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SHORT COMMUNICATION

Growth response and extracellular enzyme activity of *Ulocladium botrytis* LPSC 813 cultured on carboxy-methylcellulose under a pH range

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Abstract The fungus *Ulocladium botrytis* was isolated from *Scutia buxifolia* leaf litter and its growth was evaluated on both liquid and solid medium with sodium-carboxy-methylcellulose (CMC, 0.5%) as sole C source at a pH range between 4.0 and 10.0 and the synthesis of cellulose-degrading enzymes on litter. Growth on CMC-agar medium was maximum at pH 6.0, while in liquid CMC cultures, the highest biomass levels were found at pH 8.0 in both cases after 7 days of incubation. Cellulose-degrading enzyme activities such as β-glucosidase (2.40 U dry leaf g^{-1}), cellobiohydrolase (3.92 10^{-3} U dry leaf g^{-1}), and endoglucanase (2.01 U dry leaf g^{-1}) activities were detected in water-soluble fractions of inoculated leaves after 30 days of incubation. Endoglucanase activity was maximum at pH 6.0 and relatively stable as the pH increase, being 100 and 60% stable at pH 7 and 8, respectively. As a consequence of these enzyme activities, leaf mass was reduced by 5.8%. Our findings suggest that *U. botrytis* contains a cellulose-degrading enzyme complex that, unlike other cellulolytic systems, can degrade recalcitrant plant litter under alkaline conditions.

Keywords Alkaline soil · Endoglucanase · Growth · Leaf litter · *Ulocladium botrytis*

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Introduction

Extreme environments are the most interesting places to look for organisms and/or metabolic pathways active under stressful conditions. An example of environments like this is a native xerophilic ecosystem located on the eastern part of the Buenos Aires province (Argentina). This area is characterized by alkaline-calcareous soils (Rendolls), whose vegetation is dominated by two tree species *Celtis tala* Gill ex Planch (Ulmaceae) and *Scutia buxifolia* Reiss (Rhamnaceae; Arturi et al. 1996). Therefore, leaves of both species are major sources of soil organic matter. *S. buxifolia* is, unlike *C. tala*, a perenifolia species, whose leaves once fallen in the soil are particularly resistant to degradation, turning to a dark-brown color because of their content of phenolic compounds. Several *Fungi Imperfecti* are associated to *S. buxifolia* leaf litter from those forests (Allegrucci et al. 2005).

Ulocladium botrytis G. Preuss (*Fungi Imperfecti*) is an ubiquitous fungus that is frequently found in plant debris or soil, paper, and textiles (Farr et al. 1989). Saparrat et al. (2005) isolated from leaf litter of *S. buxifolia* a strain of *U. botrytis* LPSC 813 with an outstanding cellulolytic ability. However, there is not available information on its cellulolytic enzyme system and physiology.

Polysaccharides such as cellulose, which is one of the most important components of leaf litter, are degraded by an enzyme complex formed by a group of enzymes like cellobiohydrolase, endoglucanase, and/or β-glucosidase. Generally, fungal cellulases activity is optimal under acid pH (Magnelli and Forchiassin 1999; Pointing et al. 1999). Although a considerable amount of information regarding cellulase activity at low pH is available (Enokibara et al. 1993; Magnelli and Forchiassin 1999; Pointing et al. 1999; Tanaka et al. 1999), only a few reports described cellulose

degradation by cellulases at alkaline pH (Enokibara et al. 1993; Yamanaka 2003), which is an amenable metabolic activity for several industrial processes (Jackson et al. 1996; Bajpai 1999).

The aim of this work was to evaluate *U. botrytis* growth on liquid and solid cultures with sodium-carboxy-methylcellulose (CMC) as sole C source at different pHs. Furthermore, the effect of the fungus on *S. buxifolia* litter under in vitro solid-state fermentation (SSF) conditions and the fungal ability to produce cellulolytic enzymes also were evaluated.

Materials and methods

U. botrytis LPSC (culture collection of the La Plata Spegazzini Institute) 813 strain was isolated from the leaf litter of *S. buxifolia*, collected from a natural dry forest located 20 km south east from Magdalena city (35°11′ S, 57°17′ W) in the province of Buenos Aires, Argentina (Allegrucci et al. 2005). Stock cultures were maintained on agar-malt extract slants at 4°C.

