I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ** XV SHEB ** XV ENZITEC FLORIANÓPOLIS ** 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
BIOPROCESS ENGINEERING

INFLUENCE OF NITROGEN SOURCE ON *TRICHODERMA HARZIANUM* CULTURE FOR CELLULOLYTIC ENZYME PRODUCTION

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ABSTRACT

The effort to produce cellulases to reduce the overall expenses associated with the second-generation ethanol process holds paramount importance within the biofuel sector. This study aimed to produce cellulases by *Trichoderma harzianum* using peptone, urea, and ammonium sulfate individually as nitrogen sources in the growth medium for the degradation of pretreated sugarcane bagasse. Statistical analysis of the findings revealed that the highest levels of FPase and β-glucosidase (0.45 and 0.63 IU/mL, respectively) activity were obtained using the APEP medium, exclusively containing peptone as the nitrogen source. In the enzymatic hydrolysis of pretreated sugar cane bagasse, APEP presented the best glucose yield (5.33 g/L). This is evidence that additional sources of nitrogen are not necessary for favorable results.

Keywords: Cellulase production. Enzyme activity. Enzymatic hydrolysis.

1 INTRODUCTION

Ethanol from the sugars in lignocellulosic material, second-generation ethanol (E2G), has been the subject of numerous investigations, since sugarcane waste material does not compete with food resources. However, an important step in the production of E2G is the conversion of cellulose into glucose, requiring the action of cellulolytic enzymes [1]. Cellulases have numerous applications in chemical industries and are mainly produced by fungi, due to their ability to produce abundant cellulases and hemicellulases, which are secreted into the culture medium, facilitating extraction and purification steps. *Trichoderma reesei* is one of the most important producers, but the scientific community has been evaluating *Trichoderma harzianum* as a potential cellulase producer [2]. The acquisition of cellulolytic enzymes is a critical factor in the hydrolysis of lignocellulosic materials to produce fermentable sugars, as they represent a considerable part of the production costs [3].

Cellulases are classified into three main groups: Endoglucanase (CMCase), Cellobiohydrolase, and β-Glucosidase, which work together to hydrolyze cellulose. CMCase acts on the internal linkages of the cellulose chain. Cellobiohydrolases act on the ends of the cellulose chains with release of cell-oligossaccharides (COs). β-glucosidase acts on both COs and cellobiose with release of the glucose monomer [4].

Since improving cellulase production in an economical way is of fundamental interest to promote industrial application, the choice of microorganism, carbon and nitrogen source is essential to increase the efficiency of cellulase production [5]. This study investigated cellulase production by *T. harzianum* using three different nitrogen sources. The aim is to achieve high levels of enzymatic activity and increase the yield of fermentable sugars in enzymatic hydrolysis, to produce E2G with a low-cost approach.

2 MATERIAL & METHODS

T. harzianum CFAM 422 from the Amazon Fungi Culture Collection was employed in this study. A spore suspension was prepared by adding 10 mL of 0.9% saline and 1% Tween 80 to petri plates grown on 2% malt extract agar at 30 ºC for 7 days. A initial concentration of 10^6 spores/mL was inoculated into 100 mL Erlenmeyer flasks containing 20 mL of Mandels medium [6], consisting of the following components (g/L): 2.0 KH_2PO_4, 0.3 $CaCl_2$, 0.30 $MgSO_4 \cdot 7H_2O$, 12.143 NaH_2PO_4, 3.2 $Na_2HPO_4 \cdot 2H_2O$, 17.9 microcrystalline cellulose (Avicel® PH-101 - Fluka, Ireland) as a carbon source (A), and trace elements (mg/L): 5.0 $FeSO_4 \cdot 7H_2O$, 20 $CoCl_2 \cdot 6H_2O$, 1.6 $MnSO_4 \cdot 4H_2O$, 1.4 $ZnSO_4 \cdot 7H_2O$. The culture medium was modified in terms of the concentration of nitrogen sources, prepared based on the percentage of carbon and nitrogen to maintain a C/N ratio of 10, an optimal ratio observed in previous studies by the research group. The following mediums (g/L): 17.9 Avicel + 1,71 Urea (AU), 17.9 Avicel + 3,76 $(NH_4)_2SO_4$ (AAS), 17.9 Avicel + 7.97 Peptone (APEP), and Mandels medium with Avicel and three nitrogen sources (g/L): 17.9 Avicel + 0.3 urea, 3.6 peptone and 1.4 $(NH_4)_2SO_4$, standard medium (ASM) and standard medium with 0.1% (w/v) of Tween 80 (ASMT) were evaluated for cellulolytic enzyme production. Tween 80 was added to the standard medium to observe homogenization of the fungus. Flasks were incubated in an orbital shaker (Infors AG CH-4103 Bottmingen) at 30°C, 200 rpm for 5 days [7]. Samples were collected and centrifuged at 6 x 60 g/10 min (Centrifuge MPW 351R5418 - MPW MED Instruments). The supernatant was used to measure enzymatic activities and to hydrolyze the sugarcane bagasse. All cultures were carried out in triplicates.

