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Determination of protease and chitinase activities from *Paecilomyces variotii* NV01 isolated from Dak Lak pepper soil

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Abstract:

The filamentous fungus *Paecilomyces* is currently being developed as a biocontrol agent against plant parasitic nematodes, which often cause black leaf disease in pepper and coffee trees. Nematode eggs and cuticles are the infection sites for biocontrol agents that penetrate those areas by the production of lytic enzymes. *Paecilomyces variotii* NV01 was isolated from pepper soil from the Dak Lak province. The purpose of this work is to study the activity of protease and chitinase in the strain *Paecilomyces variotii* NV01. The protease activity was determined to be 82.3 U/ml in a 0.65% casein substrate concentration after 84 hours of culture, which was 6 folds higher than with a basal medium (12.7 U/ml). Exo-chitinase activity was found to be 37.28 U/ml after 84 hours of culture in a chitin substrate concentration of 0.75%, which is 4 folds greater than the basal medium. It was found that adding Ca^{2+} to the medium drastically altered chitinase activity, increasing it by 10.5%. A 5 mM concentration of Ca^{2+} was found to affect chitinase activity but not protease activity. Furthermore, the percentage of *Meloidogyne* sp. nematodes killed by *Paecilomyces variotii* NV01 after 96 hours of culture was 50.4%. Further study should be carried out in order to use this fungal strain to control plant parasitic nematodes.

Keywords: **chitinase, *Meloidogyne* sp., *Paecilomyces variotii* NV01, protease.**

Classification numbers: **3.1, 3.5**

Introduction

Valuable industrial crops such as pepper and coffee trees are often attacked by nematodes. Peppers growing in areas such as Dak Lak are often infected by nematodes affecting them with yellow, curly leaves and swollen roots that are unable to get nutrients. There is increasing opposition to the use of chemical pesticides for these nematodes because of undesirable environmental side effects. Thus, other methods of biological control are of interest to scientists. For example, fungus can be used as traps, nematode egg parasites, or as drop toxins to kill nematodes. Specifically, *Paecilomyces* sp. has been found as a parasitic egg nematophagous-fungi [1]. Similarly, Dackman, et al. (1989) [2] investigated fungal egg parasites, isolated from the eggs of the cyst nematode *Heterodera avenae*, with respect to their ability to infect cyst nematode eggs of *H. scbachtii*.

The mechanism of the infection process by egg-parasitic fungi may be either mechanical, enzymic, or both. Extracellular enzymes, including serine proteases and chitinases, are shown to be important virulence factors that can degrade the main chemical constituents of the nematode cuticle and eggshell [3]. These enzymes, which play an especially important role during fungal infection against nematodes, can break down the physical and physiological integrity of the cuticles of nematodes and help fungal penetration and colonization [4, 5]. To date, few attempts have been made to determine the effects of purified fungal enzymes on the eggshell of the root knot nematode. Stirling & Mankau (1979) [6] studied the enzymes of nematode egg-parasitic fungus *D. oviparasitica* in a liquid culture supplemented with colloidal chitin. These authors found chitinase activity and suggested this enzyme play a role in penetrating the eggshell, which consists partly of chitin. The cuticle degrading protease P32 was identified from the nematode egg-parasitic fungus *Pochonia rubescens* (syn. *Verticillium suchlasporia*) in 1990 [7].

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In a previous article, we isolated and sequenced based on the ITS1- 5.8s - ITS2 of NV01 and determined the ability to produce chitinase and protease extracellular enzyme by means of diffusion on agar of *Paecilomyces variotii* NV01. The purpose of the present study is to investigate whether isolated protease and chitinase might play a role in the penetration of eggshells and therefore would be vital to the use of fungi as biological control agents.

Materials and methods

Materials

Paecilomyces variotii NV01 was isolated from pepper soil in the Dak Lak province as per Binh, et al. (2017) [8]. The stock culture was maintained on PDA (Potato-Dextrose-Broth) slants kept at 4^0C.

Meloidogyne sp.: Institute of Ecology and Biological Resources - Vietnam Academy of Science and Technology.