The fungus was cultured on a mineral salt medium (Kreisel and Schauer 1987) supplemented with 0.5% CMC (BDH Chemicals) as the sole C source, either on liquid or solid media. After autoclaving, the pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 by using several buffers ($NaH_2PO_4{\cdot}2H_2O/Na_2HPO_4$//$NaHCO_3/Na_2CO_3$; Nagai et al. 1995). Media were prepared by mixing nine parts either of liquid or solid CMC medium with one part of one of the sterile buffer solutions. Plates with 20 ml of agar-CMC medium and 100-ml Erlenmeyer flasks filled with 40 ml of CMC medium at a desired pH between 4.0 and 10.0 were inoculated with one or three mycelium 6-mm plugs, respectively, which were cut from cultures grown on agar-malt extract medium. Three replicates per treatment were incubated in the dark at 25±1.5°C and liquid cultures in a rotary shaker (150 rev min^{-1}). Fungal growth was estimated after 7 and 14 days of incubation by both colony diameter on agar cultures and biomass (dry weight, mg 100 ml^{-1}) on liquid cultures after drying the pellets overnight at 90°C. Extracellular cellulolytic activity was measured on agar cultures at pH 6.0, 7.0 and 8.0 according to the CMC-clearing method (Magnelli and Forchiassin 1999) and expressed as the ratio of cellulolysis diameter to that of colony according to Choi et al. (2005). The statistical analysis consisted in a one-way analysis of variance; means were contrasted by means of Tukey's test.

The fungus was cultured under solid-state fermentation (SSF) conditions at 25±1.5°C for 30 days in 100-ml Erlenmeyer flasks containing 2 g of sterile dry leaves of *S. buxifolia* and 5 ml of water. The leaves were previously washed with hot water to eliminate soluble compounds and then, they were analyzed for their composition according to Chapman and Pratt (1973). Uninoculated control flasks were incubated under identical conditions. Cellulolytic enzyme activity of the water-soluble fraction (WSF) of inoculated leaves (expressed as U dry leaf g^{-1}, see below) and substrate mass reduction were measured according to Saparrat and Guillén (2005). The WSF of inoculated or uninoculated leaves was obtained by shaking leaves with 50 ml of water at 50 rev min^{-1} for 2 h at 4°C; the supernatant was filtered and centrifuged at 10,000×*g* and 4°C for 30 min. In addition, the pH and concentration of total soluble-reducing carbohydrates (TSRC) of the WSF from fungal cultures and control flasks were determined. TSRC were assayed by the Somogyi (1945) and Nelson method using glucose as standard.

β-1,4 Endoglucanase (E.C. 3.2.1.4) activity was estimated by measuring the reducing sugars released from CMC degradation (Magnelli and Forchiassin 1999). Cellobiohydrolase (E.C. 3.2.1.91) activity was estimated by measuring the reducing sugars released from crystalline cellulose (Sigmacell, type 20) degradation (Tanaka et al. 1999). β-glucosidase (E.C. 3.2.1.21) activity was assayed using, as substrate, *p*-nitrophenyl β-D-glucopyranoside (Magnelli and Forchiassin 1999). All enzyme reactions were carried out in triplicate and control assays were performed with boiled WSF from SSF cultures. One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 μmol reaction product min^{-1} under the conditions of assay.

The effect of pH on the endoglucanase activity of WSF from SSF cultures was determined on 1% CMC using Mc Ilvaine buffer (pH 2.1–9.0) at 50±1.5°C. The effect of the reaction buffer on enzyme activity was also determined using 150 mM sodium tartrate, sodium acetate and Mc Ilvaine solutions (pH 5.0). Stability of endoglucanase enzyme activity under different pH values was estimated by preincubating the WSF from SSF cultures at 25°C in Mc Ilvaine buffer at pH 3.0, 5.0, 6.0, 7.0, 8.0, and 9.0 for 24 h. Then, endoglucanase activity on 1% CMC was measured at 50±1.5°C in the presence of 150 mM sodium tartrate buffer, pH 6.0.

