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Vol. 14(17), pp. 1496-1503, 29 April, 2015
DOI: 10.5897/AJB2015.14447
Article Number: C5A5D0152718
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Activity of the fungus *Pleurotus ostreatus* and of its proteases on *Panagrellus* sp. larvae

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Received 25 January, 2015; Accepted 22 April, 2015

Biological control has been shown to be one of the possible biotechnological applications of fungi and their proteases. The objective of this study was to evaluate the nematicidal activity of the fungus *Pleurotus ostreatus* and its proteases on *Panagrellus* sp. larvae. Proteolytic activity of *P. ostreatus* (PLO 06) was measured and characterized at different pHs, temperatures and in the presence of a inhibitor (PMSF). Daily samples of culture medium were collected in order to determine the period of maximum enzyme production. A zymogram showed the profile of several proteases. Predatory activity of the fungus *P. ostreatus* (PLO 06) was evaluated on *Panagrellus* sp. larvae (assay A) as well as the nematicidal activity of PLO 06 proteases on the same larvae (assay B). At pH 9 and 60°C, the activity of the proteases reached the maximum. In the presence of inhibitor, there was no proteolytic activity. A sample collected on the fifth day of incubation showed the highest enzyme activity. *P. ostreatus* demonstrated capture activity on larvae *Panagrellus* sp. The values of the reduction of the larvae (Assay A) were: day 1 (65.6%); day 2 (77.4%); day 3 (95.2%). The reduction of the larvae (Assay B) was 42%. *P. ostreatus* (PLO 06) and its proteases were very effective against *Panagrellus* sp. larvae, demonstrating great potential for use in integrated biological control.

Key words: *Pleurotus*, protease, *Panagrellus* sp., biological control, nematicidal.

INTRODUCTION

Biological control assumes increasing importance in integrated management programs of agricultural pests (Parra et al., 2002) and thus is a "strategic component" in the fight against gastrointestinal nematode parasites and

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Abbreviation: PMSF, Phenylmethylsulfonyl fluoride.

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phytonematodes (Hibbett and Thorn, 1994; Grønvold et al., 1996; Eira, 2004). Living organisms that act as biological control agents constitute the group of natural enemies, which is formed by parasitoids, predators and pathogens. Accordingly, a wide variety of fungi have been studied for this purpose: nematophagous fungi (Larsen, 1999; Lopez-Llorca et al., 2008) and specifically here, fungi of the genus *Pleurotus* (Okorie et al., 2011). The genus *Pleurotus* (class of Basidiomycetes) hosts several species with important biological characteristics. Among these we can highlight: (1) ability to colonize and degrade a wide variety of lignocellulosic residues, (2) high value medicinal and gastronomical properties, (3) require shorter time of cultivation when compared to other edible genera (Cohen et al., 2002; Bonatti et al., 2004; Satou et al., 2008; Okorie et al., 2011) and (4) nematicidal activity against phytonematodes. In relation to this fact, in general, fungi of this genus exhibit a unique method of capturing nematode, which can be described as the production of toxic metabolites that cause the decrease of helminth head and subsequent paralysis (Thorn and Barron, 1984; Hibbett and Thorn, 1994). This toxin has been identified as trans-2-decenedioic acid (Kwok et al., 1992). The species *P. ostreatus* are known to prey on nematodes. However, in relation to predatory activity, this has not been fully elucidated, thus requiring more studies from biological models under laboratory conditions.

Extracellular proteases produced by nematophagous fungi are directly involved in the stages of the infection. They act in the digestion of nematode's protection barrier, rich in protein (Braga et al., 2010). However, there is a lack of studies aimed at elucidating the molecular mechanism of interaction of the proteases from fungi of the genus *Pleurotus*. Thus, the objective of this study was to evaluate the nematicidal activity of the fungus *Pleurotus ostreatus* and its proteases.

