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Effect of Different Carbon Sources on Endochitinase Production by *Colletotrichum gloeosporioides*

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Abstract. The present work analyzes the production of endochitinase by *Colletotrichum gloeosporioides*, a phytopathogenic fungus, using six different carbon sources and two pH values. For quantitative assay of endochitinase activity in solution, the synthetic substrate 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside was used. The major productions were obtained at pH 7.0 and 9.0, when colloidal chitin and glucose were used, whereas xylose and lactose were not good carbon sources. When testing different concentrations of colloidal chitin, glucose and glucosamine, colloidal chitin 0.5% was the best substrate, giving values of 2.4 U at the fifth day. When using glucose, best production occurred at 0.3% concentration, after 5 days growth, with values of 1.31 U. Endochitinase production was markedly decreased in high levels of glucose and in all glucosamine concentrations tested. SDS-PAGE co-polymerized with glycol-chitin analysis showed three major activity bands of 200, 100, and 95 kDa, when incubated at 50°C.

Chitin, a linear polymer of β-1-4-N-acetylglucosamine, is one of the most abundant substances of biological origin found in nature, being a primary constituent of fungi cell walls and shells of crustaceans and insects [5, 26]. All organisms that have chitin in their composition produce chitinases [13, 14] and many microorganisms can synthesize these enzymes, including bacteria [9, 19], fungi [6, 21], and protozoa [16]. Some microorganisms produce chitinases to degrade the polymer and use them as nutrient [10, 30]. Other organisms produce these enzymes to protect themselves against microorganisms that have chitin in their composition [11]. Some plants also produce chitinases, probably in response to infections by microorganisms containing chitin [1]. Consequently, chitinases are of enormous importance to the biosphere, performing an important physiological and ecological role in ecosystems [5].

Chitin degradation is performed by a two-enzyme system that consists of endochitinase (EC 3.2.1.14) and exochitinase (EC 3.2.1.52). The degradation occurs in two consecutive steps: first the hydrolysis by endochitinase to oligomers (mainly dimmers) followed by their degradation to free N-acetyl-glucosamine by exochitinase [29]. Finally, some chitinases are also described as having proteolytic activity [28].

Colletotrichum gloeosporioides is a phytopatogenic fungus that affects many cultures of commercial importance in agriculture. It has been observed causing anthracnose in malva, avocado, strawberry, soybean, north joinvetch, and passion fruit [27]. In passion fruit cultures, *C. gloeosporioides* infects primarily leafs, and then flowers and fruits, sometimes leading to death [15, 17, 18, 20, 22, 37]. *C. gloeosporioides* was used as a commercial mycoherbicide to control north joinvetch in rice and soybean fields from 1982 to 1992 [3]. Many factors are suggested to be involved in biological control. Enzymes such as chitinases may play an important role on the control of pathogenic organisms that contain chitin as the major component [21, 10, 12]. Previously, we reported, for the first time, the production of endochitinase and exochitinase by *Colletotrichum gloeosporioides* when grown in the presence of chitin as the sole carbon

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source [32]. Then, a 45-kDa endochitinase has been purified and characterized, showing significant biochemical properties, useful in biotechnological applications. Although considerable levels of endochitinase have been obtained in the presence of chitin, regulation of its synthesis remains obscure.

Considering the importance of fungal chitinases, the aim of the present work is to analyze the production of endochitinases by *C. gloeosporioides* using different carbon and nitrogen sources.

Material and Methods

Strain and culture conditions. The fungus *Colletotrichum gloeosporioides* Cg-10 was maintained on yeast extract-malt extract agar plates, containing (g/L) 10 malt extract; 4 yeast extract; 4 glucose, and 13 agar [31]. Plates were incubated at 28°C for 5 days and then stored at 4°C. Every 15 days, new plates were inoculated. Experimental media were inoculated with standard inoculum containing 1.0×10^7 conidia/mL, counted in Neubauer chamber, harvested from 5-day-old yeast extract-malt extract agar plates, centrifuged at 3,000 *g* and ressuspended in 0.85% (wt/vol) saline [39, 41].

