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Strategies to increase cellulase production with submerged fermentation using fungi isolated from the Brazilian biome

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ABSTRACT. Studies on new microbial sources of cellulase and accurate assessment of the steps that increase cellulase production are essential strategies to reduce costs of various processes using such enzymes. This study aimed at the selection of cellulase-producing filamentous fungi, and at the research of parameters involving cellulase production by submerged fermentation. The first test consisted of selecting the best cellulase-producing microorganisms (FPase) in Erlenmeyer flasks containing 200 mL of specific growth medium. The next test was designed to further investigate the enzyme production in fermentation with four types of soluble sugars: glucose, lactose, sucrose and xylose. In bioreactor tests, three different inoculation strategies were analyzed. The best FPase activity was presented by the strain *Trichoderma* sp. CMIAT 041 (49.9 FPU L^{-1}) and CMCase by the fungus *Lasiodiplodia theobromae* CMIAT 096 (350.0 U L^{-1}). Sucrose proved to be the best option among the soluble sugars tested, with higher rates of FPase activity (49.9 FPU L^{-1}) and CMCase (119.7 U L^{-1}). The best inoculation strategy for the bioreactor was a spore suspension obtained from a semi-solid state fermentation of wheat bran for 72h.

Keywords: cellulolytic enzymes, enzyme induction.

Estratégias para produção de celulases através de fermentação submersa utilizando fungos isolados do bioma brasileiro

RESUMO. Estudos sobre novas fontes microbianas e análises mais acuradas das etapas que compõem a produção de celulases são essenciais como estratégias para diminuir os custos gerados pelo uso de celulases nos processos de obtenção de açúcares fermentescíveis. O trabalho teve como objetivo a seleção de fungos filamentosos produtores de celulases e a investigação de parâmetros que envolvem a produção enzimática em fermentação submersa. O primeiro teste consistiu em selecionar os melhores fungos produtores de celulases totais em frascos Erlenmeyer contendo 200 mL de meio de cultura específico. O teste subsequente teve o intuito de investigar a produção enzimática com quatro tipos de açúcares solúveis: glicose, lactose, sacarose e xilose. Nos testes em biorreator foram analisados três diferentes estratégias de inoculação. Na etapa de seleção a melhor atividade de FPase foi apresentada por *Trichoderma* sp. CMIAT 041 (49,9 FPU L^{-1}) e CMCase pelo fungo *Lasiodiplodia theobromae* CMIAT 096 (350,0 U L^{-1}). O uso de sacarose mostrou-se ser a melhor opção dentre os açúcares solúveis testados, apresentando os maiores valores de atividade de FPase (49,9 FPU L^{-1}) e CMCase (119,7 U L^{-1}). A melhor estratégia de inoculação foi a suspensão de esporos obtidos a partir de fermentação em farelo de trigo, no tempo 72h.

Palavras-chave: enzimas celulolíticas, indução enzimática.

Introduction

The intensive search for alternative energy sources has gained prominence since the 1970's, when considerable research about bioethanol produced from biomass was developed (GUSAKOV, 2011). Production of bioethanol and biodiesel, made from renewable raw materials, are frequent topics in the literature (CORAZZA et al., 2003; SUKUMARAN et al., 2009). Given the growing need for alternative non-fossil energy, the synthesis of bioproducts and bioenergy generation, from renewable raw material such as lignocellulosic materials, at low cost, is important for sustainable development (DUTTA et al., 2008). In this scenario, stand out bioethanol and biodiesel produced from renewable raw material, and are known to reduce emissions of carbon monoxide in the atmosphere (ASHBY et al., 2005).

The enzymatic hydrolysis of cellulose involves a synergistic action of more than one type of cellulolytic enzymes, which together exhibit specificity for the glycosidic linkages β - 1,4. The enzymes degrades cellulose by the synergistic action of three distinct groups: endoglucanases (endo 1,4-β-glucanases), exoglucanases (exo 1,4-β-glucanases) and β-glycosidases. The first enzyme acts randomly on amorphous cellulose, thus reducing the degree of polymerization and as final products, glucose and cellobiose. Exoglucanase is active on crystalline cellulose hydrolysis and starts at the end of the chain, releasing cellobiose from the reducing and non-reducing end of the chain. Finally, cellobiase acts by cleaving the β - 1,4 glycosidic bond of cellobiose and small oligosaccharide molecules with release of monomeric sugars (WOOD; McCARE, 1979; BHAT, 2000). The final glucose produced can serve as raw material for fermentable sugars and converted into the final product, such as ethanol.

