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Evaluation of Synthetic and Semi- synthetic Culture Media for Endo-1,4-β- Glucanases Secretion by *Trichoderma koningiopsis*

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Abstract

The actual demand of energy and the environmental concerns together with the reduced fossil fuel reserves have played an important role to convert the second generation bioethanol production into an attractive research area. To convert lignocellulosic biomass to bioethanol the cellulosic components must be hydrolyzed to fermentable sugars. *Trichoderma* fungi secrete large amounts of enzymes of industrial interest such as cellulases, able to degrade holocellulose in the saccharification of lignocellulosic biomass. In this work we evaluated endo-1.4-β-glucanases enzymatic secretion of *Trichoderma koningiopsis* from Misiones province, in synthetic medium, with carboxymethylcellulose as carbon source; and semi-synthetic medium, with pine sawdust as carbon source. Higher values of endo-1.4-β-glucanases were reached when the semi-synthetic medium was used. It could be concluded that pine sawdust seems to be a good candidate for utilization as carbon source in culture media aiming to obtain good enzyme secretion, being also an economic and easily available substrate.

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Keywords: Endo-1,4-β-glucanases; Carboxymethylcellulose; Pine sawdust; *Trichoderma koningiopsis*

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1. Introduction

The limited availability of fossil fuel reserves worldwide, the increasing energy demand, the concern in climate change and mankind's role therein, have led to the development of new alternative energy sources more environmentally friendly, that can replace fossil fuels. Many countries have initiated extensive studies and developed programs on the production of fuels from different sources, including food crops, called first generation biofuels; or those that use lignocellulosic biomass for bioethanol production, called second generation biofuels, making plant feedstock a sustainable and renewable energy source [Gray et al. 2006; Himmel et al. 2007].

Bioethanol is emerging as a good alternative fuel candidate for the needs previously detailed. It is a renewable energy, because it is derived from plant biomass having good balance of carbon dioxide emissions [Jeuland et al. 2004]. Also, bioethanol can reduce gas emissions that cause the greenhouse effect, reducing environmental impacts and so climate change, and it provides many economic and strategic benefits [Hahn-Hägerdal et al. 2006].

The world's largest production of 1st generation bioethanol focuses primarily on two countries (Brazil and the U.S.), and usually is produced from the juice of sugar cane or corn starch [Dashtban et al. 2009; Sanchez 2009].

However, the use of sugar cane and corn for ethanol production is controversial. First, because the cost of its production is relatively high, which makes it difficult to compete with fossil fuels, and second, this bioethanol production require agricultural crops used for food.

Therefore, a rising feedstock in the production of bioethanol with low cost and that eliminates the conflict concerning the use of food crops is the use lignocellulosic biomass like crop residues, grasses, sawdust, wood chips and animal solid waste [Sun and Cheng 2002].

The lignocellulose is a renewable organic material and is the major structural component of plants [Dashtban et al. 2009]. It is mainly composed of three structural polymers: cellulose, hemicellulose and lignin [Martinez et al. 2005].

To convert the lignocellulosic material to ethanol is necessary that cellulosic components (cellulose and hemicellulose) were hydrolyzed to fermentable sugars, therefore saccharification is one of the main steps in obtaining bioethanol. This stage is the main economic difficult hindering profitable utilization of this abundant energy source and actually turns this process in slow and expensive [Hahn-Hägerdal et al. 2006].

To maximize the yield of degradation of cellulose into fermentable sugars, and thereby reducing costs and making the process economically viable, the optimization of cellulase production would provide innovative solutions for the application of this technology. Many microorganisms secrete a large range of enzymes of industrial interest, able to use cellulose and hemicellulose as carbon and energy source, of which fungi are the major responsible for this degradation [Sanchez 2009]. Many authors [Mandels and Reese 1957; Deshpande et al. 1984; Reczey et al. 1996; Mekala et al. 2008; Fujii et al. 2010] cited strains of the genus *Trichoderma* as potential cellulases productors.