Medium: Czapeck-Dox (CD); C PDB (Potato-Dextrose-Broth); CMA (Corn-Meal-Agar) (Merck); pH 7; T1: CD added 0.65% casein; T2: CD added 0.75% chitin and 5 mM $CaCl_2$ Chitin (BioBasic); Casein: 0.25-0.85% (BioBasic); $CaCl_2$ (Merck).

Chemicals (to determine enzyme activity): Trichloro Aceticacid (TCA) (Merck); DNS (3,5 - Dinitrosalicylic acid (BioBasic, Canada); Folin-Ciocalteau reagent (Merck); Na_2CO_3 (BioBasic, Canada); Coomassie Brilliant Blue G-250 (Merck); Ethanol (Prolab).

Preparation of colloidal chitin: colloidal shrimp cuticle was used as substrates for chitinase activity production. Shrimp cuticle and commercial chitin were treated with phosphoric acid (10 g/100 ml) overnight. Then, several water washings and filtrations were performed until a pH 7 was reached. Substrates were then sterilized and kept at 4^0C (Chávez-Camarillo and Cruz-Camarillo, 1984) [9].

Methods

Enzyme qualification: Paecilomyces variotii NV01 was cultured on PDB, CMA, Czapeck-Dox media at 28-30^0C for 3-5 days in shaking flasks at 200 rpm. The crude enzyme was obtained by centrifuging the culture broth at 10,000 rpm to remove biomass.

Chitinase activity was found through the measure of the reducing sugars using DNS (3,5 - Dinitrosalicylic acid) method (Miller, 1959) [10]. Chitinase activity was measured at 540 nm using a spectrophotometer (SHIMADZU) and calculated by a standard curve based on different concentrations of N-acetyl-glucosamine (Sigma). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 mmol/l of N-acetyl-glucosamine ($y = 0.001x + 0.041$), where y is optical density - OD, x is N-acetyl glucosamine concentration (µg/ml) with $R^2=0.9939$.

Protease activity was essentially determined according to Manachini, et al., 1988 [11] at 37^0C, pH 7.5 in 50 mM phosphate buffer. Dialyzed sample (1 ml) was added to 5.0 ml of casein (0.65%) solution and incubated at 37^0C for 10 min. The reaction was arrested after the incubation period by adding 5.0 ml of Trichloro Acetic acid (110 mM). The residual protein was precipitated after 30 min of incubation and then was pelleted by centrifugation at 1000 x g for 5 min. The supernatant (2 ml) was utilized for a color reaction using Na_2CO_3 (5 ml) and Folin-Ciocalteau reagent (1 ml). The blue color developed was read at 660 nm on UV spectrophotometer. One unit of protease activity is defined as the amount which liberates 1 µg of tyrosine min^{-1} under experimental conditions ($y = 0.904x + 0.0017$), where y is the optical density - OD and x is tyrosine concentration (µg/ml).

Protein content of the culture suspension was determined according to the method of Bradford (1976) [12] using BSA as the standard.

Investigation of the effect of casein concentration on protease activity: the culture medium was supplemented with casein concentrations between 0.25-0.85%. From those experiments, the casein concentration with the highest protease activity was selected. The culture solution was shaken in a 500 ml capacity flask with 100 ml of shaking solution at a speed of 200 rpm at a temperature between 28-30^0C. Each experiment was repeated 3 times.

Effect of incubation P. variotii NV01 *in protease production*: the culture suspension was collected at various times between 24 and 120 h to determine the protease activity and protein content.

Investigation of the effect of chitin concentration on chitinase activity: similar to protease activity experiments, the culture medium was supplemented with chitin concentrations between 0.25-0.85%.

Detection of nematode killing ability (Khan, et al., 2006) [13]: *Paecilomyces variotii* NV01 strain was cultured on T1 and T2 medium, respectively, shaking for 84 h at 30±2^0C at 200 rpm. The culture broth was filtered using Whatman N°1 paper to remove the mycelium. Then, 300 nematodes of *Meloidogyne* sp. were added to 5 ml of culture broth and kept for 96 h at 30^0C. The number of nematodes were counted every 24 h by using a counting dish and counting machine under the microscope. Microscope slides of nematodes

were prepared to determine the dead nematodes, which had a violet colour after staining with Phloxin B reagent.

