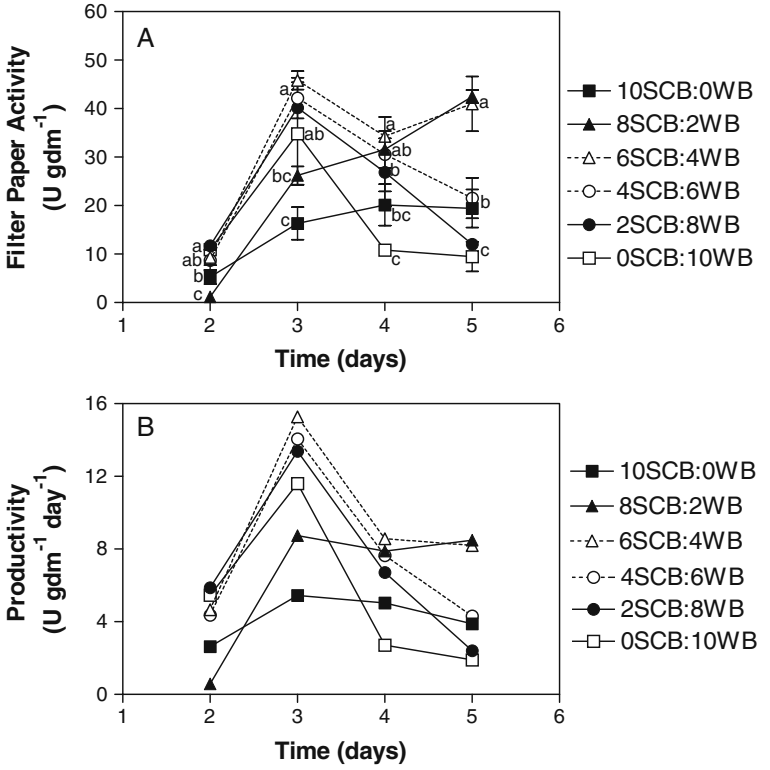

Fig. 1 FPA (**a**) and productivity (**b**) in media formulated with different proportions of non-treated SCB and WB, in SSF, using the 9A02S1 strain of *P. echinulatum*. The *numbers* shown in the legend indicate the proportion of each medium component used. Values (averages) with the *same letters* for the same day do not differ significantly by Tukey's test ($p>0.05$)

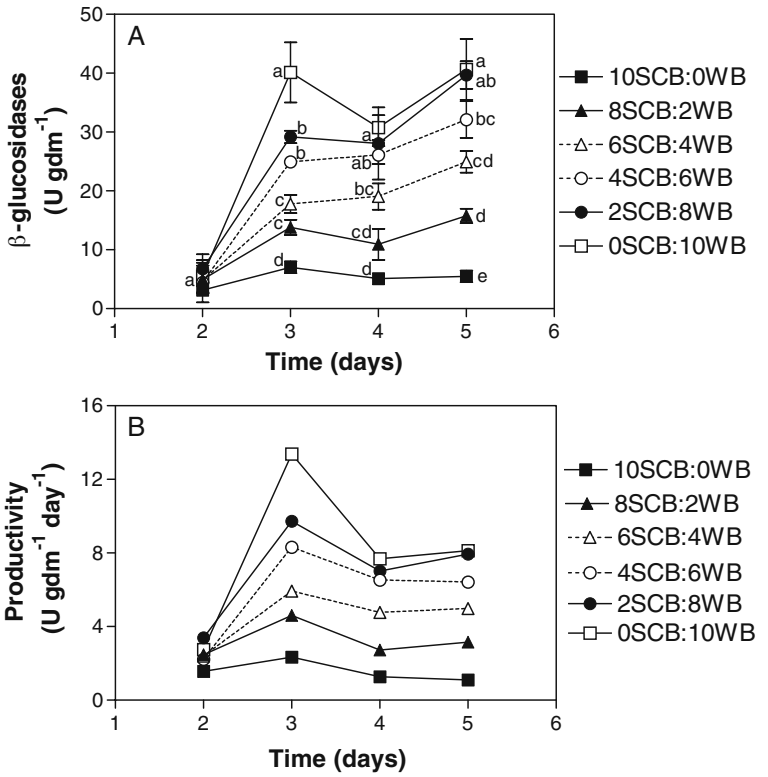

Fig. 2 β-glucosidases activity (**a**) and productivity (**b**) in media formulated with different proportions of nontreated SCB and WB, in SSF, using the 9A02S1 strain of *P. echinulatum*. The *numbers* shown in the legend indicate the proportion of each medium component used. Values (averages) with the *same letters* for the same day do not differ significantly by the Tukey's test ($p>0.05$)