MATERIALS AND METHODS

Fungi

One fungal isolate was used; *P. ostreatus* (PLO 06, GenBank accession number KC782771). This fungi is derived from the soil of Viçosa, Brazil, and belong to the Nucleus Collection of the Department of Microbiology/UFV. These organisms have been maintained by means of continuous transfer to solid medium containing 2% water-agar (2% WA) in the Department of Molecular Biology and Biochemistry, Federal University of Viçosa, Brazil. Subsequently, the fungus was again replicated in Petri dishes for removal of the inoculum in the form of disks of 2 cm and has grown on 28°C, in the dark. Free-living nematodes of the genus *Panagrellus* were used in the experiment. These organisms have been kept in Petri dishes in moistened and kneaded oat flakes medium, at the Laboratory of Parasitology, Department of Veterinary, Federal University of Viçosa, Brazil. For the test trial, these nematodes were extracted with a Baermann apparatus and collected in hemolysis tubes after 6 h of decanting.

Proteases production

Fungal mycelia of PLO 06 were transferred to flasks (250 ml) previously autoclaved containing 50 ml of liquid medium according to the methodology described by Soares et al. (2013). The liquid medium was composed of glucose 10 g /l; yeast extract, 10 g/l; K_2HPO_4, 5 g/l; and $MgSO_4$, 0.10 g/l. The fungal inoculum was grown in the flasks under agitation at 120 rpm at 28°C for seven days. Then, the fermented medium was filtered using filter paper Whatman No. 1 at 4°C and then centrifuged for 10 min, at 10,000 g, 4°C, to obtain the protease.

Activity assay

The proteolytic activity of *P. ostreatus* (PLO 06) was measured according to the methodology described by Soares et al. (2013). One protease unit was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the conditions used in the tests.

Proteases characterization

All characterization experiments were performed with three replications.

Effects of pH and temperature

The proteolytic activity was characterized at different pH values. Thus, the 50 mM phosphate-citrate buffer was used for pH 3, 4, 5, 6, 7, 8 and 9. The assay temperature was 40°C. Evaluation of the effect of temperature on the proteolytic activity was performed at the pH with the maximal activity obtained in the above assay at different temperature values (30, 40, 50, 60, 70 and 80°C).

Effect of Inhibitors

Proteases were incubated with the inhibitor phenylmethylsulfonyl fluoride (PMSF), at the pH and temperature that showed the highest activity in the previous assays.

Proteolytic activity during the days of incubation

Samples of culture medium were collected every day in order to measure the proteolytic activity over time in order to obtain the time of highest enzyme production.

Zymogram

A zymography was performed using casein as the substrate (casein-SDS-PAGE) (Hummel et al., 1996) to obtain the profile of the proteases produced by *P. ostreatus* (PLO 06). Samples were subjected to gel electrophoresis on 10% polyacrylamide containing 1% casein and mixed with gel-native sample buffer (glycerol 30% (v/v), bromophenol blue 1% (p/v), Tris- HCl 0.25 M). After the electrophoresis procedure, the gel was incubated in a Triton X-100 2.5% solution for 30 min for removal of SDS. Then, the gel was washed three times with water. Subsequently, it was incubated in Tris-HCl 50 mM (pH 9.0) for 1 h at 60°C. In the developing procedure, the gel was stained with Coomassie Brilliant Blue R-250 and then was immediately destained with decolorizing solution (10% acetic acid and 50% alcohol aqueous solution).

