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FODDER RADISH CAKE (*Raphanus sativus* L.) AS AN ALTERNATIVE BIOMASS FOR THE PRODUCTION OF CELLULASES AND XYLANASES IN SOLID-STATE CULTIVATION

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Abstract - Fodder radish (FR) is an oilseed crop with a high potential for biodiesel production due to its high productivity and the quality of its seed oil. FR oil extraction results in a residue that is rich in protein and fiber. In this study, FR cake (FRC) was evaluated as carbon and nitrogen source for the production of cellulases and xylanases using *Penicillium echinulatum* S1M29 during solid-state cultivation. It was determined that it is possible to partially replace wheat bran (WB) by FRC, resulting in 24.22 ± 0.25U/g Filter Paper Activity (144 hours), 210.5 ± 5.8U/g endoglucanase activity (144 hours), 22.62 ± 0.01U/g β-glucosidase activity (96 hours) and 784.7 ± 70.19U/g xylanase activity (120 hours). These values are equal or higher than the enzymatic activity obtained using WB. These results may contribute to the reduction of the cost of enzymes used in the production of cellulosic ethanol or other biotechnological applications.

Keywords: Cellulases; Xylanases; solid-state cultivation; Fodder radish cake; *Penicillium echinulatum.*

INTRODUCTION

In recent decades, the enzymatic hydrolysis of cellulose from lignocellulosic residues has been widely studied because cellulosic biomass is a possible resource for alternative fuel production. Additionally, this process has a great potential for reducing carbon dioxide emissions, thereby contributing to reducing global warming. However, to convert lignocellulosic biomass into biofuel, specifically ethanol, it is necessary to perform hydrolysis on the biomass, which requires enzymes, particularly cellulases and xylanases (Basso et al., 2014; Mabee et al., 2011; Reis et al., 2014).

Cellulases are enzymes that hydrolyze 1,4-linked β-glycosidic cellulose, yielding glucose, cellobiose and cello-oligosaccharides as primary products. This enzyme complex comprises endoglucanases, cellobiohydrolases and β-glucosidases. β-glucosidases are not considered legitimate cellulases, but they have an important function during cellulose hydrolysis (Martins et al., 2008; Padilha et al., 2015). These enzymes act synergistically to convert cellulose into glucose (Mabee et al., 2011). Xylanases hydrolyze xylan polysaccharides, which are the major constituents of hemicellulose. Therefore, xylanases increase the efficiency of enzymatic hydrolysis, whereas hemicellulose inhibits cellulose degradation (Herold et al., 2013).

Both cellulases and xylanases can be produced in solid-state cultivation (SSC) or submerged cultivation (SC). Commercially, most cellulases and xylanases are produced

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by SC because the production factors are easier to control. However, the SC method can be complex because it involves mixing, aeration, and control and monitoring of temperature, pH, dissolved oxygen and other factors (dos Reis et al., 2014; Reis et al., 2015), and the SC method requires specific equipment. Using SSC for enzyme production has attracted interest because it is a lower cost technology and has a relatively high enzyme production capacity (Camassola and Dillon, 2007; Camassola and Dillon, 2016; Macedo et al., 2013; Pirota et al., 2013; Yoon et al., 2014).

Among the microorganisms that can potentially produce cellulases that generate second-generation ethanol, *Penicillium echinulatum* mutants are notable because they have high enzymatic titers (Dillon et al., 2011) and produce appropriate proportions of FPA and β-glucosidase (Martins et al., 2008).

A major advantage of solid-state cultivation is the use of agricultural and agro-industrial residues (Camassola and Dillon, 2010). Agro-industrial residues include residues from the production of biodiesel, like fodder radish. Fodder radish has a rapid growth and high capacity to recycle nutrients, particularly nitrogen and phosphorus, developing reasonably in poor soils with acidity problems. It is important for crop rotation and produces great amounts of dry weight and is excellent for the practice of direct planting. Fodder radish enters the crop rotation system, minimizing soil compaction, producing green mass and reducing weed infestation during the fallow season of agricultural areas (Sluszz and Machado, 2006). Fodder radish produces 20 to 35 t / ha green mass, 3.5 to 8 t / ha of dry matter and 0.5 to 1.5 t / ha grains. The oil productivity is around 150 to 550 kg / ha (Ávila and Sodre, 2012). Seeds of fodder radish (*Raphanus sativus* L.) derive 40-54% of their mass from oil (Domingos et al., 2008) and the post-extraction residue of oil for biodiesel production contains approximately 39% (w/w) protein and 5% (w/w) minerals (Ávila and Sodré, 2012).