Results and disscusion

According to the results of the previous article [8], the NV01 strain has extracellular chitinase and protease activity. By diffusion on CD agar plates, the valuable characteristics of this strain can be seen. In this study, we continue to shed light on chitinase and protease activity as well as factors related to the culture medium's affects on enzyme activities.

Selection of protease biosynthesis medium

The selection of optimal culture medium for protease biosynthesis in the NV01 strain is the aim of this study. The protease activity results from the PDB, Czapeck-Dox, and CMA culture media under the culture conditions described in the methods section are presented in Fig. 1. The CD medium had the highest protease activity. The results showed that the CD medium contains a number of mineral elements necessary for protease biosynthesis, which increases protease activity.

The CD medium with the highest protease activity was selected for further studies to find the optimal casein substrate concentration for the NV01 strain.

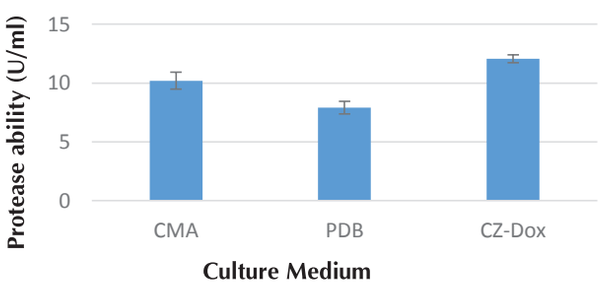

Fig 1. Protease biosynthesis ability.

Effect of casein substrate concentration of Paecilomyces variotii NV01

Figure 2 shows that the NV01 strain has the highest activity with 82.3 U/ml in a concentration of 0.65% casein after 84 h of incubation (Fig. 3). These results are 6 folds higher than with a basal medium (12.7 U/ml) and 1.5 folds higher than the results of Deore, et al. (2013) [14]. In that work, the strain *Paecilomyces variotii* PR-4 was used and it showed that protease activity was 53 U/ml between 96-120 h. Our results suggest that the protease activity of NV01 is the basis for research into applications that kill nematodes or decompose organic matter in soil into an easily absorbed organic ion form.

According to the studies of Khan, et al. (2004) [15] the ability to kill nematodes using the strain *Paecilomyces lilacinus* 251 was based on both protease and chitinase activities.

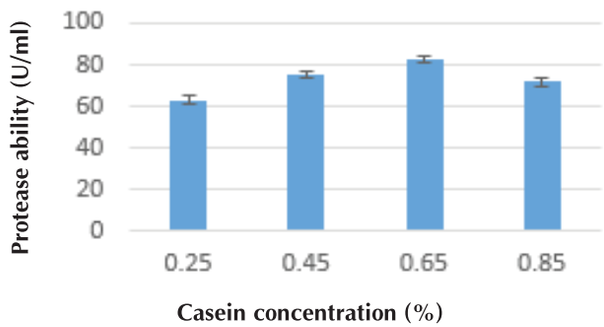

Fig. 2. Effect of casein concentration.

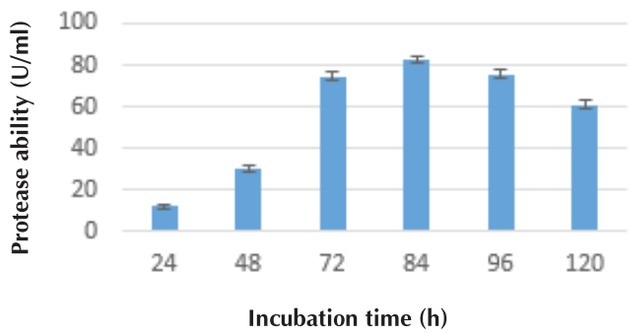

Fig. 3. Incubation time effect.

