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INFLUENCE OF CULTURE CONDITIONS ON CELLULASES PRODUCTION BY *TRICHODERMA HARZIANUM*

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ABSTRACT

Minimizing the costs of producing second-generation ethanol is of industrial interest, with enzymatic hydrolysis of lignocellulosic material being a crucial step. Optimizing the cultivation medium can reduce enzyme production costs and enhance enzyme activity. This study aimed to optimize the production of cellulases by *Trichoderma harzianum* using crystalline cellulose, glycerol as a carbon source and Tween 80 for enzyme stability. Cultivations were conducted in Erlenmeyer flasks and a benchtop bioreactor at 30 °C, 200 rpm, for 5 and 6 days, respectively. Statistical analysis and cost reduction considerations identified APEPT medium (17.9 g/L Avicel + 6.3g/L peptone + 0.1% Tween 80) as providing the best FPase results at 0.87 IU/mL in Erlenmeyer flasks. Bioreactor production using APEPT medium increased endoglucanase activity by 194.3%.

Keywords: Cellulolytic enzymes. Enzymatic hydrolysis. Cellulose. Lignocellulose.

1 INTRODUCTION

The biotechnological development of cellulolytic enzymes for second-generation ethanol (E2G) production has achieved significant scientific interest. A crucial step in sustainable E2G production is the enzymatic degradation of lignocellulosic material, a low-cost feedstock [1]. Enzymatic hydrolysis of cellulose involves three types of enzymes: Endoglucanases, which cleave the cellulose chain internally; cellobiohydrolases, which release cello-oligosaccharides from the chain ends; and β-glucosidases, which convert cellobiose and cello-oligosaccharides to glucose [2]. However, the enzymes used to hydrolyze this material represent a significant part of the process cost [3]. These enzymes (cellulases) can be produced by filamentous fungi from *Trichoderma* genus, which have proven to be adapted to biotechnological processes [4].

Studies aimed at optimizing the medium to reduce the cost of production of the enzymes are crucial. One strategy is to increase yields using sustainable substrates. Cellulose, an abundant renewable biopolymer, is one of the main carbon sources for the induction of cellulase production by filamentous fungi [5]. On the other hand, the presence of easily assimilated substrates such as glucose represses cellulase gene expression, compromising the process [6]. Among the substrate alternatives, glycerol stands out as a neutral carbon source that does not suppress cellulases and has the potential to promote *Trichoderma* growth. This is advantageous given the high biomass concentration required to maximize cellulase volumetric productivity and achieve high yields [2]. The literature reports that adding surfactants to the culture medium can increase the hydrolysis rate of pure cellulosic materials by protecting the enzymatic activity from the stress caused by agitation and heat, as well as reducing the adsorption of non-reactive cellulases to cellulose [3].

Cellulase production is a multivariable process influenced by the composition of the culture medium and environmental factors (temperature, pH), which can significantly enhance production efficiency and reduce costs for the industrial application of these enzymes in second-generation ethanol (E2G) production. This study evaluated the effects of culture medium components on enzyme production by *T. harzianum* in order to identify the optimal medium for maximizing cellulase production efficiency.