Production of endochitinases in different carbon and nitrogen sources. Initially production of endochitinases was carried out at pH values of 5.0 and 7.0, in 250-mL Erlenmeyer flasks containing 25 mL of a minimal medium comprising (g/L): 2 $NaNO_3$; 1 K_2HPO_4; 0.5 $MgSO_4 \cdot 7H_2O$; 0.5 KCl; 0.01 $FeSO_4 \cdot 7H_2O$ [2]. The effect of the different carbon sources was evaluated in this medium supplemented with the tested substrates, including 0.5% (wt/vol) glucose, 0.5% sucrose, 0.3% xylose, 0.5% glucosamine, 0.3% lactose and 1% colloidal chitin. When using 1% colloidal chitin as carbon source, other 0.2% (wt/vol) nitrogen sources besides $NaNO_3$ were also tested: yeast extract, glutamic acid, urea, tryptose, bacto casitone, ammonium sulfate and casaminoacids. After inoculation, flasks were incubated at 28°C, on a rotatory shaker at 180 rpm, for 5 days. At each day, flasks were collected in triplicates, their whole content being filtered onto Whatman filter paper n° 1 and centrifuged at 10,000 g. Supernatants were stored at −4° C until use.

In another set of experiments, the inductive effect of colloidal chitin, as well as the repressor effect of glucose and glucosamine on chitinase production were evaluated. The fungus was inoculated in 50 mL erlenmeyer flasks, containing 10 mL of the same medium, with different concentrations of these carbon sources, followed by the same procedure as above.

Enzyme assay. Endochitinase and exochitinase activity were determined by estimating the amount of methylumbelliferone released when enzyme preparations were incubated at 37°C with 4-methylumbelliferyl-β-D-N,N',N"-triacetylchitotrioside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively. Reaction mixtures, containing 150 μL of supernatants / 5 μL of 50 μM substrate / 145 μL of Tris-HCl buffer pH 7.4, were read in a Fluoroskan multiplates auto reader (Fluoroskan II version 6.3, excitation 355 nm, and emission 460 nm), using methylumbelliferone as standard. Experiments were performed in intervals of five minutes, during one hour, and the results are mean of triplicates of the best incubation time for endochitinase activity [23, 25, 35]. One unit of enzyme activity (U) was defined as the amount of enzyme able to release one μmol of methylumbelliferone per minute under standard assay condition. Protein concentration was determined using the method of Bradford [4].

Chitinase activity in gel. Supernatants exhibiting endochitinase activity were concentrated by ultrafiltration in AMICON system (cut-off 10,000) and applied at the same protein concentration on SDS-PAGE 10% using 0.01% glycol-chitin as substrate. After electrophoresis, gels were incubated with Triton X-100 sodium acetate 1% buffer for 1 h with shaking, and then incubated with 50 mM Tris-HCl buffer pH 7.5 for 18 h at 30°, 50°, and 90°C. The gels were then stained with 0.01% (wt/vol) Calcofluor white M2R in 0.5 M Tris-HCl, pH 8.9, and the resulting lytic zones visualized fluorometrically on UV transillumination [36].

Results and Discussion

A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce chitinolytic enzymes when chitin is present in the growth medium [5]. The production of chitinases by microorganisms is thought to be controlled by a repressor-inducer system in which chitin or products of degradation (oligomers) serve as an inducer [24]. More recently, Tsujibo and co-workers [38] showed that the production of an extracellular chitinase of *Streptomyces lividans* is regulated by a two-component sensor-regulator system. Usually, the production of chitinases is reduced when glucose, a known catabolic repressor not only for chitinases but for many other inducible enzymes, is present in the medium.

To verify the importance of chitin and some other carbohydrates on endochitinase production in *C. gloeosporioides*, an experimental system was developed, in which different carbon sources were used. As shown in Figure 1, synthesis of endochitinase was influenced by the carbon source. Although endochitinase production by *C. gloeosporioides* has been detected in every carbon source and pH tested, the major production, around 1.0 U, was observed when using colloidal chitin or glucose, at pH 7.0, after the 4th and 5th day. At pH 5.0, enzymatic activity was markedly decreased (Fig. 1). In our previous experiments, *C. gloesporioides* produced a maximum of 0.5 U after 4 days incubation in Czapek medium at 28°C [23], whereas Tikhonov and co-workers [34] observed much lower levels (1.66×10^{-3} U) produced by two species of *Verticillium*, being the major activities detected only after the 18th day. Although much more endochitinase production has been observed for *C. gloeosporioides*, it must be stressed that different enzyme assays have been used.

Lactose, glucosamine, xylose, and sucrose have shown to be not such good carbon sources, inducing lower levels of chitinase production, when compared to those detected when chitin and glucose were used (Fig. 1). These results suggest that the lower production of endochitinase may be due to a constitutive production, since this enzyme is involved in some stages of fungal development [11].

