

Production of extracellular enzymes in the entomopathogenic fungus *Verticillium lecanii*

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Abstract:

This study investigates the mechanisms as well as strategies for purification and characterization of potential enzymes involved in pathogenesis of entomopathogenic fungi. The test strain of *Verticillium lecanii* that was screened, during the present investigation, proved to be an efficient producer of protein and polysaccharide degrading enzymes (amylase, protease, and lipase), hence indicating versatility in biochemical mechanisms. Halo zones produced colony growth of *V. lecanii* on agar confirmed activity of protease, amylase and lipase enzyme by the *V. lecanii* isolate. Enzymatic Index (EI) observed were: Protease – 2.195, Amylase- 2.196, Lipase- 2.147. Spectrophotometric analysis of enzymatic activity of *V. lecanii* at five different pH – 3, 5, 7, 9, 11 revealed that highest proteolytic activity of the *V. lecanii* isolate was reported at pH 7 and 9 whereas proteolytic activity was minimum at acidic pH 3. Maximum amylolytic activity of *V. lecanii* on the 7th day of inoculation was at pH 3 i.e. in an acidic environment in contrast to neutral pH 7. Maximum lipolytic activity of *V. lecanii* was found at pH 7. Since enzyme production in entomopathogenic fungi is specific and forms an important criterion for successful development as well as improvement of mycoinsecticides, hence a significant conclusion from the present analysis is the degree of variation in secretion of enzymes in test strain of *Verticillium lecanii*.

Key words: *Verticillium lecanii*, Mycoinsecticides, Protease, Amylase, Lipase, Enzymatic Index.

Background:

One of the major challenges in limiting pest population below threshold level is enhanced resistance of insects and pests towards chemical pesticides. The pests mutate and develop resistance to newer molecules. Biological control of insect pests has generated interest among farmers for sustainable management. Entomopathogenic fungi possess the ability to active penetration through cuticle by mechanical pressure of invasive hypha and production of proteo-, chitino- (exo- and endochitinases) as well as lipolytic enzymes, which provide nutrients for subsequent development of fungus. Important barriers to invading micro organisms include enzymes, chitin and associated lipids, found in insect integument [1]. Pathogenesis requires secretion of hydrolytic enzymes like protease, amylase, lipase, for penetration through these barriers [2]. Entomopathogenesis entails involvement of proteases, chitinases and lipases [3], involved in hydrolysis of ester bonds of fats and waxy layers of the insect integument. Due to

lipolytic activity, free fatty acids are released, thus contributing to host adhesion. Lipids are primarily first barriers in epicuticle acting against pathogenic microbes, thus reinforcing the importance of these enzymes in penetration and hence infection. The aim of this work is to explain the mechanisms involved in extraction and purification of extracellular enzymes produced during pathogenic action in entomopathogenic fungi.

Methodology:

Isolation of the pathogen

Routine collection of water samples was made at regular intervals from insect breeding sites. Isolation of the entomopathogenic fungus *Verticillium lecanii* from natural water was frequently made on SMYA medium. Cultures for preparation of conidia were seeded onto SMYA (contained (in g⁻¹) peptone 10; maltose 40; yeast extract 10 and agar-agar 20) plates and incubated at 28 ± 1°C for 7-10 days

Identification

Slide culture technique was adopted to identify the pathogen. A loopful of conidial suspension was inoculated on plates and incubated at $28 \pm 1^\circ\text{C}$ for 7-10 days. On sporulation, slides were stained with cotton blue and examined under Nikon microscope for identification.

Biochemical studies

Fungal enzymes play an important role in host infection since they can be localized on infection structures of both necrotrophic and biotrophic mycoparasites [4, 5]. Hence, quantitative assay was performed to estimate activities of protease, amylase and lipase enzymes of *Verticillium lecanii*.