2 MATERIAL & METHODS

The *Trichoderma harzianum* CFAM 422 strain from the Amazon Fungi Culture Collection was used in this study. The fungus was grown in petri plates with Malt Extract Agar 2% medium at 30 ºC for 7 days. Spores from the petri plate were resuspended in saline solution 0.9%, and inoculated an initial concentration of 10^6 spores/mL in 100 mL Erlenmeyer flasks containing 20 mL of adapted Mandels medium [7]. The culture mediums were prepared with reagents common to all experiments, such as (in 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), and trace elements (in mg/L): 5.0 $FeSO_4 \cdot 7H_2O$, 20 $CoCl_2 \cdot 6H_2O$, 1.6 $MnSO_4 \cdot 4H_2O$, 1.4 $ZnSO_4 \cdot 7H_2O$. Specific components of each cultivation medium evaluated were prepared based on a variation of the control medium Avicel + Soy Peptone (APEP), selected in previous studies by the research group. The following elements were weighed (in g/L): 17.9 Avicel + 6.3 soy peptone and 0.8 $(NH_4)_2SO_4$ (APEPA); 17.9 Avicel + 7.97 soy peptone and 1 Tween 80 (APEPT); 17.9 Avicel + 7.97 soy peptone and 5. 97 glycerol (APEPG); 17.9 Avicel + 8.6 soy peptone, 0.8 $(NH_4)_2SO_4$ and 5.97 glycerol (APEPAG); 17.9 Avicel + 8.6 soy peptone, 0.8 $(NH_4)_2SO_4$, 5.97 glycerol and 1 Tween 80 (APEPAGT). The concentration of the carbon and nitrogen sources was calculated to maintain a C/N ratio of 10, an optimum ratio observed in previous studies by the research group. The flasks were then incubated at 30 ºC at 200 rpm for 5 days in orbital shaker (Infors AG CH-4103 Bottmingen) [8]. The

samples were centrifuged, and the supernatant was used to analyze activity and hydrolyze enzymatic, and to determine total proteins. The pellets were submitted to the gravimetric quantification method. After drying, the pellets were subjected to digestive degradation of the fungus using 80% acetic acid and 100% nitric acid in a 10:1 ratio at 100 ºC for 30 min. The mass was dried again to quantify Avicel and dry weight of fungus (DWF). Enzymatic assays were conducted in 96-well PCR microplates based on a modified Ghose method[9]. FPase, Endoglucanase, and β-glucosidase activities were evaluated using 7 mm diameter filter paper, 2% CMC, and 15 mM cellobiose as substrates, respectively, and calculated according to international units (IU), Eq 1. Reactions were carried out in a thermal cycler at 50 °C for 60 minutes for FPase activity and at 50 °C for 30 minutes for Endoglucanase and β-glucosidase activities. The released reducing sugar was measured by spectrometry at 540 nm (SpectraMax® 190 microplate reader) with the dinitrosalicylic acid (DNS) method [10] for FPase and Endoglucanase activities (at 95°C for 10 minutes), and at 505 nm for the β-glucosidase assay using Glucose Monoreagent (Quibasa BioClin-K082-2, Brazil) at 37°C for 10 minutes. Total proteins were measured using the bicinchoninic acid method [11].

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

A 2.0 L stirred tank bioreactor (Minifors 2 Benchtop Bioreactor, Infors HT) with automatic control of temperature, pH, agitation, and aeration, an operating volume of 1 L, was inoculated with a suspension of 10^6 spores/mL. The bioprocess was maintained at 30 ºC and pH 6.0, with a stirring of 200 rpm and an air flow rate of 0.6 L/min for 6 days. The pH was adjusted using 1 M NH_4OH and 1 M HCl, and foaming was controlled manually with sterilized anti-foaming agent. The produced enzyme extract was used for hydrolyzing sugarcane bagasse, which was dried at 40 °C for 48 h and pretreated in a high-pressure reactor (Parr 4555, Instrument Company) using the organosolv method at 180 °C for 3 h with ethanol/water (1:1 v/v) as the solvent [12]. Enzymatic hydrolysis was conducted with a bagasse/liquid ratio of 1:16 (w/v) in 0.1 M sodium citrate/citric acid buffer (pH 5.0) at 50 °C for 48 h in 24-well deep well plates on a shaker (Infors AG CH-4103 Bottmingen). Sugars were quantified by high-performance liquid chromatography (HPLC) (Agilent - 1260 Infinity II LC system) with a refractive index detector and an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad) at 45 °C, using 5 mM H_2SO_4 as the eluent at 0.6 mL/min flow rate. Experiments were performed in triplicate, and statistical analyses were conducted using Minitab 21 statistical software, with significance determined by ANOVA followed by Tukey's test ($\alpha = 0.05$).

