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Enzyme activity of cellulases produced by *Moniliophtora perniciosa*, the causing factor of "witch–broom" in cacao plants.

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Abstract : Cellulases are applied in food and textile industry, in the production of alcoholic beverages, bioconversion of agronomic residues and, increasingly, for production of biofuels. The fungi of cacao plants are very resistant and difficult to combat. On the other hand, enzymes produced by them have numerous biotechnological applications. The present work aimed to evaluate isolates of *Moniliophtora perniciosa* representatives from the *Reconcavo* region of Bahia, in Brazil, regarding the production of cellulases. Thirty two *M. perniciosa* strains were evaluated while growing in the presence of cellulose as the only carbon source. They were initially screened using the Congo red assay, following halo formation, after growth in solid medium containing one of the substrates mentioned before. Of the tested strains, 948F showed the greatest potential for the hydrolysis of non-crystalline cellulose. This strain was further tested for its ability to produce cellulases in minimal liquid medium in the presence of carboximetil cellulose (CMC) as the only carbon source. The maximal growth range was between 168 and 192 h. The highest activity values were observed between 240 and 246 h, maximum value being 10.5 U/mL x 10^{-3}. The zymogram analysis showed the presence of three bands with endoglucanase activity. Although the results obtained are preliminary, they may open the way for future investigations of this strain aiming optimization of conditions for production of cellulases and other hydrolytic enzymes with potential biotechnological applications.

Key words: *Theobroma cacao*, Endoglucanases, Hydrolytic enzymes.

Atividade enzimática de celulases produzidas por *Moniliophtora perniciosa*, fator causador da "vassoura de bruxa" em plantas de cacau

Resumo: As celulases são utilizadas nas indústrias têxtil e alimentícia, na produção de bebidas alcoólicas, na bioconversão de restos da produção agrícola e, de maneira crescente, na produção de biocombustíveis. Os fungos da planta de cacau são muito resistentes e de difícil combate. Por outro lado, as enzimas por eles produzidas têm muitas aplicações biotecnológicas. O presente trabalho buscou avaliar isolados de *Moniliophtora perniciosa*, representativos da região do Recôncavo da Bahia, em relação à produção de celulases. Avaliou-se o crescimento de 32 cepas de *Moniliophtora perniciosa*, tendo a celulose como sua única fonte de carbono. As cepas foram examinadas, inicialmente, por meio do teste vermelho de Congo seguindo-se à formação do halo, após crescimento em meio sólido contendo um dos substratos antes mencionados. Das cepas testadas, a 948F apresentou o maior potencial para a hidrólise de celulose não cristalina. Este cepa foi depois testada em sua capacidade de produzir celulases em meio líquido mínimo, na presença de celulose carboximetil (CMC) como única fonte de carbono. A maior variação de crescimento se deu entre 168 e 192 h. Os valores mais altos de atividade foram observados entre 240 e 246h, o máximo sendo 10,5 U/ml x 10^{-3}. A análise zimogramática mostrou a presença de três faixas com atividade de endoglucanase. Embora os resultados sejam preliminares, podem abrir caminho para futuras investigações desta cepa, com vistas à

otimização das condições de produção de celulases e de outras enzimas hidrolíticas com potencial de aplicação na biotecnologia.

Palavras chave: *Theobroma cacao*, Endoglucanases, Enzimas hidrolíticas.

Introduction

The majority of the enzymes used in industrial processes are produced by microorganisms that secrete them in the medium to hydrolyze different biological compounds such as pectin, lignin, cellulose, lipids, and quitin. Among them, hydrolytic enzymes are key proteins secreted specially by filamentous fungus (BATH, 2000). Filamentous fungus have been largely used in the production of hydrolytic enzymes (SIQUEIRA et al., 2010). Among them, cellulases and hemicellulases have received special attention, in great part, due to their enormous biotechnological potential (HA et al., 2011).

Moniliophtora perniciosa (Stahel) Singeris is a fungus that attacks cacao plants (*Theobroma cacao* L.) and is responsible for the disease usually known as “witch-broom” (vassoura-de-bruxa) (LUZ et al., 1997). The disease described in 1985, caused economic and social prejudice to many countries producers of cacao in South and Central America (PURDY & SCHMIDT, 1996). In Brazil, after staying confined to the Amazonic region, the disease was introduced in 1989 to Southwest Bahia (PEREIRA et al., 1989), one of the greatest regions for production of cacao in the world, causing serious economic, social and ecological lost.