Enzymatic saccharification is one of the most costly steps for the production of cellulosic ethanol, so it is necessary to produce enzymes at low cost, since this step is crucial in the enzymatic conversion of biomass to ethanol (CASTRO; PEREIRA JÚNIOR, 2010). Studies involving potential sources of enzymes are common, and one of the strategies is the investigation of new microorganisms, potential producers of cellulases, required for complete and effective solubilization of lignocellulosic material (GUSAKOV, 2011). However, more studies on new microbial sources as well as more accurate assessment of the steps that make up the cellulase production process are essential to reduce the costs of using the enzyme complex. Currently, the most commonly used strains for cellulase production are the genera *Trichoderma* and *Aspergillus* (SUKUMARAN et al., 2005; FENG et al., 2009).

Therefore, this study aimed at the selection of filamentous fungi, producers of the cellulolytic complex, and at the research of parameters involving cellulase production by submerged fermentation.

Material and methods

Microorganism and maintenance

In an initial step, it was used six fungal strains from the Microorganisms Collection for Tropical Agrobusiness Interest (CMIAT), belonging to Embrapa Agroindústria Tropical (Fortaleza, Brazil). The strains, which were selected in previous experiments, have not yet been disclosed: *Fusarium* sp. CMIAT 137, *Lasiodiplodia theobromae* CMIAT 096, *Trichoderma* sp. CMIAT 038, *Trichoderma* sp. CMIAT 041, *Trichoderma* sp. CMIAT 046, and *Trichoderma* sp. CMIAT 054. Stock cultures were maintained on Potato Dextrose Agar (PDA) slants at 4°C.

Strain selection by fermentation in shake flasks

Submerged fermentation tests were performed in culture medium adapted from Mandels and Reese (1957) (g L^{-1}): $CaCl_2.2H_2O$, 0.4; $CoCl_2.6.H_2O$, 0.0037; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $FeSO_4.7H_2O$, 0.005; $MgSO_4.7H_2O$, 0.3; $MnSO_4.H2O$, 0.0016; $ZnSO_4.7H_2O$, 0.0014; sucrose, 10.0; microcrystalline cellulose (Sigma, St. Louis, Missouri State, USA). The initial pH of the medium was adjusted to 5.0. All fermentation tests were performed in triplicate.

Tests were conducted in Erlenmeyer flasks of 500 mL capacity containing 200 mL of the reaction medium, in orbital shaker at 150 rpm and 30°C. To each flask, 1.0 mL of spore suspension was inoculated, corresponding to a final concentration of 10^6 spores/mL in the fermentation medium. For testing, the spores were produced on PDA, whose cultures were incubated at 30°C for seven days. After the incubation period, the tubes containing the grown culture were washed with 10 mL of sterile Tween 80 solution (0.3%, v v^{-1}) to obtain the spore suspension.

Different soluble sugars in fermentation media for cellulase production

This step consisted of evaluating four different sources of soluble sugars used on the enzyme production media at a concentration of 10 g L^{-1} of glucose, lactose, sucrose and xylose. Thus, the strain that preliminarily achieved the best results of total cellulase production in previous tests was selected in such step. The experiments were conducted under the same growing conditions from the previous step, changing the source of soluble carbon for each test.

Tests in Bioreactor

The strain that showed the highest total cellulase production (FPase) in the previous steps was subjected to tests in bioreactor. The fermentation process was conducted in a 3.0 L BioFlo 115 fermenter (New Brunswick Scientific Co, USA) with an operating volume of 2.0 L. The process temperature was maintained at 30°C and shaking at 250 rpm. The pH was adjusted to 5.0 ± 0.2 and thereafter was controlled by automatic addition of 0.5M NaOH or 0.5M H_2SO_4. Samples were taken at regular intervals of 24 h for seven days, and the enzyme extract was obtained from centrifuging the fermentation broth (9600g for 10 min.) and used for enzymatic analysis. The composition of the culture

medium was the same as the one used in the Erlenmeyer flask tests.

The influence of three strategies of pre-incubation of the inoculum on enzyme production was investigated. For production of spores on PDA, it was used the methodology adopted for obtaining spores on PDA medium.

A second strategy was to produce spores in wheat bran medium. The activation followed the same steps of the item activation on PDA medium. It was inoculated an aliquot of 1.0 mL spore suspension from PDA in culture medium containing 4.6 g wheat bran with 6 mL of peptone solution (5.6% w v^{-1}) maintained in a 125 mL Erlenmeyer flask. The seeded media were then incubated for five days at 30°C. The spores formed were extracted by homogenization with 50 mL of sterile Tween 80 (0.3% v v^{-1}), and inoculated into the reactor at a final concentration of 10^6 spores mL^{-1} of reaction medium.

The growth in predetermined submerged fermentation was also tested. The culture was produced in 500 mL Erlenmeyer flasks with 200 mL culture medium. The fungus was initially activated in PDA medium, and then inoculated on spore production medium at a final concentration of 10^6 spores mL^{-1}. The inoculated medium (pre-cultivation) was incubated under shaking at 150 rpm at 30°C for 72h. Thereafter, the preculture was used directly to inoculate the bioreactor medium. An aliquot of 200 mL of the preculture corresponded to 10% (v v^{-1}) of the total fermentation medium.

