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Production of endo-1,4-β-D-glucanase by *Curvularia pallescens*

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Abstract

Studies have been performed on the production and thermostability of endo-1, 4-β-D-glucanase enzyme produced by *Curvularia pallescens*. The strain of the microorganism was grown on a mineral medium suitable for cellulolytic fungi. Different cellulose concentrations, carbon sources, solid ammonium sulfate saturations, time and temperatures were used in order to determine the best conditions for cellulase production by this species. It was observed that it is capable of producing extracelular endoglucanase with a specific activity of 0.474 UI/mg. Protein was grown on the optimized liquid medium with microcrystalline cellulose as carbon source/enzyme inducer and the enzyme is stable at 45–60°C/20 min. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The biomass that is produced as industrial, urban and agricultural waste every year represents a great loss of energy resources. The use of both high and low molecular products from cellulosic materials can be one of the most viable alternatives to supply the increasing demands for food, fuel and chemical production. Cellulose may also be re-built into a number of products via enzyme hydrolysis and fermentation of the product glucose.

The complete enzyme degradation of cellulosic substrates relies upon the production of a complex system of cellulolytic enzymes. This is accomplished by three different types of cellulases: endoglucanase (1, 4-β-D-glucan 4 glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1, 4-β-D-glucan glucohydrolase; EC 3.2.1.74), and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21). These enzymes have different specificities and modes of action and interact synergistically to solubilize crystalline cellulose (Wood and McCrae, 1979; Markhan and Basin, 1991).

In the culture collection of the Mycology Department/UFPE there are many samples of Hyphomycetes (Deuteromycotina) with biotechnological characteristics still not studied. Among the fungi, the genus *Curvularia* is of interest since there is the possibility of its use as a biodegrading agent. This genus has many saprophytes and a high number of plant pathogenic species which occur mainly in tropical and subtropical areas. These species are found on a wide variety of substrates such as soil, organic matter and hosts such as plants, animals and even on man (Domsch et al., 1980; Travis et al., 1991).

Some species of *Curvularia* have been characterized as producers of cellulose degrading enzymes. The properties of cellulases produced by other species of *Curvularia* as well as the factors that affect their production have been shown by many authors (Jyoti and Sing, 1985; Tan et al., 1985; Pramod et al., 1989; Banerjee, 1990; Nitharwal et al., 1991; Banerjee and Chakrabarti, 1992). This paper presents results regarding production and thermostability of the endo-1, 4-β-D-glucanase produced by *Curvularia pallescens*.

2. Materials and methods

2.1. Organisms

Cultures of *C. pallescens* (3296); *C. prasadii* (3274); *C. brachyspora* (3297); *C. clavata* (3273); *C. tuberculata* (2354); *C. lunata* (2432); *C. eragrotydis* (2376), isolated from cellulosic substrates were maintained under mineral

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oil or lyophylized at the culture collection of the Department of Mycology of the Federal University of Pernambuco (Micoteca URM).

2.2. Screening

Disks (10 mm dia.) of 10-day old mycelia of *C. pallescens*, grown on potato dextrose agar (PDA) and originating from monosporic cultures, were transferred to Petri dishes containing a mineral medium for cellulolytic fungi (MMC) comprising ($g\,l^{-1}$): 7.0 KH_2PO_4; 2.0 K_2HPO_4; 0.1 $MgSO_4{\cdot}7H_20$; 1.0 $(NH_4)_2SO_4$; 0.6 yeast extract; 10 microcrystalline cellulose; 15 agar; 1 l of distilled water. The pH was adjusted to 5.5 with HCl 1 M. Colony diameters were measured after 6 days. The sample with better growth and higher degradation ability was chosen for the experiments. Three replicates were used for each experiment and the culture conditions were held at 25°–28°C and 12 h day/night lighting.

2.3. Growth on MMC with different cellulose concentrations

Disks of an 8-day old mycelium of *C. pallescens* were transferred to MMC with different concentrations of microcrystalline cellulose: 10, 20, 50, 100 g/l. After 6 days the diameter of colonies was measured in order to select the best cellulose concentration for the growth medium.