Since the cell wall of the fungus is composed by quitin, it is not possible to fagocitate the substrates used as nutrients. In addition to that some of its nutrients are polimeric compounds (eg., cellulose, hemicellulose and proteins), and the fungus needs to break down those substrates through the action of secreted hydrolytic enzymes, allowing them to cross the cell wall (BHAT, 2000). This characterizes the initial process of infection in cacao plants. The strategy for plant infection by this microorganism brought attention to the possible potential of *M. perniciosa* in producing hydrolytic enzymes. Here we focused our work on the cellulases, specifically endoglucanases.

Cellulases are enzymes capable of degrading cellulose, the main constituent of plant cell wall. They cellulases have been applied in the food and textile industry, in the production of alcoholic beverages, bioconversion of agronomic rejects and in the recent years increasingly for production of biofuels (HECK et al., 2002; CUNHA et al., 2014). Different fungi species have been evaluated regarding to its capability to produce cellulolytic enzymes, fungi of genus *Trichoderma* (JIANG et al., 2011; ZHANG et al., 2012), *Penicillium* (CAMASSOLA & DILLON, 2012; GUSAKOV & SINITSYN, 2012), *Aspergillus* (BANSAL et al., 2012; NARRA et al., 2012) and *Neurospora* (LI et al., 2014) have been extensively used. In the other hand, many studies have been performed in order to ameliorate the efficiency of bioconversion of cellulosic materials in biofuels (CAMASSOLA & DILLON, 2014; CUNHA et al., 2014; ZHANG et al., 2012). Although major progress has been made to reduce costs for cellulase production, it is still an economic issue, especially for biofuels production.

The present work aimed to evaluate isolates of *M. perniciosa* representatives of the southwest and recôncavo region of Bahia regarding the production of cellulases, select the isolate showing highest potential for production of these enzymes, characterize the enzyme activity as function of mycelial growth in different time gaps by spectrophotometry and also total protein profile by SDS-PAGE and zymograms.

Material and methods

Initially, 32 isolates of *M. perniciosa* representative of the southwest and *recôncavo* region were used. Mycelium were present in the form of 1cm agar discs, conditioned in glass flasks containing 15 mL of sterile distillated water.

To obtain the matrix plate, each isolate was transferred to 10cm diameter Petri dishes, containing Potato Dextrose Agar (BDA) media (w/v) (10% *Solanum tuberosum*, 1% glucose and 1.5% agar, pH 6.0) previously autoclaved at 121 °C for 20 min. After seeding under sterile conditions, the plates were maintained in aseptic chamber for

144 hours at 27 ± 2 °C, and after this period, harvested at 4 °C.

To determine the isolate with higher cellulolytic activity in solid media, the Congo test was used. For each matrix plate, mycelium of 0.8 cm were removed, and afterwards, transferred to Petri dishes containing 30mL of minimum culture media (CHAVES, 1982), 5g/L of carboximethylcellulose CMC (Sigma) as only carbon source, and 1.5 % (w/v) agar. Two Petri dishes were used per isolate and, after seeding, all plates were maintained in aseptic camera for 192 h at 27 ± 2°C. After this period, each plate was covered with a solution of 1% (v/v) red Congo for 1h staining following serial washings with 1M NaCl per 30 minutes each (ZHANG et al., 2006). The isolate showing the biggest halo, corresponding to the distance between the border of the colony and the border of the most stained region, was selected for further tests. These tests included obtaining the mycelium growth curve, measuring cellulosic activity in liquid media and getting gel profiles of total protein and enzyme activity of the cellulases (zymograms).

For determination of mycelium growth (g) in minimum liquid media, considering different time intervals, fifteen mycelium disks of 0.8 cm were removed from the matrix plate corresponding to the isolate 948F and five mycelium disks were inoculated in three Erlenmeyers containing 400 mL of sterile liquid media (CHAVES, 1982) with 0.5 % (w/v) of CMC (Sigma). Following that, the erlenmeyers were incubated in an orbital shaker at 130 rpm, 28^0C for 360h. During the incubation period, 12 mL aliquots of each Erlenmeyer were collected at regular intervals for 24 h starting at time 0h, totalizing 16 samples for each repetition. Each aliquot containing pre-determined weight was filtered in Filtrax Ø 110 mm paper and the filtrates obtained were harvested in plastic tubes of 50 mL before frozen for later glucose determination.

