RESUMO: Fungos do solo do cerrado foram prospectados por sua atividade celulolítica. A linhagem CCIBt #1488 de Penicillium citrinum foi selecionada por sua atividade endoglucolítica superior após cultivo a 30º. A atividade sobre CMC e hemicelulose foi estabelecida ao longo do tempo de cultivo, temperatura e pH. A atividade sobre CMC máxima (7958 VU ml\(^{-1}\)) ocorreu após seis dias de cultivo, entre 40 e 50º C, desaparecendo a 60º C. Enquanto o crescimento e a produção de glicose foi maior em pHs ácidos, a atividade celulolítica sobre CMC foi estável entre pH 3 e 8. As atividades máximas sobre xiloglucano e galactomannano foram 460 e 1233 VU ml\(^{-1}\), respectivamente. Cerca de 90 % da atividade de CMCase foi mantida após armazenamento do extrato bruto durante 150 dias a 5º C. Os resultados sugerem que P. citrinum #1488 tem potencial para produzir enzimas para saccharificação de materiais lignocelulósicos bem como para outras aplicações industriais.

PALAVRAS-CHAVE: Penicillium citrinum, celulase, endoglucanase, xiloglucanase, galactomannanase.

ABSTRACT: Fungi species from Brazilian savannah soil (cerrado) were screened for cellulase activity. Penicillium citrinum (CCIBt #1488) was selected by its superior endoglucanase (CMCase) activity. Its activity over cellulose and hemicellulose was established at a range of growth time, temperature and pH. Maximal CMCase activity (7958 VU ml\(^{-1}\)) was produced after 6 days between 40 and 50º C, vanishing at 60º C. While P. citrinum 1488 growth and glucose production was higher at low pH, CMCase activity was kept rather stable between pH 3 and 8. Crude extract assayed for xyllo glucanase and galactomannanase activities presented maximal points of 460 and 1233 VU ml\(^{-1}\), respectively. About 90 % of CMCase activity was retained after crude extract storage for 150 days at 5º C. The results suggest P. citrinum #1488 has potential to produce enzymes for saccharification of lignocellulose as well as other industrial applications.

KEYWORDS: Penicillium citrinum, cellulase, endoglucanase, xylloglucanase, galactomannanase.
INTRODUCTION

Lignocellulose is the most abundant biological resource in nature accounting for c. 90% of plant dry biomass. Vital for the whole food chain, cellulose is also directly important for industry as a raw material employed in building, food, paper and textile industries, among others. It has also been longwise used as source of energy as firewood and, more recently, for production of ethanol (Santos et al. 2011). Cellulose is a linear polymer of glucose linked in 1,4 position. The hydrolysis of cellulose requires a cellulase system with at least three kind of hydrolases, namely endo-β-1,4-glucanase (E.C.3.2.1.4), cellobiohydrolase or exo-β-1,4-glucanase (E.C.3.2.1.91) and β-glycosidase or cellobiohydrolase (E.C.3.2.1.21). Fungi from some species of *Trichoderma*, *Aspergillus* and *Penicillium* are the main cellulase makers found in nature (Karboune et al. 2008). Among them, wild and mutant strains of *Penicillium* have been important model for investigation on expression, regulation and metabolism of cellulases *in vivo* (Camassola & Dilon, 2007, Sun et al. 2008) as well as *in vitro* assays in order to characterize (individual and synergic) kinetic properties of cellulases (Bhiri et al. 2008, Andersen et al. 2008, Karboune et al. 2008). *Penicillium funiculosum* (van Wyk 1997, Karboune et al. 2008) and *P. citrinum* cellulase (Dutta et al. 2008) and hemicellulase (Dutta et al 2007a, 2007b) activities has been shown to present increased tolerance to temperature and pH, properties of industrial interest.