The ability of some species, such as *T. viride* and *T. reesei* to produce large amounts of hydrolytic enzymes such as cellulases, is commercially applied in many industries like in the production of laundry detergents in the textile industry, the pulp and paper industry and in the production of biofuels [Griffin 1994; Kubicek-Pranz 1998; Schuster and Schmoll 2010].

Organisms capable of degrading crystalline cellulose secrete a set of cellulases with different specificities and mode of action involved in cellulose hydrolysis. This set of enzymes is called cellulolytic system or complex [Macarron Larumbe 1992]. These cellulases include endoglucanases (EGs) or endo-1,4-â-glucanases, cellobiohydrolases (CBHs) or exoglucanases and â-glucosidases (BGLs). The cellulases catalyze the conversion of insoluble cellulose to water soluble cellulose and simple products [Jahangeer et al. 2005]. EGs initiate randomly the attack by cleaving â-1,4 glucosidic bonds mainly in the amorphous regions in the interior of the cellulose molecules, producing new nonreducing termini. The CBHs gradually attack the cellulose molecules in the nonreducing termini releasing cellobiose subunits. Finally, BGHs hydrolyze cellobiose to glucose. Therefore, the total degradation of cellulose involves the three cellulase enzyme components acting in a synergistic mode [Vilches Paz 2002].

Biotechnology can use the planet's biodiversity to develop new products and processes [Venegas and Suarez 2004], however to evaluate and select new native microbial strains capable of produce cellulases first is essential to optimize nutritional and operating parameters such as media types, operating conditions, pH, temperature, cofactors, etc. to ensure maximum yields in the production of enzymes.

To determine quantitatively the cellulolytic activity [Miller 1959], it is necessary to grow the microorganisms in a culture medium containing a source of carbon and nitrogen to enable extracellular secretion of cellulase. Several

authors consider carboxymethylcellulose (CMC) as a carbon source for optimal cellulases secretion [Sazci et al. 1986; Venegas and Suarez 2004; Doolotkeldieva and Bobusheva 2011], and the Mandels culture medium as a nitrogen full complex that induce cellulases secretion [Mandels and Reese 1957; Mandels 1975; Guerra et al. 2006]. Some methods have been proposed for the detection of cellulolytic activity in microorganisms, both qualitatively and quantitatively [Miller 1959; Mandels et al. 1976; Hankin and Anagnostakis 1977; Smith 1977; Mahasneh and Stewart 1980; Sazci et al. 1986]. Currently the most commonly used methods are: a qualitative method using a medium containing CMC as sole carbon source, revealing the presence of cellulases by adding a solution of congo red azo dye [Sazci et al. 1986; Magnelli et al. 1997; Yoon and Kim 2005; Kasana et al. 2008; Doolotkeldieva and Bobusheva 2011]; and a quantitative method that determines the release of reducing sugars during enzymatic hydrolysis of CMC, by the technique of Dinitrosalicylic Acid (DNS) [Miller 1959; Wood 1988; Andersen et al. 2008; Dashtban et al. 2010; Jurick et al. 2012]. However, in order to obtain large amounts of enzyme secretion to decrease the cost of cellulolytic preparations used in the saccharification step at industrial level, it is very important to evaluate other carbon sources which should be renewable, cheap and locally available, as could be in Misiones the pine sawdust.

Therefore, in this study we evaluated the endo-1,4-â-glucanases enzymatic secretion of a native strain of *Trichoderma koningiopsis* from Misiones province, in solid and liquid synthetic medium, with CMC as sole carbon source; and a semi-synthetic medium, with pine sawdust as sole carbon source.

2. Experimental Procedure / Methodology

2.1. Microorganisms and maintenance conditions

We worked with a strain belonging to the genus *Trichoderma*, isolated from a natural ecosystem in Misiones province (Table 1).

