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Original Article

Potential of edible mushroom *Pleurotus* spp. for controlling root-knot nematode (*Meloidogyne incognita*) and their cuticle degrading enzyme production♣

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Abstract

The antagonistic potential of Genus *Pleurotus* for controlling *Meloidogyne incognita* under laboratory condition was examined and the effective species were selected. The results showed the egg hatching was significantly inhibited by *Pleurotus* sp. from Hungary (PH) and *Pleurotus* sp. from Bhutan No. 3 (PB-3) as 13.33 and 15.00%, respectively within 48 hours. Both species caused mortality of infective juveniles as 23.33 and 18.33%, respectively within 24 hours. The optimal growth temperature for PH and PB-3 (selected *Pleurotus*) on PDA was 28 ± 2 ºC. Nematotoxin droplets were found on their hyphae within 12 hours. The selected *Pleurotus* could produce protease on protein-enriched media. The protease was 0.27 U/mg protein when cultured PH on PDB with 1% casein and 0.17 U/mg protein when PB-3 was cultured on PDB with 1% skim milk. Furthermore, the potential of PH and PB-3 to control root-knot nematode disease in the field need to be conducted.

Keywords: *Pleurotus*, antagonistic fungi, root- knot nematode, protease

1. Introduction

Root-knot nematodes *Meloidogyne* spp. cause serious damage and yield losses in a wide range of crops throughout the world (Vestergård, 2019). In order to control root-knot nematodes, the uses of chemical nematicides are useful but they can cause side effects on the environment and human health (Kalaiselvia *et al.*, 2019). Thus, the use of biological methods for controlling nematodes are focused. A wide variety of fungi has been studied for nematode control purposes, such as genus *Pleurotus* is reported as nematophagous fungi (Sufiatea *et al*., 2017).

Biocontrol by using the antagonist fungi can reduce the use of chemicals and as a guideline for sustainable agriculture. The nematophagous fungi are divided into four groups: (1) Nematode-trapping fungi, a fungus that creates a special structural filamentary trap to capture nematodes. (2) Endoparasite is an internal parasitic fungus conidia adheres to the nematode surface, producing enzymes to digest the body wall. (3) Egg-parasitic fungi, a fungus that destroys the nematode's eggs. (4) Toxin-producing of secondary metabolites such as antibiotics, toxins, and enzymes (Barron, 1978).

The oyster mushroom (genus *Pleurotus*), a common edible mushroom, was found to have antagonistic effects when nematodes contacted the hyphae of *Pleurotus*. Various metabolites from *Pleurotus* spp. exhibit nematicidal activity which has been explored as a possible alternative control of nematodes (Degenkolb & Vilcinskas, 2016). As reported by Palizi, Goltapeh, Pourjam and Safaie (2009); Abbasi, Torkashvan and Rahanandeh (2014); Sufiatea *et al.* (2017) or, more recently, by Youssef and El-Nagdi (2020), *Pleurotus* spp. have high potential to control many species of plant nematodes, e.g., *Heterodera schachtii*, *M. javanica*, and *M. incognita. Pleurotus* sp. produced protease to degrade cuticle and caused 90% mortality of nematodes within 24 hours (Sufiatea *et al*., 2017). The secretion of protease by an

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antagonist is believed to be an important pathogenic factor affecting the attachment of eggshell and cuticle of nematode (Khan, Williams, & Nevalainen, 2004). Thus, the purpose of this research was to select the effective *Pleurotus* sp. for controlling root-knot nematode (*Meloidogyne incognita*) in laboratory condition and to look for its biological characteristic, i.e., protease production.

