SEÇÃO 8
BIOQUÍMICA

PENICILLIUM CITRINUM POLYSACCHARIDASES

Wanderley Dantas dos Santos[1], Adriana Froutman Lopes[2], Ana Maria Baroni[2], Paulo Henrique Netto Alcântara[2] e Marcos Silveira Buckeridge[3]

[1]Universidade Estadual de Maringá, Departamento de Ciências Agronômicas, Estrada da Paca s/n, Fazenda UEM, São Cristóvão, ZIP 87501-190, PO Box 65, Umuarama, PR. E-mail wdsantos@uem.br
[2] Jardim Botânico de São Paulo, Av. Miguel Stéfano, 3687, Água Funda, ZIP 04301-902, PO Box 68041, São Paulo, SP
[3]Universidade de São Paulo, Instituto de Biociências, Departamento de Botânica, Laboratório de Fisiologia Ecológica de Plantas, R. Matão, 277, Cidade Universitária, Butantã, ZIP 05510-001, PO Box 1461, São Paulo, SP. +55 11 3091 7592. E-mail msbuck@usp.br.

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 50° C, desaparecendo a 60° 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 galctomanano 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 5° C. Os resultados sugerem que P. citrinum #1488 tem potencial para produzir enzimas para sacarificação de materiais lignocelulósicos bem como para outras aplicações industriais.

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

PENICILLIUM CITRINUM POLYSACCHARIDASES

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 50° C, vanishing at 60° 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 xyloglucanase 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 5° 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, xyloglucanase, 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 galctomananase 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 $= 100/t_{0.5}$). 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 descript 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 45° 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.
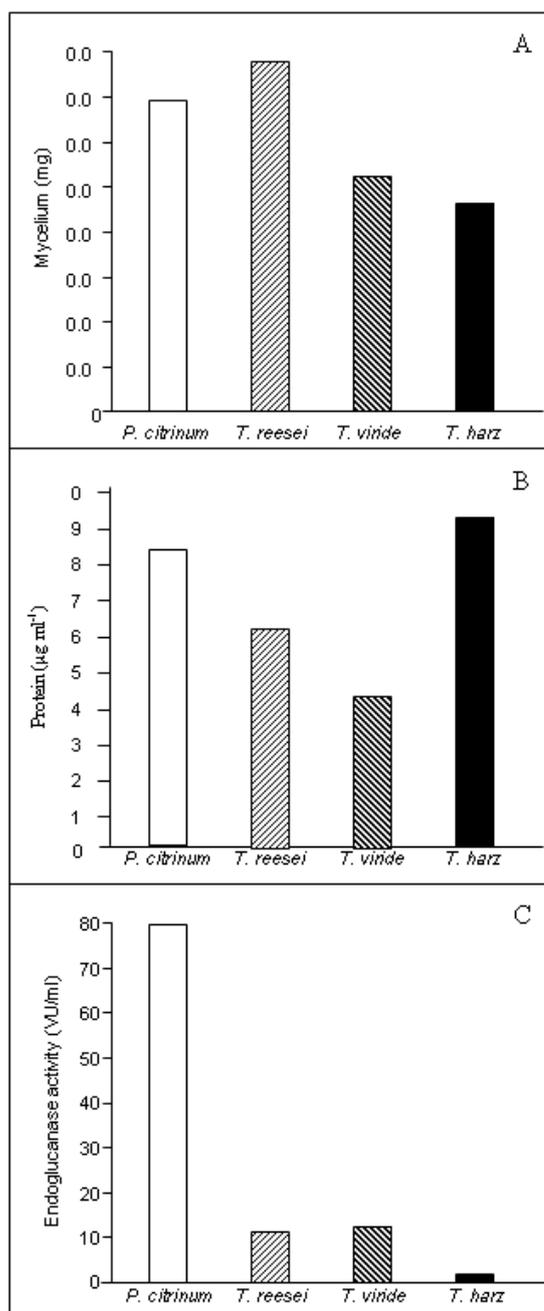
Figure 1 – Development indicators of three species of *Trichoderma* and *P. citrinum* in a medium with cellulose as the only source of carbon. A) Dry mycelium biomass, B) protein and C) endoglucanase activity.

