Quantification of Endoglucanase Activity based on Carboxymethyl Cellulose in Four Fungi Isolated from an Aerated Lagoon in a Pulp and Paper Mill

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The aim of this study was to identify cellulolytic fungal strains capable of degrading cellulose from an aerated lagoon in a pulp and paper mill. Four fungal strains that were found to be highly active were isolated on carboxymethyl cellulose (CMC) and suggested to be CMCase/endoglucanase. The identified strains were *Aspergillus niger*, *Penicillium* sp., *Aspergillus fumigatus*, and *Mucor* sp. All the strains were studied in terms of cultural morphological characteristics and microscopic examinations. The endoglucanase with the highest isolate production was *Penicillium* sp., which also showed the highest qualitative endoglucanase activity (1.3 cm), in addition to the main activity of endoglucanase with 297 mmol/mg.min after 116 h. The results indicated that CMC is able to induce endoglucanase enzyme production and that the fungal isolates showed significant cellulose degradation properties.

Keywords: Quantification; CMC; Activity endoglucanase; Cellulose; Pulp and paper; Aerated lagoon

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INTRODUCTION

The bioprospecting of microorganisms, enzymes, and genes involved in cellulose degradation is still cutting-edge research in applied microbiology and biotechnology. The quantification of cellulolytic activity is a challenge in enzymology because of the complexity of the enzyme system involved and the heterogeneity of the techniques and units used to report it by different laboratories (Ohmiya *et al.* 2003; Aro *et al.* 2005).

The degradative system comprises several cellulolytic enzymes including endoglucanases, exoglucanases (cellobiohydrolases), and glycosidases (Diorio *et al.* 2003). Endoglucanases are widely used, and one of the most important components of the cellulolytic enzyme system is the endo1,4-β glucanase enzyme, which hydrolyzes the cellulose chains at random. This enzyme is found in a wide range of organisms, from bacteria to cellulolytic fungi. Among the substrates that have been evaluated are carboxymethylcellulose (CMC), cellulose pretreated with alkali or acid, and crystalline cellulose (Avicel or cotton fiber) (Aro *et al.* 2005).

The qualitative technique used was that reported by Tanaka *et al.* (2005), which is based on the association of Congo red and CMC generating a strong color that fades with

the depolymerizing activity of the endoglucanase. Congo red has a high affinity for polysaccharides and has been used for the diagnosis of amyloidosis (Bély and Makovitzky 2006), the differentiation of pathogenic strains of *Escherichia coli* (Berkhoff and Vinal 1986), and the isolation of *Azospirillum* (Hernández *et al.* 2000) as vital dye and roots for describing fungal tissues (Guzmán *et al.* 1999). It is necessary to assess the amount of glucose produced by the endoglucanases from CMC. Endoglucanases are produced by fungus in the first incubation. In the second incubation, no fungal biomass was used to avoid incorporating the glucose generated (Brasil *et al.* 2004).

The species of cellulolytic fungi most frequently studied belong to the genus *Trichoderma* and have been found to be the best producers of cellulases. However, other genera or species of fungi have been studied, including *Aspergillus* (Bastawde 1992), *Cladosporium* (Abrha and Gashe 1992), *Penicillium* (Keskar 1992), and *Neurospora crassa* (Yazdi *et al.* 1990). Edible fungi including *Lentinula edodes*, straw mushroom, and *Pleurotus* sp. (Poutou *et al.* 1994; Buswell *et al.* 1996) also produce cellulases. The fungi are characterized by the rapid colonization of substrates, enzyme secretion into the medium, the efficient removal of hydrolysis products, and the diversity of their cellulolytic systems. A variety of studies on fungi have been conducted to better understand the process of the enzymatic hydrolysis of cellulose and to use it for industrial purposes. The endoglucanases from fungus have been recognized as being potentially important. The objectives of this study were to isolate fungi from an aerated lagoon and evaluate their enzymatic activity (endoglucanase).

EXPERIMENTAL

Isolation of Fungi in the Aerated Lagoon

The microorganism used in this study was obtained from the Bio-PAPPEL SAB CV industry; the culture was maintained on dry sand under freezing conditions (-18 °C). Microorganism activation was carried out using basic agar medium slants incubated for seven days at 32 °C, which had been isolated from an aerated lagoon (Ordaz-Díaz *et al.* 2014, 2016). The samples were collected and transported in sterile glass bottles in coolers for preservation. They were planted in sterile, triplicate plates containing agar PDA (Potato dextrose), then incubated at a suitable temperature of 28 °C, and were evaluated periodically to monitor the growth of microorganisms in the environment. The result was a diverse microflora, which was evaluated and analyzed under a hand lens.