Hemicelluloses have been used as interesting source of raw material to cosmetics and other chemical application (Rodrigues & Guirardello 2008) including bioethanol (Shaw et al. 2008). Hemicellulose and pectin are also important because they cover cellulose microfibrils and obstruct the access to cellulose (Buckeridge et al. 2010). Thus, to obtain access to cellulose and hemicellulose monomers and oligomers, efficient chemical and biochemical approaches must be developed.

Brazilian savannah (cerrado) is a rich and singular biome, but is also poorly studied and present several species threatened of extinction. In current study we performed a first approach investigation over cerrado soil in order to identify fungi species able to digest cellulose. We identified the most effective as *P. citrinum* #1488 and characterized its cellulase and hemicellulase properties.
MATERIAL AND METHODS

Fungi were isolated from soil of cerrado rizosphere in the Campininha Farm ecological station, Mogi Guaçú, São Paulo, Brazil, using modified Czapek media in which 1.5% (w/v) of sucrose or carboxymethyl cellulose (CMC; Sigma) were employed as carbon and energy sources. Plates were incubated at 30°C for 12 days. The isolated mycelia were selected and transferred to a new plate. Five fungi colonies identified as *Trichoderma viride*, *T. harzianum*, *T. reesei*, *T. lignorum* and *Penicillium citrinum* were subcultured in 250 ml of liquid Czapek-CMC in Erlenmeyer flasks under continuous shaking for until 8 days. Fungi were confirmed by the Section of Mycology. *P. citrinum* was deposited in the collection of fungi cultures from Botanical Institute of São Paulo – CCIBt #1488.

Enzyme extraction and storage

Soluble content was separated from mycelia mass by vacuum filtration and considered crude extract. Mycelia were dried in oven at 80°C for 2 days and weighted. Crude extract was stored at -18°C with 0.02% (w/v) sodium azide to avoid contamination. One liter of *P. citrinum* inoculum was prepared to be cultured in a bioreactor, concentrated about 5 times and used in laundry assays. For storage stability viscosimetry assays were performed after storage at -80, -18 and +5°C for 7, 75 and 150 days.

Enzyme assay

Endoglucanase (CMCase) xyloglucanase and galctomannanase activities were assayed by viscosimetry. Media assay was 50 µl crude extract, CMC 1.67% (w/v) or hemicellulose 0.83% (w/v), 4 mM sodium acetate pH 4.5, and incubated at 45°C. Viscosity was measured as the time required for 100 µl solution to be released from a 200 µl graduated pipette. A viscosimetric unit (VU) was defined as the inverse of the incubation time required for the enzyme extract to reduce the viscosity of the solution to half of its initial value times 100 (VU = 100/t0.5). Initial viscosity was obtained by replacing the crude extract with water (Alcântara, 1995). Thermal stability of crude extract was assayed by pre-incubating the enzyme extract for 5, 20, 30, 40, 50 and 60°C for 20, 40, 60 and 80 min in each temperature. After, the extract was cooled at 0°C and assayed at 30°C as described above.
Carbohydrate Assays

Crude enzyme extract (100µl) was incubated with 500µl of 1% xyloglucan (from *Copaiphera langsdorf*) or galactomannan (from *Sesbania marginata*) in ammonium sulfate 1 M, pH 4.5 at 45º C for 24 hours. Afterwise, 300 µl of solution were diluted in 1 ml deionized water. Oligosaccharides were separated by High Performance Anion Exchange Chromatography (HPAEC; Dionex® model DX-500) in a linear gradient from 30 to 150 mM sodium acetate throughout 50 min with 88 mM NaOH, in a Carbo Pak PA100 column (Dionex®) and analyzed in a pulse amperometric detector (PAD) using an automatic sampler (AS3000).

Protein and sugar assays

To quantify protein concentration crude extract was boiled for 10 min and reacted with 1% (w/v) coomassie brilliant blue, with 1% (w/v) serum albumin as standard (Bradford, 1976). Total free sugar content was measured by phenol-sulfuric method as described by Dubois et al. (1956). Reductive sugars were determined by Somogy method (1945) as described by Conn and Stumpf (1981).