2.4. Effect of carbon source on endoglucanase production

Innocula (10 ml suspension/10^6 conidia/ml) were transferred to 1 l Erlenmyer flasks with 200 ml of MMC and incubated for 15 days on a rotary shaker (150 rpm) without pH control. Fractions of 5 ml were then centrifuged (12100***g***, 4°C, 10 min) and the pH, protein and substrate levels and enzymatic activity were determined. Microcrystalline cellulose and glucose were used as carbon sources (10 g/l).

2.5. Protein fractioning

Cellular extracts of *C. pallescens* were centrifuged and the supernatant was treated with solid ammonium sulfate to give 20, 40, 60 and 80% saturation (Vilela et al., 1973) to precipitate out the enzyme protein. After precipitation, samples were filtered and dialysed overnight against sodium citrate buffer 0.05 M, pH 4.8.

2.5.1. Optimum time and temperature of hydrolysis

In order to determined the best time (10, 15, 20, 35 and 40 min), and temperature (30, 40, 50, 60, 65 and 70°C) for enzyme activity, systems using 0.5 mol of carboxymethlcellulose (CMC) at 1% plus 0.5 ml of crude extract of *C. pallescens* grown on MMC were prepared. For each assay the enzymatic activity was tested immediately.

2.5.2. Enzyme assay

Endoglucanase activity was assayed by following the release of reducing sugars estimated by the dinitrosalicylic acid method (Miller, 1959) using glucose as the standard. The culture filtrates were diluted with 0.05 M sodium citrate buffer, pH 4.8. The reaction mixture containing 0.5 ml of rehydrated culture filtrates and 0.5 ml of 1% CMC (w/v) solution was boiled for 5 min. One unit of CMCase is defined as the amount of enzyme that produces 1 μmol of glucose from the standard assay conditions and is expressed as UI/l of medium. Cellulase specific activity (CU) was expressed as μmol glucose mg^{-1} protein h^{-1}, released under conditions of pH 4.8 and 50°C. The protein concentration of culture filtrates was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

3. Results

Among the tested cultures, *C. clavata*, *C. tuberculata*, *C. lunata* and *C. brachyspora* were not able to develop well on cellulosic media while *C. prasadii* did not grow under the cultural conditions established in this assay (MMC containing microcrystalline cellulose as carbon source, pH 5.5). Cultures of the species *C. pallescens* and *C. eragrotides* developed large colonies on MMC. However, microscopic observations showed that the growth of *C. eragrotides* was mainly vegetative, while the development of *C. pallescens*

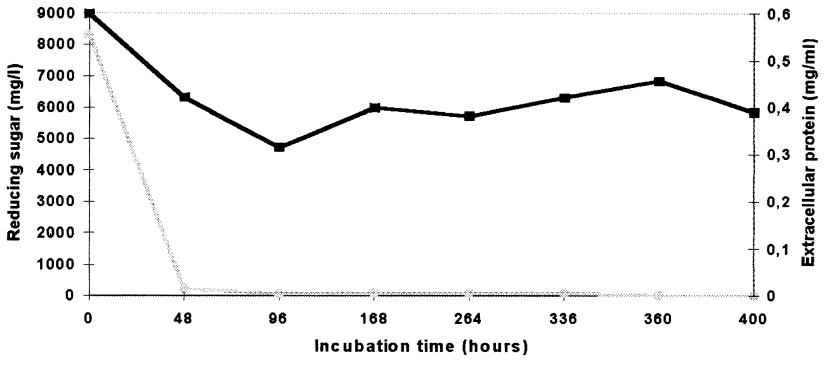

Fig. 1. Amount of reducing sugar ○ and production of extracellular protein ■ in the culture filtrate of *Curvularia pallescens* grown in glucose.

was complete, with production of extensive mycelium and high number of spores. Regarding these results, *C. pallescens* (URM 3296) was the strain chosen to be studied.

On investigation of the possible effects of induction and repression of cellulase synthesis by the strain *C. pallescens*, the effect of the initial concentration of cellulose was observed after 6 days of incubation. The results showed that when the amount of cellulose was increased (from 10 to 100 g/l), the size of the colonies formed by the strain was smaller, with diameters of 8.7, 6.2, 4.3 and 4.0 cm, respectively, at 10, 20, 50, and 100 g/l of cellulose. Extracellular protein (0.456 mg/ml) was obtained at the 15th day (360 h) of fermentation (Fig. 1).