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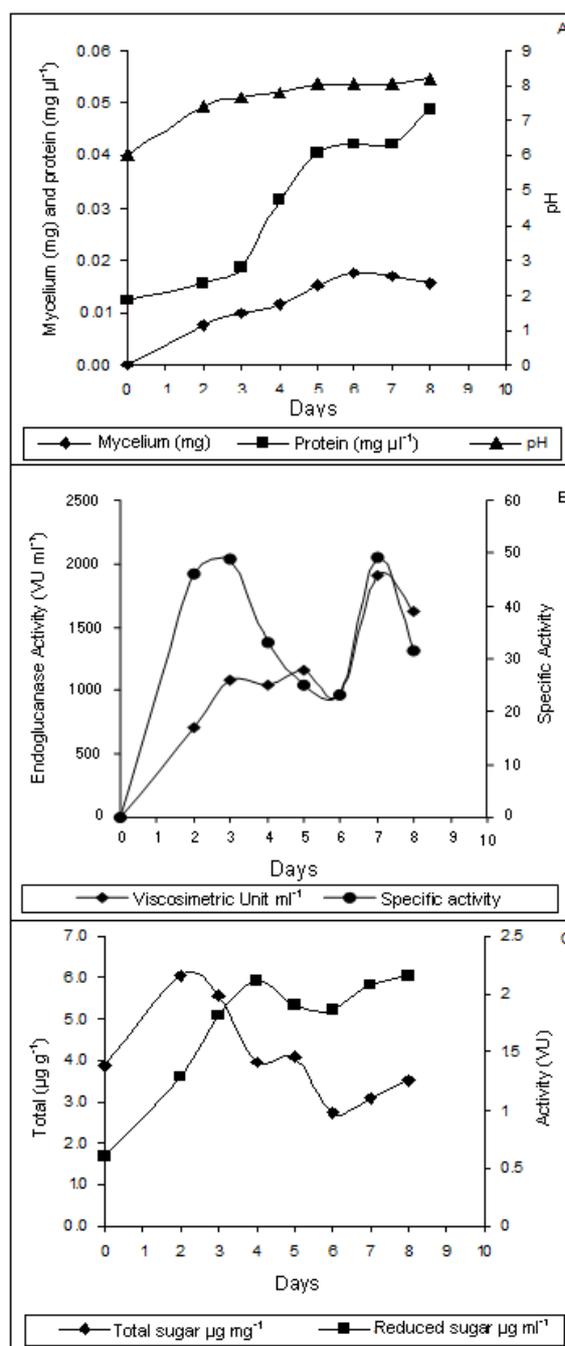
Figure 2 – Development indicators of *P. citrinum* colony in function of time. A) Dry mycelium biomass, protein and pH variation; B) endo-β-glucanase activity and specific activity; and C) total and reduced sugar production.

The supposed relationship between protein production an 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*

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.
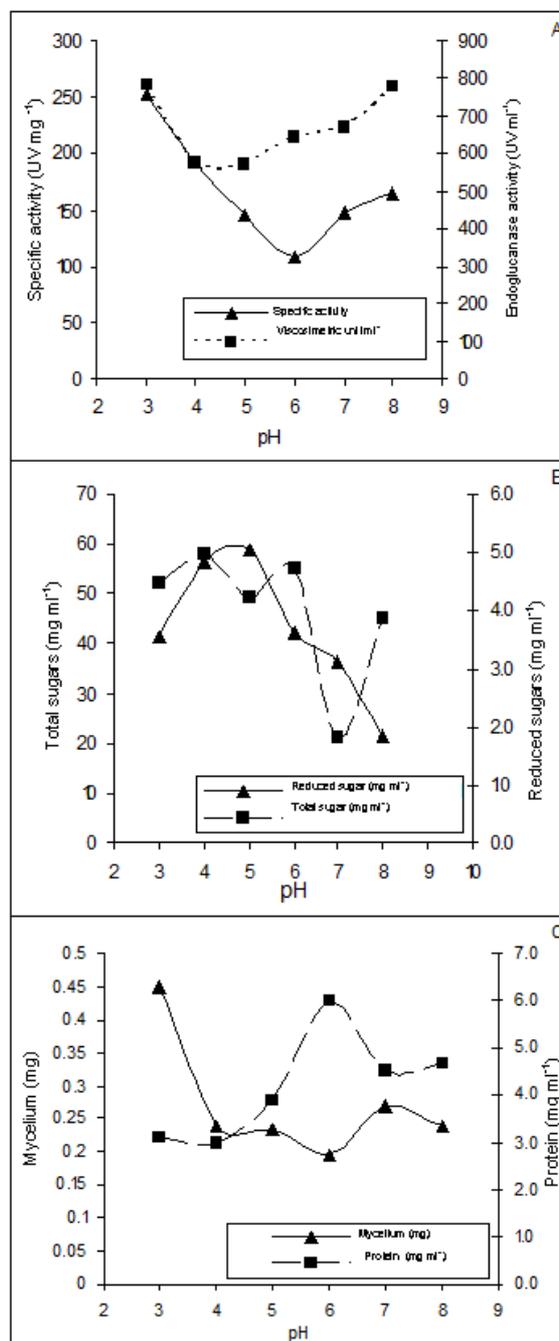