2. Materials and Methods

2.1 *Pleurotus* spp. preparation

Eight isolates of genus *Pleurotus* including *Pleurotus* sp. from Bhutan No.1 (PB-1), *Pleurotus* sp. from Bhutan No.2 (PB-2), *Pleurotus* sp. from Bhutan No.3 (PB-3), *Pleurotus* sp. from Bhutan No.4 (PB-4) , *P. sajor-caju* (PS), *P. cystidiosus* No.1 (PC-1), *P. cystidiosus* No.2 (PC-2) and *Pleurotus* sp. from Hungary (PH) were obtained from the Plant Protection Research and Development Office, Department of Agriculture, Bangkok, Thailand and were selected for use as the antagonist in this study. *Pleurotus* spp. were cultured on potato dextrose agar (PDA) for 7 days. The mycelium disc (0.5 cm diameter) of each *Pleurotus* sp. was transferred to new PDA and incubated at room temperature (28 ± 2 °C). The 7-day-old of *Pleurotus* spp. were used for all experiments.

2.2 Root-knot nematodes preparation

The roots of the tomato plants that presented galls of *Meloidogyne incognita* were washed and cut to 1-2 cm pieces then washed with water through a coarse sieve. The roots with mature egg masses were soaked in 0.6% sodium hypochlorite (NaOCl) solution for three minutes and shaken vigorously. Poured the solution through a 500-mesh sieve, washed with distilled water four times for two minutes and poured down in a sterilized beaker. After that the nematode eggs were incubated at room temperature (30 ± 2 °C) until the infective juveniles (IJs) were hatched and used for all experiments (Marla, Huettel, & Mosjidis, 2008).

2.3 Antagonistic effect of *Pleurotus* spp. on *M. incognita*

Pleurotus spp. were performed to assess the potential control in laboratory scale against *M. incognita*, eggs hatching and mortality of IJs. Ten microliters of sterilized water containing 15 eggs or IJs per replication were pipetted to 1% (w/v) of water agar (WA) in 24-well plates. Eggs and IJs were treated with a mycelial plug (3 mm diameter) of each *Pleurotus*, or without *Pleurotus* as control. Tested plates were incubated at room temperature (28 ± 2 °C) in the dark. The experimental design used was completely randomized design (CRD) with five replicates per treatment and the experiment was repeated 3 times. Numbers of hatched eggs and dead IJs were observed every 24 hours directly under an inverted microscope (DMi1, Leica, Germany) for three days. The hatching percentage was calculated using the formula:

$$\text{Percentage of hatching} = (J/E) \times 100$$

where J is the number of hatched juveniles and E is the total number of eggs.

The mortality was calculated from the count of dead IJ by using the formula:

$$\text{Percentage of mortality} = (D/N) \times 100$$

where D is the number of dead juveniles and N is the total number of tested juveniles.

After that, the *Pleurotus* spp. with high potential to inhibit egg hatching and kill IJs were selected for determining their biological characteristic and nematode cuticle degrading enzyme production.

2.4 Optimization of incubation temperature

Each mycelium disc (5 mm diameter) of selected *Pleurotus* was cultured on PDA medium and incubated in the dark condition at various temperatures, i.e., 25, 28, 30 and 35 ± 2 °C to optimize temperature for fungal growth. The diameter of the mycelium expansion was measured for seven days.

2.5 Detection of nematode cuticle- degrading enzyme production

The 7-day-old of selected *Pleurotus* was cultured on specific agar media for chitinase and protease activity detection by agar diffusion method. One liter of colloidal chitin agar (CCA) contained: 0.3 g of $MgSO_47H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar powder, 200 mL of Tween-80, 4.5 g of colloidal chitin and 0.15 g of bromocresol purple (Agrawal & Kotasthane, 2012) was used for chitinase detection. Skim milk agar was as follows: 200 g of potato, 20 g of glucose, 17 g of agar powder, 1% skim milk and 1,000 mL distilled water (Pundir, Rana, & Tyagi, 2012) was used for protease detection. The chitinase production was positive when the color of CCA was changed from yellow to purple around the fungal colony. The protease production was detected as a formation of a clear zone around the colony on skim milk agar medium.