Analytical determinations

The determination of the activity of the total cellulase (FPase) and the endo-β-1,4-glucanase (CMCase) was performed according to Ghose (1987). In the first analysis, a mixture of 0.5 mL of the crude enzyme extract, 1.0 mL of citrate buffer (50 mM, pH 4.8) and 50 mg of Whatman n.1 (1.0 x 6.0 cm) was incubated for 60 min. at 50°C. The CMCase activity was measured from the reducing sugars produced in the reaction of 1.5 mL of enzyme extract with 0.5 mL of carboxymethyl cellulose solution (CMC) (2% w v^{-1} in sodium citrate buffer, pH 4.8) incubated at 50ºC, during 30 min.

The determination of total reducing sugars was made by the DNS method (3,5- dinitrosalicylic acid) (MILLER, 1959). The reducing sugars were quantified by spectrophotometry (540 nm) and expressed in g L^{-1}. Enzyme activities were determined by measuring reducing sugars released in the reaction. One unit of activity was defined as the amount of enzyme capable of releasing 1 micromole of glucose equivalents per minute under the reaction conditions. Activities were reported as FPU L^{-1} and U L^{-1} for FPase and CMCase, respectively. Dry biomass of the fungus was determined by measuring the dry weight of the solid, after centrifugation of 20 mL liquid culture at 9600 g for 20 min. and the resulting material oven-dried at 90°C for 48h.

The comparison of three replicates was made by Analysis of Variance (ANOVA) using Statistic 7.0 software. Means were compared by Tukey's test and the level of significance was set at $p \leq 0.05$.

Results and discussion

Tests in shake flasks

The first step of the experiments was conducted in shake flasks, in which we compared the enzymatic production of six strains of filamentous fungi, which were pre-selected from over a hundred wild fungi isolated from different biomes (unpublished data). For this step, FPase was used as a parameter. *Trichoderma* sp. CMIAT 041 presented the highest activity of FPase after 72h of fermentation (49.9 FPU L^{-1} ± 0.013^a) followed by *Trichoderma* sp. CMIAT 038 (38.0 ± 0.029^{ab} FPU L^{-1}) (Figure 1). ANOVA evidenced significant differences in cellulase activity between CMIAT 041 and CMIAT 038 samples. Similarly, a study on the selection of cellulolytic fungi also showed higher production of FPase and CMCase in strains of the genus *Trichoderma* (SADDLER, 1982).

Despite the production of cellulolytic enzymes is concentrated on *Aspergillus* and *Trichoderma*, the identification of various potential microbial sources producing cellulase is a strategic interest for the cellulase market (FENG et al., 2009). Furthermore, as highlighted by Maheshwari et al. (2000), cellulase produced by these two genera only work efficiently in a narrow pH range, reinforcing the need for research with new microorganisms producing these enzymes. Investigations of possible sources of cellulolytic enzymes are recurrent, focusing on the search for microorganisms capable of producing a mixture of cellulases, necessary for full and effective solubilization of cellulose (DAS et al., 2007; GUSAKOV, 2011).

Values of endoglucanase enzyme activity, through analysis of CMCase, show a different profile. Under the established conditions, *L. theobromae* CMIAT 096 stood out from the others, with CMCase activity of 351.3 ± 0.38^a U L^{-1}. The isolate of *Fusarium* sp. CMIAT 137 was the fungus with the least potential for enzyme production (12.1 ± 0.32^a U L^{-1}).