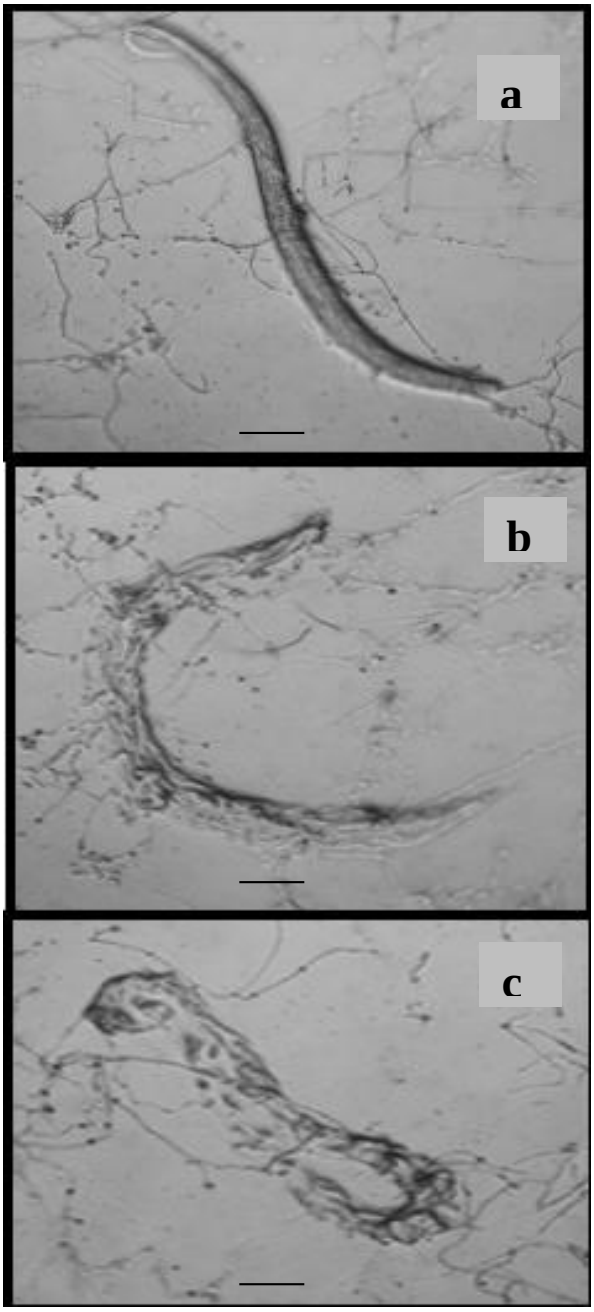

Figure 1. a. Initial action of hyphae of the fungus *Pleurotus ostreatus* on larvae *Panagrellus* sp. **b.** Destruction of larvae and colonization by hyphae of *Pleurotus ostreatus.* **C.** Destruction and/or digestion of *Panagrellus* sp. larvae by *Pleurotus ostreatus.* Bars: a-7,000 µm; b-4,500 µm and c-6,000 µm.

Experimental assays

Two assays (A and B) were performed. In the assay A, predatory activity of the fungus *P. ostreatus* (PLO 06) was evaluated on *Panagrellus* sp. larvae. In the assay B, the nematicidal activity of *P. ostreatus* (PLO 06) proteases was evaluated on larvae of *Panagrellus* sp.

Assay A

Two groups were formed on Petri dishes 4.5 cm containing 10 ml of 2% WA; one treated group and one control group, with six replicates for each group. The Petri dishes were previously marked in fields of 4 mm in diameter. In the treated group, each Petri dish contained 1000 *Panagrellus* sp. larvae and the fungus. The control group (without fungi) contained only 1000 *Panagrellus* sp. larvae. Both groups of plates were incubated in a BOD incubator chamber in the dark at 28°C. For three days, every 24 h, 10 random fields of 4 mm in diameter in each plate of the treated and control groups were observed under an optical microscope at 10x objective by counting the number of non-destroyed larvae in each, according to the methodology adapted from Braga et al. (2010). Photomicrographs were taken for proof of capture activity and subsequent destruction of nematodes.

Destruction efficiency of *Panagrellus* sp. larvae compared to the control was assessed by the Tukey test at 1% probability. The data were interpreted by analysis of variance at significance levels of 1 and 5% probability (Ayres et al., 2003). Subsequently, the average reduction percentage of the larvae was calculated according to the following formula:

$$\text{Reduction (\%)} = \frac{\text{Average larvae recovered from control} - \text{Average larvae recovered from treatment}}{\text{Average larvae recovered from control}} \times 100$$