β-Glucosidases

In relation to the enzymatic activity of β-glucosidases (Fig. 2a), it was found that the higher the concentration of WB employed in the cultures, the greater the activity of this enzyme, with the enzymatic behavior proportional to the amount of WB added. Peaks of β-glucosidase activity were observed on the third and fifth days. On the third day, the cultures 0SCB/10WB, 8SCB/2WB, and 4SCB/6WB showed activities of 40.45±5.11, 29.49±1.06, and 25.25±0.77 U gdm^{-1}, respectively. On the fifth day, these cultures showed activities of 40.39±5.16, 39.43±2.33, and 31.89±3.11 U gdm^{-1}, respectively. However, when comparing the data for the productivity of these cultures on the third day (13.48±1.70, 9.83±0.35, and 8.42±0.25 U gdm^{-1} day^{-1} for cultures 0SCB/10WB, 8SCB/2WB, and 4SCB/6WB, respectively) in relation to those obtained on the fifth day (8.07±1.03, 7.88±0.46, and 6.37±0.62 U gdm^{-1} day^{-1}, respectively), it can be said that the third day is recommended for the interruption of the process (Fig. 2b).

Endoglucanases

Regarding the endoglucanases activities (Fig. 3a) on the second day of the process, all cultures were statistically similar, although culture 8SCB/2WB had the highest average. In

this sample collection, culture 8SCB/2WB also showed its greatest productivity (76.99± 14.19 U gdm^{-1} day^{-1}; Fig. 3b) during this experiment.

On the third day, cultures 4SCB/6WB and 6SCB/4WB were the most notable with activities of 261.84±20.58 and 229.27±43.40 U gdm^{-1}, respectively. These two cultures also had the greatest productivity values for endoglucanases on this day (87.28± 6.86 U gdm^{-1} day^{-1} for 4SCB/6WB and 76.42±14.47 U gdm^{-1} day^{-1} for 6SCB/4WB). On the fourth day, all cultures formulated with mixtures of SCB and WB had activity values higher than the controls, which had been formulated with isolated lignocelluloses. Finally, on the last day of this experiment, the cultures 8SCB2/WB, 6SCB/4WB, and 4SCB/6WB had activities significantly greater than the other cultures.

Xylanases

The enzymatic activities obtained for xylanases are shown in Fig. 4. It was found that the cultures formulated with mixtures of WB and SCB had greater activities than the control cultures, in at least one of the sample collections carried out between the third and fifth days of cultivation.

On the second day, the control culture formulated only with WB (0SCB/10WB) showed the greatest xylanase activity (7.02±0.48 U gdm^{-1}). The greatest xylanase activities on the

