



CUTICLE DEGRADING ENZYME PRODUCTION BY SOME ISOLATES OF THE ENTOMOPATHOGENIC FUNGUS, *METARHIZIUM ANISOPLIAE* (METSCH.)

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Abstract

Context: Entomopathogenic fungi have been recognized as viable alternate options to chemicals in insect pest control. Unlike other potential biocontrol agents, fungi do not have to be ingested to infect their hosts but invade directly through the cuticle. Entry into the host involves both enzymic degradation of the cuticle barrier and mechanical pressure. Production of a range of cuticle degrading enzymes is an important event in the interaction of entomopathogenic fungi and host. Enzyme secretion is believed to be a key contributor for the virulence of a fungal isolate.

Objectives: The potentiality of nine isolates of *M. anisopliae* were tested to produce to produce three important cuticle degrading enzymes, viz., chitinase, protease and lipase.

Materials and Methods: Nine isolates of *M. anisopliae* were evaluated for chitinase, protease and lipase enzyme production by determining the enzyme index and activities.

Results: Chitinase index of these isolates were ranged from 1.5 to 2.2 and chitinolytic activity from 0.525 to 1.560 U/ml. The isolates showed protease index in the range of 1.2 to 3.3 and the activity ranged from 0.020 to 0.114 U/ml. Lipase index ranged from 1.15 to 7.0 and the enzyme activity ranged from 0.153 to 0.500 U/ml. A strong relationship was observed between virulence of the isolates and cuticle degrading enzyme production as increased enzyme production was observed for virulent isolates.

Conclusion: In the present study three isolates as (MIS2, MIS7 and MIS13) demonstrated cuticle degrading enzyme (CDE) that indicate higher virulence based on the bioassay conducted earlier by the authors as strongly substantiating the role of CDEs is considered the virulence of *Metarhizium* isolates. So, these isolates may be as ecofriendly insect-pest control agent in future.

Key words: *Metarhizium*, CDE, Chitinase, Protease, Lipase.

Introduction

The increasing use of chemical products has generated negative aspects for the biotic complex of nature, affecting plants, animals and humans resulting in a growing demand for alternatives to chemical control. Entomopathogenic fungi have already been recognized as a viable alternate control option for chemicals. Myco-biocontrol is an environmentally sound and effective means of mitigating insect-pests. Past researches have shown fungi being a potential biological control agent mainly due to their high reproductive capabilities, target specific activity, short generation time and resting stage producing capabilities that can ensure their survival for a longer time when no host is present (Sandhu *et al.* 2012). Entomopathogenic fungi need to penetrate through the cuticle into the insect body to obtain nutrients for their growth and reproduction.

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Penetration of the insect cuticle requires both mechanical pressure by penetrated hyphae and enzymic degradation by a range of extracellular cuticle degrading enzymes, including chitinases, lipases and at least four different classes of proteases (Hegedus and Khachatourians 1995, St. Leger *et al.* 1996). Proteases play an important role in providing nutrients before and after the cuticle is penetrated. On the other hand chitinase is required only for a brief period during penetration of host cuticle and is tightly regulated by chitin degradation products. Chitinolytic enzymes usually act after the pathogen proteases have significantly digested the cuticle protein and unmasked the chitin component of the cuticle (St. Leger *et al.* 1998).

Enzyme secretion is believed to be a key factor in determining the virulence of the isolate and this is considered as a rationale for the enhance virulence of certain isolates (Mustafa and Kaur 2009). Highly pathogenic strains show detectable amounts of extracellular chitinase, lipase, and protease activities Samuels *et al.* (1989). In this study the potentiality of nine *M. anisopliae* were assessed to produce chitinase, protease and lipase enzymes by determining of chitinase, protease and lipase index and activities.

Materials and Methods

Fungus

Among the nine fungal isolates (MIS1 to MIS25) used in this study, 6 were isolated either from soil or from infected insects and 3 procured from different institutions.

Enzyme Assay

Preparation of Culture filtrate

Pure cultures of the isolates were prepared on potato dextrose agar yeast (PDAY) plates. After 4 days of incubation, suspension of the different isolates with a concentration of 1×10^7 spores ml^{-1} were prepared and inoculated (5 ml) into 500 ml of Potato Dextrose Broth (PDB) fortified with 1% Yeast extract and incubated for 7 days at $28 \pm 1^\circ\text{C}$ and 90% RH in dark. The mycelia were filtered through Whatman No.1 filter paper and the suspensions were further filtered through $0.22 \mu\text{m}$ filter (Millipore) before use.

Chitinase assay

Chitinase index was assessed (De Boer *et al.* 2004, Valadares-Inglis and Azevedo 1997) by measuring the clear zone produced by degradation of chitin in Chitin yeast extract agar (CYEA) in combination with (2% colloidal chitin, 0.05% yeast extract, 2% agar and 0.01% congo red). Ten milli meter well was cut in the centre of the CYEA plate and 50 μl of crude culture filtrate was added to the well. The zone of clearance around the well was measured after four days of incubation. Each plate served as a replicate with three replications per treatment. Enzymatic index was calculated based on diameter of the halos with well, divided by well diameter.