Assay B

Two groups were formed in microtubes. One treated group in which about 50 *Panagrellus* sp. larvae were incubated with the proteases and one control group, in which about 50 larvae *Panagrellus* sp. were incubated with denatured proteases (boiled for 1 h). Six replicates were performed for each group. Both groups were incubated at 28°C for 24 h. After this period, the total number of larvae was counted (Soares et al., 2013). The data were interpreted by analysis of variance in significance levels of 1 and 5% probability. The efficiency of the destruction of the larvae in the control was evaluated by the Tukey test at 1% probability (Ayres et al., 2003). The average reduction percentage of the larvae was calculated according to the following equation:

$$\text{Reduction (\%)} = \frac{\text{Average larvae recovered from control} - \text{Average larvae recovered from treatment}}{\text{Average larvae recovered from control}} \times 100$$

RESULTS AND DISCUSSION

The fungus *P. ostreatus* (PLO 06) showed high *in vitro* capture activity on larvae *Panagrellus* sp. After 24 h, an interaction of hyphae of the fungus with nematodes was observed, as shown in Figure 1a. Figure 1b shows the destruction of the larvae, as well as colonization by fungal hyphae. Figure 1c shows the complete destruction and or digestion of the larvae by the tested fungi. Figure 2b illustrates the production of toxic droplets by the fungus *P. ostreatus*. The tested fungi were effective in destroying *Panagrellus* sp. larvae, as shown in Table 1. On the first day of the experiment, the percentage reductions

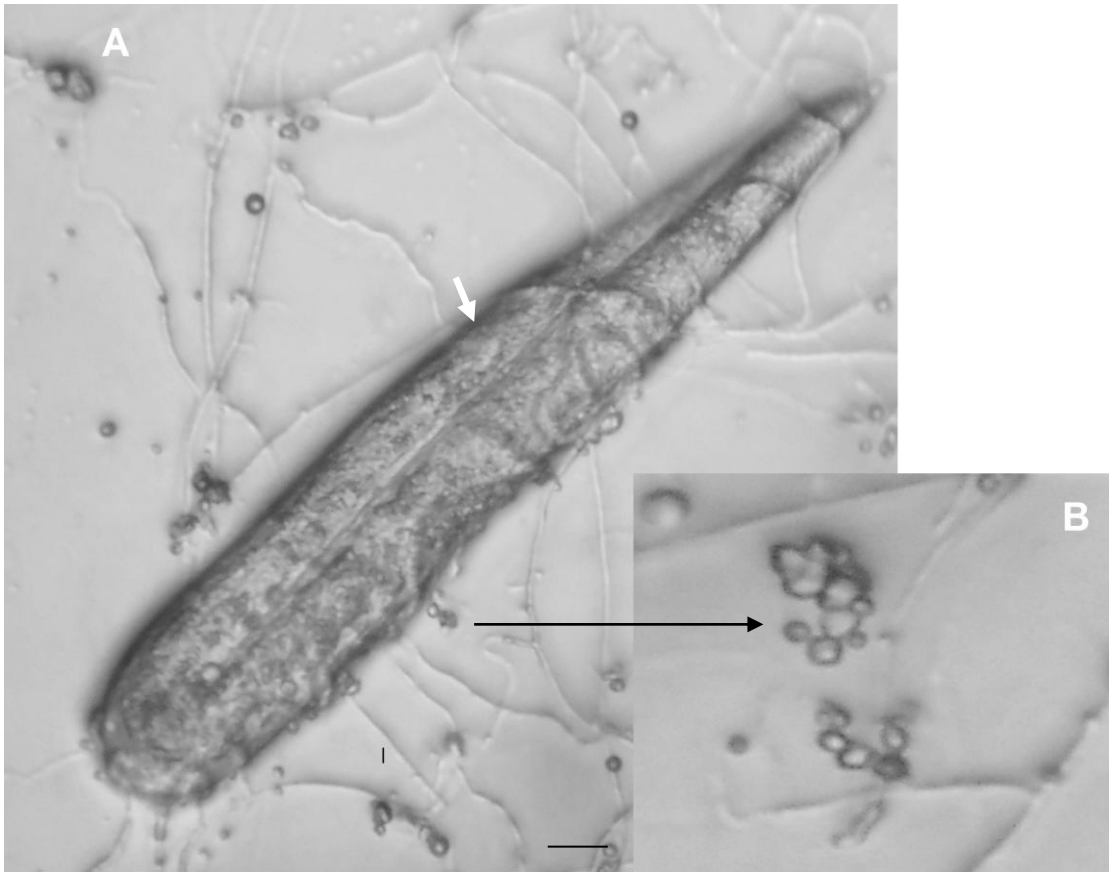

Figure 2. a. *Panagrellus* sp. larvae (white arrow). **b.** Toxic droplet production by the fungi *Pleurotus ostreatus* (black arrow / detail). Bar: a-15,000 µm.