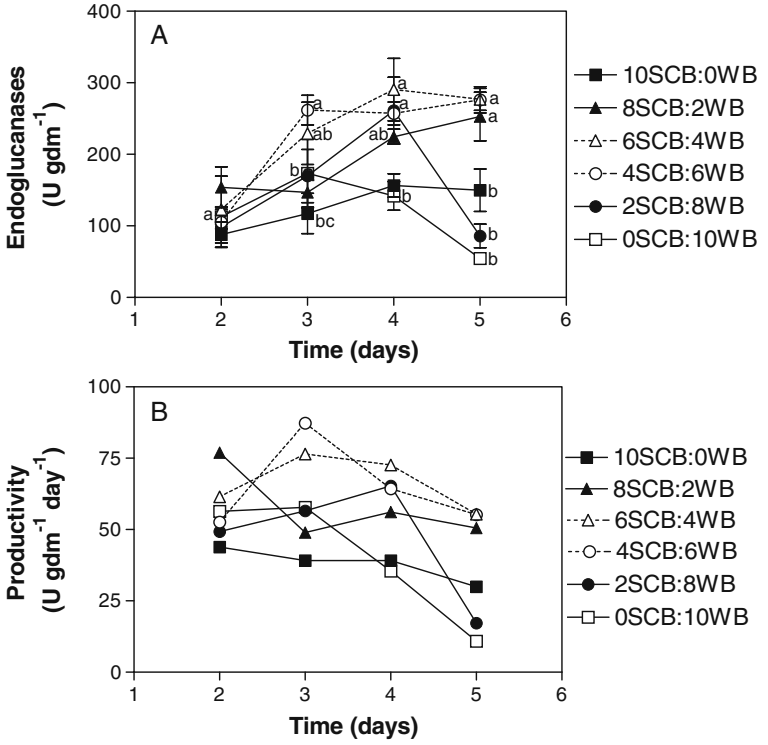

Fig. 3 Endoglucanase activity (**a**) and productivity (**b**) in media formulated with different proportions of nontreated SCB and WB, in SSF, using the 9A02S1 strain of *P. echinulatum*. The *numbers* shown in the legend indicate the proportion of each medium component used. Values (averages) with the *same letters* for the same day do not differ significantly by Tukey's test ($p>0.05$)

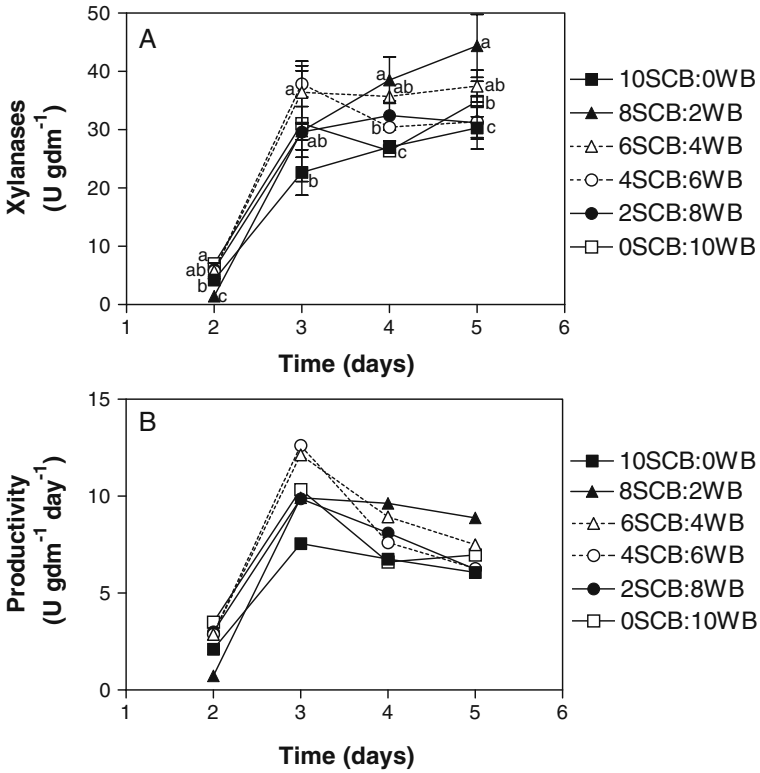

Fig. 4 Xylanase activity (**a**) and productivity (**b**) in media formulated with different proportions of nontreated SCB and WB, in SSF, using the 9A02S1 strain of *P. echinulatum*. The *numbers* shown in the legend indicate the proportion of each medium component used. Values (averages) with the *same letters* for the same day do not differ significantly by Tukey's test ($p>0.05$)

third day were determined for cultures 6SCB/4WB (36.38±5.38 U gdm^{-1}) and 4SCB/6WB (37.87±2.26 U gdm^{-1}). These cultures had higher averages than the cultures 0SCB/10WB (31.05±9.95 U gdm^{-1}) and 2SCB/8WB (29.61±4.36 U gdm^{-1}), and they differed statistically from culture 10SCB/0WB (22.67±3.90 U gdm^{-1}). The greatest xylanase productivities were obtained on this day; cultures 6SCB/4WB (12.13±1.79 U gdm^{-1} day^{-1}) and 4SCB/6WB (12.62±0.75 U gdm^{-1} day^{-1}) showed the highest values, which were even higher than those obtained in the control culture 0SCB/10WB (10.35± 3.32 U gdm^{-1} day^{-1}).