Estimation of proteolytic activity of *Verticillium lecanii*

Protease activity of *Verticillium lecanii* was determined by two methods: Spectrophotometric analysis – Kunitz (1947) [6]; Plate assay method [7]: Production of proteolytic enzymes by *Verticillium lecanii* was detected by using gelatin as protein source in growth medium. The fungal strain was spot inoculated in petridishes with nutrient agar medium supplemented with 1% gelatin (Peptone, 5g; Beef extract, 3g; NaCl, 5g; Agar, 15g; Distilled water, 1 liter, pH 6). Prior to inoculation, the petridishes were incubated at $28 \pm 1^\circ\text{C}$ for 7 days. After a week of incubation, gelatin degradation was observed as a clearing zone around fungal colonies. This zone of gelatinolysis was seen clearly upon flooding the plate with aqueous saturated solution of mercuric chloride reagent (15g HgCl_2 dissolved completely in 20 ml 7M conc. HCl, then raised to 100 ml with sterile distilled water). Mercuric chloride solution reacted with gelatin to produce a white precipitate which made the clearing zone visible. The clearing zone was measured indicative of the extracellular protease activity of the fungal strain. Enzyme activity was measured by the following formula: $\text{EA} = \text{D} - \text{d}$; D- diameter of colony plus clearing zone; d- diameter of colony.

Spectrophotometric (Quantitative) analysis (Kunitz 1947)

Minimal broth supplemented with 1% gelatin was used as the growth medium. Erlenmeyer flasks with capacity 150 ml were dispensed with 25 ml broth each. These flasks were inoculated with spore suspension of *Verticillium lecanii* and were incubated at $28 \pm 1^\circ\text{C}$ in an incubator. The enzyme activity was measured at the end of 7th, 14th and 21st day. Aliquots were aseptically removed from the flasks and were centrifuged at 5000 rpm for 5 minutes. The supernatant was used as crude enzyme extract (CEE). To determine extracellular protease activity, reaction mixture was prepared by mixing crude enzyme extract- 1 ml and Buffered casein stock solution (1g casein in 100 ml of 0.1 M PO_4 buffer)- 1 ml. This reaction mixture was incubated in water bath set at $36 \pm 1^\circ\text{C}$ for 60 minutes. At the end of one hour of incubation, the reaction was terminated by addition of 3 ml of TCA which precipitated the unhydrolysed casein. Precipitate was removed by centrifugation and to the supernatant, 5 ml Na_2CO_3 (0.4 M) and 0.5 M Folin's reagent were added. These were incubated at room temperature for 20 minutes and enzyme activity was read as absorbance at 660 nm in UV- VIS Spectrophotometer. Control was prepared by taking 1 ml distilled water in place of crude enzyme extract.

Characterization of Fungal Proteases

Protease assay was performed by varying the cultural conditions in terms of pH of medium keeping the temperature

constant. Minimal broth supplemented with 1% gelatin was employed as the growth medium and pH of the broth was maintained at 3,5,7,9 and 11 respectively. The minimal broth was inoculated with 1 ml spore suspension of *Verticillium lecanii* and all flasks were incubated at $28 \pm 1^\circ\text{C}$. Proteolytic activity of the fungal strain was measured quantitatively at the end of 7th, 14th and 21st day and absorbances were observed in spectrophotometer at 660 nm.

Estimation of amylolytic activity of *Verticillium lecanii*

Production of amylase by the fungal strain was assessed by two parameters viz Plate assay method and Spectrophotometric method.

Plate assay method

Starch was employed as a carbohydrate source in the medium. The fungal strain was spot inoculated in petridishes with minimal media supplemented with 1% starch. These plates were incubated at $28 \pm 1^\circ\text{C}$ for one week. At the end of incubation period, colony diameter was measured and plates were flooded with iodine reagent (65 mg Iodine crystals and 130 mg KI in 100ml sterile distilled water). A clearing zone developed immediately around the colony which was measured. Enzymatic activity was measured by the following formula: $\text{EA} = \text{D} - \text{d}$; D- diameter of colony plus clear zone; d- diameter of colony.

Spectrophotometric (quantitative) analysis

Growth medium employed 1% starch supplemented in minimal broth. The broth was dispensed in Erlenmeyer flasks (150 ml capacity), autoclaved and inoculated with equal concentration of conidia of *Verticillium lecanii*. These flasks were incubated at $28 \pm 1^\circ\text{C}$ and enzyme activity was measured at the end of 7th, 14th and 21st day of incubation. Aliquots were aseptically removed from flasks and were centrifuged at 5000 rpm for 5 minutes. The supernatant was used as a crude enzyme extract (CEE). This appropriately diluted enzyme was mixed with equal amount of starch solution (1 ml) and reaction was allowed to proceed by incubation at 27°C for 15 minutes. At the end of incubation period, reaction was stopped by addition of 6 ml water. Enzyme activity was observed as absorbance at 540 nm and readings were recorded. Control tubes were prepared by addition of distilled water in place of CEE.