Results and discussion

Extreme environmental conditions such as high or low pH may inhibit fungal growth as well as enzyme activity and stability mainly because of the effect that H^+/OH^- concentration has on the availability and ionic forms of nutrients, the ionization of the enzymes active sites, and/or protein denaturation (Rosset and Barlocher 1985; Nagai et al. 1995; Pointing et al. 1999).

Growth of *U. botrytis* on solid and liquid CMC media at a pH range between 4.0 and 10.0 after seven incubation days is presented in Fig. 1. While growth on solid medium was maximum at pH 6.0 but fully inhibited at pHs above

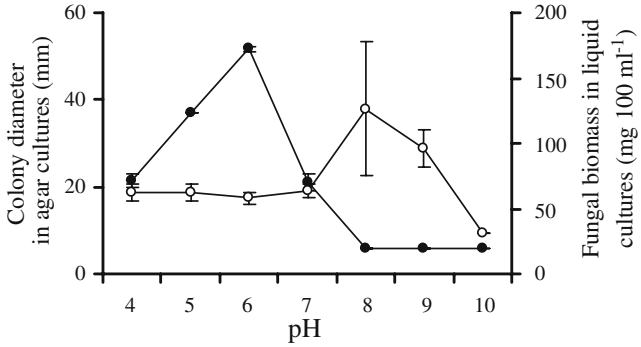

Fig. 1 Growth of *U. botrytis* on solid (*closed symbols*) and liquid (*open symbols*) cultures supplemented with 0.5% CMC and different buffer solutions after seven incubation days. All values are means of three replicate cultures; *error bars* represent mean standard deviation. Data from liquid cultures at pH 10 were not significantly different from the initial inoculum biomass

7.0, at pH 4.0, there was a 60% reduction. The ability of the fungus to degrade CMC on solid media at pH 6.0 and 7.0 was determined by the CMC-clearing method; the ratio of cellulose degraded halo and that of the colonies was about 1.2 (data not shown), confirming its outstanding degradative ability. Although in liquid medium fungal biomass was higher after 14 than after 7 days of culture, the fungal response to pH was identical. While biomass production at pH values ranging between 4.0 and 7.0 was of only 64 mg 100 ml^{-1} of liquid culture or even less, it was higher than 120 mg 100 ml^{-1} at pH 8.0 after 7 days of incubation, which was not significantly different from the biomass obtained with cultures grown at pH 9.0. The higher fungal biomass achieved on CMC at pH 8.0 might have been due to the effect of pH on nutrients availability. Roth-Bejerano et al. (2004) and Dix and Webster (1995) found that the environmental H^+ concentration influenced the ionization of salts in solution and/or the permeability of the plasmalemma of the hyphae.

U. botrytis biomass production in liquid and solid cultures had different optimal pH values: 6.0 and 8.0–9.0, respectively. In agreement with our findings, Rosset and Barlocher (1985) reported that growth of several mitosporic isolates was higher in liquid than in solid culture. The growth differences observed in liquid and solid cultures might be due to nutrient availability in the culture medium. Agar, which is the only different component between liquid and solid cultures, is known to complex a variety of metal ions, thus reducing their availability to the fungus (Hurst et al. 1997).

Because *U. botrytis* was unable to grow at pH 10.0, it is classified as an alkalophobic strain (Nagai et al. 1995).

S. buxifolia leaves had 95.5% (w w^{-1}) of dry matter, 72.7% of organic matter, 42.2% of organic C, 2.273% of total N (Kjehldahl) and a 18.57 C/N ratio. When *U. botrytis* was cultured for 30 days on sterile leaves, substrate biomass was reduced by 5.8±2.1% compared to control uninoculated flasks, which is a higher reduction than those observed for other fungi (Osono and Takeda 2002). The WSF from SSF cultures had a higher pH (6.6±0.2) than that of the uninoculated controls (5.7±0.1). This increase in pH might be due to the release of ammonium ions as a result of fungal metabolism as well as the appearance of degradation byproducts and fungal metabolites synthesized during the incubation period. Wainwright (1992) suggested that the increment in soil pH was probably due to the release of NH_4^+ as a result of fungal degradation of organic N.