On the fourth day, it was found that the cultures formulated with mixtures of WB and SCB had greater activities than did the control cultures (0SCB/10WB and 10SCB/0WB), On the fifth day, the cultures 8SCB/2WB and 6SCB/4WB showed the greatest xylanases activities.

At the beginning of the process, there were drops in the pH values for all cultures, but from the third day onward, there were increases in this parameter. Furthermore, it was found that the cultures with WB had higher pH values in relation to the culture formulated without this substrate (10SCB/0WB). To a certain extent, the behavior of this parameter is associated with the quantity of WB in the medium, and the greater the quantity of WB, the higher the pH (Fig. 5a).

The quantity of mycelial mass estimated by means of quantity of *N*-acetylglucosamine is shown in Fig. 5b. It was found that at the beginning of the process (second day), the cultures formulated with WB and SCB mixtures had greater quantities of mycelial mass than did the control cultures (10SCB/0WB and 0SCB/10WB). However, from the third day onward, the culture formulated only with WB showed greater mycelial mass in relation to the other cultures.

Discussion

The results obtained for FPA, endoglucanases, β-glucosidases, and xylanases allow us to conclude that the production of these enzymes is favored in culture media formulated with mixtures of SCB and WB, carried out in SSF. Thus, these results indicated that such substrates have great potential for the generation of enzymes and also enable the reduction of production related costs.

It was found that the fungus *P. echinulatum* has great potential for enzyme secretion, since a FPA value of 45.82±1.88 U gdm^{-1} for the culture 6SCB/4WB was obtained on the third day. Although the substrates and methodologies of enzymatic activity analysis are not the same, it can be seen in Table 1 that the value for this study is higher than those shown

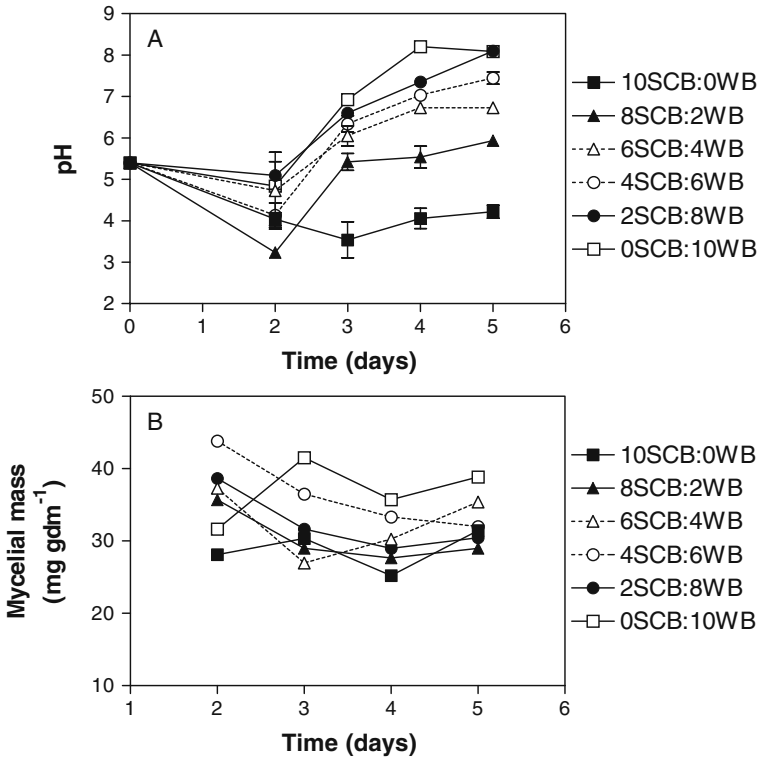

Fig. 5 Variation of the pH (**a**) and mycelial mass (**b**) in media formulated with different proportions of nontreated SCB and WB, in SSF, using the 9A02S1 strain of *P. echinulatum*. The *numbers* shown in the legend indicate the proportion of each medium component used

Table 1 Comparisons of enzymes productions from different fungi grown on lignocellulosic materials.