Figure 3 – Developmental indexes of *P. citrinum* colony in function of pH. A) Activity and specific activity of endo-β-glucanase; B) total and reductive sugars; C) dry mycelium biomass and protein concentration.

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.
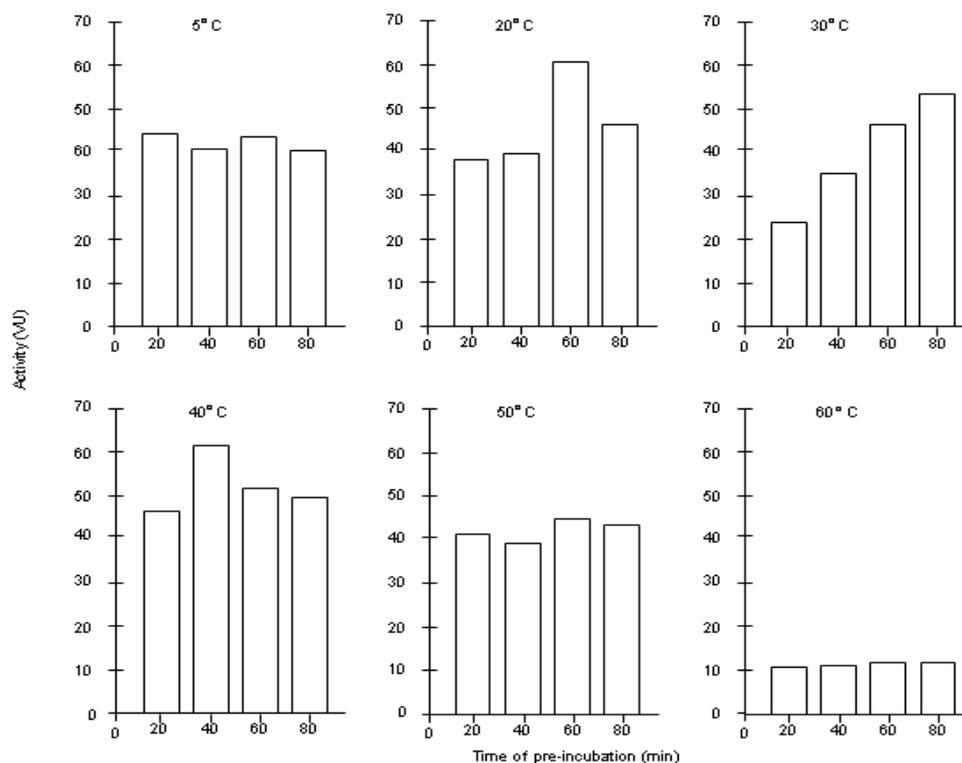
Figure 4 – Activity of *P. citrinum* endo-β-glucanase at 30° C after pre-incubation over 5, 20, 30, 40, 50 and 60° C for periods of 20, 40, 60 and 80 min.