Table 1: Natural ecosystem of microorganisms isolated.

Isolate (code)	Place of isolation	GPS location	Species
NAN-11	Ñacanguazu, Gobernador Roca, Misiones	Latitude: S 27º 06’ 57’’ Longitude: O 55º 22’ 37’’	*Trichoderma koningiopsis*

The strain was maintained on potato dextrose agar slants under controlled conditions of temperature (4ºC) and photoperiod.

2.2. Culture media

2.2.1. Synthetic solid medium

Composed with CMC 0.5% (w/v) as sole carbon source, 1.7% agar (w/v) and Mandels medium as nitrogen full complex [Mandels and Reese 1957]. The components were mixed, sterilized and autoclaved at 121 °C and 1 atmosphere for 15 minutes and then were dispensed on Petri dishes. This culture medium was used to perform a primary qualitative test of enzyme secretion by the strain under study.

2.2.2. Synthetic liquid medium

Composed with CMC 0.5% (w/v) as sole source of carbon and Mandels medium as nitrogen full complex [Mandels and Reese 1957], the pH was adjusted adding sodium succinate buffer 100 mM at pH 4.8. This medium was used as an experimental test for the quantitative assay. The components were mixed and distributed into 250 ml Erlenmeyer flasks containing 50 mL of medium previously cited plus 5 mL of sodium succinate buffer. The medium was sterilized and autoclaved at 121 °C and 1 atmosphere for 15 minutes.

2.2.3. Semi-synthetic culture medium

Composed of sawdust of pine (*Pinus* sp.) (from a local Sawmill located in Garupá, Misiones province) as carbon source, and Mandels medium as nitrogen full complex [Mandels and Reese 1957], pH was adjusted adding sodium succinate buffer 100 mM at pH 4.8. This medium was used as an experimental test for the quantitative assay. Components were mixed and distributed into 250 ml Erlenmeyer flasks containing 20 g of substrate with a 75% humidity adjusted incorporating Mandels medium previously prepared.

2.2.4. Mandels medium as nitrogen full complex [Mandels and Reese 1957] composition

Composed by $(NH4)_2SO_4$ 1.4 g/L, KH_2PO_4 2 g/L, $CaCl_2.2H_2O$ 0.4 g/L, $MgSO_4.4H_2O$ 0.3 g/L, $FeSO_4.7H_2O$ 0.005 g/L, $MnSO_4.4H_2O$ 0.0016 g/L, $ZnSO_4.7H_2O$ 0.0014 g/L, $CoCl_2.6H_2O$ 0.02 g/L, urea 0.3 g/L and yeast extract 0.25g/L.

2.3. Culture conditions

The incubation conditions tested in all cases were an incubation temperature of 29 ± 1 °C with constant photoperiod for 10 days.

In the synthetic solid culture medium the inoculum used was a 10 mm diameter plug of fresh mycelium taken from the strain under study reactivated in Petri dishes containing potato dextrose agar (PDA 3.95% w/v) as culture medium. The plugs of fresh mycelium were taken from the edge of the growing colony with a circular punch of 5 mm radius, and were transferred to Petri plates containing the culture medium for the qualitative determination of enzyme secretion.

A spore suspension (10^7 spores/mL) from the culture media was used as inoculum for quantitative assays (synthetic liquid medium and semi-synthetic medium). An aliquot of 1 ml of the culture supernatant was extracted every 2 days to determine its enzymatic activity.

The spore suspension was prepared in sterile distilled water with the addition of Tween 80 to a final concentration of 0.1% (v/v). The spore count was performed using the technique proposed by Alves and Farias (2010), making the counts in the central square of the Neubauer chamber.

All assays were performed in duplicate.