Table 1. Daily average, standard deviation and percentage reduction of *Panagrellus* sp. larvae in water-agar (2% WA) during the period of three days in the treatments with the fungus *Pleurotus ostreatus* and the control group without fungi.

Days	*Pleurotus ostreatus*	Control	Reduction (%)
1	10.2^{a} ±4.3	29.1^{b} ±8.92	65.6
2	6.2^{a} ±3.4	15.7^{b} ±10.6	77.4
3	1.4^{a} ±0.8	29.4^{b} ±11.15	95.2

Averages followed by the same lowercase letter in the lines do not differ statistically of (p>0.01).

observed were higher than 50%, reaching values higher than 90% on the third day. The results of this study have demonstrated the nematicidal activity of fungi of the genus *Pleurotus* on the free-living nematode *Panagrellus* sp. (Table 1). Fungal species tested have activity against phytonematodes, however, these isolates have never been tested on the real possibility of biological control. In this sense, it can be argued that among the various species of fungi there are variations between the action of different isolates, which must be constantly tested (Mendoza-de Gives, 1999). This information is also in line with those of Okorie et al. (2011) who tested the nematicidal activity of two species of fungi of the genus *Pleurotus* and demonstrated no distinction in their activities. Hibbett and Thorn (1994) mention that the basic mechanism of the activity of predatory fungi of the genus *Pleurotus* is the production of small droplets of anti-nematode toxin derived from linoleic acid. It is further described that the paralyzed nematodes are subsequently invaded by hyphae. These results are in agreement with the present work's results, where PLO 06 showed nematicidal activity and production of these toxic droplets against *Panagrellus* sp. larvae (Figure 2a, b). Some works that targeted *in vitro* control of plant parasitic