Effect of chitin substrate concentration

Based on the survey of protease-producing medium, the Czapeck-Dox (CD) medium had a higher protease activity than PDB and CMA. In this experiment, the CD medium was the control medium. According V.N. Nguyen, et al. (2009) [16], the chitinase activity from the culture liquid of *P. variotii* DG-3 was 3.8 U/ml after 12 days with 0.5% chitin added. On that basis, we study the effect of chitin substrate concentration on the chitinase biosynthesis ability of strain NV01. Based on the results of protease activity from NV01, a chitin concentration of 0.25-1% was added to the CD medium.

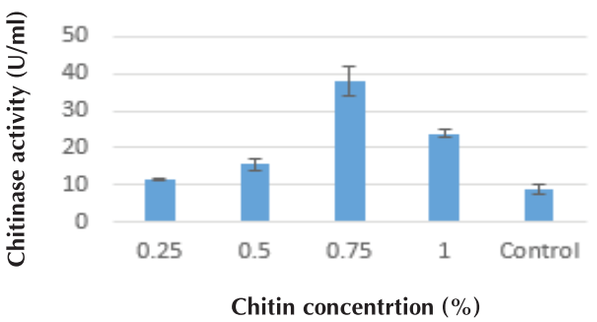

Fig. 4. Chitin substrate concentration.

The results in Fig. 4 show that the chitinase activity of NV01 in the medium supplemented with 0.75% chitin was highest after 84 h of incubation, which is 4 folds higher than the control CD medium. The chitinase activity of the 0.5% chitin substrate was 12.3 U/ml after 84 h. This result is 3 folds higher than what was reported by V.N. Nguyen, et al. (2009) [16] using a 0.5% substrate supplement and 10 folds higher than the 0.75% substrate. The culture time was shortened by 1/3 (for research by V.N. Nguyen, et al. (2009) [16] was 12 days.

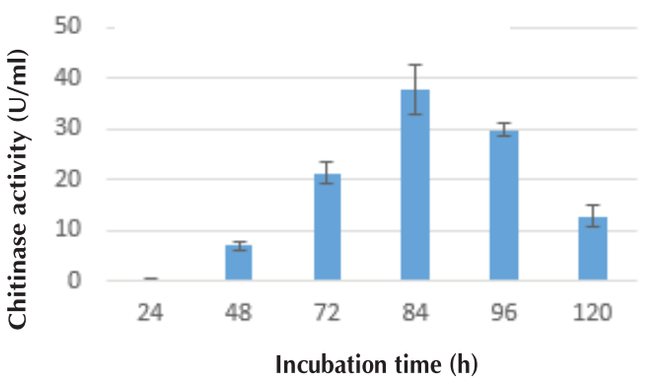

Fig. 5. Incubation time effect.

In Fig. 5, from 72 h to 84 h of culture, the chitinase activity of NV01 strain increased. The enzyme activity decreased after 96 h of culture. The harvest time for chitinase is 84 h. After 84 hours of culture, the metabolites in fungi product is made up of many chitinase activity inhibitors. At the same time, due to the metabolic process of fungi, some trace elements in the medium, which the device needs, has been exhausted [17, 18].

Effect of Ca^{2+} on chitinase and protease activity

Due to its innate ability to produce extracellular enzymes, *Paecilomyces variotii* provides eco-friendly solutions for a variety of biotechnological applications and is a potential source of industrial bioproducts. In addition to substrate concentration and culture conditions, typical trace elements such as Ca^{2+} play an important role in the enzyme activity of microorganisms. Therefore, the culture medium of strain NV01 was supplemented with 5 mM of $CaCl_2$.

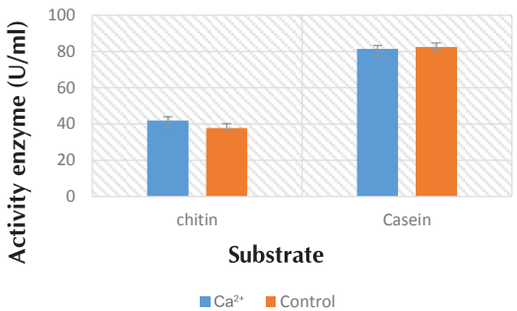

Fig. 6. Influence of Ca^{2+} on enzyme activity of NV01 strain.