3 RESULTS & DISCUSSION

APEPT and APEPAGT mediums presented the highest FPase activities, 0.87 IU/mL and 0.86 IU/mL, respectively (Figure 1A). According to the Tukey test, adding $(NH_4)_2SO_4$ and glycerol to APEPAGT did not significantly increase FPase activity compared to APEPT ($p > 0.05$). Average dry weight of fungal (DWF) was not significantly different between the mediums evaluated, except APEPT, with the lowest concentration (7.78 g/L DWF). On the other hand, adding 0.1% Tween 80 to the control medium (APEP) increased FPase activity, likely by improving enzyme stability [3]. For Endoglucanase activities, there was a significant difference ($p < 0.05$) between the APEPT and APEPAGT mediums, with APEPT yielding the highest results. The β-glucosidase activities did not show any significant differences between the different mediums ($p > 0.05$) (Table 1).

Sugarcane bagasse pretreated by the organosolv method was characterized and 60% cellulose was determined. The APEPT, APEPAG and APEPAGT mediums presented the highest values of glucose released upon enzymatic hydrolysis of sugarcane bagasse (Figure 1B), converting 20%, 20.1% and 23.3% of cellulose respectively. All mediums presented an average cellobiose residue of 0.26 g/L at the end of hydrolysis, which could increase glucose yields with increasing hydrolysis time.

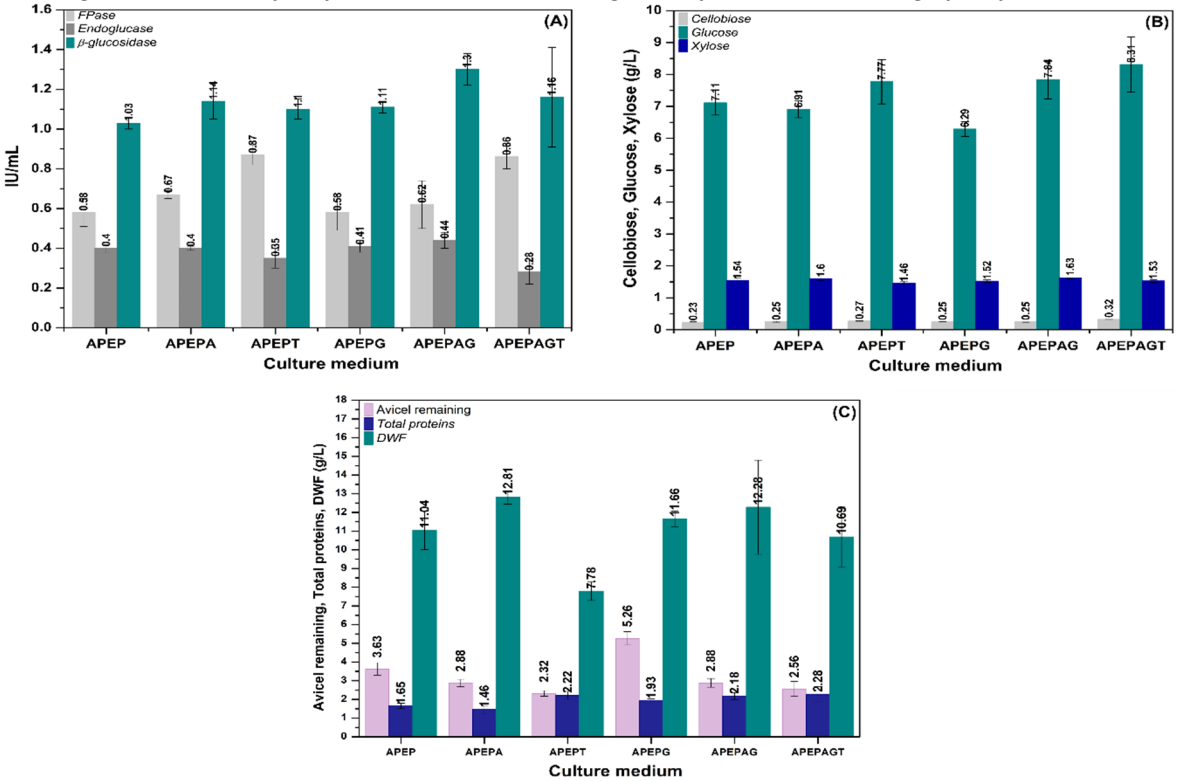

Figure 1 FPase, Endoglucanase, β-glucosidase activities (A). Cellobiose, glucose and xylose data from the enzymatic hydrolysis of pretreated sugarcane bagasse (B), Remaining avicel, total proteins and DWF (C).