Additionally, fodder radish cake (FRC) is a promising residue that can be used in combination with lignocellulose for enzyme production. This study evaluated the potential of using FRC, alone and in combination with wheat bran (WB), as a substrate for the production of cellulase and xylanase during solid-state cultivation with *P. echinulatum.*

MATERIALS AND METHODS

Microorganism

The strain S1M29 of *P. echinulatum* was obtained from the strain 9A02S1 after several steps of hydrogen peroxide mutagenesis and selection in medium containing 2-deoxyglucose (Dillon et al., 2006). This strain was stored in the cultivation collection of the Enzymes and Biomass Laboratory, Institute of Biotechnology, Caxias do Sul, Rio Grande do Sul, Brazil. This strain was grown on C-agar slants (Dillon et al., 2011) for up to 7 days at 28°C until conidia formed and then was stored at 4°C until further use.

Enzyme production

WB and FRC were used as the support and primary carbon and nitrogen sources. The cultivation media consisted of a mixture of various ratios of WB and FRC, as specified in Table 1. The controls were WB and FRC alone.

The SSC was performed as described in Camassola and Dillon (2007, 2010) (Camassola and Dillon, 2010; Camassola and Dillon, 2007). The cultivation flasks were incubated at 28 °C and 90% humidity for 6 days. Four replicate experiments containing 2 g of dry mass biomass were performed for the same strains and each incubation time. The extraction of enzymes was done according to Camassola and Dillon (2007), the contents of each flask were separately added to a 125 mL-Erlenmeyer flask containing 10 mL distilled water, and the pH was measured. Next, 17 mL of 0.05 M citrate buffer (pH 4.8) was added, mixed, incubated under agitation for 30 min at 4 °C and then centrifuged at 3220 × g for 20 min. Enzyme assays were performed on the broth samples as described below.

Enzyme assay

The enzymatic activity was analyzed on filter paper (Filter Paper Activity - FPA) according to Camassola and Dillon (2012). The β-glucosidase activity was determined using p-nitrophenyl-β-D-glucopyranoside as the substrate (Daroit et al., 2008). The endoglucanase activity was determined according to the method outlined by Ghose (1987) using 2% (w/v) carboxymethyl cellulose solution

Table 1. Formulations used in the solid-state cultivation to produce xylanases and cellulases from *Penicillium echinulatum* S1M29.

	WB (%)	FRC (%)
Medium 1 (control)	100	0
Medium 2	0	100
Medium 3	50	50
Medium 4	75	25
Medium 5	25	75

WB – wheat bran; FRC – fodder radish cake.

in citrate buffer. The xylanase activity was determined using the same method as the endoglucanase activity assay, except that 1% (w/v) xylan from oat spelt solution was used as the substrate in place of carboxymethylcellulose. The reducing sugar was estimated as either the xylose or glucose equivalent using the dinitrosalicylic acid (DNS) method (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 minute under assay conditions. The enzymatic activities are expressed as units per gram of dry medium (U/g).

Mycelial Mass Determination

Growth determinations were performed by determining the amount of N-acetyl-D-glucosamine present in the mycelium and converted fungal biomass according to the method described by Novello et al. (2014).

Statistical tests

The results were statistically analyzed using analysis of variance (ANOVA) and Tukey post-test using the Prism GraphPad program (GraphPad Software, Inc., USA). A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Production of endoglucanases, β-glucosidases, FPA and xylanases

The endoglucanase production was the lowest in solid-state cultivation containing only FRC (100% FRC) compared with the other cultures (Figure 1A). However, the 96-hour cultivation in which the WB was replaced by 25% FRC (75% WB + 25% FRC) had similar enzyme activity compared with the control cultivation. There were higher endoglucanase activity values in 144-hour (210.5 ± 5.8 U/g) cultivation; however, the cultivations were not significantly different (100% WB, 75% WB + 25% FRC or 50% WB + 50% FRC).

For β-glucosidase (Fig. 1B), the highest activity was observed at 96 hours (22.62 U/g) in the 75% WB + 25% FRC medium. At 120 hours, there was an increase in β-glucosidase activity in the 50% WB + 50% FRC and 100% FRC media; however, the cultures were not significantly different. At 144 hours, the β-glucosidase enzyme activity was statistically indistinguishable for all media, with the exception of the FRC only medium (FRC 100%), which had decreased enzyme activity compared with 120 hours.

The FPA of the 75% WB + 25% FRC media was superior to the control (100% WB) at 120 hours. Although the average of the 75% WB + 25% FRC was higher at 144 hours (24.22 ± 0.25 U/g), the results were not significantly different from the 100% WB and 50% WB + 50% FRC treatments. The lowest FPA was observed using 100% FRC and 25% WB + 75% FRC (Figure 1C).