However, residual glucose in the medium decreased to 3% of the original concentration within 48 h of incubation. This was associated with a fast decrease of pH, from 6.0 to 2.8 (Fig. 2). No production of carboxymethylcellulase was detected during this experiment.

In contrast to the rapid growth on the glucose assay, *C. pallescens* grew slowly on cellulose media with lower pH variation from 6.0 to 4.0 (Fig. 2). The highest protein content (0.392 mg/ml) was obtained after the 15th day (360 h) of fermentation (Fig. 3) and maximum endoglucanase activity (101.2 UI/l) on the 14th day (336 h), but the highest specific activity (0.472 U/mg protein) on the 11th day (264 h) of fermentation (Fig. 3).

During fractionation studies with sulphate ammonium, the highest protein content (0.132 mg/ml) and highest specific activity (0.044 U/mg protein) were obtained with 20% of ammonium salt.

A temperature of hydrolysis of 70° for 20 min reduced endoglucanase activity by 65%, compared with the activity at 45°C (Fig. 4). An activity of 180.7 UI/l at 45°C changed to 59.2 UI/l when the system was at 70°C. The endoglucanase produced by *C. pallescens* was stable when the temperature was maintained between 45 and 60°C/20 min.

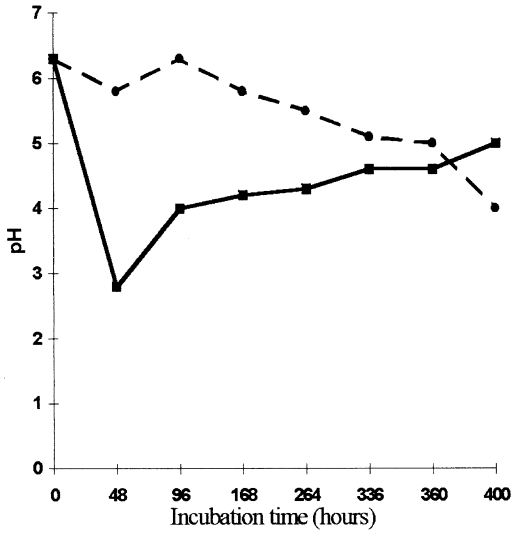

Fig. 2. Changes in pH of the culture filtrate of *C. pallescens* grown in cellulose ● and glucose ■ (25–28°C).

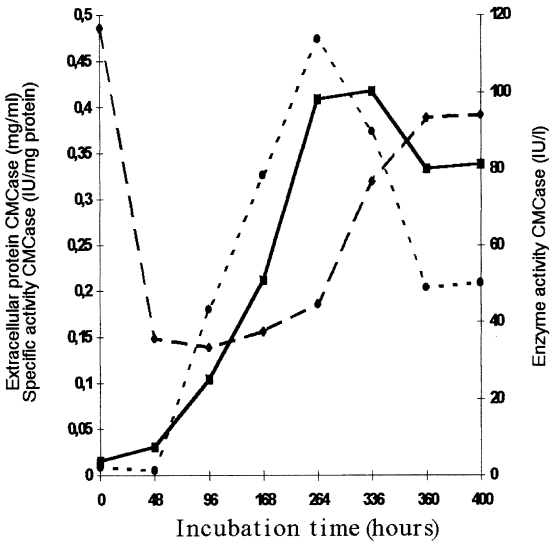

Fig. 3. Specific activity CMCase (–●–), extracellular protein (— —●— —) and CMCase activity (—■—) of *C. pallescens* grown in cellulosic mdedia (25–28°C).

(Fig. 4). High endoglucanase activity (160.7 UI/l) was obtained when the reaction system was hydrolyzed at 50°C for 20 min, but when the system was incubated during a 40 min period, there was a decrease of 35% on enzyme activity (Fig. 5).

4. Discussion

Most of the strains of *Curvularia* used in this study did not develop well on cellulosic medium, which may indicate they were not able to produce cellulolytic enzymes to allow growth. *C. prasadii* did not develop under the cultural conditions of this assay. Cellulosic activity of this fungus, if it does exist, was not expressed probably because it was maintained on mineral oil which may have produced metabolic alterations in the strain.