Morphological Characterization

Selection criteria were used for morphological characterization. As a basic procedure, one can visibly differentiate bacteria based on the appearance of their colonies. Next, description of a colony's morphology includes its shape, the margins or edges of the colony, its color, and surface features. Some colonies are round and smooth; others can have wavy edges and a wrinkled appearance. These were characterized by being very well-defined and identified for each member of this research, making for an objective analysis of the results.

Qualitative Endoglucanase Activity of Fungi in CMC

The amount of glucose produced by the endoglucanases from CMC was evaluated. Endoglucanases are produced by the fungus in the first incubation. In the second

incubation, no fungal biomass was used to avoid incorporating the glucose generated. One milliliter of sample was taken and serial dilutions of 10^{-1} to 10^{-5} in 0.1% (w/v) nutrient broth were performed. Fungi were maintained on PDA agar plates for five days until sporulation. A sterile solution with spores was harvested with a surfactant, Tween® 0.1%, and a suspension with 106 spores/mL was obtained. This was followed by growth in 1% CMC agar (w/v), incubated at 28 °C for 48 h. After the incubation time, Congo red 1% (w/v) was added, and after 15 min the excess was removed and 0.1 M NaCl was added for 15 min. Fungal strains were identified by microscopic observation (mounting tape) (Theather and Wood 1982) and morphological keys (Domsch *et al.* 1980; Barnett and Barry 1998). The macroscopic study was used to evaluate the color of the colony, topography, texture, *etc.* Microscopic studies were carried out to evaluate the type of hyphae, conidia, *etc.* (Usnayo 2007). In each case, the growth of the colony and degradation halo around it at 116 h was measured. Halo intensity values were categorized from 1 to 3, with 1 being a weakly visible halo and 3 being a visible halo, to give an idea of the intensity of the endoglucanase activity of the specific substrate (Pedroza *et al.* 2007). The cellulolytic isolates were stored at -20 °C in 25% glycerol v/v under a procedure described by Poutou *et al.* (1994).

Quantitative Determination of Endoglucanase Activity in the Fungi

To determine endoglucanase activity, CMC was used as a substrate in 50 mL of 0.8% CMC in a citric acid buffer at pH = 5.0, with 50 µL of supernatant from each microorganism. Both reactions were incubated at 40 °C for 1 h and were stopped at 4 °C for 10 min. Then, 50 mL of this solution was mixed with 50 µL of a solution of 1% DNS (3,5 dinitrosalicylic);16% NaOH and 43.8% sodium potassium tartrate in distilled water for the determination of reducing sugars were released. The mixture was incubated for 5 min at 90 °C and then cooled for 10 min at 4 °C. Then the mixture was added to 50 mL of the above solution with 250 mL of distilled water. Absorbance readings were taken at 540 nm, with one unit of enzyme activity (IU) corresponding to 1 mol of glucose released per 123 mg of enzyme per minute during hydrolysis (Adrado *et al.* 2005).

RESULTS AND DISCUSSION

The strains grown on the plates are shown in Fig. 1, and the results of the microscopic observation and morphological keys are shown in Table 1.

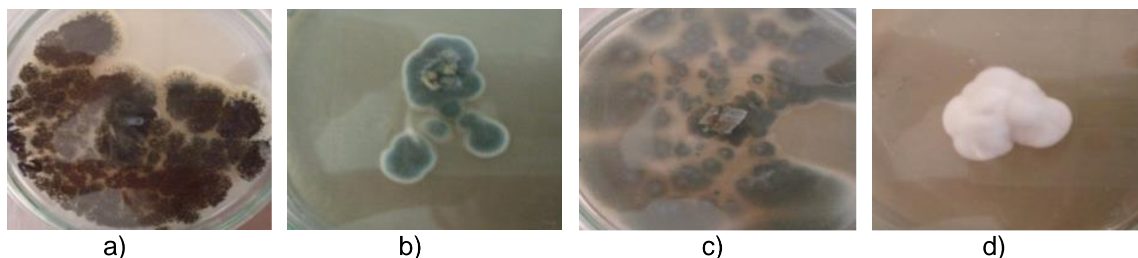

Fig. 1. a) *A. niger*, b) *Penicillium sp.*, c) *A. fumigatus*, and d) *Mucor* sp.
The following fungi were identified: *Aspergillus niger*; *Penicillium* sp.; *Aspergillus fumigatus*; and *Mucor* sp. They are fungi that colonize and exploit the paper supports, in addition to having cellulolytic capacities.