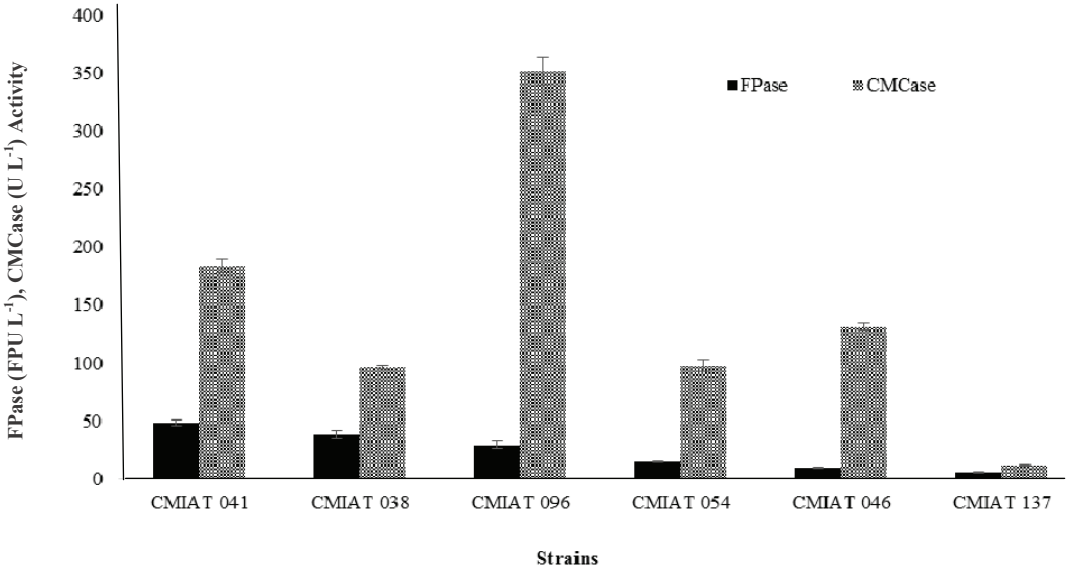

Figure 1. Profile for FPase and CMCase enzyme strains in submerged fermentation tests in Erlenmeyer flasks (72h).
Source: prepared by the authors.

Although the parameter used for microorganism selection has been the analysis of FPase, which provides the profile of total cellulase production, it is worth emphasizing the importance of analyzing the potential production of specific enzymes such as endoglucanases (CMCase). As distinct genera of fungi produce different amounts of enzymes of the cellulolytic complex, only those producing appropriate levels of endoglucanase, exoglucanase and glucosidase are able to effectively degrade natural cellulose (SUKUMARAN et al., 2005; KUMAR et al., 2008). While several fungi have the ability to metabolize cellulose as an energy source, only some strains are capable of secreting all enzymes of the cellulolytic complex. The cellulolytic system of *Trichoderma reesei*, for example, comprises up to 80% exoglucanases, 20-36% of endoglucanases and is deficient in β-glucosidases, with production of only 1%. While the fungus *T. reesei* synthesizes low levels of β-glucosidase, fungi of the genus *Aspergillus* have limited levels of endoglucanases (MUTHUVELAYUDHAM; VIRUTHAGIRI, 2006).

In assessing the cellulolytic potential of diverse fungi for the production of second generation biofuels, it is necessary to take into account the parameters involved in the fermentation process, such as the strain ability to spread, viscosity of the culture medium, final protein concentration and the final cost of enzyme production and not only the hydrolytic potential of each microorganism (GUSAKOV, 2011). Despite the increased production of CMCase by *Lasiodiplodia*, it was decided to choose the strain with the highest total cellulase production (CMIAT 041) in subsequent tests.

Different soluble sugars in fermentation media for cellulase production

Cellulases are enzymes that need to be induced and among the aspects generating costs in industrial production of the enzyme is the careful choice of the components of the culture medium, including production inducers. While production of most of microbial cellulases is induced in the presence of cellulose, the uniqueness of this carbon source may not be sufficient to trigger an enzymatic response, because it is an insoluble molecule. Studies have reported that a basal level of cellulase is produced in the presence of an inducer of lower molecular weight. Thus, soluble saccharides can be used as inducers such as cellobiose, glucose, lactose, and sucrose (CARLE-URIOSTE, 1997; BON et al., 2008).

Figure 2 shows the values of CMCase and FPase activities for four types of soluble sugars: glucose, lactose, sucrose and xylose. Sucrose, as a component of the culture medium, increased the production of FPase, presenting activity of 50.0 ± 0.013^{a} FPU L^{-1} with significant differences in enzyme activity between fermentation with xylose and glucose (respectively, 40.1 ± 0.04^{ab} and 38.7 ± 0.03^{ab} FPU L^{-1}).

The same soluble carbon source was also reported as the best inducer for the production of cellulases. Delabona et al. (2012) used *Trichoderma harzianum* strain grown in Erlenmeyer flasks with liquid medium at 29°C, and reported that the use of sucrose cause a higher activity of FPase (420 FPU L^{-1}) than lactose at the same concentration.