Characterization of fungal amylases

Amylolytic capability of the fungal strain was assessed at different pH (keeping the temperature constant). pH of minimal broth (supplemented with 1% starch) was adjusted to 3,5,7,9 and 11 respectively. Inoculation of the broth was done with equal concentration of spore suspension followed by incubation at $28 \pm 1^\circ\text{C}$. Quantitative estimation of amylase activity was done at the end of 7th, 14th and 21st day of incubation by the protocol described above and observations were reported in terms of absorbances.

Estimation of lipolytic activity of *Verticillium lecanii*

Production of the enzyme lipase by the test fungal strain was also considered as an important parameter in biochemical analysis. Sorbitan monolaurate (Tween-20) was used as a lipid substrate. Lipolytic activity of *Verticillium lecanii* was analysed by the following two methods: A) Plate assay method; B) Titrimetric assay.

Plate assay method

Production of the enzyme lipase by the fungal strain was confirmed through this method. Growth medium supplemented with 1% Tween-20 contained peptone 10; NaCl 5; CaCl₂ 0.1; Agar 20 g l⁻¹. Petridishes with sterile solidified media were spot inoculated with *Verticillium lecanii* and was incubated at 28 ± 1°C for one week. Zone around the fungal colony developed as a visible precipitate. This zone formation was attributed to the production of fatty acids from lipid substrate due to lipolytic activity of the fungus. Diameter of this zone was noted.

Titrimetric method

For determination of lipase activity, the test fungal strain was grown on basal medium (KNO₃ 0.1g, MgSO₄.7H₂O 0.025g, ZnSO₄.7H₂O 0.022g, FeSO₄.7H₂O 0.055g, Tween -20 1% , Distilled water 50 ml). This medium was inoculated with equal amounts of inoculum. Lipase activity was determined by titrimetric assay [8] with 0.05 M NaOH using emulsified oil as substrate. 1 ml of appropriately diluted enzyme was added to 5 ml of emulsion containing 25% (v/v) olive oil, 75% (v/v) gum acacia and 2 ml of 10 mM phosphate buffer at pH 7. The assay was carried at 37°C and 30 minutes incubation. Reaction was stopped by addition of 15 ml of acetone /ethanol (1:1) (v:v). The amount of fatty acid liberated due to enzyme activity was then titrated against 0.05M NaOH till the colour of reaction mixture changes to pink. One unit of lipase was defined as the enzyme that liberated 1µmol of fatty acid per minute at 37°C, pH 7. One ml of titration volume is equal to 2.5 units of lipase. Observations were recorded at the end of 7th, 14th and 21st day. Substrate buffer (5 ml) contained Olive oil 1.25 ml, Gum acacia 0.25g, Distilled water 3.75 ml whereas Indicator contained Phenolphthalein in alcohol + water (1:1).



Figure 1: Colony Morphology and Growth characteristics of *V. lecanii*.

Discussion:

Growth studies of *Verticillium lecanii*

Integrating an organism for microbial control in a pest management strategy requires basic studies such as isolation, ISSN 0973-2063 (online) 0973-8894 (print) Bioinformation 9(5): 238-242 (2013)

culturing, biological testing and prediction of it's effects on the pest population and the environment. A greater adoption will require, among other important aspects, a predictable performance under challenging environmental conditions, for example cool or warm weather, and a higher production efficiency [9]. Studies on biochemical aspects of *V. lecanii* have been done by Lopez- Llorca et al (1999) [10] and substantial results have been obtained.

Identification of *Verticillium lecanii*

V. lecanii attacks a wide range of insects and is grouped into the extremely diverse aggregate species. The present isolate of *V. lecanii* was maintained on SMY agar at 28±1 °C. It was observed that colonies of *V. lecanii* on SMY agar media grow white or pale yellow, cottony or velvety, turning cream in colour with mealy appearance and gradually to purplish pink pigmentation (Figure 1). Mycelium was composed of septate, branching hyphae, hyaline or light coloured, conidiophores erect, septate, simple or branched. Phialides formed either singly or in whorls of 3-4.