The strain of *C. pallescens* was the only one capable of

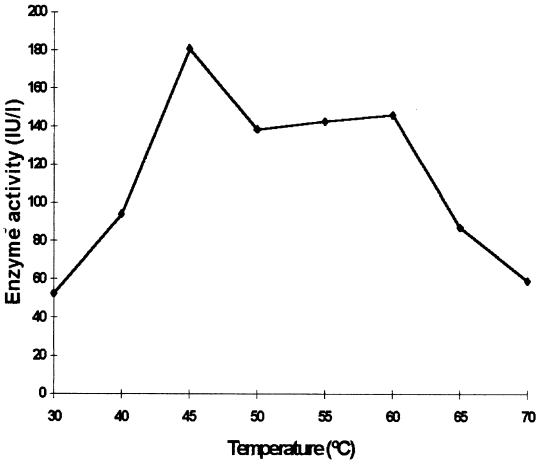

Fig. 4. Stability of CMCase activity of *C. pallescens* for 20 min, at different temperatures.

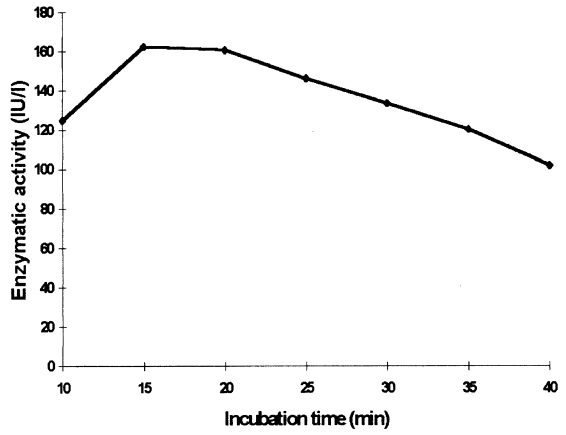

Fig. 5. Stability of CMCase activity of *C. pallescens* at different hydrolysis times.

normal vegetative and reproductive development. However, it was observed that inhibition of growth of this strain occurred when levels of cellulose were increased above 10 g/l. The inhibition probably occurred because of glucose accumulation on the medium, inducing a catabolic repression. Repression of cellulase synthesis was observed with *Trichoderma viride* after adding glucose to the medium. This confirms the repression effect of carbon sources that are easily metabolized (Sanyal et al., 1988).

When a liquid medium containing glucose as carbon source was used, maximum content of extracellular protein was obtained at the 15th day of fermentation, while glucose was exhausted after 48 h of incubation. When the cellulosic medium was employed, the strain of *C. pallescens* showed slow growth and highest protein content was obtained after the 15th day of fermentation.

No detection of carboxymethylcellulase during the experiment indicates that the glucose consumed by the strain was used for growth promotion. In the presence of rapidly metabolized carbon sources, such as glucose, the cellulase complex of enzymes is repressed. This reaction may be due to a rapid decrease of the pH level (Maninder et al., 1984; Eriksson, 1979). During studies of the production of endo-glucanase by *C. lunata* on dextrose, yeast extract and peptone media, Banerjee and Chakrabarti (1992) observed activity of 6.71 UI/l after the 4th day of growth. These results show the constitutive characteristic of some cellulases produced by fungi. However, the results obtained under the conditions of this assay with *C. pallescens*, growing on medium with glucose, indicated that the endo-glucanase produced by this strain is regulated by an induction mechanism.

The high levels of protein at time 0 resulted from the yeast extract added to the MMC medium. Values of endo-glucanase activity obtained in this assay were higher than those produced by *Aspergillus niger* (74 U/l). However, they were low compared with fungi such as *Trichoderma koningii*, *Lenzites troba* and *Myrothecium verrucaria*, which produced, respectively 700, 800 and 4375 U/l (Herr et al., 1978). It could be considered that the conditions used on different assays make it difficult to compare our data with those of other authors. One hypothesis that can be raised is that *C. pallescens* may increase its enzyme production once more suitable growth conditions occur. Further studies are necessary to determine the growth conditions that allow *C. pallescens* to express its full capacity for cellulose degradation.