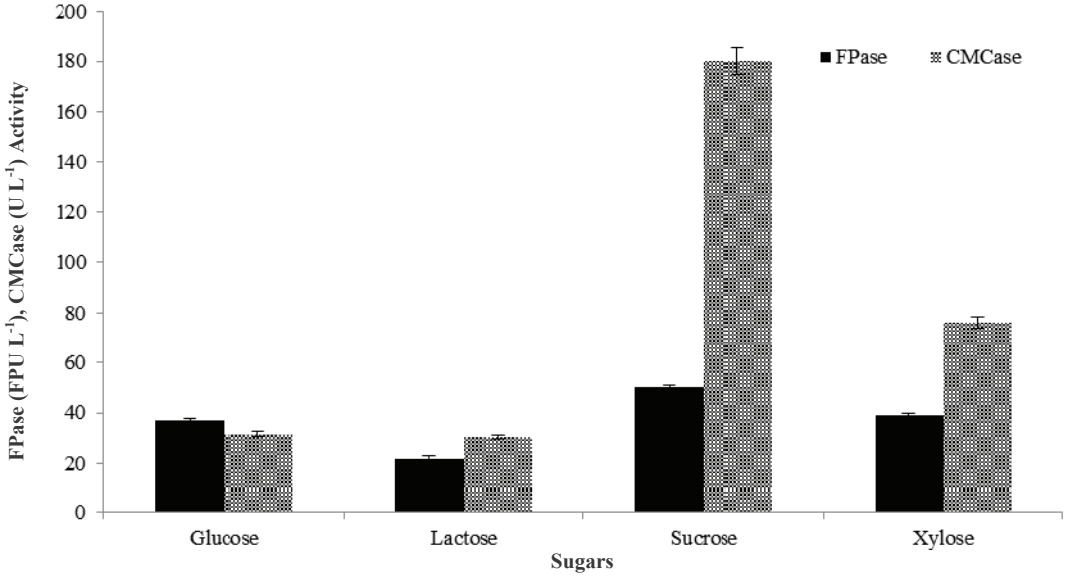

Figure 2. Profile of cellulase production by *Trichoderma* sp. CMIAT 041 using different sources of soluble sugars (72h).
Source: prepared by the authors.

Lee et al. (2010) tested culture media with different combinations of nitrogen sources and soluble sugars and observed increased production of CMCase in medium supplemented with sucrose using a strain of *Bacillus subtilis*.

Muthuvelayudham and Viruthagiri (2006) analyzed the influence of different carbon sources such as glucose, lactose and xylose in submerged fermentation using three isolates of *T. reesei*, and verified that the combination of cellulose with xylose led to the best production of FPase with activities of 7.02, 24.86 and 23.48 FPU L^{-1}, respectively to the strains 94144, 97177 and Tm3 in 10 days of fermentation. The same trend was also found in the production of CMCase. The authors found that cellulase activity was low when glucose was used as carbon source, possibly due to its inhibitory effect.

Lactose is an excellent inducer of cellulase for use in commercial scale, particularly due to the low cost of the material (KARAFFA et al., 2006). Despite recognized as a good inducer, in the conditions established in this study, the performance of the inducer was not relevant, since the use of this disaccharide was related to lower values for activities of FPase (22 FPU L^{-1}) and CMCase (30.4 U L^{-1}).

In fermentation with lactose, it was observed fungal growth with formation of pellets. Possibly, according to the requirements, the use of lactose may have been deleterious to the enzyme production compared with other sources of sugars. Singhania et al. (2010) stated that, although the relationship between productivity and morphology of the fungus is not yet clearly established, the behavior of the microorganism may vary with changing components of the medium hindering the adherence of cells to the culture medium and consequently, the production of metabolites. In a study evaluating the influence of nutritional conditions on the morphology of *T. reesei* RUT C30 on cellulose production, Domingues et al. (2000) observed the formation of pellets in the medium with lactose as the carbon source and lower FPase production due to this behavior of the fungus. On the other hand, in the same culture medium added with Tween 80, without pellets, there was an increased FPase production within 72h. The authors emphasized that the growth in the form of hyphae in liquid medium facilitates the adherence of fungi to the cellulosic material, assisting in reaching a higher productivity of cellulolytic enzymes. This phenomenon is possibly related to the limited oxygen transfer within the pellets.

Domingues et al. (2001), in an analysis using *T. reesei* RUT C30, observed an increased production of cellulases by using lactose as carbon source compared with the use of glucose. Nevertheless, the authors emphasize that the combination of these two sugars has increased enzyme production, suggesting that the combination of lactose and glucose in the fermentation medium promotes economic benefits to the process.

Even being an efficient supply for cell growth in the liquid medium, the use of glucose in the production of various enzymes and their regulation has been studied mainly for its known repressive effect (LAMBERT; MEERS, 1983). This effect was not observed in the present study. The activity of

FPase and CMCase using glucose were, respectively, 37.0 FPU L^{-1} and 31.5 U L^{-1}, being the third best profile of cellulase production. Domingues et al. (2001) also reported the production of FPase (0.9 FPU L^{-1}) in 96 h fermentation with *T. reesei*.

Tests in Bioreactor

The strain *Trichoderma* sp. CMIAT 041, selected as the best producer of FPase on previous tests, was subjected to submerged fermentation in a bioreactor to investigate the best strategy for obtaining the pre-inoculum for cellulase production. Figures 3, 4 and 5 show the enzyme production profile (FPase and CMCase), reducing sugars and total biomass in all three tests. The enzymatic activity (denoted by FPase test) had an increase from the first day of fermentation and achieves maximum values on the third day. The soluble sugar concentration decreased significantly within 72h of fermentation accompanying the total cellulase production.