The APEPT, APEPAGT, APEPG and APEPAG mediums produced similar total protein values with an average Avicel (microcrystalline cellulose) consumption of 85%, as shown in Table 2.

Table 1 Comparisons between means using Tukey's 95% confidence method.

		FPase (IU/mL)	Endoglucanase (IU/mL)	β-glucosidase (IU/mL)
Culture medium	**N***	**Average**	**Average**	**Average**
APEPT	3	0.87 ± 0.05[a]	0.35 ± 0.02[ab]	1.10 ± 0.05[a]
APEPAGT	3	0.86 ± 0.06[a]	0.28 ± 0.06[b]	1.17 ± 0.25[a]
APEPA	3	0.67 ± 0.02[ab]	0.40 ± 0.01[a]	1.14 ± 0.09[a]
APEPAG	3	0.62 ± 0.12[b]	0.44 ± 0.04[a]	1.53 ± 0.08[a]
APEP	3	0.57 ± 0.07[b]	0.40 ± 0.02[a]	1.03 ± 0.03[a]
APEPG	3	0.58 ± 0.09[b]	0.41 ± 0.03[a]	1.11 ± 0.03[a]

**Number of replicates. Averages that do not share the same letter are significantly different.*

Table 2 Overall yield of DWF and product for substrate consumed, $Y_{X/S}$ and $Y_{P/S}$, respectively, and overall yield of product per DWF $Y_{P/X}$.

Culture medium	$Y_{X/S}$	$Y_{P/S}$	$Y_{P/X}$
APEPT	0.50 ± 0.04	0.14 ± 0.01	0.27 ± 0.04
APEPAGT	0.70 ± 0.13	0.15 ± 0.02	0.22 ± 0.07
APEPA	0.85 ± 0.04	0.10 ± 0.01	0.12 ± 0.02
APEPAG	0.82 ± 0.18	0.14 ± 0.01	0.18 ± 0.05
APEP	0.77 ± 0.09	0.12 ± 0.01	0.15 ± 0.03
APEPG	0.92 ± 0.01	0.13 ± 0.02	0.16 ± 0.02

X is dry weight of fungus produced (g/L); S is Avicel concentration consumed (g/L) and P is proteins concentration (g/L).

Among the evaluated mediums, APEPT was selected for scaling up in a benchtop bioreactor. Compared to the small-scale results, FPase and β-glucosidase activities decreased by 39% and 15%, respectively, in the bioreactor cultures. However, Endoglucanase activity increased by 194.3% (Tables 1 and 3). The total protein yield was 2.24 ± 0.1 g/L, similar to that obtained in Erlenmeyer flask cultures. Despite the reduced FPase and β-glucosidase activities in the bioreactor-produced enzyme extract, bagasse hydrolysis released 14.5 g/L glucose, 186.6% more than small-scale production (Table 3), and converted 38.3% of cellulose at an enzyme load of 7.9 FPU/g bagasse.

Table 3 Data on the enzymatic activity and hydrolysis of the APEPT culture medium on a bench-scale bioreactor.

Enzymatic activities (IU/mL)			Enzymatic hydrolysis (g/L)		
FPase	**Endoglucanase**	**β-glucosidase**	**Glucose**	**Cellobiose**	**Xylose**
0.53 ± 0.01	1.03 ± 0.2	0.93 ± 0.04	14.5 ± 0.85	0.24 ± 0.03	1.37 ± 0.1

4 CONCLUSION

Considering the general reduction of reagents in the culture medium, the results observed in this study suggest that the cellulolytic complex produced by *T. harzianum* using APEPT medium has excellent potential for second-generation ethanol production.

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