2.6 Protease production under protein-enriched media

The selected *Pleurotus* was cultured in potato dextrose broth (PDB) medium supplemented with 1% casein or 1% skim milk and incubated at room temperature (28 ± 2˚C) for seven days on a rotary shaker. The cell free culture filtrate (CF) of each medium was collected by centrifugation at 10,000 rpm, 4 °C and filtered through a 0.45 µm membrane filter (Sartorius Stedim Biotech, Germany). The protease activity and total protein of CF were determined by spectrophotometry method.

The protease activity was determined by the method modified from Anson (1938) by using 0.65% casein in 50 mM potassium phosphate buffer (pH 7.5) as the substrate, incubated at temperature of 37 °C for ten minutes, 0.5 mL of enzyme was added and the mixture was incubated at 37 °C for five minutes. After incubation, the reaction was stopped by addition of 2.5 mL of 110 mM Trichloroacetic acid and incubated at 37 °C for 30 minutes. The mixture was proceeded with 10,000 rpm centrifugation at 10 °C for one minute and the supernatant was collected, then 2.5 mL of 500 mM

Na_2CO_3 and 0.5 mL of 0.5 mM Folin and Ciocalteu's Phenol reagent were added to the supernatant and incubated at 37 °C for 30 minutes. The absorbance of the blue color compound was measured at 660 nm against a reagent blank using the tyrosine standard. The protease activity was calculated using the formula:

Protease activity (U/ml) = (Ty × Vt) / (Ve × T × V)

where Ty is µmole tyrosine equivalents released, Vt is total volume (in mL) of assay, Ve is volume of enzyme used (in mL), T is time of assay (in min) as per the unit definition, and V is volume (in mL) used in colorimetric determination.

The concentration of total protein was determined according to the procedure of Bradford (1976) by bovine serum albumin (BSA) as the standard. The test tube was added 20 µL of sample followed by 1,580 µL of Coomassie Brilliant Blue G-250 (CBB) mixture then mixed by vortex mixer after 5-10 minutes incubation, and the developed color is measured at 595 nm. Protein concentration (in mg/mL) in the sample was determined by using a calibration curve of a BSA standard and used for specific activity calculation. The specific activity of enzyme sample was calculated using the formula: Specific activity (U/ mg protein) = protease activity (U/mL) / total protein (mg/mL).

2.7 Statistical analysis

All results were analyzed by one-way analysis of variance (ANOVA) and subjected to the Dancan's new multiple range test (DMRT) test ($p < 0.05$) using SPSS version 11.5.

3. Results and Discussion

The effect of *Pleurotus* spp. on egg hatching and mortality of IJs of root-knot nematodes (*M. incognita*) were observed. The result found that all *Pleurotus* spp. in this study inhibited egg hatching of *M. incognita*. After 48 hours, PB-3 and PH showed the percentages of egg hatching as 13.33 ± 3.44% and 15.00 ± 3.75%, respectively with statistically significant difference (p<0.05) when compared with control (30.00 ± 5.16%) (Table 1). PB-3 and PH affected IJs of *M. incognita* as well. The percentage of mortality were 23.33 ± 3.85% and 18.33 ± 2.85%, respectively at 24 hours post inoculation with significantly (p<0.05) higher than control (6.67 ± 2.43%) (Table 2). Therefore, the mushroom PB-3 (*Pleurotus* sp. from Bhutan No. 3) and PH (*Pleurotus* sp. from Hungary) were selected for the next experiments.

Table 1. Effect of *Pleurotus* spp. on *M. incognita* egg hatching

Treatment	Percentage of egg hatching [1/]		
	24 h	48 h	72 h
PH	8.33 ± 2.85^{a}	15.00 ± 3.75^{ab}	31.67 ± 2.85^{a}
PC-1	13.33 ± 4.22^{a}	18.33 ± 2.85^{abc}	35.00 ± 5.09^{ab}
PC-2	15.00 ± 3.75^{a}	26.67 ± 5.44^{abc}	45.00 ± 3.75^{abc}
PS	10.00 ± 1.72^{a}	16.67 ± 1.72^{abc}	33.33 ± 2.43^{ab}
PB-1	15.00 ± 2.85^{a}	25.00 ± 2.85^{abc}	41.67 ± 4.47^{ab}
PB-2	16.67 ± 3.85^{a}	28.33 ± 4.47^{bc}	50.00 ± 7.10^{bc}
PB-3	5.00 ± 1.49^{a}	13.33 ± 3.44^{a}	28.33 ± 4.47^{a}
PB-4	10.00 ± 3.85^{a}	23.33 ± 3.84^{abc}	33.33 ± 4.87^{ab}
Control	16.67 ± 3.85^{a}	30.00 ± 5.16^{c}	60.00 ± 4.87^{c}