After the reduction in the amount of soluble sugar, we detected a decline in enzyme production from 72h. It might be due to the depletion of macro- and micronutrients in the fermentation medium during consumption of the carbon source more easily assimilated than cellulose. This can induce physiological stress on the microorganism and thus a depletion of the mechanism of secretion of cellulolytic enzymes (NOCHURE et al., 1993).

The profile of the test using spores grown on PDA (Figure 3) showed an increased production of FPase in 72h of fermentation, with activity 155.5 FPU L^{-1}. At the same time interval, it was observed the highest microbial growth, denoted by dry biomass (10.13 g L^{-1}) and a pronounced reduction in the level of sugar in the fermentation medium. As shown in the Figure 3, from 4 days, CMCase production is increased until reaching the maximum production at 144h (193.0 U L^{-1}).

Figure 4 illustrates the profile of the fermentation in which spores were produced in semi-solid wheat bran medium.

At the time 72h, in which the FPase production is maximum (164.0 FPU L^{-1}) as well as its biomass (11.4 g L^{-1}), a small amount of sugar still remained in the fermentation medium (2.25 g L^{-1}). From that time on, the production profile of CMCase distinguished from FPase production, which exhibited a decreasing trend, and the maximum production of CMCase occurred at 144h, with activity of 285.0 U L^{-1}.

In the test using a subculture of the fungus in a liquid medium (Figure 5), the CMCase production profile was similar to the test with inoculum from spores developed on PDA and a maximum production at 144 h (246.0 U L^{-1}).

The use of waste for fungal spore production, compared with synthetic culture medium, is a potential strategy to reduce the cost of cellulase production. The cellulose fraction of biomass can assist in inducing enzyme production and obtaining higher yields. In order to classify the inoculation strategies that presented higher enzyme production, the mean results of FPase time of 72h were statistically compared using the Tukey test at 5% significance. The results for wheat bran, PDA and liquid medium were 164.6^{a}, 155.5^{b} and 143.9^{c}, respectively.

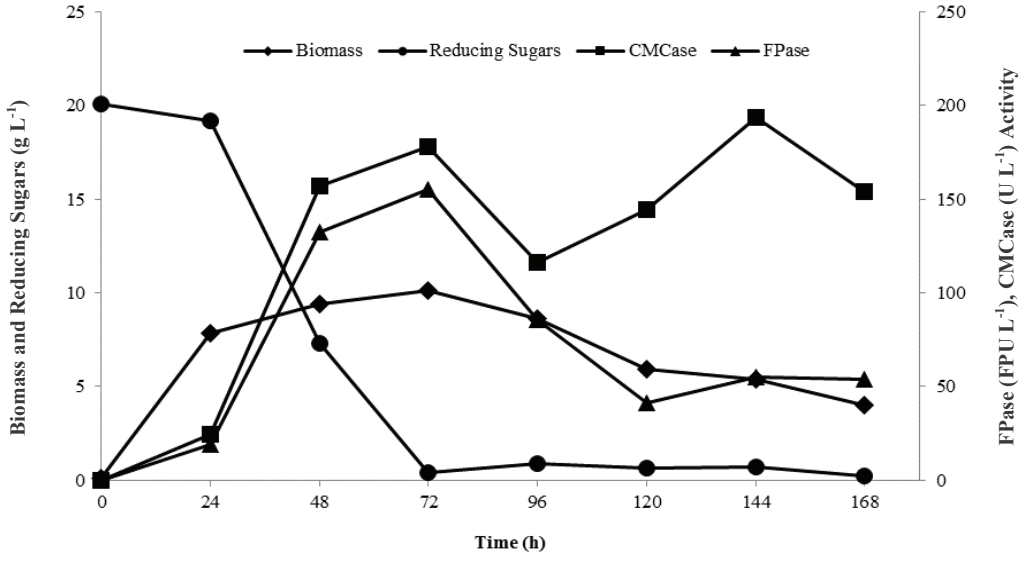

Figure 3. Production of FPase, CMCase, biomass and sugar consumption for 168h of fermentation with spores grown on PDA.
Source: prepared by the authors.