The filter paper used that retained the fungus mycelium was dried at room temperature to obtain the mycelium dry mass at different time intervals. The initial weight of each filter paper was subtracted from it the filter paper after filtering and drying, and the average values were used for elaboration of the growth curve from the mycelium isolate.

Glucose conversion was measured as following. 250 µL of sample was mixed to 500 µlL of 100 mM Sodium Acetate Buffer (CH_3COONa), pH 5.0 containing 1% CMC (w/v) (Sigma).The mixture was incubated for 1h at 28 °C. The reaction was interrupted by the addition of 1.5 mL dinitrosalysilic acid (DNS) and boiling at 100 °C for 5 min. After boiling, distillate water was added up to a final volume of 10 mL. The absorbance of each sample was read in spectrophotometer (METERTEK SP-850) at 550nm. Cellulase units (endoglucanase activity) were expressed as the amount of glucose released per minute.

SDS-PAGE protein gel was prepared in the concentration of 10% (ALFENAS, 1998), applying 80µL of the aliquots from each growth peaks per well, equivalent to the period between 168 and 192h, considering two repetition for each time point. Electrophoretic running was performed with tris-glicine buffer (Tris 0,25 M; glicine 1,92 M; SDS 1% - pH 8,3) at 100V for 1h, following silver staining. For that, the gel was immersed in a 50% (v/v) methanol solution for 30 min, and then washed five times with deionized water for 5 min. After that the gel was immersed in a DTT solution 1,0 $mol.L^{-1}$ (6.6µL in 50ml water) for 15 min, and then immersed in a Silver Nitrate solution (300µL of $AgNO_3$ 20% (w/v) in 50ml of distillate water) for 15 minutes, being washed again three times with deionized distillate water. Development of the gel was done in Sodium Carbonate (1.5g), 25µL of forlmadehyde and 50 ml of deionized water. The reaction was blocked by addition of 10% (w/v) citric acid.

All the experiments were done in triplicate, and data is reported as their average and respective standard deviation

Results and discussion

All the 32 isolates evaluated by the Congo red assay showed presence of cellulosic activity at different levels (Figures 1a and 1b showing the 2 major endoglucanase producers). The 948F isolate developed the biggest halo (1.04 cm and 1.55 rate halo/colony - Figure 1a). Based on that, this isolate was chosen to further studies for cellulosic activity initial characterization.

The fact that different isolates have grown in solid culture media containing carboximetil cellulose (CMC) as only source of carbon indicates that those isolates are capable of producing cellulases. This result was not surprising, since *M. perniciosa* is a fungus that presents hemibiotrophic

life cycle (HEDGER et al., 1989), i.e., it owes a saprophytic phase that decomposes wood, a material rich in cellulose.

The cultivation of isolate 948F in liquid media, containing CMC as only carbon source, demonstrated the capacity of this isolate on secreting cellulases, more specifically, endoglucanases. The production of more than one endoglucanase (or other cellulases) by filamentous fungi is commonly described in the literature. In this study it was initially observed a mycelial growth in the first 24h, however, the growth was more significant after 96h. The growth peak happened in the interval between 168h and 192h of incubation. After 216h it was observed a tendency of decline followed by stabilization in the growth curve (Figure 2). This stabilization may be explained by reduction in the availability of cellulose and other nutrients in the medium.

Figure 1 - Enzyme activity in solid media stained by congo red test of isolates. (a) Isolate 948F and (b) 1149G, the arrows indicate the halo of degradation correspondent to the action zone of cellulolytic activity.