Extracellular cellobiohydrolase, endoglucanase, and β-glucosidase activities of WSF from SSF cultures were 3.92 10^{-3}, 2.01, and 2.40 U dry leaf g^{-1} levels, respectively, suggesting that the cellulolytic enzyme complex was triggered. Probably for this reason, the substrate sugars were a 22% of the concentration of the control flasks.

Endoglucanase activity of WSF from SSF cultures was maximal at pH 6.0 in the presence of Mc Ilvaine buffer (2.1–9.0; Fig. 2). Other enzymes belonging to cellulolytic enzyme complexes of several ammonia and mangrove fungi showed high activities at pH 6.0 (Enokibara et al. 1993; Pointing et al. 1999). Endoglucanase activity was dependent upon the buffer used, being higher in 150 mM sodium tartrate (pH 5.0) than in sodium acetate (66%) or Mc Ilvaine (borate-citrate-phosphate, 29%) buffers. Higher levels of endoglucanase activity in an extract from a *Quercus ilex* L. litter were observed on acetate buffer compared to citrate one and this was suggested to be due to the ionization effect on the substrate and enzyme active site and therefore on enzyme stability (Criquet 2002). Collins et al. (1998) and Gómez-Toribio et al. (2001) found that active species of oxygen are generated by the oxidation of organic anions such as acetate and oxalate buffer by trace metals, but this did not occur when tartrate buffer was used. As active species of oxygen might alter proteins, the production of free radicals in the reaction mixtures using

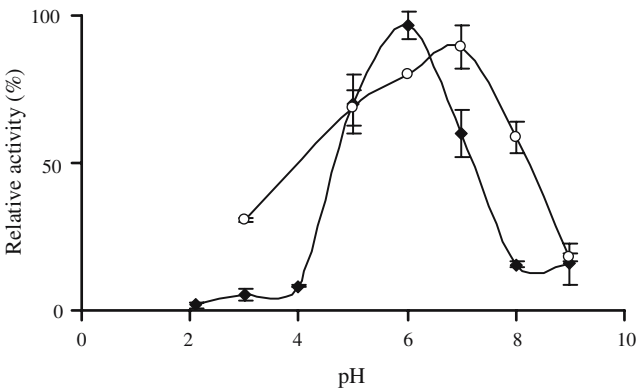

Fig. 2 Effect of pH on *U. botrytis* endoglucanase activity (*closed symbols*) and stability (*open symbols*). All reported values are means of three replicate assays. *Error bars* represent the mean standard deviation. Maximum relative endoglucanase activity (consigned as 100%) is 64.4 mU ml^{-1} WSF from SSF cultures

acetate and oxalate buffer might be an explanation of the higher endoglucanase activity detected on sodium tartrate.

According to the pH of the WSF from inoculated *S. buxifolia* leaves (6.6), endoglucanase activity was stable at pH 7.0 (Fig. 2), decreasing to 80 and 60% after 24 h at pH 6.0 and 8.0, respectively. At pH 9.0, the remaining activity was only 20%. Yamanaka (2003) found that the optimum pH for fungal growth was directly related with the pH of soil from which the fungus was isolated.

The ability of *U. botrytis* to grow on CMC in liquid cultures at pH 8.0 and 9.0, as well as the stability of endoglucanase activity in WSF at pH 8.0, indicate that the cellulolytic enzyme complex of this fungus might be active in alkaline soils with *S. buxifolia* litter.

In conclusion, although *U. botrytis* was isolated from an alkaline environment, it is an alkalophobic species as high pH affected its growth. In vitro *U. botrytis* LPSC 813 grew in liquid culture but not on agar at pH 8.0–9.0 with CMC as single C source. To our knowledge, this is the first report about the physiology of cellulose degradation by this fungus and its extracellular enzyme system. The ability of the fungus to degrade leaves of *S. buxifolia* with the concomitant production of cellulolytic enzymes suggests that the organism participates in decomposition of the *S. buxifolia* leaf litter in alkaline soils. The findings of endoglucanases highly active at high pH have also important biotechnological implications.

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