2.4. Enzymatic activity determination

After incubation at 29 ± 1 °C under constant photoperiod for 5 days, the cellulolytic potential in the qualitative assay in solid medium was determined by adding a solution of the azo dye congo red (0.1% w/v) to each plate for 15 minutes with gentle agitation. Finally the reaction was stopped with repeated washes with tap water, observing on the surface of the plate the formation of degradation halo. This halo is formed due to the absence of CMC, which had been degraded by the action of the cellulase complex of the fungal strain [Teather and Wood 1982; Magnelli et al. 1997].

The enzyme activity determination of EGs in the quantitative assays was performed with 2% crystalline cellulose as substrate, and quantified on a Shimadzu UV-3600 spectrophotometer with an absorbance of 540 nm. The amount of reducing sugars produced during the test was measured by DNS Acid assay [Miller 1959] using glucose as standard. The mixture reaction was incubated at 50 °C for 30 min. The EGs secretion was measured in International Units. An International Unit (U/L) of EGs enzyme activity was defined as the amount of enzyme which released one µmol reducing sugars per minute per Litre.

2.5. Statistical Analysis

ANOVA analysis and Bonferroni test were performed using Graph Pad Prism version 6.0 for Windows (Graph Pad Software, San Diego, CA, USA).

3. Results and Discussion

3.1. Qualitative assay of cellulases secretion

Tests using Congo red azo dye 0.1% in Petri dishes were performed to qualitatively characterize the enzymatic secretion of *Trichoderma koningiopsis* strain NAN11, allowing the visualization of the potential cellulolytic activity (Figure 1).

From visual observations of degradation halos, under the conditions of study of this paper, it was inferred that the strain of *T. koningiopsis* NAN11 presented a significant enzyme secretion. Following this confirmation, the next step in our work was to continue with the quantitative assays of cellulases enzymatic secretion.