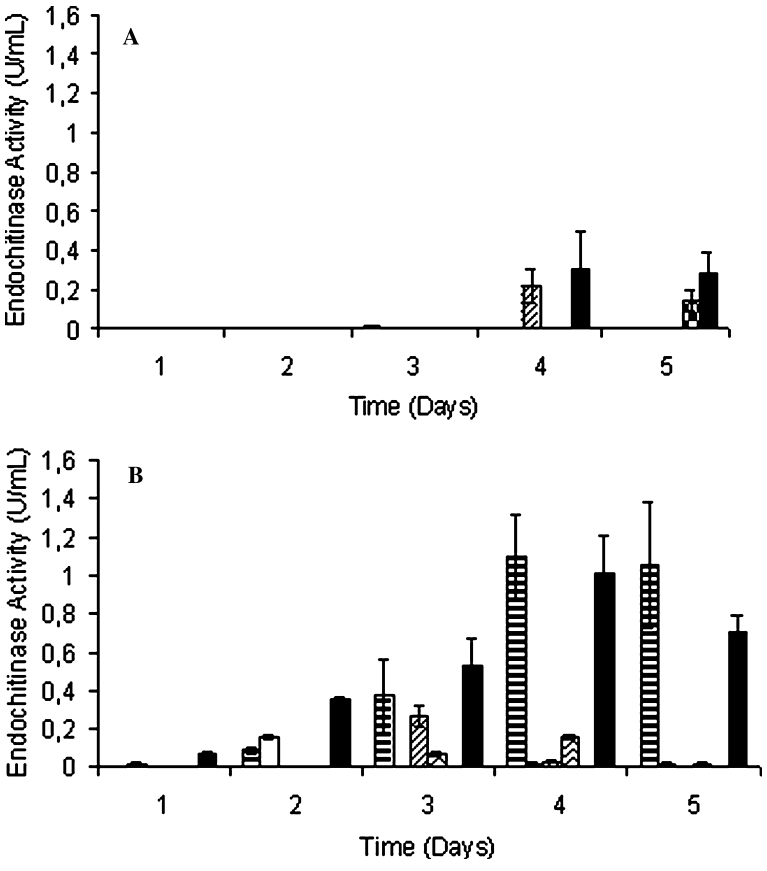

Fig. 1. Production of endochitinase by *Colletotrichum gloeosporioides* in different carbon sources and pH values during 5 days at 28°C/180 rpm. (A) pH 5.0; (B) pH 7.0. ▤ 0.5% Glucose, □ 0.5% Sucrose, ▨ 0.3% Xylose, ▩ 0.5% Glucosamine, ▦ 0.3% Lactose, ■ 1% Chitin.

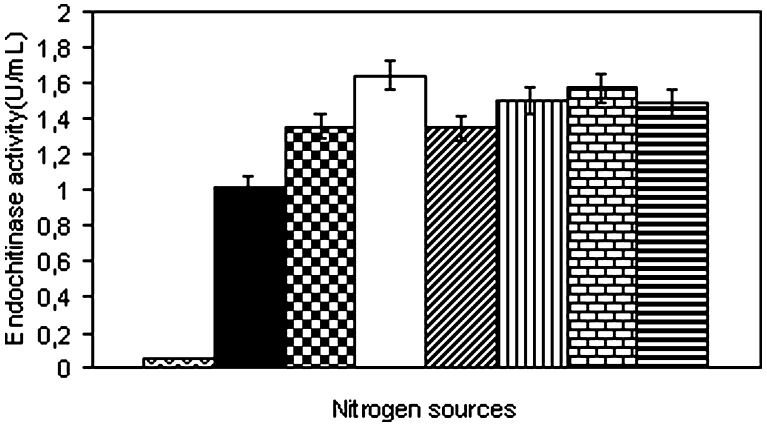

Fig. 2. Effect of organic and inorganic nitrogen sources on endochitinase production by *Colletotrichum gloeosporioides* after 4 days of growth, at 28°C/180 rpm/pH 7.0. ▩ Ammonium sulfate, ■ Sodium nitrate, ▩ Glutamic acid, □ Yeast extract, ▨ Urea, ▥ Casitone, ▦ Casoamino acids, ▤ Peptone.

Among the nitrogen sources tested, the organic ones gave the best results in chitinase production, when compared to the inorganic ones (Fig. 2), and complex sources appeared to induce more endochitinase production than simple ones such as glutamic acid and urea. This was also observed by Vaidya and co-workers for *Alcaligenes xylosoxydans* [42].