The highest xylanase enzyme activity (Figure 1D) was obtained with the 100% WB medium after 144 hours of cultivation (1137.59 ± 4.76 U/g). All cultures containing FRC had lower activities, with increasing FRC concentrations resulting in lower xylanase production.

Evaluation of fungal mass and pH of the cultures

The mycelial masses in various SSCs using the fungus *P. echinulatum* S1M29 are shown in Figure 2. The mycelial mass was estimated indirectly by analyzing the production of N-acetyl-D-glucosamine from the enzymatic hydrolysis of chitin in the fungal cell wall after 120 hours of cultivation.

The mycelial mass is proportional to the ratio of WB present in the medium; as the WB concentration increased, the mycelial mass increased. It was determined that that there was no direct relationship between enzymatic activities and mycelial mass for the evaluated enzymes (Figure 2).

The pH profile was measured in the experimental cultures (Figure 3). It was determined that for 48 hours there was no pH change; however, at 96 hours, there was acidification in all cultures. During this period, there was potentially carbon source consumption in the medium that resulted in acidification, with the pH reaching values near 4.0. After 96 hours, when there was increased enzymatic activity, the pH values increased to between 6.0 and 7.0. These data indicate that there is a correlation between pH and growth; these findings are according to Sternberg and Dorval (1979). They interpreted the pH variation in *Trichoderma reesei* cultures as a period of growth and NH_3 uptake, which released H^+ into the medium, while the pH increase at the end of the cultivation was due to NH_3 secretion by the fungus. This hypothesis is potentially corroborated by the results of the present work because the 100% WB cultures had the lowest pH and had the highest growth (Figure 2). There were lower pH values in the WB only medium after 48 hours of cultivation. These results are contrary to the results observed by Camassola and Dillon (2007); however, in that study, the WB was complexed with cane sugar bagasse.

Identical results can be observed when comparing enzymatic production using *A. niger* NRRL 567 (Dhillon et al., 2012); however, the FPA values were very high compared with other studies. A mixed culture of *T. reesei* and *A. niger* (Dhillon et al., 2011) had relatively similar enzyme concentrations compared with *P. echinulatum* S1M29 in 75% WB + 25% FRC medium, demonstrating the potential use of FRC for the production of enzymes that mediate lignocellulose hydrolysis (Table 2).