RESULTS AND DISCUSSION

Microorganism

Among fourteen fungi species isolated from cerrado soil samples, only four species from *Trichoderma* and *Penicillium citrinum* were able to grow in a medium with cellulose (CZAPEK-CMC) as the sole source of carbon and energy – fig. 1a e 1b. *Penicillium citrinum* showed a superior endoglucanase (CMCase) activity when compared with *Trichoderma* species – fig. 1c.
To be able to use cellulose as food (fig 1), fungi must be able to release free glucose from cellulose and so they are supposed to produce the three major components of the complex responsible to complete hydrolysis of cellulose: endoglucanase, exoglucanase and β-glycosidase. When assayed on an industrial laundry, *P. citrinum* crude extract performed a stone washed action similar to commercial products (data not shown).

*Penicillium citrinum* culture
Figure 2a shows an almost constant increase in *P. citrinum* mycelium (dry mass) until the maximum biomass was reach, around the sixth day. Protein content grew up fast mainly between the third and fifth days accompanied by a soft reduction in mycelium growth rate by the forth day. This qualitative assay suggested that *P. citrinum* endoglucanase might lack the cellulose binding domain (CBD) responsible for anchoring some kind endoglucanases onto the cellulose microfibril. The absence of CBD reduces the enzyme efficiency over microcrystalline cellulose, but provides a more stochastic enzymatic action over the fibers what might be associated with the stone washed effect observed.

Data presented in fig. 2, suggest that as the CMC was metabolized by the fungi, decreasing total sugar and increasing free sugar content (fig 2C) employed to build proteins (fig. 2A). By the forth day, while total sugar still dropping, reduced sugar stabilized and protein content is in fully ascension as we can be seen in fig. 2A. Nonetheless, the increase in protein content can not be directly correlated with an increase in endoglucanase activity (fig. 2B). Although the endoglucanase activity tended to increase with the time of cultivation and growth of the colony (fig. 2B) it rather stabilized between the third and sixth days while the specific activity strongly decreased during this period. The increase in protein production might be tentatively related to alkali protection. As the colony grew, pH strongly increased from six to about 7.5 stabilizing in approximately eight by the fiftieth day, when the endoglucolytic activity returned to be correlated with protein production (fig. 2B). This hypothesis was corroborated by results from pH assays (fig. 3). As above mentioned, reduced and total sugar content was, some kind, inversely proportional to pH and endoglucolytic activity as can be inferred from fig. 3B. In some extension, this can be explained in terms of fungal ability to hydrolysis CMC until glucose and employ it in its metabolism, thus decreasing the amount of sugar available. In fact, as the pH rose above 4, there was a rapid increase in protein production (fig. 3C) not entirely related with endoglucolytic activity as we see in fig. 3A.
The supposed relationship between protein production and alkali protection, once the endoglucolytic activity, although had been lower in the intermediary pH, was completely recovered at pH 8 – fig. 3A (left). On the other hand, the activity recover might be related with the two peaks of activity, at pH 5.5 and 8.0, observed by Dutta et al. (2008) in a partially purified enzyme extract of *P. citrinum*. The increased biomass and diameter of *P. citrinum*

Figure 2 – Development indicators of *P. citrinum* colony in function of time. A) Dry mycelium biomass, protein and pH variation; B) endo-β-glucanase activity and specific activity; and C) total and reduced sugar production.

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CCIBt 1488 mycelium was higher in low pH (fig. 3C and D) what is in agreement with its natural habitat in the acidic soil of cerrado.

Figure 3 – Developmental indexes of *P. citrinum* colony in function of pH. A) Activity and specific activity of endo-β-glucanase; B) total and reductive sugars; C) dry mycelium biomass and protein concentration.

A maximum point of specific activity occurred at the third day in face of the fall in protein content, but highest total endoglucanase activity occurred by the seventh day – fig. 2b.
Concentration of reduced sugars increased until the forth day – fig. 2c. The medium pH increased fast from 6 to 7.5 by the second day and still increasing slower until reach pH 8 by the eighth day.