[1/] Mean of 5 replicates ± standard error and mean values indicated by the same letters within one column are not significant differences ($p > 0.05$) according to Duncan's new multiple range test. Where, *Pleurotus* sp. from Bhutan No.1 = PB-1, *Pleurotus* sp. from Bhutan No.2 = PB-2, *Pleurotus* sp. from Bhutan No.3 = PB-3, *Pleurotus* sp. from Bhutan No.4 = PB-4, *P. sajor-caju* = PS, *P. cystidiosus* No.1 = PC-1, *P. cystidiosus* No.2 = PC-2 and *Pleurotus* sp. from Hungary = PH

Table 2. Effect of *Pleurotus* spp. on *M. incognita* IJ mortality

Treatment	Percentage of mortality [1/]		
	24 h	48 h	72 h
PH	18.33 ± 2.85^{bc}	28.33 ± 4.22^{bd}	66.67 ± 11.67^{c}
PC-1	11.67 ± 3.75^{ab}	23.33 ± 2.98^{abc}	40.00 ± 2.43^{ab}
PC-2	10.00 ± 1.72^{ad}	21.67 ± 2.85^{abc}	30.00 ± 1.72^{ab}
PS	13.33 ± 2.43^{abc}	25.00 ± 3.75^{bcd}	45.00 ± 2.85^{b}
PB-1	11.67 ± 1.49^{ab}	21.67 ± 2.85^{abc}	31.67 ± 3.75^{ab}
PB-2	8.33 ± 2.85^{ab}	16.67 ± 3.85^{ab}	36.67 ± 3.85^{ab}
PB-3	23.33 ± 3.85^{c}	35.00 ± 3.85^{d}	71.67 ± 9.22^{c}
PB-4	13.33 ± 4.22^{abc}	23.33 ± 3.85^{abc}	41.67 ± 2.85^{ab}
Control	6.67 ± 2.43^{a}	11.67 ± 2.85^{a}	21.67 ± 4.47^{a}

[1/] Mean of 5 replicates ± standard error and mean values indicated by the same letters within one column are not significant differences ($p > 0.05$) according to Duncan's new multiple range test. Where, *Pleurotus* sp. from Bhutan No.1 = PB-1, *Pleurotus* sp. from Bhutan No.2 = PB-2, *Pleurotus* sp. from Bhutan No.3 = PB-3, *Pleurotus* sp. from Bhutan No.4 = PB-4, *P. sajor-caju* = PS, *P. cystidiosus* No.1 = PC-1, *P. cystidiosus* No.2 = PC-2 and *Pleurotus* sp. from Hungary = PH

In previous studies, *Pleurotus* spp. proved to be effective for controlling root- knot nematodes under *in vitro* condition by affecting juvenile mortality and egg hatching (Abbasi *et al*. 2014; Sufiatea *et al*. 2017). Moreover, the applications of *Pleurotus* spp. to control plant nematodes under *in vivo* condition were evaluated by Wille, Gomes, Minotto, and Nascimento (2019), the aqueous extracts of *P. pulmonarius* was applied to control *M. incognita* on lettuce seedling and found that the nematode reproduction was reduced at 70%, and it could reduce damage of lettuce roots. Analogously Youssef and El-Nagdi (2020), fifteen grams of fresh fruits of *P. ostreatus* per 100 mL distilled water achieved the highest percentages of reduction of *M. incognita* reproduction and galls of 86.4% and 92.4% on cowpea and improved plant shoot, root growth, and consequently cowpea yield as well.