Biochemical profile of *V. lecanii*

Protein, chitin and lipids form the major composition of insect cuticle, the prime barrier to infection. For degradation of chemical constituents in cuticle, extracellular enzymes are secreted by entomopathogenic fungi. Infection process is facilitated by cuticle degrading enzymes. Entomopathogenic fungi produce protease, chitinase and lipase which can degrade insect cuticle [11, 12]. During fungal infections, a range of hydrolytic enzymes are secreted to help promoting host colonization. Depending on the ecological niche occupied by each fungus, a particular set of enzymes mainly composed of proteases and carbohydrases, are displayed to degrade specific tissues and scavenge for nutrient sources [13]. Because these enzymes work outside the fungal cell, activity as well as mechanisms that control synthesis and secretion are under the influence of several environmental factors such as ambient pH [14].

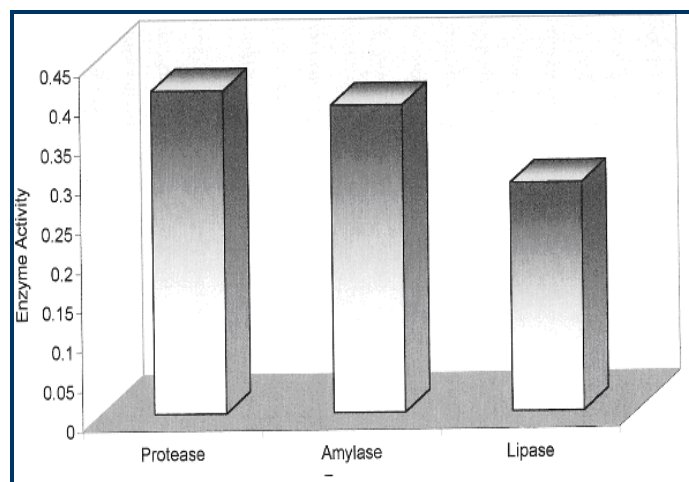


Figure 2: Comparative analysis of extracellular enzyme activity (Amylase, Protease and Lipase) in *V. lecanii*

Enzyme assay at different pH

Differential expression of gene governing survival within a particular niche, is regulated by pH as an important environmental factor. In the present investigation, enzyme activities (protease, amylase and lipases) were assessed at

different pH conditions of the medium as pH has a considerable effect upon enzyme production and hence enzyme activity of the fungus.

Protease activity at different pH

Proteases are very important enzymes required in large amounts for complete digestion of insect cuticle complex. Activity of proteases in the test fungal strain was estimated by two methods:

Method of Hankin and Anagnostakis (1975)

Enzyme production by the *V. lecanii* isolate was confirmed by using gelatin as substrate whose degradation could be evaluated by addition of HgCl₂ on solid medium. Halo zones produced colony growth of *V. lecanii* on agar confirmed activity of protease enzyme by the *V. lecanii* isolate. These halo zones were measured as indices of protease activity (Figure 2). This result is in accordance with the results given by Lopez Llorca *et al.* (1999) [10] who determined production of extracellular enzymes by *V. lecanii* and found out that *V. lecanii* showed high proteolytic activity. Enzymatic activity (EA) was calculated by the following formula (Jackson *et al.*, 1985):

$$\text{Enzyme activity (EA)} = \frac{\text{Colony Diameter}}{\text{Diameter of halo zone}}$$

Lopez Llorca *et al.* (1999) [10] also deduced that proteolytic activity with respect to time was highest for *Verticillium lecanii* i.e. reaching peak values at only two days after inoculation on the plates. The results obtained in the present investigation are also in accordance with results of Gomez- Santiesteban *et al.* (2004) [15] who showed that after 16 hours of fermentation of *V. lecanii*, proteolytic activity was reported in the form of degradation zones on agar.