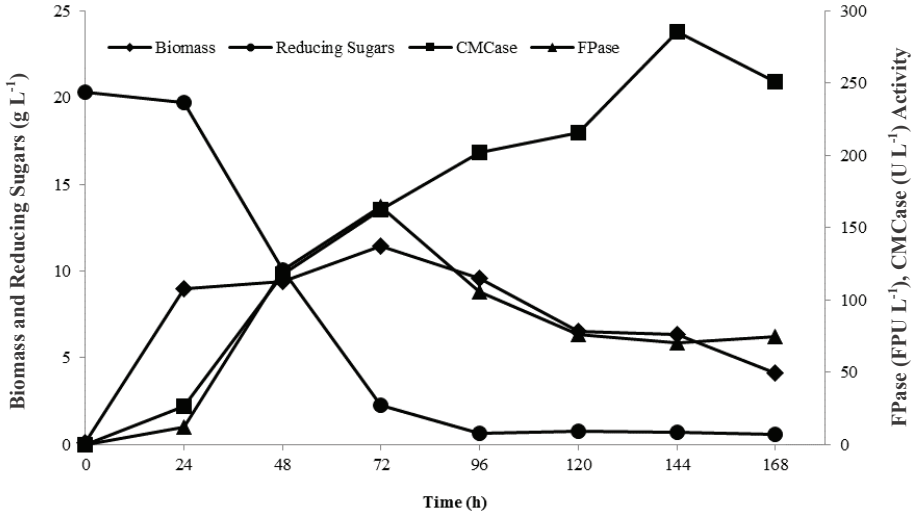

Figure 4. Production of FPase, CMCase, biomass and sugar consumption for 168h of fermentation with spores grown on wheat bran medium.
Source: prepared by the authors.

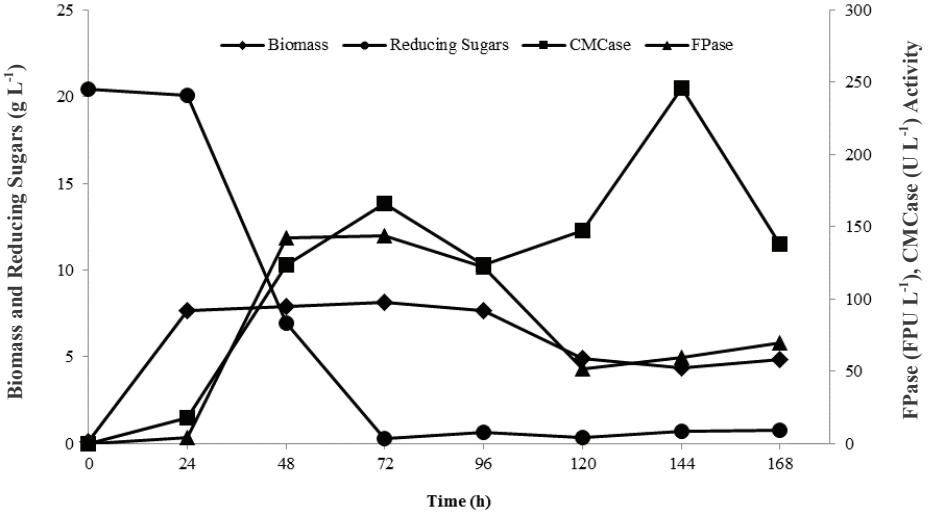

Figure 5. Production of FPase, CMCase, biomass and sugar consumption for 168h of fermentation with spores grown in liquid medium.
Source: prepared by the authors.

Conclusion

The fungal isolate from the Amazon forest were efficient producers of cellulase compared with the other strains. The best FPase activity was presented by *Trichoderma* sp. CMIAT 041 and CMCase by *Lasiodiplodia theobromae* CMIAT 096. The use of sucrose has proven to be the best option from the soluble sugars tested , with higher rates of FPase and CMCase activity. The best inoculation strategy bioreactor has been obtained from a suspension of spores from a solid state fermentation with wheat bran in 72 hours of fermentation.

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References

ASHBY, R. D.; NUÑEZ, A.; SOLAIMAN, D. K. Y.; FOGLIA, T. A. Sophorolipid biosynthesis from a biodiesel coproduct stream. **Journal of the American Oil Chemists' Society**, v. 82, n. 9, p. 625-630, 2005.

BHAT, M. K. Cellulases and related enzymes in biotechnology. **Biotechnology Advances**, v. 18, n. 5, p. 355-383, 2000.

BON, E. P. S.; GÍRIO, F.; PEREIRA JÚNIOR, N. Enzimas na produção de etanol. In: BON, E. P. S.; FERRARA, M. A.; CORVO, M. L. (Ed.). **Enzimas em biotecnologia**: produção, aplicações e mercado. Rio de Janeiro: Interciência, 2008. p. 241-271.

CARLE-URIOSTE, J. C.; ESCOBAR-VERA, J.; EL-GOGARY, F.; TORIGOI, E.; CRIVELLARO, O.; HERRERA- ESTRELLA, A.; EL-DORRY, H. Cellulase induction in *Trichoderma reesei* by cellulose requires its own basal expression. **The Journal of Biologic Chemistry**, v. 272, n. 15, p. 10169-10174, 1997.

CASTRO, A. M.; PEREIRA JÚNIOR, N. Produção, propriedades e aplicação de celulases na hidrólise de resíduos agroindustriais. **Química Nova**, v. 33, n. 1, p. 181-188, 2010.