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.
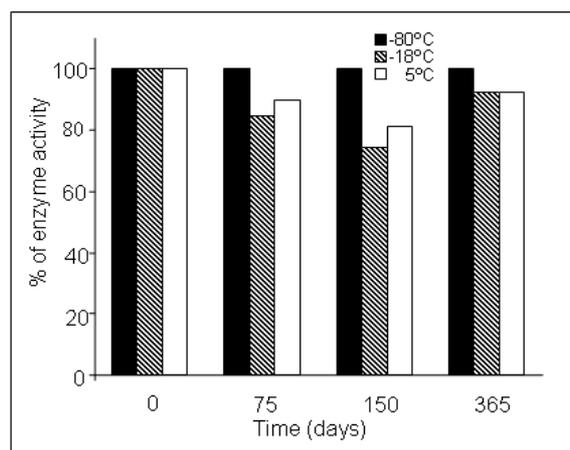

Figure 5 – Effect of temperature and time of storage in endoglucanase crude extract of *P. citrinum* at minus 80° C (*black bars*), minus 18º C (*crosshatched bars*), and minus 5° C (*white bars*).

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 $45^{\circ}C$ – fig 6c.
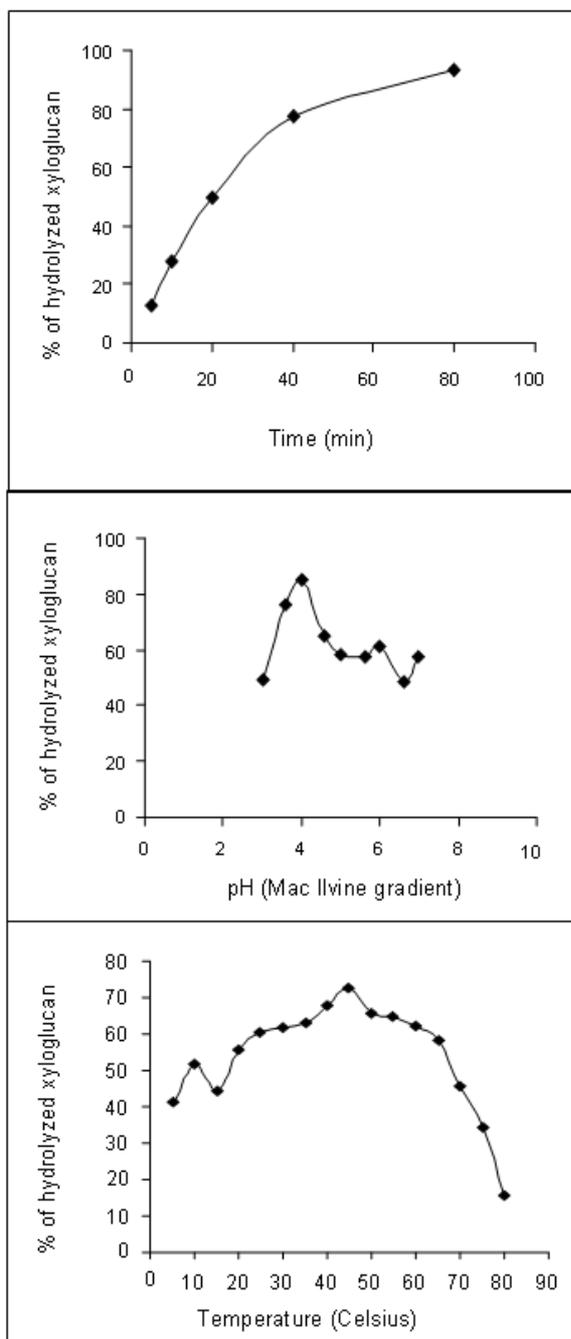

Figure 6 – Xyloglucanase activity of *P. citrinum* crude extract in a range of time, pH, and temperature of incubation.

