β-glucosidase production by *Trichoderma reesei* and *Thermoascus aurantiacus* by solid state cultivation in sugarcane bagasse

P.A. Casciatori[1], F.P. Casciatori[2], R. Silva[1]

[1]Department of Chemistry and Environmental Sciences, Institute of Biosciences, Letters and Exact Sciences, São Paulo State University (UNESP), Cristóvão Colombo 2265, 15054-000, São José do Rio Preto – SP, Brazil
[2] Department of Chemical Engineering, Federal University of São Carlos (UFSCar), Rodovia Washington Luís km 235 (SP 310), 13565-905, São Carlos – SP, Brazil
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Presenting author email: roberto.silva@unesp.br

The global concern about the depletion of fossil fuel deposits and greenhouse gas effects demands research about renewable energy alternatives. Hence, the large-scale production of clean fuels, such as bioethanol, is generating interest worldwide. The solid state cultivation (SSC) is a process that provides high concentrations of enzymes, however, this technique still requires studies to be elected as a viable industrial process. The response of the process of production compared to the use of thermophilic and mesophilic filamentous fungi, as well as the effect of substrate composition and initial moisture content are important variables that can contribute to the improvement of this technique.

In Brazil, sugarcane bagasse (SCB) represents an abundantly available lignocellulosic agro-industrial by-product for conversion to second generation ethanol. Therefore, it is a potential source of fermentable sugars (Pandey et al., 2000a). The amount of sugarcane harvested in Brazil during the 2017/2018 season was approximately 641 million tons (UNICA, 2018) which generated around 160 million tons of SCB. However, the production of ethanol from SCB is limited by the recalcitrance of this biomass. The most ecologically friendly alternative to produce biofuel is through the enzymatic route, which requires cellulolytic and hemicellulolytic enzymes to depolymerise the biomass into fermentable sugars. Nevertheless, the cost of commercial enzymes is the main limiting factor for making bioethanol production via the enzymatic route viable (Mishima et al., 2006). In this context, studies on obtaining cellulases and hemicellulases and optimizing the production process are greatly important. Cellulolytic and hemicellulolytic enzymes produced by filamentous fungi in solid-state cultivation (SSC) present high activities because SSC mimics the natural habitat of filamentous fungi, which release high levels of enzymes to the extracellular solid medium (Pandey, 2003). The utilization of agro-industrial by-products as substrates is another great advantage of SSC, due to the low cost of this kind of substrate (Pandey et al., 2000b).

In this context, the aim of this work was to study the production of cellulolytic enzymes of fungi *Thermoascus aurantiacus* CBMAI 756 (thermophilic) and *Trichoderma reesei* QM9414 (mesophilic) by solid state cultivation for the production of β-glucosidase, using agroindustrial waste as substrates. Varied the proportion of bagasse for wheat bran in substrate composition and the substrate moisture content wet basis.

SSC experiments were done in plastic bag received 5 g of substrate (dry weight). The sets of plastic bags and substrates were sterilized in an autoclave at 121 ºC for 20 min. For inoculation, spores were suspended with nutrient solution enriched with mineral salts (Zanelato et al., 2012). The spore concentration was fixed at 10^7 spores/mL and 1 mL was added to each bag. The moisture content of the substrate was adjusted by adding nutrient solution to the solid media. *Thermoascus aurantiacus* was cultivated at 50 ºC for 120 h; *Trichoderma reesei* at 28 ºC for 168 h.

Minitab 15.0 (Minitab Inc., State College, USA) was used for experimental design and statistical analysis, in which the response was the β-glucosidase activity. The chosen experimental design for solid-state cultivation was a full-factorial design with random blocks and 2 factors with 3 levels each one. Factors were moisture content of the substrate, with levels 70, 75 and 80 % (wet basis), and composition of the substrate, with levels SCB:WB 1:1, 3:1 and 9:1 w/w. The fungi were treated as blocks of the design. ANOVA analysis and means comparisons by Tukey test were performed in order to find the conditions that provided highest β-glucosidase activities. All statistical significance levels were 95 %. For samples destined for enzymatic activity quantification, distilled water was added to the fermented material (20 mL/g of initial dry substrate), followed by agitation at 100 rpm for 30 min at room temperature and centrifugation at 10^4 g for 15 min at 5 ºC. The supernatant was denoted as enzymatic raw extract. β-glucosidase activity was quantified according to Leite *et al* (2007) at 45 ºC for *Trichoderma reesei* and 65 ºC for *Thermoascus aurantiacus*.