Organism	Substrate	Enzyme activities (IU gdm^{-1}) Substrate used to enzymatic assay				Reference
		FPA[a]	β-Glucosidase	Endoglucanase [b]	Xylanase	
P. echinulatum 9A02S1	WB and untreated SCB	45.82	40.13[c]	290.47	37.87[d]	Present study
T. reesei	Delignified wheat straw[d]	250	212[c]		5.400[f]	[1]
P. echinulatum 9A02S1	WB and pretreated SCB	32.89	58.95[c]	282.36	10[g]	[15]
T. reesei RUT C30	SCB	25.6				[37]
P. decumbens	Wheat straw and WB	17.7	52.8[d]			[27]
T. reesei and *A. phoenicis*	SCB	13.4	18.1[h]	73.8	2.842[f]	[38, 39]
T. reesei	SCB	5.3	7.7[h]	18.8	1.968[f]	[38, 39]
Myceliophthora sp.	Rice straw	2.44	7.48[h]	32.9	900[i]	[28]
Myceliophthora sp.	Bagasse	0.7	2.01[h]	6.62	620.1[i]	[28]
A. niger	Wheat straw and WB			14.8		[23]
A. ustus	Rice straw	6.51	15.8[h]	12.6	740[j]	[40]
A. ustus	WB	2	60[h]	11.8	615[j]	[40]
Thermoascus aurantiacus	Untreated wheat straw		105[h]	1235		[32]
T. aurantiacus	Alkali treated wheat straw		0.4[h]	66		[32]
T. aurantiacus	Untreated corn cobs		ND	18		[32]
T. aurantiacus	Alkali treated corn cobs		0.3[h]	68		[32]
Myceliophthora sp.	WB	0.74	3.83[h]	26.6	128.9[i]	[28]
A. awamori	Grape pomace				38[i]	[35]

[a] Measured using filter paper (Whatman No. 1)

[b] Measured using carboxymethyl cellulose

[c] Salicine

[d] Cellobiose

[e] For gram of cellulose present in delignified wheat straw

[f] Xylan

[g] Oat spelts xylan

[h] 4-Nitrophenyl β-D-glucopyranoside

[i] Birch wood xylan

[j] Larchwood D-xylan

ND not detected

previously, with the exception of those obtained by Chahal [1] for *Trichoderma reesei*, who found values of 250 U g^{-1} for cellulose of delignified wheat straw.

The greatest activities of β-glucosidases were 40.13±5.10 U gdm^{-1} obtained on the third day for culture 0SCB/10WB and 29.17±1.06 U gdm^{-1} for culture 2SCB/8WB, whose values (as seen in Table 1) were higher than those shown in several studies, but lower than the values obtained by Chahal [1] for *T. reesei*.

For endoglucanases, the values were higher than those seen in Table 1. The greatest activities were 290.47±43.57 and 276.84±15.47 U gdm^{-1} for the 6SCB/4WB culture on the fourth and fifth days, respectively.

The greatest xylanase activities were found on the third day for the cultures 6SCB/4WB (36.38±5.38 U gdm^{-1}) and 4SCB/6WB (37.87±2.26 U gdm^{-1}), whose values are not in agreement with the data given in Table 1.

It is important to note that the results presented in this study are preliminary. Future experiments will be carried out in a bioreactor, using the fungus *P. echinulatum* and the culture medium compositions selected in this study, monitoring parameters such as aeration, shaking, temperature, and humidity.

According to obtained results, it was found that the greatest activities were detected in the media formulated with mixtures of WB and SCB. The mixture of substrates allows a greater availability of nutrients for the mycelial development and the presence of enzyme inductors.