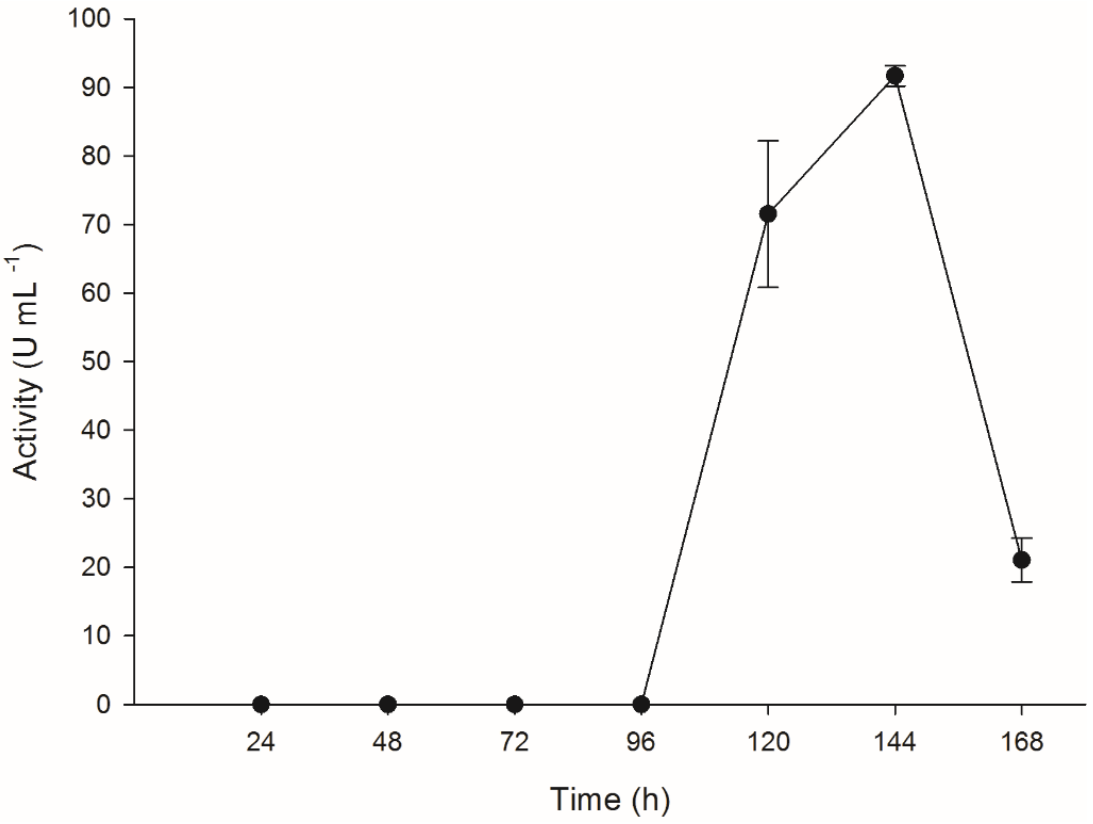

Figure 3. Assay of proteolytic activity during the incubation period of 168 h (7 days). Samples of culture medium were collected every day in order to measure the proteolytic activity along the days, to obtain the time of higher enzyme production.

nematodes have been performed with the species *P. ostreatus* (Okorie et al., 2011). However, based on previous studies involving other groups of nematophagous fungi, the present experimental assay has shown to be "elucidative" with regard to the reduction of nematodes in Petri dishes in medium 2% WA, since these plates were observed for three days and every 24 h. This premise is important to elucidate the predatory mechanism of this species on free-living nematodes (Thorn and Barron, 1984). The model organism *Panagrellus* sp. has been used for observation of predatory activity of fungi with potential nematophagous, since they are "agile" and provide the production of traps by fungi (Eren and Pramer, 1965). This type of model is important since it provides the basis for other experiments aimed at reducing nematodes that are harmful to animals, humans and plants (Jaffee and Muldoon, 1997; Tavela et al., 2012). From this point, comparatively, the carnivore fungus *Monacrosporium* spp., were shown to be effective in capturing and destroying *Panagrellus* sp. and *Meloidogyne incognita*, phytonematodes, under laboratory conditions (Gomes et al., 1999). In that work, the authors showed that *Panagrellus* sp., was the most susceptible ($p<0.05$). Those results are in agreement with the present work, demonstrating that the species *P. ostreatus* has reduced *Panagrellus* sp. larvae in Petri dishes by more than 90% at the end of 3 days, thus suggesting the enormous biocontrol potential of these species.

In another context, the literature has mentioned the realization of some studies with nematophagous fungi with potential for the production of extracellular enzymes with nematicidal activity (Braga et al., 2012b; 2013; Soares et al., 2012; 2013). In this study, it was observed that the fungus *P. ostreatus* (PLO 06) successfully produced proteases. The fifth day of incubation was that in which the major proteolytic activity was observed (Figure 3). Furthermore, highest enzyme activity occurred at pH 9 and 60°C (Figure 4). In the assay with the inhibitor, total proteolytic activity was inhibited by PMSF (Table 2), suggesting that proteases produced by addition to *P. ostreatus* are serine proteases. Shin and Choi (1998) have purified and characterized a cysteine protease from the fungus *P. ostreatus* after the formation of the fruiting body. In this study, the fungus in fruiting body stage was not used, suggesting that in different morphological stages there is the production of different proteases. The proteolytic profile of proteases produced