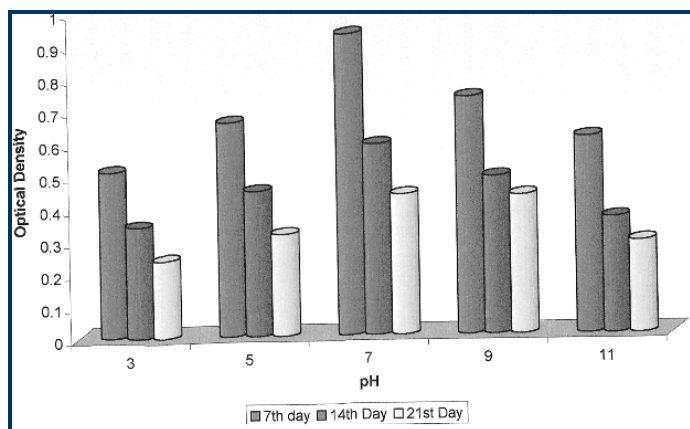


Figure 3: Protease activity of *Verticillium lecanii* at different pH

Spectrophotometric analysis by Kunitz (1947)

Proteolytic activity of *V. lecanii* isolate was measured spectrophotometrically at five different pH – 3, 5, 7, 9, 11 on 7th, 14th and 21st day of incubation. Highest proteolytic activity of *V. lecanii* isolate could be seen at pH 7 and 9 on the 7th day of inoculation whereas proteolytic activity was minimum at acidic pH 3 (Figure 3). Hence the test strain portrayed an increasing trend in protease production, starting from the acidic range, thereby reaching a maximum at neutral pH and declining again on the alkaline side only upto 7th day of incubation and then gradual decrease in enzyme activity upto 21st day. St. Leger *et al.* (1999) [16] investigated the influence of *M. anisopliae* on the environmental pH and its influence on protease production.

They concluded that protease production reduced by acidification of medium (acidic pH).

DeMoraes *et al.* (2003) [17] analyzed secretion of proteases by *M. anisopliae* in single and combined C/N sources and detected good proteolytic activities in all carbon sources tested. Yang *et al.* (2005) [18] produced an extracellular protease (Ver112) from *Lecanicillium psalliotae* on SDS-PAGE with a molecular mass of 32kDa. Optimum activity of Ver112 was at pH 10 and 70°C.

Amylase activity at different pH

Extracellular enzymes capable of carbohydrate degradation in plant tissues are produced by pathogenic fungi. Plant tissues are analogous in structure to insect cuticle (containing fibrous chitin or cellulose within a matrix of protein, pectic substances or hemicelluloses). All enzymes are secreted in a pH dependent manner by all isolates. Ambient pH seems to be a general factor controlling enzyme secretion in fungus – host interactions through a conserved genetic circuit [19]. In the present investigation, amylase activity was assessed by two methods:

Hankin and Anagnostakis (1975)

This method of plate assay employed starch as the substrate whose breakdown was confirmed by iodine solution, hence appearance of blue colour upon addition of iodine to the colonial growth of *V. lecanii*. Amylolytic activity of *V. lecanii* was confirmed by appearance of the halo / clearing zone whose diameter was measured (Figure 2).

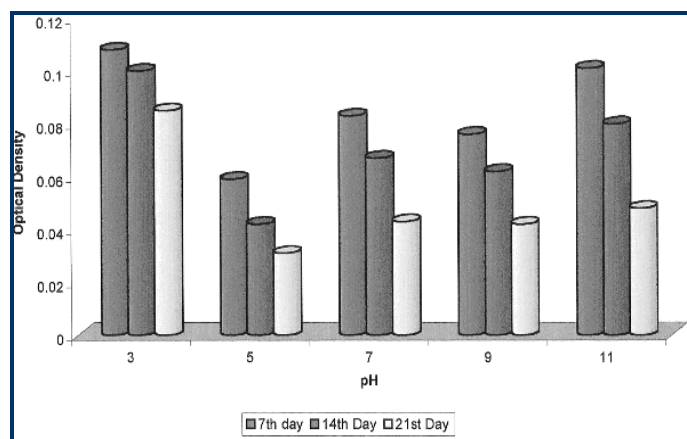


Figure 4: Amylase activity of *Verticillium lecanii* at different Ph

Miller (1959) - Dinitrosalicylic acid method

Spectrophotometric analysis was conducted for quantitative estimation of amylase activity in the *V. lecanii* isolate at five different pH – 3, 5, 7, 9, 11 on 7th, 14th and 21st day of incubation. It was observed that maximum amylolytic activity of *V. lecanii* on the 7th day of inoculation was at pH 3 i.e. in an acidic environment in contrast to neutral pH – 7. Activity gradually decreased upto 21st day (Figure 4). Valadres –Inglis *et al.* (1997) [20] estimated the amylolytic activity of *M. anisopliae* and found that amylase activity showed no variation even after recombination and formation of prototrophic recombinants. Maccheroni Jr. 1 *et al.* (2004) [19] tested amylase activity in *Colletotrichum spp.* and observed that amylase activity was not seen at an alkaline pH and starch was degraded at neutral and acidic pH. Lopez- Llorca *et al.* (1999) [10] deduced the same result on extracellular amylase production by *V. lecanii* strains. He found that amylolytic activity was scarcely studied in *V. lecanii* strains.