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).
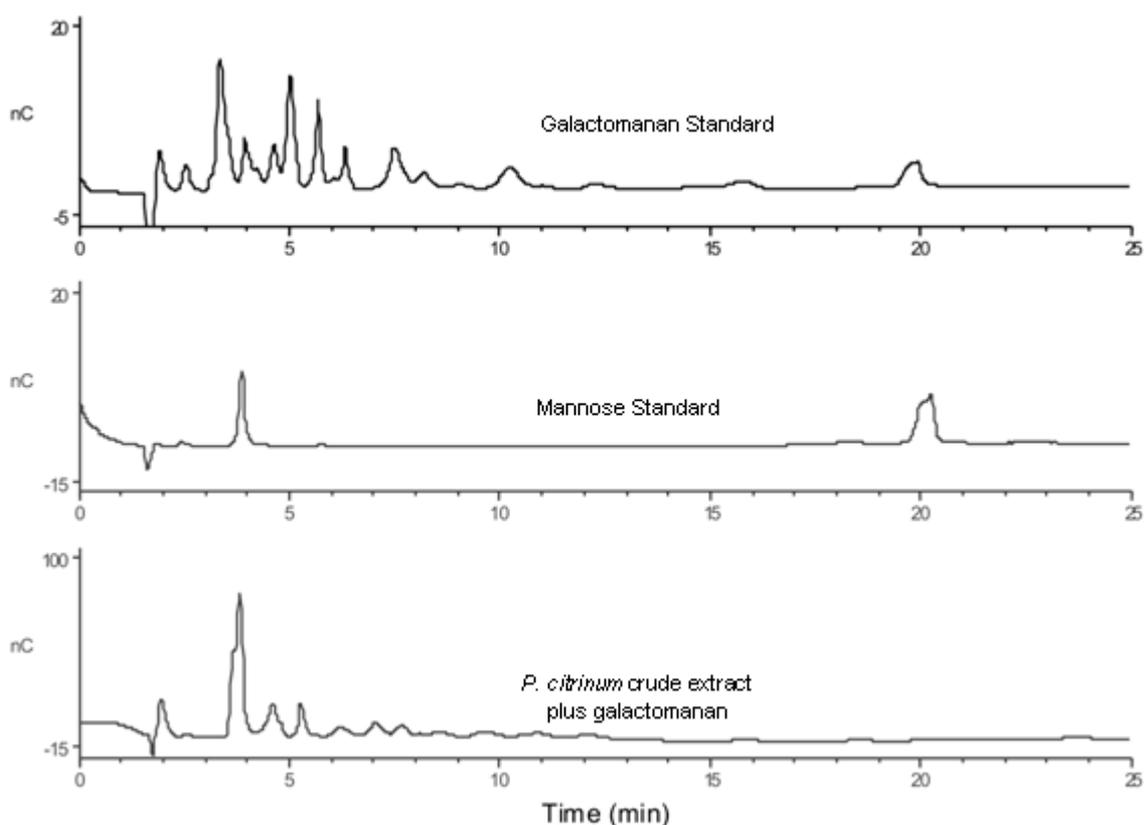

Figure 7 – HPLC analysis of galactomanan oligosaccharides. Above: oligosaccharides of galactomanan from *Sesbania maginata* seed extracted with pure endo-mananase; middle: standard of mannose; below: galactomanan from *S. maginata* seed hydrolyzed for 24 h with *P. citrinum* crude extract.

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.

REFERENCES

Alcântara, P. H. N. **Purificação e caracterização da β-galactosidase de sementes de *Copaifera langsdorfii* e suas relações com o metabolismo de xiloglucano**. 1995. 139p. Dissertação (Mestrado em Biologia Molecular). Universidade Federal de São Paulo, São Paulo, 1995.

Andersen, N., Johansen, K. S., Michelsen, M. Stenby, E. H., Krogh, K. B.R.M., Olsson L. Hydrolysis of cellulose using mono-component enzymes shows synergy during hydrolysis of phosphoric acid swollen cellulose (PASC), but competition on Avicel. **Enz. and Microb. Technology**, v.42, p. 362–370, 2008.

Assamoi, A.A., Destain, J., Delvigne, F., Lognay, G., Thonart, P. Solid-state fermentation of xylanase from *Penicillium canescens* 10–10c in a multi-layer-packed bed reactor. **Appl. Biochem. Biotechnol,** v.145, p. 87–98, 2008.