Observations on fungal mycelium growing at the surface of the water agar, some liquid droplets were presented on the hyphae after 12 hours post incubation (Figure 1A) and these droplets involved nematode movements. The hyphae penetrated to the egg and IJ of the *M. incognita* and grew inside after inoculating 24 hours (Figure 1B, C). In observation of similar kinds by Barron and Thorn (1987) and Khan, Saifullah, Iqbal, and Hussain (2014), the droplet produced from *Pleurotus* spp. was toxic to nematodes (nematotoxin) called ostreatin. The toxins had nematode-stunning activity and the nematode was immediately paralyzed, after that the fungus secreted protease, a cuticle degrading enzyme, and fungal hyphae colonized the nematode and eventually digested the nematode (Barron & Thorn, 1987; Genier *et al*., 2015). Interestingly, the important mechanism of *Pleurotus* spp. when penetrating their mycelia to the nematode was releasing proteases to degrade proteins, the components of nematode cuticle, which affected the nematode growth and survival (Inácio *et al*., 2015).

Fungal mycelia of selected *Pleurotus* (PB-3 and PH) grew rapidly on PDA within three days with colony diameters of 7.0 ± 0.13 and 6.9 ± 0.07 cm, respectively when incubated at 28 °C (Figures 2-3). Thus, the optimal incubation temperature for the mycelial growth of selected *Pleurotus* was 28 °C when compared with 25, 30 and 35 °C. It was similar to the result reported by Hoa and Wang (2015) that the optimal temperature for mycelial growth of *P. ostreatus* and *P. cystidiosus* were obtained at 28 °C on PDA and yam dextrose agar (YDA), when observed at various temperatures, i.e., 16, 20, 24, 28, 32 and 36 °C.

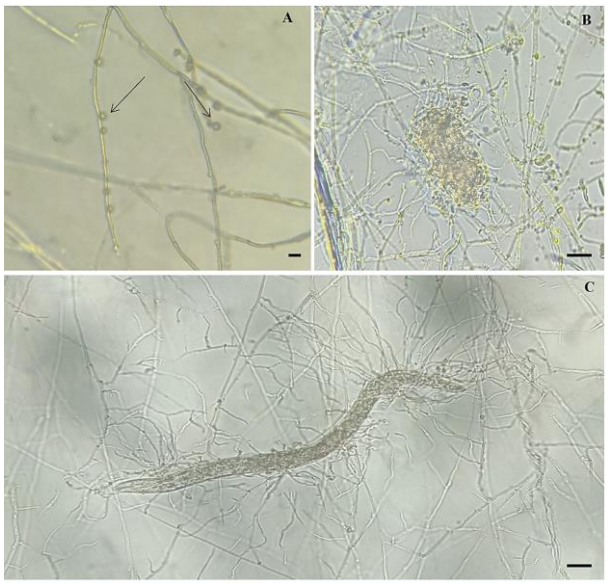
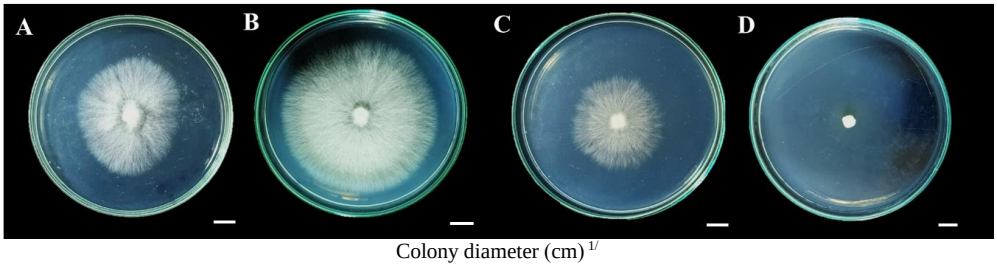


Figure 1. Hypha of *Pleurotus* sp. from Bhutan No. 3 (PB-3) that infected egg and infective stage juvenile of the *M. incognita* at 48 hours post inoculation. (A) the arrows point to droplets of toxin (B) infected eggs and (C) infected juvenile (Bar = 20 µm).