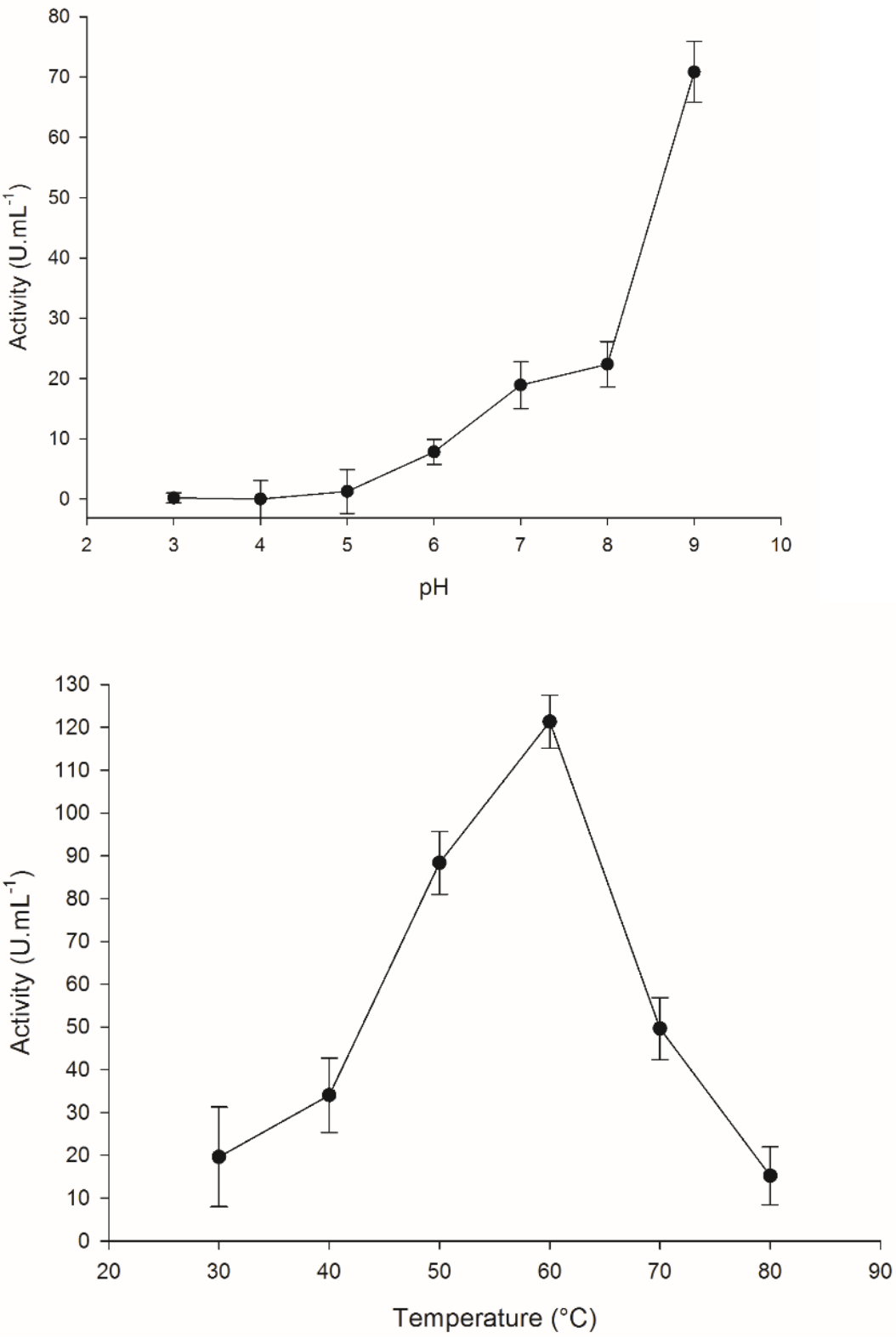

Figure 4. a. Effect of pH on activity of the *Pleurotus ostreatus (*PLO 06) proteases. Citrate-phosphate 50 mM buffer was used throughout the pH range. The assay was conducted at temperature of 40°C. **b.** Effect of temperature on activity of the *Pleurotus ostreatus (*PLO 06) proteases. The tested temperatures were: 30, 40, 50, 60, 70 and 80°C. The pH used in the test was the one with maximum activity obtained in the above test.

Table 2. Influence of inhibitor PMSF (1 mM) on the relative activity (%) of *Pleorotus ostreatus* (PLO 06) proteases in the optimum values of pH and temperature found in previous experiments.