Production of endochitinases by *C. gloeosporioides* was also evaluated in different concentrations of colloidal chitin, glucose, and glucosamine at pH 7.0. In these conditions, 0.5% colloidal chitin was the best substrate, giving values of 2.4 U at the fifth day (Fig. 3A), 1.3- to 1.9-fold higher than those obtained when using 1 or 0.25%, respectively. When using glucose as substrate (Fig. 3B), the best endochitinase production occurred at 0.3% concentration, enzyme production decreasing as glucose concentration increased, similar to other results described in the literature [41, 8]. After 5 days growth, at this glucose 0.3% concentration, values of 1.31 U were obtained. The production of endochitinases using glu-

cosamine as a carbon source was usually low, in all concentrations tested (Fig. 3C), a better result being obtained at the 4th day when using 0.8% concentration (0.5 U). The synthesis of various catabolic enzymes in microorganisms is repressed when glucose or other readily metabolizable compounds are added to the culture. Tsujibo and co-workers [38] observed that production of chitinase in *Streptomyces thermoviolaceus* was almost undetectable when glucose was added to the culture medium, even when chitin was also present. Similar results were also observed with chitinase produced by *Alcaligenes xylosoxydans*, in which the highest chitinase levels were detected when chitin was used as carbon source, but when glucose was added the production was markedly affected [40].

According to our results, in a general way, the production of endochitinase increased with the increase of chitin concentration, and decreased when the concentration of glucose or glucosamine was higher. Ulhoa and co-workers [40] have also observed a decrease in chitinase activity when fourteen *Trichoderma* strains were grown on chitin-containing medium supplemented with 0.3% (wt/vol) glucose. Endochitinase from *C. gloeosporioides* seems to be an inducible enzyme, the level obtained on chitin being higher than that obtained on any other carbon sources.

Chitinase activity was also analyzed by SDS-PAGE using gels containing glycol chitin. Colloidal chitin and glucose appeared to induce different chitinase isoforms. Figure 4 shows two distinct hydrolytic zones of glycol-chitin degradation at 100 and 200 kDa, at 30°, 50°, and 90°C, the best zones being observed at 50°C, in which a third hydrolytic zone, of 95 kDa, was also detected. Although the best hydrolytic zones were observed at 50°C, the chitinolytic enzymes were also able to digest glycol-chitin at 90°C, suggesting a biotechnological application. The results obtained (Fig. 4) confirm the major chitinolytic activity being detected when using colloidal-chitin and glucose as the sole carbon source, since no hydrolytic zones were detected when the other carbon sources were analyzed.

Fluorogenic substrates have been widely used in the literature due to its high sensibility. More recently, some authors have shown that 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside is unable to distinguish between endochitinase and exochitinase [33]. In the present work, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide was used to quantify exochitinase (N-acetyl-β-D-glucosaminidase) and this activity was not detected (data not shown). Then, it was concluded that the enzyme herein described is an endochitinase.

In previous studies, *C. gloeosporioies* [32] yields on endochitinase production have suggested a promising