Although considered a mesophylic fungus, the endo-glucanase produced by *C. pallescens* was more stable at temperatures between 45 and 60°C/20 min. Similar results were also observed with *Trichoderma harzianum* (Wojtezak et al., 1987).

As shown, cellulase production by *C. pallescens* occurred when the fungus was grown on medium with micro-crystalline cellulose. However, this production can be increased and other studies should find the optimal cultural conditions for *C. pallescens* to become a high cellulase producer.

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References

Banerjee, U. C. (1990). Production of beta-glucosidase (cellobiase) by *Curvularia*. sp. *Lett. Appl. Microbiol., 10,* 197–199.

Banerjee, U. C., & Chakrabarti, S. (1992). Production and properties of carboxymethylcellulase (endo-1, 4-β-glucanse) from *Curvularia lunata. World J. Microbiol. Biotech., 8,* 423–424.

Domsch, K. H., Gams, W., & Anderson, T. H. (1980). *Compendium of soil fungi. I* (p. 859). San Francisco, CA: Academic Press, 1980.

Eriksson, K. E. (1979). Biosynthesis of polysaccharases. In R. C. W. Berkeley, G. W. Gooday & D. C. Ellwood (Eds.), *Microbiol polysaccharids and polysaccharases* (pp. 285–293). New York: Academic Press.

Herr, D., Luck, G., & Dellweg, H. (1978). Formation of cellulases and degradation of cellulose by several fungi. *J. Ferment. Technol., 56,* 273–278.

Jyoti, S., & Sing, R. P. (1985). Temperature relation and cellulolytic activity of weed fungi occurring in the beds of *Pleurotus sajor caju*. *Ind. J. Plant Pathol, 3,* 7–17.

Lowry, O. H., Rosenbrough, N. V., Farr, R. V., & Randall, R. V. J. (1951). Protein measured with folin phenol reagent. *J. Biol. Chem., 193,* 265.

Maninder, K. K., Mohinderjit, S. S., Dhanwant, K. S., & Rajindar, S. S. (1984). Production and regulation of cellulases in *Trichoderma harzianum*. *Appl. Microbiol. Biotechnol., 20,* 427–429.

Markhan, P., & Basin, J. M. (1991). Decomposition of cellulose by fungi. In D. K. Arora, B. Rai & K. G. Mukergi (Eds.), *Handbook of applied mycology* (Vol. 1, pp. 379–424). New York: Marcel Dekker.

Miller, G. L. (1959). Use of denitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem., 31,* 426–428.

Nitharwal, P. D., Gour, H. N., & Agarwal, S. (1991). Effects of different factors on the production of cellulase by *Curvularia lunata*. *Folia Microbiol., 36,* 357–361.

Pramod, K., Mishra, U. S., & Kumar, P. (1989). Production of cellulolytic (Cl) enzyme by fungi causing serious diseases of sesame. *Adv. Plant Sci., 2*, 291–294.

Sanyal, A., Kundu, R. K., Sinha, S. N., & Dube, D. K. (1988). Extracellular cellulolytic enzyme system of *Aspergillius japonicus*, 1. Effect of different carbon sources. *Enz. Microbiol Technol., 10*, 2.

Tan, T. K., Yeoh, H. H., & Tian, K. E. (1985). Celluloytic fungi isolated from wood shavings. *Mycopathologia, 90*, 97–99.

Travis, W. D., Kwon-Ghung, K. J., Kleiner, D. E., Geber, A., Lawson, W., Pass, H. I., & Henderson, D. (1991). Unusual aspects of allergic bronchopulmonary fungal disease. Report of two cases due to *Curvularia organisms* associated with allergic fungal sinusitis. *Human Pathol., 22*, 1240–1248.

Vilela, G. G., Bacila, M., & Tastaldi, H. (1973). *Técnicas e experimentos de bioquímica*. Rio de Janeiro: Guanabara-Koogan.

Wojtezak, G., Breuil, C., Yamada, J., & Saddler, J. N. (1987). A comparison of the thermostability of cellulases from various thermophilic fungi. *Appl. Microbiol. Biotechnol., 27*, 82–87.

Wood, T. M., & McCrae, S. I. (1979). Synergism between enzymes involved in the solubilization of native cellulose. *Adv. Chem. Ser., 181*, 181–209.