Table 1. Morphological Identification of Isolated Fungi

Identification	Size	Color	Form	Strain
ITDOD-0001	Unlimited (covers all medium)	Yellow-white and then black	Purulent unpigmented	*Aspergillus niger*
ITDOD-0002	Unlimited (covers all medium)	Green, white halo on the periphery	Velvety-powdery flat	*Penicillium sp.*
ITDOD-0003	Unlimited (covers all medium)	Green, white mycelial halo (sometimes pink)	Dry flat velvety	*Aspergillus fumigatus*
ITDOD-0004	Unlimited (covers all medium)	White 3 days after white-gray	Cottony fluff-dry	*Mucor sp.*

Aspergillus niger is present in papermaking effluent and helps with the removal of color and COD, together with bacteria (Tezel *et al.* 2001). *Penicillium* sp.; *Cladosporium* sp., and *Scopulariopsis* sp. are fungi that colonize and exploit paper supports and have acceptable cellulolytic capacity, especially if secondary fiber (recycled paper or cardboard) is used in the manufacture of the paper. Among the fungi's increased production of *T. reesei* cellulases are several species of *Penicillium* and *Aspergillus*, whose activity has been evaluated in different substrates as wheat chaff and bagasse (Guevara and Zambrano 2006) and palm tusa oil (Rodríguez *et al.* 2007). The use of dilutions in the plate is one of the most common sporulation methods used to isolate a large number of fungi (Mueller *et al.* 2005). In a previous study (Kumari *et al.* 2011), isolated ground *Aspergillus fumigatus* was shown to produce cellulase and optimize production based on physicochemical parameters.

Determining the growth and hydrolysis based on the halo allows the visual differentiation of organisms that use glucose, introducing discoloration in areas due to the breakdown of β 1-4 bonds of cellulose. According to the qualitative tests of the cellulolytic activity performed, the zone diameters degradation of the four fungal isolates ranged from 0.4 to 1.3 cm, as shown in Table 2.

Table 2. Qualitative Test Endoglucanase Activity in Fungi

Strain	Growing Period	Halo Diameter Hydrolysis (cm)	Intensity Halo *
Aspergillus niger	48 h	0.7 ± 0.13	2.5
Penicillium sp.	48 h	1.3 ± 0.05	3
Aspergillus fumigatus	48 h	0.5 ± 0.11	2
Mucor sp.	48 h	0.4 ± 0.14	1

* 1 = halo faintly visible, 2 = visible halo, 3 = very visible halo
* Average of three replicates ± S. D.

Four degrading fungi were applied to endoglucanase semi-quantitative tests. The results showed that all fungi exhibited cellulolytic activity, revealing a clearance halo (Valencia *et al.* 2007). The fungus with the highest hydrolysis halo diameter was

Penicillium sp., followed by *A. niger*, while the less active fungus was *Mucor* sp. The ability of strains of *Penicillium* sp. to produce cellulases indicates that this strain has great potential for the production of the enzyme for biotechnological applications. Cardona *et al.* (2009) obtained degradation halos similar degrading fungi to values between 1.05 and 3.11 for *P. chrysosporium* and *A. discolor* expressing at least one of the lignolytic enzymes. It has also been shown that *Penicillium citrinum* has the ability to produce thermostable cellulases and is tolerant to alkaline conditions, which indicates its great potential in the industry (Dutta *et al.* 2008).

The participation of microorganisms in paper bio-deterioration processes is given in terms of the chemical composition of the material (fibrous and non-fibrous) and the effectiveness of microorganism multi-enzyme systems for use in various nutritional and environmental conditions. Comparing the results of the qualitative tests with quantitative evidence highlights the fact that some fungi, which are the best lightening on board, are not the best lightening in liquid medium. A test was conducted to compare the endoglucanase activity in the four cellulolytic fungal isolates obtained from the lagoon; control experimental condition is the same as in fungi (Fig. 2).