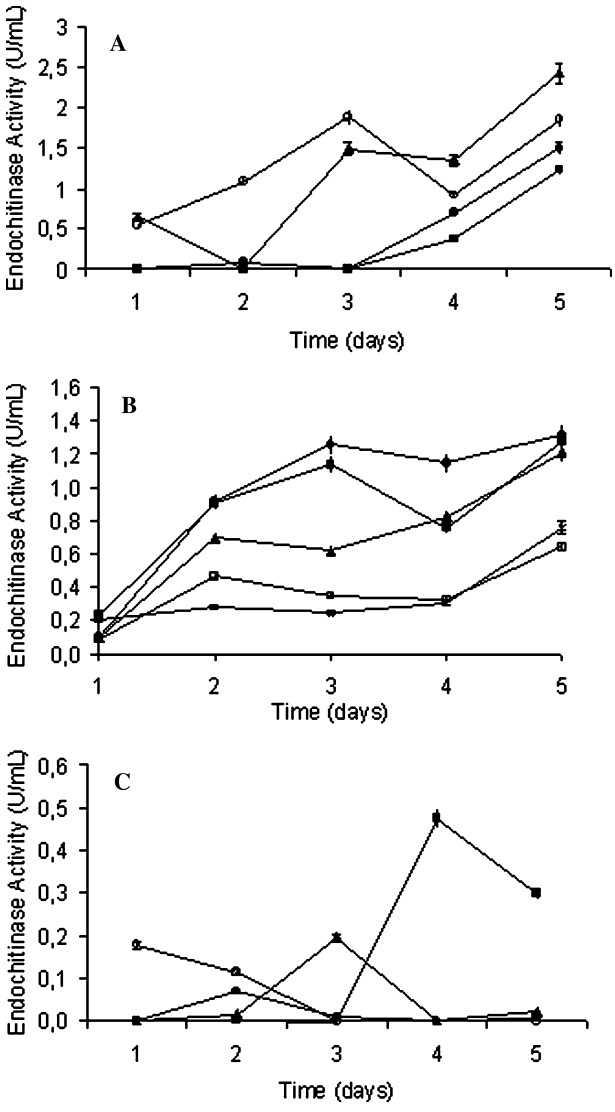

Fig. 3. Production of endochitinase by *Colletotrichum gloeosporioides* in different carbon sources concentrations during 5 days of growth, at 28°C/180 rpm/pH 7.0. (A) Colloidal chitin: 0.1% (-●-); 0.25% (-■-); 0.5% (-▲-); 1% (-○-). (B) Glucose: 0.3% (-●-); 0.5% (-■-); 1% (-▲-); 2% (-×-); 3% (-□-). (C) Glucosamine 0.1% (-●-); 0.2% (-○-); 0.4% (-▲-); 0.8% (-■-).

biotechnological application. In the present work, the influence of different carbon and nitrogen sources in chitinase production by this organism have been described, contributing to the understanding of the induction-repression system of this fungus.

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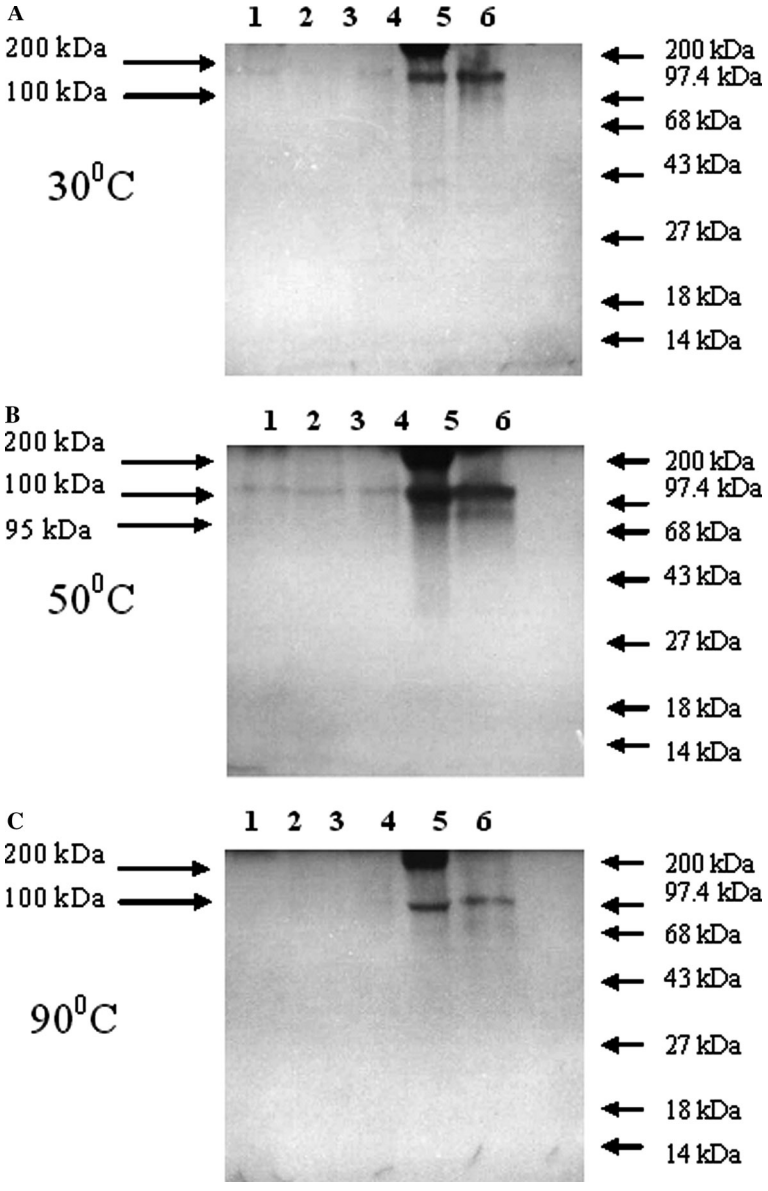

Fig. 4. Detection of endochitinase activity of *C. gloeosporioides* in gel of polyacrylamide co-polymerized with glycol-chitin. Supernatants from growth on: lane 1, 0.5% sucrose; lane 2, 0.3% lactose; lane 3, 0.3% xylose; lane 4, 1.0% colloidal chitin; lane 5, 0.5% glucose; lane 6, 0.5% glucosamine. The amounts loaded in the gel contained 0.5 mg of protein. The MW markers used were pre-stained and are shown on the left side of each gel.

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