Chitinase was not exhibited from PB-3 and PH on 3-day post incubation, because a negative result was found on colloidal chitin agar medium (Figure 4). On the other hand, mushroom PB-3 and PH could produce protease with clear zones of 4.5 ± 0.2 and 1.9 ± 0.04 cm, respectively when observed on skim milk agar within three days (Figure 5). Similarity report of Bano, Dahot and Naqvi (2016) that *P. eryngii* could produce protease on solid agar. Genus *Pleurotus* produce proteases for their metabolic pathways, cellular signaling, cell growth, and immune defense in similarity to many organisms. In addition, *Pleurotus* spp. released protease when penetrated their mycelia to the nematode for degrading the components of nematode cuticle (Inácio *et al*., 2015) that collagen and collagen-like proteins (80% of total protein) constitute the vast majority of cuticular structural components (Page *et al*., 2014). The eggshells of root- knot nematode contained 50% protein and 30% chitin (Bird & Bird, 1991). Thus, there are many studies presented the efficacy of proteases to degrade eggshell and nematode cuticle. For



Colony diameter (cm) [1/]

25 °C	28 °C	30 °C	35 °C
4.6 ± 0.01 [b]	6.9 ± 0.07 [a]	3.9 ± 0.04 [c]	0.5 ± 0.00 [d]

Figure 2. Mycelial growth of *Pleurotus* sp. from Hungary (PH) on potato dextrose agar (PDA) within 3 days after inoculation at different temperatures; (A) 25 °C, (B) 28 °C, (C) 30 °C and (D), 35 °C (bar = 1 cm). [1/] Mean of 5 replicates ± standard error and mean values indicated by the same letters are not significant difference ($p > 0.05$) according to Duncan's new multiple range test.

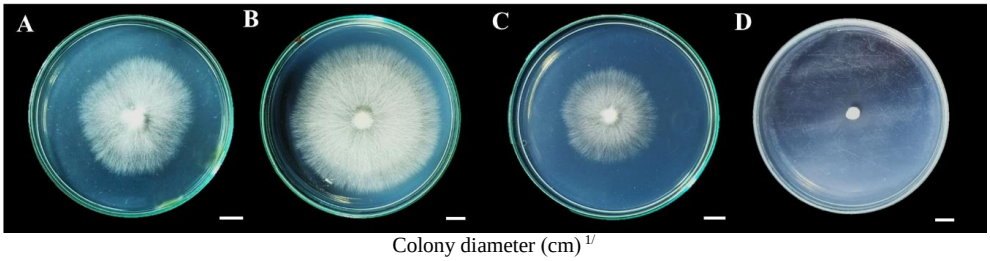


Figure 3. Mycelial growth of *Pleurotus* sp. from Bhutan No.3 (PB-3) on potato dextrose agar (PDA) within 3 days after inoculation at different temperatures; (A) 25 °C, (B) 28 °C, (C) 30 °C and, (D) 35 °C (bar = 1 cm). 1/ Mean of 5 replicates ± standard error and mean values indicated by the same letters are not significant difference ($p > 0.05$) according to Duncan's new multiple range test.

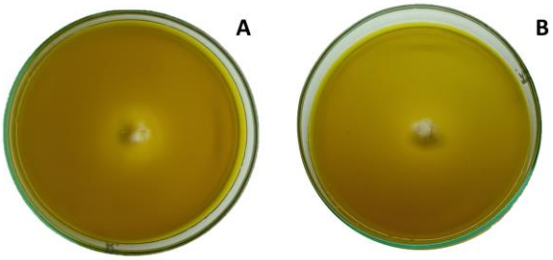


Figure 4. Detection of chitinase production on colloidal chitin agar, incubated at room temperature for 3 days; (A) *Pleurotus* sp. from Bhutan No.3 (PB-3) and (B) *Pleurotus* sp. from Hungary (PH)