Enzyme activities were measured using microassays based on Ghose's methodology, with modifications [8]. FPase, Endoglucanase, and β-glucosidase activities were evaluated using filter paper with a diameter of 7 mm, 2% CMC, and 15 mM

cellobiose as substrates, respectively, and calculated according to Eq. 1, in which, IU is the International Unit. The enzyme extract was diluted in 0.1 M citrate/citric acid buffer (pH 5) for all tests. The reactions were conducted in PCR plates incubated in a thermal cycler (Applied Biosystems Veriti™ Thermal Cycler) at 50 °C for 60 min for FPase, 50 °C for 30 min for Endoglucanase and β-glucosidase. The reducing sugar released was quantified by spectrometry at 540 nm (SpectraMax® 190 microplate reader) using DNS method[9] for the FPase and Endoglucanase activities (95 ºC for 10 min), and at 505 nm for the β-glucosidase assay using Glucose Monoreagent (Quibasa BioClin-K082-2, Brazil) at 37 °C for 10 min.

$$IU = \frac{\mu mol\ glucose\ released}{min.\ enzyme\ (mL)} \quad (1)$$

Sugarcane bagasse, previously dried at 40 ºC for 48 h, was subjected to organosolv pretreatment in a high-pressure reactor (Parr 4555, Instrument Company, Moline, United States) at 180 ºC for 3 h, using ethanol/water as a solvent in a ratio (1:1- v/v) [10] and characterized according to the protocol published by the Laboratory Analytical Procedures [11]. Sugarcane bagasse pretreated, 0.1 M sodium citrate/citric acid buffer (pH 5.0) and the enzyme extracts were added to the reaction medium at a solid: liquid ratio of 1:16 (w/v) for the enzymatic hydrolysis assays in triplicate. The reaction was carried out at 50 °C for 48 h in 24-well deep well plates incubated on an orbital shaker (Infors AG CH-4103 Bottmingen) [10]. Sugars were then determined by high-performance liquid chromatography (HPLC) (Agilent - 1260 Infinity II LC System) equipped with a refractive index detector, Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) operating at 45 ºC, 5 mM H_2SO_4 as eluent, at a flow rate of 0.6 mL/min. Analysis of variance (ANOVA) and Tukey pos-hoc test (α = *0.05*) were performed to evaluate the statistical significance between the mean of the activities and enzymatic hydrolysis of each culture medium. Analyses were performed using Origin 8.5 Pro.

3 RESULTS & DISCUSSION

Characterization of the pretreated sugar cane bagasse revealed 60% cellulose. Among the nitrogen sources evaluated for enzyme production by *T. harzianum*, the APEP, ASM, and ASMT mediums exhibited the highest levels of enzyme activity (Figure 1A). The APEP, ASM, and ASMT mediums demonstrated maximum FPase activities of 0.45, 0.27, and 0.46 (IU/mL), respectively. Additionally, β-glucosidase activities were recorded at 0.63, 0.48, and 0.61 (IU/mL) respectively. A study utilizing pretreated corn stover for cellulase production by *T. harzianum* LZ117 at 28°C for 5 days revealed an FPase activity of 0.64 IU/mL, consistent with the FPase data observed in this study for the APEP and ASMT mediums. Furthermore, the incorporation of Tween 80 in the standard medium (ASMT) demonstrated improved performance, with a 70.4% and 27% increase in FPase and β-glucosidase activity, respectively, compared to the ASM medium (Figure 1A). Including Tween in the medium offers advantages such as reducing the adsorption of non-productive cellulases to cellulose and safeguarding enzyme activity from thermal or mechanical stress, thereby enhancing enzyme efficiency [3]. However, it is noteworthy that despite the apparent increase in activity with the addition of Tween, the standard medium (ASM) requires more reagents than the APEP medium, potentially elevating the overall cost of enzyme production. Based on statistical analysis (Figure 2), there was no significant difference between the means ($p > 0.05$) of FPase, Endoglucanase and β-glucosidase activities for the APEP and ASMT mediums (Figure 2A-C).

The hydrolysis of pretreated sugarcane bagasse (Figure 1B) was only carried out for mediums that presented high FPase and β-glucosidase activities. The statistics for enzymatic hydrolysis (Figure 2D-F) indicated that the APEP and ASMT mediums did not present significant differences ($p > 0.05$) in the yield of glucose and xylose. The APEP and ASMT media had glucose yields of 5.33 and 5.27 g/l, respectively, results that were not statistically different ($p > 0.05$).

The theoretical conversion of cellulose to glucose for the dry mass of the pretreated sugarcane bagasse used in this study is 41.6 g/L. From the data on glucose produced by enzymatic hydrolysis, the APEP, ASM, and ASMT mediums presented conversions of 13.7, 8.7, and 13.5%, respectively.

These results suggest that the APEP medium could be promising for the production of cellulolytic enzymes, and degradation of sugarcane bagasse for the production of E2G, since there are fewer reagents in its composition, making the process less expensive.