Bhiri, F., Chaabouni, S. E., Limam, F Ghrir, R., Marzouki, N. Purification and Biochemical Characterization of Extracellular β-Glucosidases from the Hypercellulolytic Pol6 Mutant of Penicillium occitanis. **Appl. Biochem. Biotechnol.** v. 149, p. 169–182, 2008.

Bisaria, V. S., Ghose, T. K. Enzyme and Microbial Technologic Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. **Enz. Microb. Technol.** n. 3, p.90-104, 1981.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Analyt. Biochim.** 72, p.248-254, 1976.

Buckeridge, M.S.; Silva, G.B.; Cavalari, A. A. Parede Celular. In: Kerbauy, G.B. (Ed). **Fisiologia Vegetal**. Rio de Janeiro: Guanabara Koogan. 2008.

Buckeridge, M.; Santos, W.D.; de Souza, A.P. As rotas para o etanol celulósico no Brasil. In L. A. B. Cortez (Coord.). **Bioetanol de cana-de-açúcar: P&D para produtividade e sustentabilidade**. São Paulo, Edgard Blucher. 2010.

Camassola, M., Dillon, A.J.P. Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. **J. App. Microb.** 103, p.2196–2204, 2007.

dos Santos, W.D., Gomes, E.O., Buckeridge, M.S. Bioenergy and the Sustainable Revolution. In, p. M.S. Buckeridge and G.H. Goldman (Org). **Routes to Cellulosic Ethanol.** New York: Springer Science and Business Media, 2011, p. 15-26.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. & Smith, F. Colorimetric method for determination of sugars and related substances. **Anal. Chem.** 28, 350-356, 1956.

Dutta T, Sahoo R, Sengupta R, Sinha Ray S, Bhattacharjee A, Ghosh S. Probing the active site environment of alkaliphilic family 11 xylanase from Penicillium citrinum, p. evidence of essential histidine residue at the active site. **Enz. Microbial. Technol**. 41, p.440–446, 2007a.

Dutta T, Sengupta R, Sahoo R, Sinha Ray S, Bhattacharjee A, Ghosh S. A novel cellulase free alkaliphilic xylanase from alkali tolerant Penicillium citrinum, p. production, purification and characterization. **Lett Appl Microbiol**. 44, 206–211, 2007b.

Dutta, T. Sahoo, R., Sengupta, R. Ray, S.S. Bhattacharjee, A. Ghosh, S. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*, p. production and characterization. **J. Ind. Microb. Biotechnology**, 35, p.275–282, 2008.

Karboune, S., Geraert, P-A., Kermasha, S. Characterization of Selected Cellulolytic Activities of Multi-enzymatic Complex System from *Penicillium funiculosum*. **J. Agric. Food Chem.,** 56, p.903–909, 2008.

Rodrigues, F.A. & Guirardello, R. Evaluation of a sugarcane bagasse acid hydrolisis technology. **Chem Eng & Tech.** v.31, n.6. p. 883-892, 2008.

Segel, I. H. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. In Segel, I. H. **Enzyme Kinetics**. Canada: Jonh Wiley & Sons. 1975.p. 927-930.

Shaw, A. J., Podkaminer, K. K., Desai, S. G., Bardsley, J. S., Rogers, S. R., Thorne, P. G., Hogsett, D. A., Lynd, L. R. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. **PNAS**. v.105, n.37 p.13769-13774. 2008.

Stumpf, P. K., Conn, E. E. **The biochemistry of Plants Secondary Plant Products** (vol. 7). New York: Academic Press. 1981.

Sun, X., Liu, Z., Qu, Y., Li, X. The Effects of Wheat Bran Composition on the Production of Biomass-Hydrolyzing Enzymes by *Penicillium decumbens*. **Appl Biochem Biotechnol.** v.146, p.119–128, 2008.

Sun, X., Liu, Z., Zheng, K., Son, X., Qu, X. The composition of basal and induced cellulase systems in *Penicillium decumbens* under induction or repression conditions. **Enz. and Microb. Tech.** n.42, p.560–567, 2008.

van Wyk, J. P. H. Thermostability of cellulase from *Penicillium funiculosum*. **Biotech. Technics.** n.11, v.6, p.385–386, 1997.