Treatments	Relative activity (%)
Control[a]	100
PMSF	0

[a]50 mM citrate - phosphate (pH 9,0; 60°C).

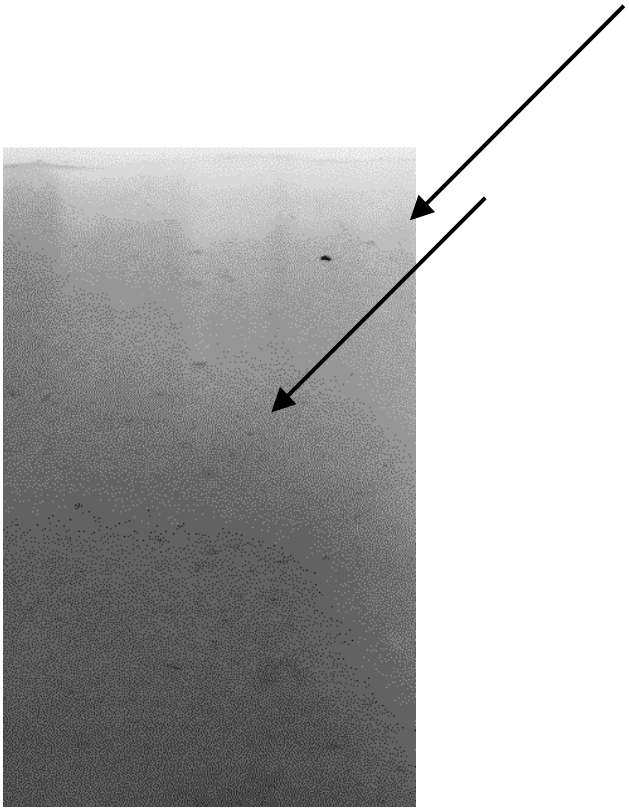

Figure 5. Zymogram of proteases produced by the fungus *Pleurotus ostreatus* (PLO 06) in polyacrylamide gel containing SDS. Arrows highlight the different halos of digestion, suggesting the existence of more than one protease.

by *P. ostreatus* was evaluated by a zymogram (Figure 5). The presence of different halos of digestion in the gel indicates the presence of more than one protease in the gel. This result confirms the experimental data obtained by Faraco et al. (2005), who demonstrated by zymography that the fungus *P. ostreatus* produces different proteases. In relation to the nematicidal assay of PLO 06 proteases, at the interval of 24 h, these enzymes have provided a significant percentage reduction ($p < 0.05$) of 42% on the number of *Panagrellus* sp. larvae, when compared to the control. Soares et al. (2013) have used the nematophagous proteases of *Monacrosporium thaumasium* (NF34) on larvae of *Panagrellus redivivus* and have observed that the purified enzymes destroyed more than 90% of the larvae subjected to the treatment. In the present work, the destruction was lower, however, it is worth mentioning that the fungus *M. thaumasium* belongs to the predatory class of fungi, while the fungus *P. ostreatus* belongs to the toxin producers class (Yang et al., 2007). This fact is important because it is known that different classes of fungi produce different metabolites and in distinct concentrations (Braga et al., 2012).

In this study, comparing the percentage reduction of *P. ostreatus* (95%) with the action of its proteases (65.6%) on the larvae of *Panagrellus sp*, there was a higher value of the action of the fungus. However, this fact can be explained by means of the interaction-time difference with the larvae in the two assays (three days in the assay with the fungus and only one day in the assay with the enzymes). The results show that the fungus *P. ostreatus* (PLO 06) and its proteases have very effective predatory activity against nematode larvae of the *Panagrellus* genus, demonstrating great potential for use in integrated biological control. Moreover, this is the first report of the use of *P. ostreatus* (PLO 06) proteases against nematode larvae of the *Panagrellus* genus, thus much remains to be studied about the action and the mechanism of these enzymes nematicidal.

Conflict of interest

The authors do not have any financial or commercial conflicts of interest to declare.

ACKNOWLEDGMENTS

The authors would like to thank CAPES, FAPEMIG, FAPES and CNPq for the financial support.

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