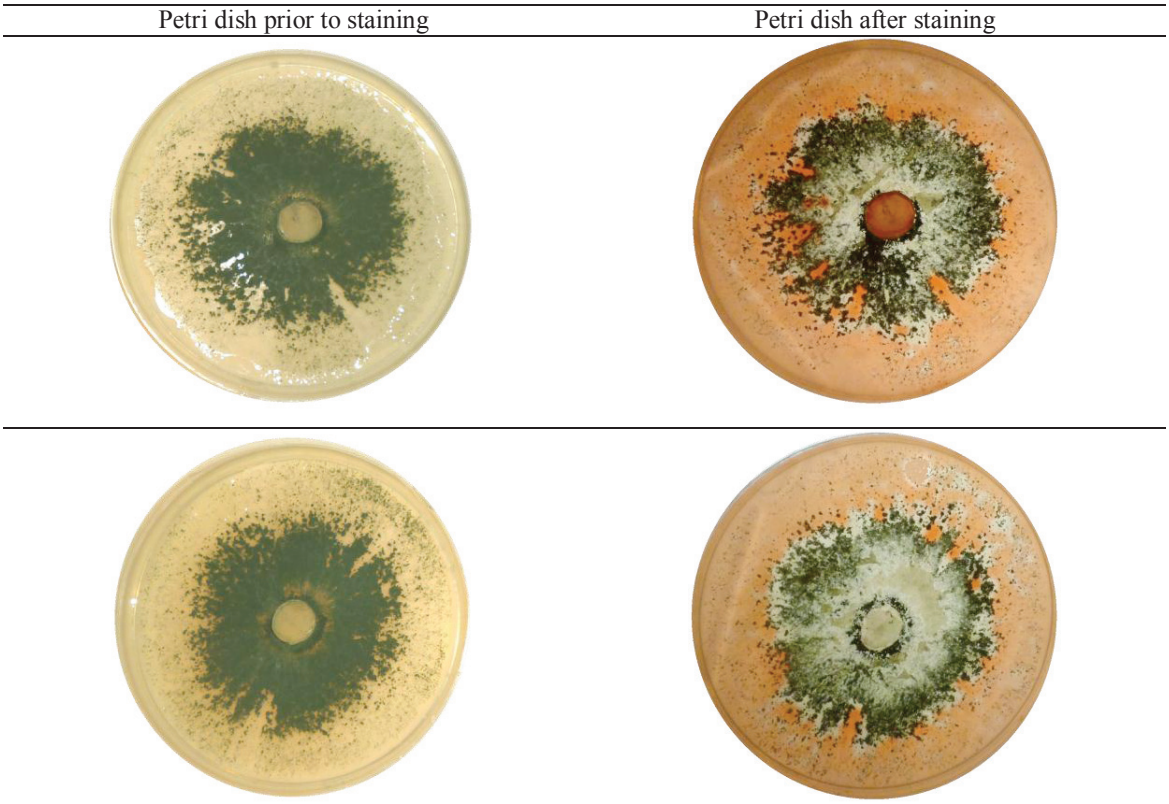

Figure 1. Qualitative evaluation of cellulases secretion by *Trichoderma koningiopsis* strain NAN11**.** Synthetic solid medium stained with dye azo Congo red (0.1% w/v).

3.2. Quantitative assay of cellulases secretion

The quantitative studies to determine the EGs secretion were performed by means of synthetic and semi-synthetic culture media.

Although the CMC is frequently cited as a good substrate enabling cellulolytic complex secretion [Sazci et al. 1986; Venegas and Suarez 2004; Doolotkeldieva and Bobusheva 2011] the results obtained here showed that when NAN 11 strain was cultured in a semi-synthetic medium, a superior secretion of EGs throughout the incubation period was attained as compared to the synthetic medium ($p<0.05$) (Figure 2). This outcome may occur because the carbon source used in the semi-synthetic culture medium (pine sawdust) is composed of a variety of components that can act as different carbon sources, and thus allowing the fungi to grow in a better way and to secrete a variety of enzymes [Colina et al. 2009; Sangjoon et al. 2011]. The potential use of pine sawdust as carbon source represents

an important advantage in the development of industrial-scale process level, because it is renewable, economical and locally available in Misiones province. And also this natural resource promotes large amounts of enzyme secretion, lowering the preparations cost in the cellulolytic saccharification process for bioethanol production at industrial level.

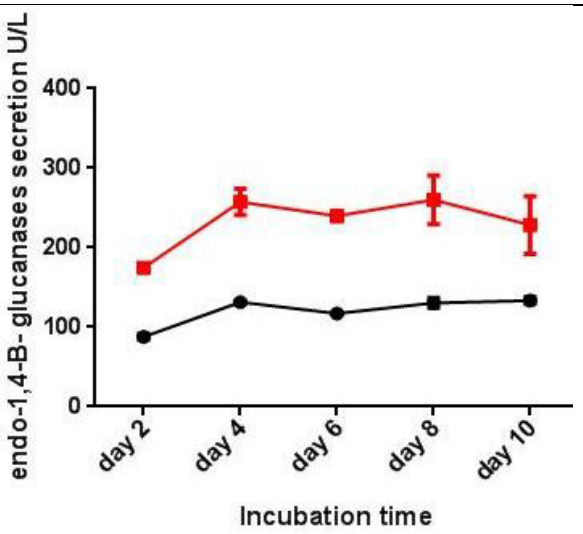

Figure 2. Secretion kinetics of EGs by NAN-11 strain. (■) Semi-synthetic culture medium, with pine sawdust. (●) Synthetic liquid medium, with CMC 0,5%. The EGs secretion was measured in International Units. EGs values are plotted in U/L per days of culture.

4. Conclusions

In the present study, it could be concluded that pine sawdust seems to be a good candidate for utilization as carbon source in culture media aiming to obtain good enzymatic secretion when compared to common synthetic medium with CMC; It should be keep in mind that pine saw dust is an economic, renewable, and easily available substrate for energy source.

Following steps in our research will be to evaluate and propose a standard protocol in the use of pine sawdust for secretion media.

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