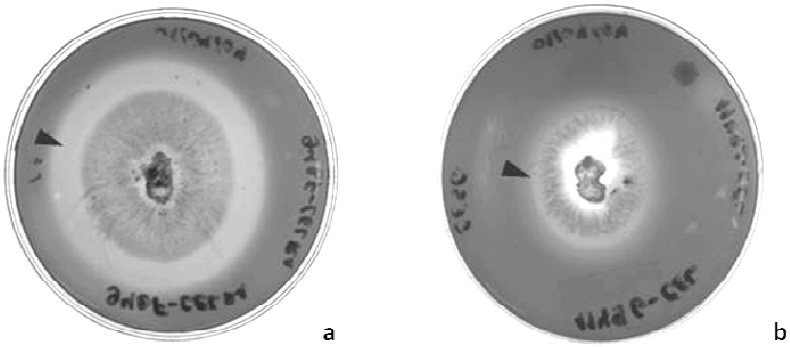
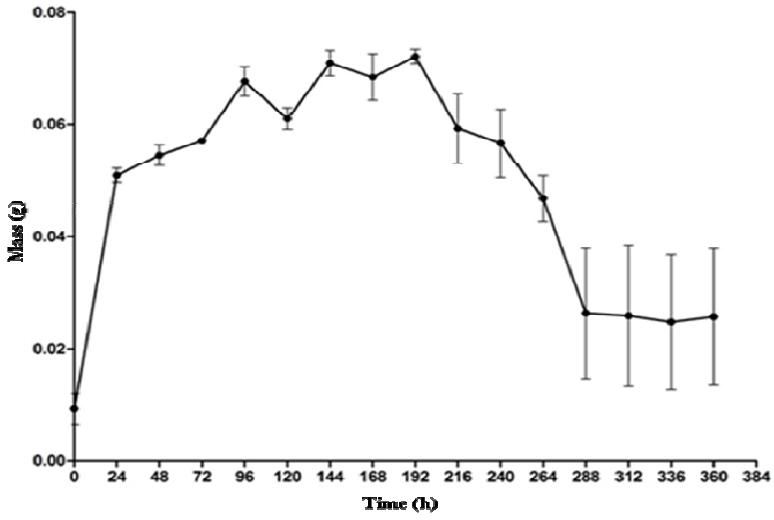


Figure 2 - Average curve of mycelial growth of isolate 948F (g) in different periods of time (h). The bars correspond to standard error.

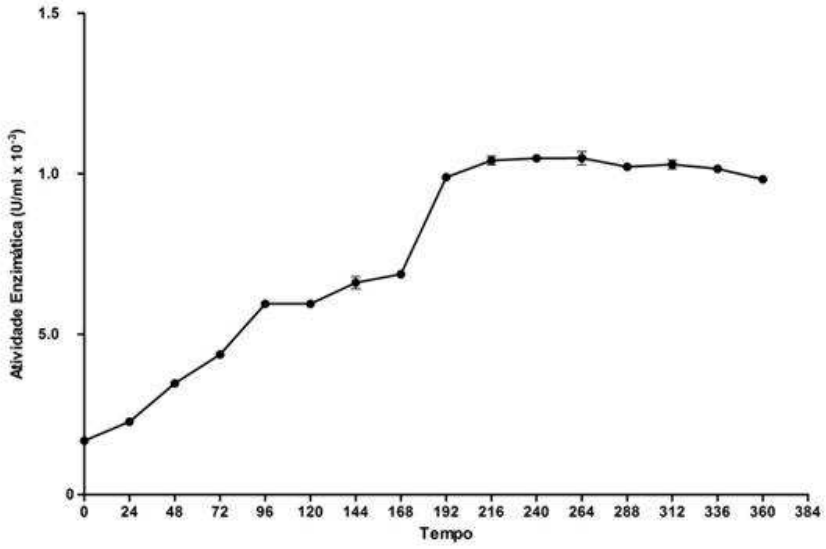


Another factor to be considered is that according to the time from inoculation, toxic substances may have also been released in the medium as a regular process of culture aging.

Literature reports indicate a variation in the peaks of growth from different phylamentous fungi varying from 48 h for *Trichoderma reesei* (TAKASHIMA et al., 1998) to 288h for *Pycnoporus cinnabarinus* (SIGOILLOT et al., 2002) and *Lentinula edodes* (JUNIOR et al., 2003).

The maximal cellulase activity of 10.5 U/mLx10^{-3} was observed in the time interval between 240h and 264h (Figure 3). These results demonstrate that *M. perniciosa* is a fungus which secrets enzymes relatively slow, since other phylamentous fungus, as *Trichoderma*, has maximum cellulosic activity between 72h and 120h after inoculation (DING & BUSWELL, 2002), practically half of the time used by *M. perniciosa*. According to Figure 3, it is possible to observe an increase in the activity from 6.9 U/mLx10^{-3} to 9.9 U/mL x10^{-3}, between 168 and 192h. When observing Figure 2 for the same period, it is noticeable that they correspond exactly to the time interval where the growth of the mycelia got to the maximum peak. This result shows a relation between the mycelial growth and the increase in enzyme concentration in the medium, which is justified by the increase in the number of hyphens during the mycelial growth of the fungus, that is dependent of the demand for glucose.