Figure 6 shows that chitinase activity increased significantly when compared to the control (without supplementing Ca^{2+}). These results also coincide with studies of chitinase from microorganisms by Kassa (2017) [19], and Annamalai, et al. (2010) [20]. However, protease activity was not influenced and protease does not require $CaCl_2$ for its stability. Our results also show that Ca^{2+} does not influence protease activities. These results are similar to those of protease activity from the strain *Paecilomyces fomosoroseus* reported by Castellanos, et al. (2008) [21].

Perveen and Shahzad (2013), Khan, et al. (2003), and Kopparapu, et al. (2012) [22-24] studied chitinase activity on the *Paecilomyces* species, which is capable of killing nematodes and is used in biological control. Chitinase from *Trichoderma atroviridae* has been introduced to control the golden potato cyst nematode. It can also be used for chitinase enzyme production, which is supposed to be used in commercial formulation [25]. This is the basis for conducting further studies, such as the investigation of nematode killing ability.

Test of killing ability of nematodes

Table 1. Insecticide results of *Paecilomyces variotii* NV01.

Number	Strain	Nematode death rate (%)				
		24 h	*48 h*	*72 h*	*96 h*	*120 h*
0	Control (H_2O)	-	-	-	-	-
1	*Paecilomyces variotii* NV01 (T1 - Protease)	-	2.08	12.74	35.8	35.8
2	*Paecilomyces variotii* NV01 (T2 - Chitinase)	-	1.75	10.7	23.65	23.65
3	*Paecilomyces variotii* NV01 (Protease and Chitinase)	-	2.31	15.6	50.4	50.4

Note: (-) did not die.

Table 1 shows that incubation of the protease and chitinase together had a higher efficiency than T1 and T2 alone. Genier, et al. (2016) [26] aimed to evaluate the action of *Paecilomyces marquandii* proteases on *Ancylostoma* spp L_3. Protease from L_3 reduced action by 41.4%. The NV01 strain between 72-96 h tested the pathogenicity of *Meloidogyne* sp. and destroyed 50.4%. According to the study by Perveen and Shahzad using *P. lilacinus*, *P. variotii*, and *P. fumosoroseus* incubated with the larvae of *M. incognita*. After 24, 48, and 72 h, the number of larvae was counted. Results showed that after 72 h, the ability to kill nematodes was 75% effective [21]. In order to be able to use the fungus for the application of plant parasitic nematode control, further research is needed to improve the activity and appropriate culture conditions. .According to Al-Assas, et al. (2011) [27], *Paecilomyces variotii* reduced the number of nematode root galls and showed the superiority of pesticides based on plant extract over chemical pesticides. The fungus showed a high effectiveness (91.5%) in controlling root-knot nematode, while it was 96.4, 99.7, and 98.9% in DMAC, diazinon and plant extract, respectively. Ahmad, et al. (2019) [28] indicated that out of the five strains of fungus *Paecilomyces variotii*, *Paecilomyces lilacinus*, *Duddingtonia flagrans*, *Trichoderma harzianum*, and *T. asperelum*, only *D. flagrans* could not kill the egg in an in vitro egg culture of *Meloidogyne* sp..Among the remaining 4 strains, *P. lilacinus* showed a significant reduction in eggs by 67.9%, meanwhile the potency of *P. variotii* could kill only 25.1% of the eggs.

Conclusions

Protease activity of *P. variotii* NV01 reached 82.3 U/ml within 84 h of incubation. When 0.65% casein was added, the protease activity was 6 times higher than the basal medium. It is interesting to note that protease activity was not significantly altered by the addition 5 mM of $CaCl_2$ concentration.

Chitinase activity of *P. variotii* NV01 reached 37.28 U/ml within 84 h of incubation with 0.75% chitin, which is 4 folds more than the basal medium. Unlike protease, the chitinase activity increased by 10.5% with the addition of 5 mM $CaCl_2$.

By the method of testing for infectious nematode *Meloidogyne* sp. with *Paecilomyces variotii* NV01, the efficiency reached 50.4% within 96 h. This is the basis for further research on the NV01 strain.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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