CORAZZA, F. C.; MORAES, F. F.; ZANIN, G. M.; NEITZEL, I. Optimal control in fed-batch reactor for the cellobiose hydrolysis. **Acta Scientiarum. Technology**, v. 25, n. 1, p. 33-38, 2003.

DAS, M.; ROYER, T. V.; LEFF, L. G. Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. **Applied and Environmental Microbiology**, v. 73, n. 3, p. 756-767, 2007.

DELABONA, P. D. S.; FARINAS, C. S.; SILVA, M. R.; AZZONI, S. F.; PRADELLA, J. G. D. C. Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. **Bioresource Technology**, v. 107, p. 517-521, 2012.

DOMINGUES, F.; QUEIROZ, J.; CABRAL, J.; FONSECA, L. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. **Enzyme and Microbial Technology**, v. 26, n. 5, p. 394-401, 2000.

DOMINGUES, F. C.; QUEIROZ, J. A.; CABRAL, J. M. S.; FONSECA, L. P. Production of cellulases in batch culture using a mutant strain of *Trichoderma reesei* growing on soluble carbon source. **Biotechnology Letters**, v. 23, n. 10, p. 771-775, 2001.

DUTTA, T.; SAHOO, R.; SENGPUTA, R.; RAY, S. S.; BHATTACHARJEE, A.; GHOSH, S. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. **Journal of Industrial Microbiology and Biotechnology**, v. 35, n. 4, p. 275-282, 2008.

FENG, Y.; JIANG, J.; ZHU, L. Recent developments in activities, utilization and sources of cellulase. **Forestry Studies in China**, v. 11, n. 3, p. 202-207, 2009.

GHOSE, T. K. Measurement of cellulase activities. **Pure and Applied Chemistry**, v. 59, n. 2, p. 257-268, 1987.

GUSAKOV, A. V. Alternatives to *Trichoderma reesei* in biofuel production. **Trends Biotechnology**, v. 29, n. 9, p. 419-425, 2011.

KARAFFA, L.; FEKETE, E.; GAMAUF, C.; SZENTIRMAI, A.; KUBICEK, C. P.; SEIBOTH, B. D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates. **Microbiology**, v. 152, n. 5, p. 1507-1514, 2006.

KUMAR, R.; SINGH, S.; SINGH, O. V. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. **Journal of Industrial Microbiology and Biotechnology**, v. 35, n. 5, p. 377-391, 2008.

LAMBERT, P. W.; MEERS, J. L. The production of industrial enzymes. **Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences**, v. 300, n. 1100, p. 263-282, 1983.

LEE, B. H.; KIM, B. K.; LEE, Y. J.; CHUNG, C. H.; LEE, J. W. Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53. **Enzyme and Microbial Technology**, v. 46, n. 7, p. 38-42, 2010.

MAHESHWARI, R.; BHARADWAJ, G.; BHAT, M. K. Thermophilic fungi: their physiology and enzymes. **Microbiology and Molecular Biology Reviews**, v. 64, n. 3, p. 461-488, 2000.

MANDELS, M.; REESE, E. T. Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. **Journal of Bacteriology**, v. 73, n. 2, p. 269-278, 1957.

MILLER, G. L. Use of dinitrosalicylic acid reagents for determination of reducing sugars. **Analytical Chemistry**, v. 31, n. 3, p. 426-438, 1959.

MUTHUVELAYUDHAM, R.; VIRUTHAGIRI, T. Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw. **African Journal of Biotechnology**, v. 5, n. 20, p. 1873-1881, 2006.

NOCHURE, S. V.; ROBERTS, M. F.; DEMAIN, A. L. True cellulase production by *C. thermocellum* grown on different carbon sources. **Biotechnology Letters**, v. 15, n. 6, p. 641-646, 1993.

SADDLER, J. N.; Screening of highly cellulolytic fungi and the action of their cellulase enzyme systems. **Enzyme and Microbial Technology**, v. 4, n. 6, p. 414-418, 1982.

SINGHANIA, R. R.; SUKUMARAN, R. K.; PATEL, A. K.; LARROCHE, C.; PANDEY, A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. **Enzyme and Microbial Technology**, v. 46, n. 7, p. 541-549, 2010.

SUKUMARAN, R. K.; SINGHANIA, R. R.; PANDEY, A. Microbial cellulases - Production, applications and challenges. **Journal of Scientific & Industrial Research**, v. 64, p. 832-844, 2005.

SUKUMARAN, R. K.; SINGHANIA, R. R.; MATHEW, G. M.; PANDEY, A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. **Renewable Energy**, v. 34, n. 2, p. 421-424, 2009.

WOOD, T. M.; McCARE, S. I. Synergism between enzymes involved in the solubilization of native cellulose. **Advances in Chemistry**, v. 181, p. 181-209, 1979.

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