Figure 3 – Average curve of cellulolytic activity of isolate 948F. The bars in each one of the points correspond to the standard deviation.



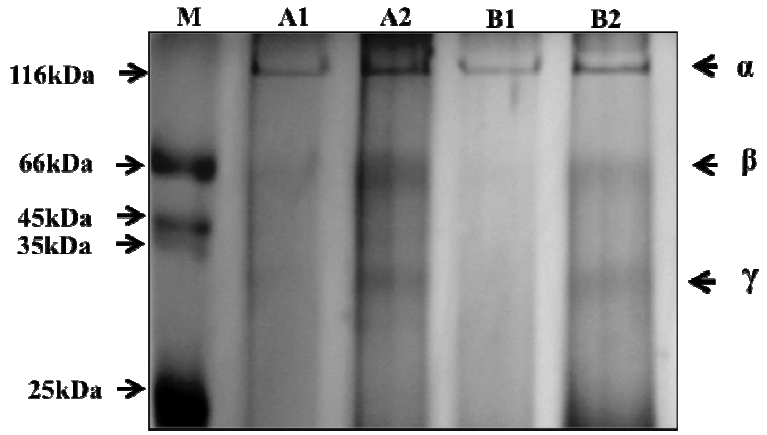
After the activity peak was obtained, there was a discreet decrease in the cellulosic activity followed by a stabilization *plateau*. As shown in Figure 2, it is possible to verify the tendency to stabilization of the growth curve after 216h, although the concentration of cellulose in the media was maintained stable in the same time interval. This phenomenon may be explained by the fact that these enzymes often have a half-life relatively long, possibly due to their glycosylation before secretion, which results in protecting them against proteolytic factors and temperature denaturation, according to what was observed by Azevedo & Poças-Fonseca (2002).

The total protein gel shows, in the time points considered, three distinct bands, denominated α, β and γ. The α band corresponded to the molecular weight (MW) of 116 kDa, the β band 66kDa and γ band between 35KDa and 25kDa (Figure 4).

The presence of different endoglucanases may provide the microorganism with improved capacity to degrade the targeted substrate. However, some cellulases and hemicellulases

show cross activity, and some of these bands showing cellulase activity could reflect that. Further characterization is needed to fully identify the nature of these proteins (CHEN et al., 2003; LI et al., 2004). The fact that those enzymes are secreted in low amounts does not impair its use in industrial or biotechnological processes, once it is possible to isolate the genes coding the enzymes produced by this isolate, and clone them, so that they can be overexpressed in different hosts (DING & BUSWELL, 2002; HYEON et al., 2010; LUO et al., 2010; TAKASHIMA et al., 1998). The most important advantage will be to show superior specific activity compared to fungi (eg. *T. reesei*, *A. niger*) commonly used for the production of available commercial enzyme preparations.

Figure 4 - Total protein gel profile. M - marker; A1 e B1: replicates of the time point 168h and A2 and B2: replicates of the time point 192h. The numbers correspond to the marker bands: 116 KDa; 66 KDa; 45 KDa; 35KDa and 25 KDa. The observed bands are indicated by α, β e γ.



Although the results obtained are preliminary, they may open the way for future investigations of this strain aiming optimization of conditions or testing different approaches for production of cellulases and other hydrolytic enzymes with potential biotechnological applications. For instance, Cunha et al. (2014) have shown that when using an approach for enzyme catalyzed liquefaction of sugarcane bagasse, this enabled submerged fermentation of *Aspergillus niger* and production of endoglucanase at a 12-fold higher yield than solid state fermentation.

Conclusion

M. perniciosa is a major plant pathogen, and according to that, a potential producer of hydrolytic enzymes. In this work, we started to investigate that, and our results show that multiple forms of endoglucanases (endo-cellulases) are possibly produced by this fungus *when* cultivated in the presence of carboximethylcellulose as substrate. Although the enzymes are expressed in low amounts, further work may help to optimize enzyme production conditions, or alternatively genes coding for enzymes showing high specific activity can be cloned and expressed in appropriated hosts.

The presence of the bands cited previously indicated the action of endoglucanases secreted by the studied isolate. The results presented in this work are significant since they indicate the potential of the studied isolate to produce enzymes for biotechnological applications (e.g. cellulases), and open the doors for future investigations.

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