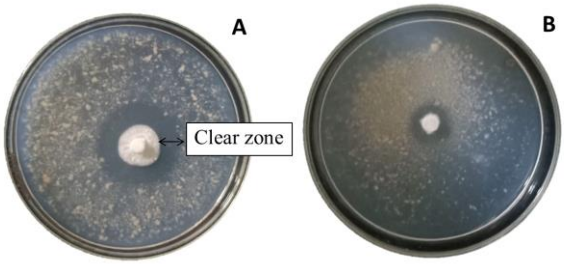


Figure 5. Detection of protease production on skim milk agar, incubated at room temperature for 3 days; (A) *Pleurotus* sp. from Bhutan No.3 (PB-3) and (B) *Pleurotus* sp. from Hungary (PH)

example, Bonants *et al.* (1995) reported that an egg-parasitic fungus, *Purpureocillium lilacinus*, secreted a protease and infected eggs of *M. hapla*. Genier *et al.* (2015) found that the number of juveniles of nematode *Panagrellus* sp. were decreased up to 95.20% within three days after inoculation with *P. ostreatus* and its proteases. Thongkaeyuan and Chairin (2018) suggested that the use of *Metarhizium* spores in combination with protease completely inhibited egg hatching of root-knot nematode *M. incognita* within 24 hour and the mortality of IJs increased to 100% at 48-hour post treatment.

In this study, the mushroom PB-3 and PH showed higher enzyme productivity under protein-enriched media cultivation. PB-3 produced 0.272 ± 0.02 U/mg protein of proteases on PDB with 1% casein. PH produced 0.171 ± 0.01 U/mg protein of proteases on PDB with 1% skim milk. These proteases activities were significantly increased from control (PDB) that proteases were not detectable (Table 3). Bano *et al*. (2016) mentioned that protease activity of *Pleurotus* sp. highly increased when it was cultured on PDB supplemented with casein at 4 days after inoculation. Likewise, Bano, Dahot, Naqvi and Qureshi (2018) studied on protease production by *P. sajor-caju* on PDB supplemented with skim milk under submerged culture and the maximum protease was observed after 72 hours post inoculation at 28 °C. In addition, Sufiatea *et al*. (2017) demonstrated that the dose of protease enzyme obtained from *P. eryngii* could kill root-knot nematodes up to 90% and degraded the nematode cuticle within 24 hours.

4. Conclusions

Pleurotus sp. from Hungary and *Pleurotus* sp. from Bhutan (No. 3) were selected for controlling *M. incognita* because they were effective against eggs and infective stage juveniles. Moreover, because of their interesting biological characteristics including (1) require short time of cultivation, (2) ability to secret nematode toxin; and especially, and (3) ability to produce protease, a nematode cuticle-degrading enzyme. Furthermore, the antagonistic potential to control root-knot nematode disease in the field needs to be conducted.

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Table 3. Protease activity, total protein and specific activity in cell-free culture filtrate of selected *Pleurotus*

Fungi	Media	Protease activity (U/ml) [1/]	Total protein (mg/ml) [1/]	Specific activity (U/mg protein) [1/]
PB-3	Control (PDB)	0.011 ± 0.01 [b]	0	0
	+ 1% casein	0.039 ± 0.01 [a]	2.195 ± 0.04 [b]	0.272 ± 0.04 [a]
	+ 1% skim milk	0.025± 0.004 [a]	2.886 ± 0.11 [b]	0.022 ± 0.001 [c]
PH	Control (PDB)	0.019 ± 0.004 [b]	0	0
	+ 1% casein	0.043 ± 0.004 [a]	9.408 ± 0.03 [a]	0.063 ± 0.00 [b]
	+ 1% skim milk	0.036 ± 0.001 [a]	0.386 ± 0.12 [c]	0.171 ± 0.01 [a]

[1/] Mean of 5 replicates ± standard error and mean values indicated by the same letters within one column are not significant differences ($p > 0.05$) according to Duncan's new multiple range test.

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