In *Fomes sclerodermeus,* the mixture of substrates (soy/WB, 1:1) induced the highest levels of hydrolases, the differences being more evident in polygalacturonase and polymetilgalacturonase activities [22]. In *Aspergillus niger,* the mixtures of milled wheat straw and WB induced the highest levels of endoglucanases [23]. This result is similar to those obtained with *T. reesei*, where enzymatic activities were generally higher during growth on mixed substrates than those obtained when single substrate was used [24].

Also, the use of WB may have influenced the results of this work. According to Archana and Satyanarayana [25], the universal suitability of WB as a substrate is that it contains sufficient nutrients and is able to remain free even in high-moisture condition providing large surface area. The biochemical composition of WB indicated that this material, when hidrolized, contains considerable amount of soluble sugars like glucose (42.5% dry wt), xylose (15.4% dry wt), arabinose (3.1% dry wt), and galactose (2.7% dry wt), required to initiate growth and to replicate the microorganism. The degree of substitution of the main xylan chains by arabinose was higher in WB [26]. It contained hemicellulose (45%), which may fulfill the role of inducers, and organic nitrogen sources (23%) that are essential for protein synthesis.

The use of lignocellulosic materials in SSF for enzyme production, especially in combination with WB, has been well documented [23, 27, 28], while the ability of SSF to minimize catabolic repression has been described previously. Some authors have suggested that the absence of catabolite repression in SSF is due to several factors, including the slow and low processes of diffusion in solid-state cultures owing to the low water activity [29]. However, all solid-state systems described as resistant to catabolite repression were developed using WB as substrate [30, 31].

The utilization of lignocellulosic material untreated to produce enzymes in SSF has being related by some authors. According to Kalogeris et al. [32], untreated wheat straw was the most effective carbon source for endoglucanase and β-glucosidase production (1235 and 105 U g^{-1} carbon source, respectively), while in alkali treated wheat straw, the production of endoglucanase and β-glucosidase was of 66 and 0.4 U g^{-1} carbon source, respectively. However, better results were obtained for corn cobs when the substrate was

alkali-treated. In experiments with corn cobs, an endoglucanase activity of 18 U g^{-1} was obtained, but β-glucosidase activity was not found. The results obtained in alkali-treated corn cobs were 68 and 0.3 U g^{-1} for endoglucanase and for β-glucosidase activities, respectively.

According to obtained results, the enzymatic secretion of *P. echinulatum* contains all enzymes that are part of the cellulose complex, including xylanase. In addition, to have a good thermal stability of FPA and β-glucosidases enzymes at 50 °C [33] is a valuable characteristic for its application in processes such as the enzymatic hydrolysis of cellulose and lignocelluloses for the production of glucose syrup, its cellulase complex presents a good proportion of FPA and β-glucosidase for efficiently hydrolyzing cellulose, when compared with the cellulases of *T. reesei* [34].

The pH decreased during the first 2 days of fermentation and reached 3.1, possibly due to microbial production of organic acids. When the concentration of soluble reducing sugars was very low, the pH increased, probably due to microbial assimilation of organic acids. Similar pH trends have been observed by Botella et al. [35] and Blandino et al. [36] during enzyme production by *Aspergillus awamori.*

The direct measurement of the fungal biomass is not possible due to the characteristics of the solid substrate as well as the association of the mycelium to the particles. The growth was estimated by measuring the *N*-acetylglucosamine (chitin) content in the dry matter. In this work, it was found that the levels of enzyme production were different according to the mixture of substrate used (Figs. 1, 2, 3, 4). Nevertheless, the biomass productions show that differential enzyme production among the different composition of medium was independent of the amount of biomass produced.

Conclusions

The fungus *P. echinulatum*, grown on a simple medium made of agricultural by-products and a low-cost mineral source in SSF, proved to be a promising microorganism for simultaneous cellulases and xylanases production. The fact that enzyme production differs depending on the substrate used makes the production of a particular group of enzymes by the selection of the substrate source rather easy in *P. echinulatum*. The incorporation of cheap sources, such as SCB into media for the production of lignocellulose enzymes, helps to reduce the production costs of enzymatic complexes capable of hydrolyzing lignocellulose residues for the formation of fermented syrups, thus contributing to the viable production of second generation ethanol. In addition, it can help to minimize the food chain competition and increase overall yields in comparison to biofuels of first generation.

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