Effect of pH on the culture of P. citrinum.

We cultivated *P. citrinum* in a range of pH in order to optimize its culture conditions – Fig. 3. The endoglucanase activity was higher in extremes of pH - fig. 3A (right). Total and reduced sugar concentrations have rather fluctuated with tendency of fall in function of pH (fig. 3B). Nonetheless, roughly, the amount of total (fig. 3B, left) and, above all, reduced sugars (fig. 3B, right) scaled inversely to pH and endoglucolytic activity. As the pH rose up to 4, there was a rapid increase in the protein production – fig. 3C. Biomass (fig. 3C) and diameter (not shown) of mycelium was higher in low pH (between 3 and 4.5).

Thermal stability of endoglucanase.

Enzyme crude extract was kept active after incubations at 50°C for 80 min. However, the endoglucolytic activity was virtually lost after incubation at 60°C (fig. 4). Tolerance to pH and temperature are relevant to industrial application of cellulases. However, differently of observed by Dutta et al. (2008), the endoglucolytic activity of *P. citrinum* CCIBt 1488 strain was virtually lost after incubation at 60°C (fig. 4). On the other hand, a residual activity might be noted after incubation at 60°C. This suggest the possibility of a phenotypic variation in the population sampled be able to provide adaptation to the extreme conditions as those found in *P. citrinum* MTCC 6489 strain.

A considerable part of the energy stored in the cell wall is in form of hemicellulases. Beyond, hemicelluloses cover the cellulose microfibril obstructing the access of cellulase system to it substrate. Possibly, a coordinate effort of chemical and biochemical technologies will be necessary to efficiently disrupt cell wall polysaccharides in order to convert it in bioethanol (Buckeridge et al. 20010). Xyloglucanase of *P. citrinum* CCIBt #1488 was significantly alkali- and thermo- stable keeping about 60% of its activity in a large range of parameters making it an interesting tool to industrial applications.
Crude extract was resistant to storage in mild conditions keeping about 90% of its initial activity after stored for until a year at 5º C – fig. 5B.

Xyloglucanase and galctomannanase activities assays.

In our experimental conditions xyloglucanase activity was linear until 20 min – Fig. 6a. The optimum pH was about 4.0 (fig 6b). The xyloglucanase presented thermostability between 20 and 65° C with an activity peak at 45° C – fig 6c.

Crude extract was able to release free mannose from galactomannan – fig. 7. However, further than xyloglucans, several other kinds of hemicelluloses form plant cell wall. Thus we
assayed crude extract of *P. citrinum* CCIBt #1488 on galactomannan – fig. 7. It was not just capable to produce free mannose from galactomannan as well, in fact, maximum galactomannanase activity was more than twice larger than that of xyloglucanase (1233 UV ml\(^{-1}\) against only 460 UV ml\(^{-1}\), respectively – data not shown).

![Graph](image)

Figure 7 – HPLC analysis of galactomannan oligosaccharides. Above: oligosaccharides of galactomannan from *Sesbania maginata* seed extracted with pure endo-mananase; middle: standard of mannose; below: galactomannan from *S. maginata* seed hydrolyzed for 24 h with *P. citrinum* crude extract.

**CONCLUSIONS**

*Penicillium citrinum* CCIBt #1488 has potential to be used in laboratorial and industrial purposes associated with decomposition of cellulose and hemicelluloses. Although original from acidic soils *P. citrinum* CCIBt #1488 enzymes (in particular xyloglucanase) maintain activity in mild alkaline medium. Crude extracts might be stocked in mild conditions and both endoglucanase and xyloglucanase activities are moderately resistant to high temperatures and pH. Thermal stability significantly differed from that observed previously in *P. citrinum* MTCC 6489 strain, although a hint of phenotypical variation had been observed. On the other
hand, preliminary investigation revealed a strong galactomannanse activity. Additional investigation must be applied in order to purify and better characterize cellulase and hemicellulase enzyme systems and phenotypical variation of *Penicillium citrinum* CCIBt #1488.

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