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Evaluation of *Penicillium chrysogenum* isolated from Brazilian Cerrado soil as potential producer of industrial enzymes

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Abstract

The possible exhaustion of fossil fuels sources, its association with environmental pollution; climate change, and global warming is driving world energy policies towards the development of green energy sources. Purposely, plant biomass (lignocellulose) gains the attention to be used in industrial processes, such as second generation bioethanol production. The present study investigates the production of plant cell hydrolases by Penicillium chrysogenum isolated from Brazilian savanna-like soil using sugar cane bagasse and straw as a carbon source. Maximal xylanase activity production was observed after 4 days of growth, for both carbon sources, and for assays carried at 40˚C and pH 5. A xylanase was completely purified after three purification steps: Ultrafiltration, molecular exclusion and anion-exchange chromatography. Fractions containing xylanase activity were pooled and analyzed by SDS-PAGE, which showed a unique protein band presenting molecular mass between 35 to 40 kDa.

Key words: Agro-residues, Enzyme purification, FPLC, Size exclusion chromatography, Xylanase

INTRODUCTION

The current demand for alternative sources of fuel and environmentally safe industrial processes has been driving a crescent interest in the development of biofuels using lignocellulose biomass as raw material, as cellulosic ethanol. This biofuel production depends of two critical steps before fermentation: pretreatment and hydrolysis to produce fermentable sugars. Enzymatic hydrolysis has gained a remarkable position to this purpose, as well as, microorganisms as source of enzymes suitable to be used in industrial processes. As plant cell wall biomass contains, lignocellulose composed by cellulose (glucose monomer linked through glycosidic bond), hemicellulose (hexoses, pentoses, glucuronic acid) and lignin (Hamann et al., 2015). Carbohydrate hydrolases (xylanase, cellulase) used to degrade these glycoside bonds gained remarkable position in market of enzyme world. In this regards xylanase have important commercial value, not only for plant cell wall deconstruction to produce bioethanol, but also due to its application in paper industry to bleach paper pulp, and in food and beverages industry for clarification of fruit juices. Many microorganism Bacteria (*Bacillus*, *Streptomyces* and *Clostridium*) and fungi (*Trichoderma, Aspergillius and*

Penicillium) have been reported for the production of glycosidic hydrolases. *Penicillium chrysogenum* is well known producer of cell wall degrading enzymes (Kubicek, 2014)

In the present work, we analyzed the use of agro industrial residues as carbon source for growth and production of plant cell wall hydrolases by *Penicillium chrysogenum* isolated from Cerrado soil, provided as a rich source of hydrolyzing enzymes In future, investigation of this specie will emphasize the high yield of theses enzymes with potential application in industry

MATERIALS AND METHODS

Penicillium sp. was isolated from Cerrado Soil cultivated and purified on MYG agar media (Yeast extract (4g-1), Malt extract (10g-1), Glucose (4g-1), Agar (2%w/v) using 0.5ml sample incubated at 28^{o}C for 48hours.

Xylanase secretion was analyzed on MYG agar medium with xylan oat-spelts (1% w/v) substrate at 28^{o}C after 4days. 3mm disk of seven days old culture were placed in the center of agar plate in three sets. The control plates were prepared without substrate in same conditions. For halo visualization all plates were flooded with 0.1% Congo red for 15 to 20 minutes and washed with 1M Nacl.

Evaluation of plant cell wall hydrolases production by *Penicillium sp.* was carried using minimal liquid media containing 1% (w/v) of sugarcane bagasse or straw or avicel and citrus as sole carbon source. Cultural flasks containing liquid media was inoculated with of *Penicillium sp*. conidia (1× 10^{6}/ml) and incubated at 28^{o}C in shaker incubator (120rpm) for 10 days. Aliquots of 5 mL were removed after 2, 4, 6, 8, 10 days. Sample was vacuum filtered and centrifuged at 10,000g for 10min at 4^{o}C and cell free supernatant was collected and stored at -20^{o}C until their use to quantify enzymatic activities (Cellulase, Xylanase, and Avicelase) and protein content. Each culture was grown in triplicates.