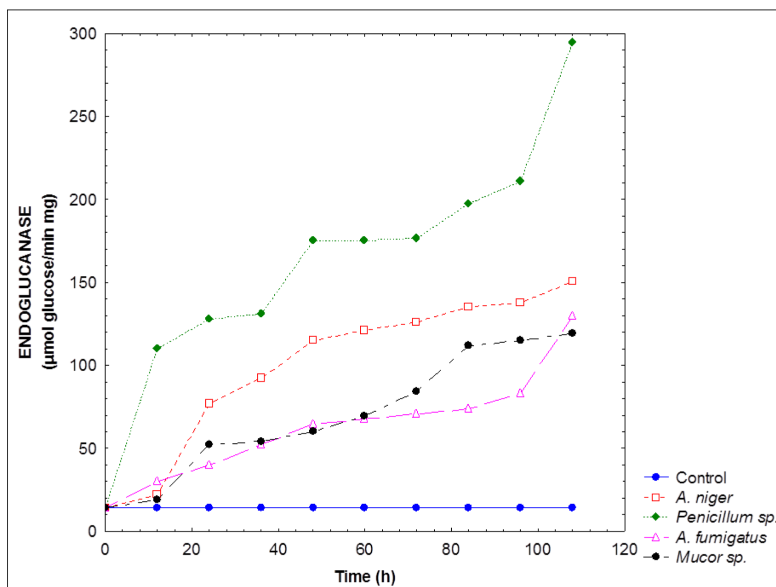

Fig 2. Specific endoglucanase activity in selected fungi

The ability to produce and release enzymes in suitable complex proportions is an important factor for cellulolytic capacity in some fungi. If the enzyme complex is deficient in the amount or activity of any of the three types of enzyme, the result can be a hydrolysis of cellulose or deficient rapid hydrolysis accompanied by the accumulation of byproducts that may be inhibitors after a certain concentration. *Tanaka et al.* (2005) and Tauk *et al.* (2009) reported the ability of *P. citrinum* to produce xylases, denoting their biotechnological potential in the food and pulp and paper industries, among others. Dutta *et al.* (2008) reported the fungus *Penicillium citrinum* to be capable of producing endoglucanase enzymes in stable high temperatures, pH, and alkali metal ion concentrations. In their study, Aguiar (2001) evaluated enzyme production by *Aspergillus niger* IZ 9 in different carbon sources showing the influence of temperature, substrate type, and concentration enzyme. Omosajola and Jilani (2008) studied the production of

cellulases from *Trichoderma longibrachiatum*, *Aspergillus niger*, and *Saccharomyces cerevisiae* and compared cellulase activity and the amount of glucose produced. *T. longibrachiatum* was found to produce higher amounts of glucose.

Soni *et al.* (2010) evaluated *Aspergillus fumigatus* and obtained an endoglucanase activity of 240 U/g substrate endoglucanase. Camassola and Dillon (2007) evaluated *Penicillium echinulatum* and obtained 32.9 U/dm/g filter paper. Usama (2008) evaluated *Aspergillus niger* and obtained 0.9 U/mg CMCase. Sohail *et al.* (2009) evaluated *Aspergillus niger* MS82 and obtained 0.3 U/mL endoglucanase. In their studies, Howard *et al.* (2003) compared the specific enzyme activity at pH = 5 for *Aspergillus niger* 194 mmol/min.mg, comparable to the 150 mmol/min.mg obtained in this investigation, and also for the *Penicillium brefeldianum* 405 mmol/min.mg, compared with 300 mmol/min.mg obtained for *Penicillium sp*.

The fungus *A. niger* had the highest enzyme activity. Furthermore, in cultures plated on the CMC sample, it was remarkable for the structures formed. However, between 96 and 116 h, a stabilization of activity was observed. Additionally, according to the observations, the fungus did not consume all the cellulose during cultivation. However, according to statistical analysis (Kruskal Wallis), significant differences ($P = 0.0001$) were demonstrated in enzyme activity during the 116 h of study using the technique of Somogyi Nelson. However, between these treatments they did not show significant differences ($P = 0.3536$). It is possible that *A. niger* did not reach the stage of maturity at which it synthesized and released into the different proteins to cellulolytic enzymes, which would decrease its calculated relative activity. To better understand the behavior of the fungus, it is necessary to cultivate it for a longer period of time to check whether the relative activity continues to increase and is effectively degrading cellulose. These data indicate that this strain is highly promising for the degradation of cellulose, though it is not a recommended consortium when mixed with the strain *Penicillium sp*., which decreased its cellulolytic activity. This suggests that these microorganisms, as well as cellulose and other compounds can degrade as gum arabic used as stabilizer, which upon hydrolysis produces arabinose, galactose, rhamnose, and glucuronic acid, which can be used as sources of carbon and energy.

CONCLUSIONS

1. Four fungi were identified as producers of endoglucanase based on their decolorizing ability: *Penicillium* sp., *Aspergillus niger*, *Aspergillus fumigatus*, and *Mucor* sp.

2. The fungus *Penicillium* sp. showed the highest qualitative endoglucanase activity (1.3 cm) in addition to the main activity of endoglucanase with 297 mmol/mg.min after 116 h.

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