Table 1 shows the results of β-glucosidase activities of the enzymatic extracts obtained from cultivation of thermophilic fungus *Thermoascus aurantiacus* CBMAI 756 and of mesophilic fungus *Trichoderma reesei* QM9414 on three combinations of substrate composition and moisture content. A average values and standard deviation of triplicates are presented, as well as the summarized results of average comparisons by the Tukey test ($\alpha = 0.05$).

Table 1. β-glucosidase activities of extracts from *Thermoascus aurantiacus* and by *Trichoderma reesei*. (*)

β-glucosidase activities ± standard deviations (U/gss)				
Thermoascus aurantiacus				
MC	SCB:WB			
(%)	1:1	3:1	9:1	← Average
70	8.0 (± 1.2)	7.9 (± 1.2)	16.8 (± 0.8)	10.9 (± 5.1) [a]
75	7.0 (± 1.0)	8.9 (± 2.6)	15.9 (± 0.2)	10.6 (± 4.7) [a]
80	6.8 (± 1.0)	12.6 (± 1.1)	15.0 (± 1.2)	11.5 (± 4.2) [a]
Average ↑	7.3 (± 0.6) [B]	9.8 (± 2.5) [B]	15.9 (± 0.9) [A]	
Trichoderma reesei				
MC	SCB:WB			
(%)	1:1	3:1	9:1	← Average
70	5.0 (± 0.1)	3.8 (± 0.2)	3.9 (± 0.5)	4.2 (± 0.7) [b]
75	5.0 (± 0.7)	4.3 (± 0.3)	4.2 (± 0.3)	4.5 (± 0.4) [ab]
80	5.4 (± 0.3)	4.6 (± 0.3)	4.3 (± 0.3)	4.8 (± 0.6) [a]
Average ↑	5.1 (± 0.2) [A]	4.2 (± 0.4) [B]	4.1 (± 0.2) [B]	

(*) Significance level $\alpha = 0.05$. Different capital letters at same row indicates significant difference by Tukey test ($p < 0.05$) due to effect of composition, for average value of activity for each moisture content (MC, % w.b.); different lowercase letters at same column indicates significant difference by Tukey test ($p < 0.05$) due to effect of MC, for average value of activity for each composition; equal capital letters at same row and equal lowercase letters at same column indicate no significant difference by Tukey test ($p < 0.05$) due to effects of composition (for average on MC) and of MC (for average on composition), respectively.

For the thermophilic fungus, the minimum β-glucosidase activity (6.8 U/gss) was obtained with SCB:WB 1:1 w/w and 80 % moisture content, while the maximum (16.8 U/gss) was found with SCB:WB 9:1 independent of moisture content. Statistical analysis based on main effects plots suggested that β-glucosidase activity is significantly affected only by substrate composition. However, ANOVA analysis pointed out statistically significant interaction effects among substrate composition and moisture content in all treatments are analysed.

For the mesophilic fungus *Tr. reesei*, Table 1 shows minimum β-glucosidase activity (3.8 U/gss) for SCB:WB 3:1 w/w and 70 % moisture content, while maximum activity (5.4 U/gss) was obtained with SCB:WB 1:1 w/w and 80 % moisture content. Statistical analysis based on main effects plots suggested that, for *Tr. reesei*, β-glucosidase activity is significantly affected by both the substrate composition and the moisture content.

As already stated, the use of two fungi with different thermal behaviors aimed to compare which system (fungus and temperature) enables to reach higher β-glucosidase activities. Enzymatic extracts obtained from the cultivation of *Th. aurantiacus* presented β-glucosidase activities approximately 3 times higher than the activities of the extracts obtained from *Tr. reesei*, reiterating that the thermophilic fungus is able to release enzymes with higher activities. The optimum conditions for production of β-glucosidase by both fungi were obtained and did not vary in the proportion of sugarcane bagasse and wheat bran 9:1 w/w with 70 % moisture content wet basis.

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