Xylanase enzymatic assay was carried out using: 50µL of cell free sample mixed with 100µL of xylan (oat spelt concentration) for xylanase, 100µL (carboxymethylcellulose) for cellulase and 100µL (Avicel PH-101) for avicelase activity, suspension in sodium acetate buffer, 100mM pH 5.0 as substrate. This mixture was incubated at 40^{o}C for 15 minutes; the reaction stopped by adding 300µL of dinitrosalicylic reagent and boiled for 10minutes as previously described by Miller (1959). Absorbance was read at 540nm in UV-VIS spectrophotometer. One unit of was defined as the amount of enzyme which produces reducing sugars equivalent to 1umoL of xylose per minute of reaction under the experimental conditions. For avicelase and cellulase one unit defined as the amount of enzyme which produces reducing sugars equivalent to 1µmol of glucose per minute of reaction under the experimental conditions Protein content was measured using Bradford method and bovine serum albumin as a standard. (Bradford, 1976)

For enzyme purification, cell free supernatant obtained from 4 days cultures was dialyzed overnight against sodium acetate buffer, 50mM pH 5.0 at 4^{o}C (10kDa cut-off), concentrated 10x fold by freeze drying and rehydrate with distilled water. An aliquot of this sample10 mL (63ug/ml protein) was applied onto a Sepharose G-75 column (3×71cm), equilibrated against 50mM sodium acetate buffer at pH-5. Proteins were eluted at flow rate of 0.3ml/min and fractions of volume 505ml containing 6ml final volume in each fraction. Each

fraction was used as protein source to measure absorbance at 280 nm and to assay for xylanase activity. Fractions that showed xylanase activity were pooled. Pooled xylanase fractions were further purified by using HiTrap Anion Qxl 1ml Column in fast protein liquid chromatography (Akta system GE health care life sciences) against sodium acetate buffer (pH.5) at flow rate 1ml/minute. Xylanase purification was verified by SDS-PAGE and Zymogram analysis.

RESULTS AND DISCUSSION

The medium containing sugarcane bagasse and straw as carbon source secreted more xylanase (U=0.56), after 4 days of growth. The avicelase activity (U=0.15) observed higher in avicel (carbon source) after 6 days of growth. Cellulase activity (U=0.09) observed higher in sugar cane bagasse at 8 day of growth. The xylanase enzyme produced in sugarcane bagasse was completely purified in three steps, (a) Ultrafiltration (b) Size exclusion chromatography (c) Anion exchange chromatography. The enzyme purification was confirmed by the presence of single band on SDS-Polyacrylamide gel (12%).

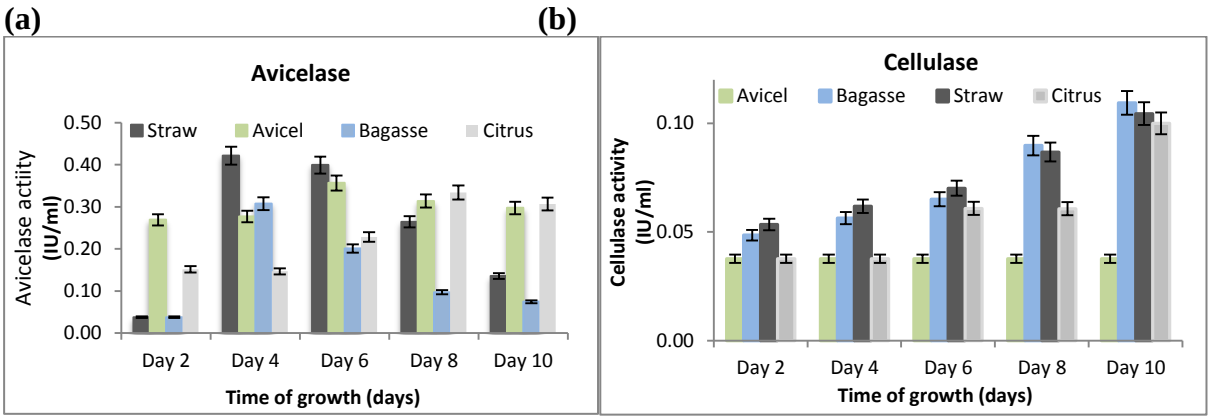

Figure 1: (a) Avicelase activity in different biomass (b) Cellulase activity in different biomass