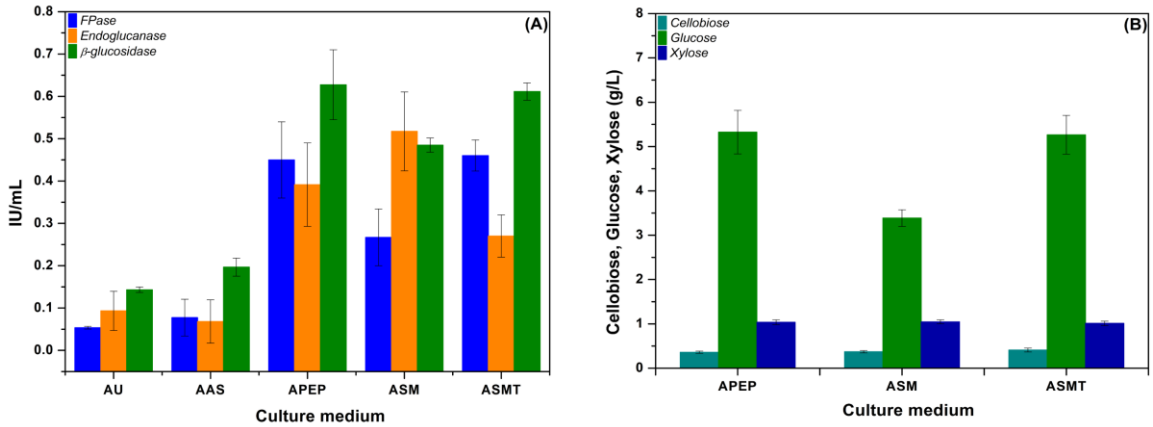
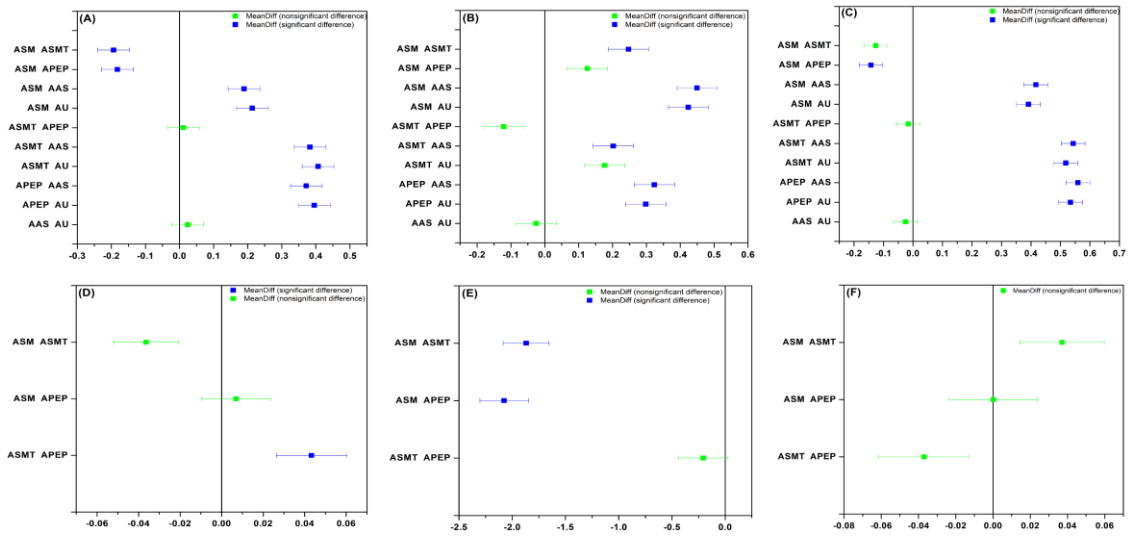


Figure 1 FPase, Endoglucanase, β-glucosidase activities (A), cellobiose, glucose and xylose data from the enzymatic hydrolysis of pretreated sugarcane bagasse (B).



Closer the averages are to zero, it means that there is no significant difference

Figure 2 Significant difference between the mean values of FPase (A), Endoglucanase (B), β-glucosidase (C) activities and difference between the mean yields of cellobiose (D), glucose (E) and xylose (F) in the hydrolysis of pretreated sugarcane bagasse.

4 CONCLUSION

APEP medium provided good results in terms of enzymatic activity and hydrolysis, and the addition of Tween to the standard medium improved FPase activity. It can be concluded that peptone meets the nitrogen requirements of *T. harzianum* and that it is not necessary to incorporate urea and ammonium sulfate to achieve satisfactory FPAse and enzymatic hydrolysis results. Therefore, to improve FPase activity and glucose yields for the subsequent production of E2G, the addition of Tween to the APEP medium could be evaluated in further studies.

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ACKNOWLEDGEMENTS

For financial support, the authors acknowledge the Brazilian National Council for Scientific and Technological Development (CNPq) (project process: 440538/2022-0 - Development of a bioprocess for the production of second-generation ethanol). Embrapa Agroenergy for its structural and technical support.