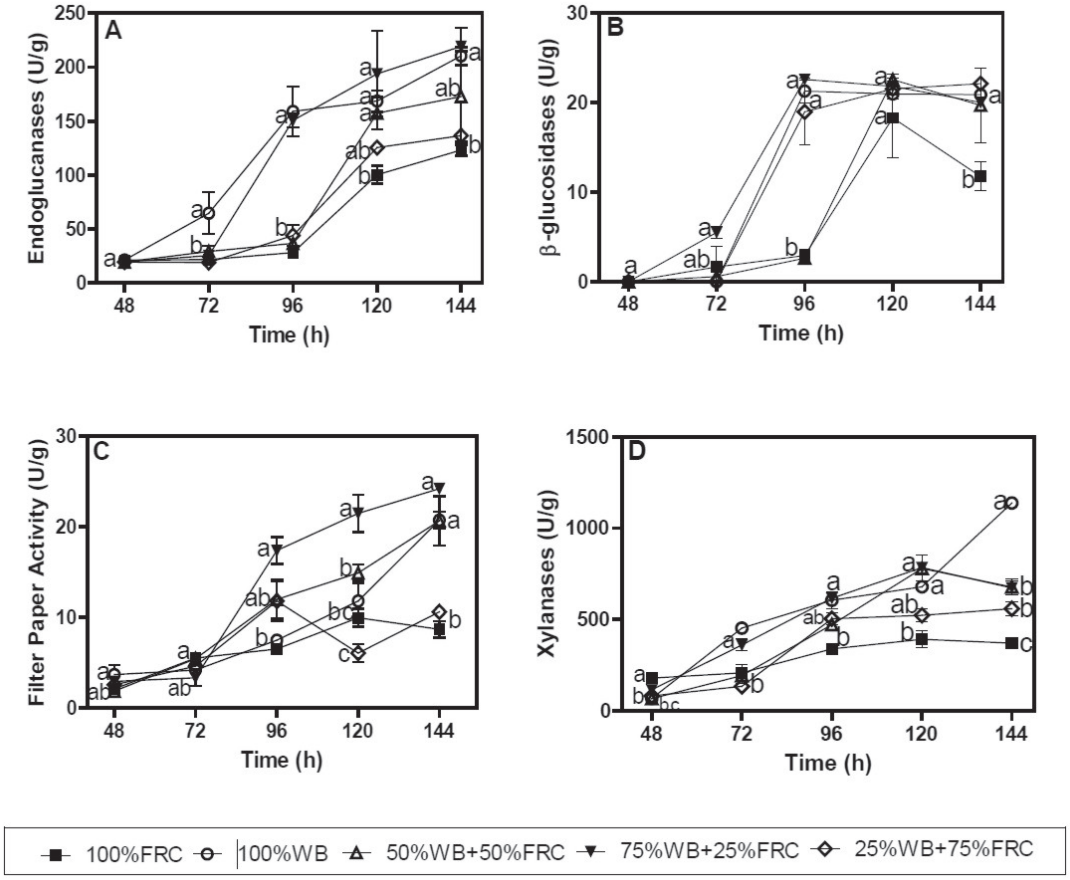

Figure 1. Activities of endoglucanases (A), β-glucosidases (B) FPA (C) and xylanase (D) in solid-state cultivation with *Penicillium echinulatum* S1M29 in various culture media using fodder radish cake and wheat bran as a substrate. The treatment means that have the same letters for the same day are not significantly different when evaluated using Tukey's test ($p > 0.05$). WB – wheat bran; FRC – fodder radish cake.

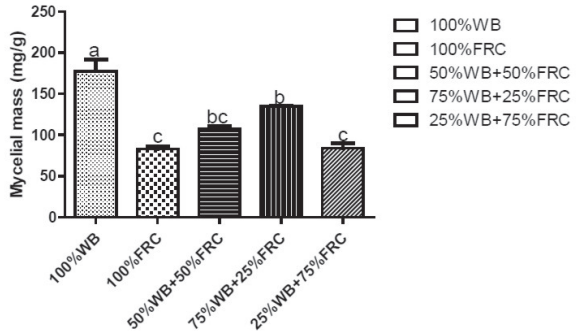

Figure 2. Mycelial mass concentrations in solid-state cultivation using *Penicillium echinulatum* S1M29 after 120 hours of cultivation in media with different formulations.

WB – wheat bran; FRC – fodder radish cake.

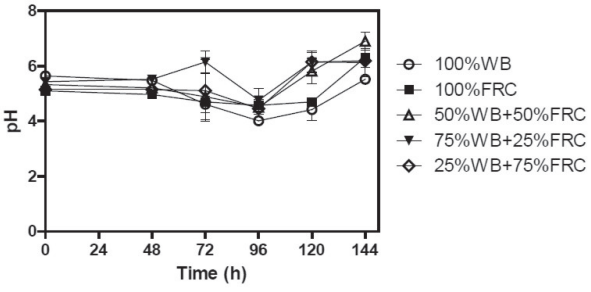

Figure 3. Variations of pH at different time points during the solid-state cultivation using *Penicillium echinulatum* S1M29.

Table 2. Comparisons of enzyme activities for different microorganisms grown on various lignocellulosic substrates.

Microorganism	Substrates	Enzyme activities (U/g)				References
		FPA	Endoglucanase	β-glucosidase	Xylanases	
P. echinulatum S1M29	75%WB+25%FRC	24.22	210.5	22.62	784.73	This Study
P. echinulatum S1M29	100% FRC	9.98	123.17	18.31	392.10	This Study
P. echinulatum 9A02S1	Sugar cane bagasse + WB	32.89	282.36	58.95	10.0	Camassola and Dillon, (2007)
A. niger NS-2	Sugar cane bagasse	3.0	5.0	0.03	-	Bansal et al., (2012)
T. asperellum SR1-7	WB	2.2	13.13	9.2	-	Raghuwanshi et al. (2014)
T. reesei + *A. niger*	WB	22.89	117.71	24.54	2.71	Dhillon et al. (2011)
A. niger NRRL 567	Rice husk + Apple pomace	113.68	172.3	60.09	-	Dhillon et al. (2012)
A. fumigatus SK1	Oil palm trunk	3.36	54.27	4.51	418.70	Ang et al. (2012)

The results indicated that FRC alone did not produce adequate cellulase and xylanase levels during solid-state cultivation. However, the replacement of 25% of the WB with FRC may be favorable for the production of cellulases because higher values were observed for FPA activity and β-glucosidases in cultures grown in this media. These experiments are relevant because they indicate that WB could be replaced by up to 25% for cellulase production in solid-state cultivation.

CONCLUSIONS

The present study has demonstrated that fodder radish cake can be employed as a component in media for cellulase production during *P. echinulatum* solid-state cultivation. Specifically, this study determined that up to 25% WB, a traditional substrate for cellulase production, may be substituted and is advantageous for cellulase production. Therefore, using FRC as an alternative carbon source to partially replace WB may lower the costs of producing enzyme complexes, which may in turn reduce the production costs of cellulosic ethanol.

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