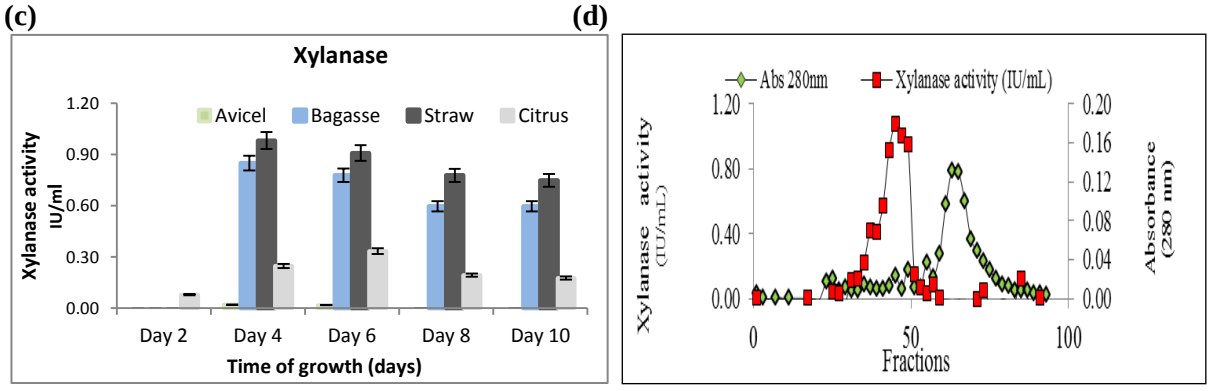

Figure 2: (c) Time course of xylanase production in different biomass **(d)** Chromatography profile by using G-75 (Sepharose column). Curves show protein content (280 nm) and xylanase activity 540nm.

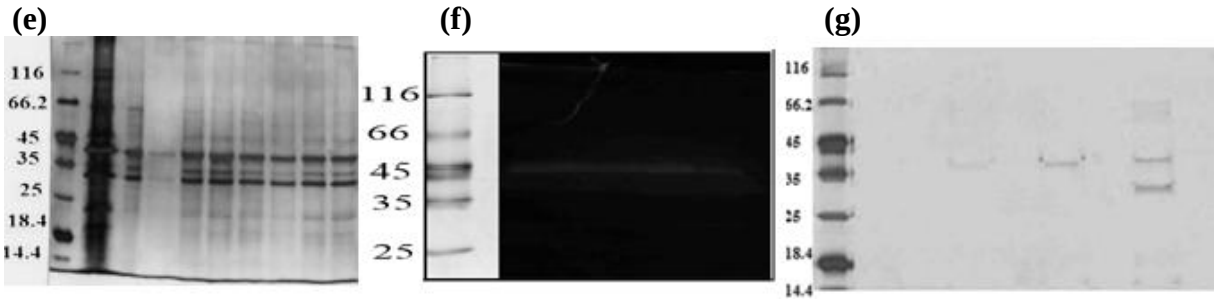

Figure 3: (e) Protein peak profile after size exclusion chromatography **(f)** Xylanse activity in zymogram gel **(g)** xylanse purification after anion exchange charomatography.

The purified xylanase have molecular weight ranged between 35-40 kDa these results are in line with (Hubertus *et a* .,1992) completely purified xylanase of 35 kDa using DEAE. Enzyme characterizations are in progress.

CONCLUSIONS

Asexual filamentous *Penicillium chrysogenum*, collected from Cerrado soil was able to grow and potentially produce hydrolases (which hydrolases) enzymes during growth in the presence of sugar cane bagasse or straw as unique carbon source. Anion exchange chromatography completely purified the xylanase. Further analysis on this isolate will be emphasized its application of biotechnological processes by using this purified enzyme.

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