Lipase activity at different pH

Exoenzymes of fungi have a role in cuticle digestion during penetration of the fungus into the insect. Evaluation of enzyme activity appears to be a promising method for screening new genotypes with potential for biocontrol. Lipase activity of the *V. lecanii* isolate was assessed by two methods:

Hankin and Anagnostakis method

The production of lipolytic enzymes by the test strain of *V. lecanii* was measured by estimation of degradative capacity of Tween-20 substrate of this enzyme. Lipolytic activity was confirmed by appearance of a transport zone around the radial colony growth of *V. lecanii*. Diameter of the transparent zone was measured as index of lipase activity (**Figure 2**)

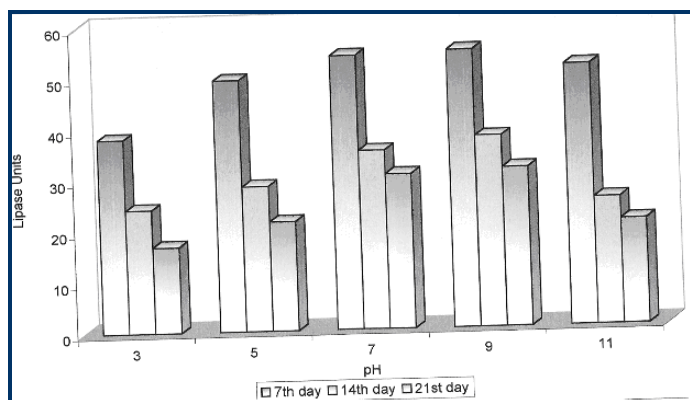


Figure 5: Lipase activity of *Verticillium lecanii* at different pH

Kaamimura et al. (2001) – Titrimetric assay

Lipolytic activity of the *V. lecanii* isolate was estimated titrimetrically at five different pH -3,5,7,9,11 on 7th, 14th and 21st day of inoculation. Maximum lipolytic activity of *V. lecanii* was found at pH 7 (**Figure 5**). This result is in accordance with the result of Maccheroni et al. (2004) [19] who deduced that lipase was absent at acidic pH and secreted at neutral and alkaline pH. Gomez-Santiesteban et al. (2004) [15] showed lipolytic activity of *V. lecanii* by development of inhibition zones on solidified agar. Lopez -Llorca et al. (1999) [10] estimated production of extracellular lipase activity. Lipolytic activity in *V. lecanii* was followed by proteolytic activity when enzymatic activity (EA) due to degradation of substrate was studied. Krikstaponis et al. (2001) [21] revealed that some of the tested strains of *Aspergillus fumigatus* are able to produce extracellular lipases and hydrolyze fatty acids of vegetable oil. Nahar et al. (2004) [22] deduced that *M. anisopliae* constitutively produced the enzyme lipase. Silva et al. (2005) [23] investigated lipase from a Brazilian strain of *M. anisopliae* and concluded that lipase activity was highest for olive oil (108.38 U/mg).

Conclusion:

Pathogenicity, being a multi component process, still, infection can be stopped by production of cuticle degrading enzymes. The test strain of *Verticillium lecanii* that was screened in the present study produced good amounts of proteolytic as well as

amylolytic and lipolytic enzymes which are instrumental in breakdown of complex organic nutrients, thus implying genetic versatility. In invitro plate screening studies, enzyme production is typically indicated by formation of zones and clearing surrounding the colony or by appearance of coloured product. The test strain of *Verticillium lecanii* showed good levels of extracellular enzyme secretion during in vitro studies. Morphological and growth studies showed that these isolates had almost similar growth rates and formed colonies with powdery and fluffy traits. Dispersal of mycopesticide occurs through powdery growth rather than fluffy colonial growth. Thus, in absolute terms, the test strain of *Verticillium lecanii* was observed to be an outstanding source of enzyme secretion and can be rationally advocated towards production of improved mycopesticides.

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