Chitinolytic activity was assayed according to the method of Valdimir *et al.* (2002) by measuring the release of reducing saccharides from colloidal chitin: A reaction mixture containing 1 ml of crude culture filtrate, 0.3 ml of 1M sodium acetate buffer (pH 4.7) and 0.2 ml of colloidal chitin was incubated at 40°C for 6h and then centrifuged at 12,225 g for 5 min at 6°C . After centrifugation, an aliquot of 0.75 ml of the supernatant, 0.25 ml of 1% solution of dinitrosalicylic acid in 0.7M NaOH and 0.1 ml of 10M NaOH were mixed in 1.5ml eppendorf tubes and heated at 100°C for 5 min. Absorbance of the reaction mixture at 582 nm was measured after cooling in room temperature. Each tube served as a replicate and three replications were done for per treatment. A calibration curve with N-acetyl D-glucosamine as a standard was used to determine the reducing sugar concentration. One unit of enzyme activity was defined as the amount of enzyme that released 1μ mole of N-acetyl D-glucosamine per min under conditions described.

Protease assay

Protease index in solid medium was tested (St. Leger *et al.* 1999, Valadares-Ingliš and Azevedo 1997) by measuring the clearing zone produced by degradation of milk protein in pH indicator medium (0.01% yeast extract, 2% agar, 0.01% bromocresol purple adjusted to pH 5.2) containing 1% skimmed milk. Ten milli meter well was cut in the centre of the medium and 50 μ l of crude culture filtrate added to the well and the zone of clearance around the well was measured after 24-48 h of incubation. Each plate served as a replicate and three replications were done per treatment. Enzymatic index was calculated based on diameter of the halos with well divided by well diameter.

Protease activity was assayed according to the method of (Hossain *et al.* 2006). The reaction mixture containing 3 ml of 1% (w/v) casein in 3 ml 0.1M citrate-phosphate buffer, pH 7.0 and 3 ml of crude culture filtrate was incubated at $40 \pm 1^\circ\text{C}$ for 1 hr. The reaction was stopped by the addition of 5 ml 20 % (w/v) TCA and the absorbance of the solution was measured at 650 nm in a spectrophotometer (SP3000 PLUS, CE OPTIMA, Tokyo, Japan). Each tube served as replicate with three replications were done for per treatment. The amount of amino acids released was calculated from a standard curve plotted against known concentrations of tyrosine. One unit of enzyme was defined as the amount of enzyme that released $1\mu\text{g}$ of tyrosine ml^{-1} of substrate.

Lipase assay

Lipase index in solid medium was measured by the clearing zone produced by degradation of lipids in tributyrin agar (0.5% peptone, 0.3% yeast extract, 2% agar, 0.01% methyl red, pH 7.5 with 1% tributyrin). One percent sterile tributyrin was added to the media after cooling to 80°C and mixed thoroughly to emulsify the tributyrin completely and poured to maintain uniform turbidity. Ten milli meter well was cut in the centre of tributyrin agar plate and 50 μ l of crude culture filtrate added to the well and the zone of clearance around the well measured after 24-48 h of incubation. Each plate served as a replicate with three replications per treatment. Enzymatic index was calculated based on diameter of the halos with well divided by well diameter.

Lipase activity was measured by a titrimetric assay with 0.05N NaOH using emulsified olive oil as substrate (Kamimura *et al.* 1999). One ml of crude culture filtrate was added to 5 ml emulsion containing 25% (v/v) olive oil and 75% (v/v) gum arabic and 2 ml 10 mM phosphate buffer at pH 7. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 15 ml acetone-ethanol (1:1v/v) and the amount of fatty acids was then titrated. Each flask served as a replicate and three replications were done for per treatment. One unit of lipase was defined as the amount of enzyme that released 1μ mole of fatty acids per min under these conditions.

Results

Qualitative Assay

The nine isolates of *M. anisopliae* were tested for chitinase. All the isolates showed positive results and chitinase index were ranged from 1.5 to 2.2 (Fig. 1). Maximum index of 2.2 was shown by the isolate MIS2 and next of index was exhibited by isolates MIS7, MIS13, MIS24, MIS20, MIS19 and MIS18. The lowest index of 1.5 was recorded for the isolates MIS1 and MIS3.

Protease index varied from 1.2 to 3.3 for the nine isolates (Fig. 2). Isolate MIS7 showed highest protease index of 3.33. Moderate index values were recorded in isolates MIS13, MIS2 and MIS19. Isolates MIS3 and MIS1 showed intermediate index value of 1.83 and the lowest was recorded in MIS24.

Lipase index varied significantly between the tested isolates (Fig. 3) and the values ranged from 1.15 to 7.0. The highest index of 7.0 was recorded in isolates MIS7 and MIS13. Moderate index values of 6 to 4.80 were recorded for isolates MIS2, MIS24 and MIS18 respectively and the lowest index of 1.15 was exhibited for isolate MIS1.

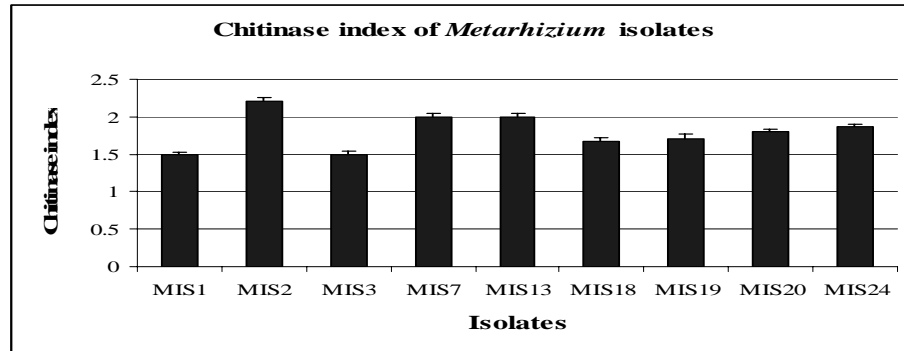


Fig. 1. Chitinase index of *Metarhizium* isolates.

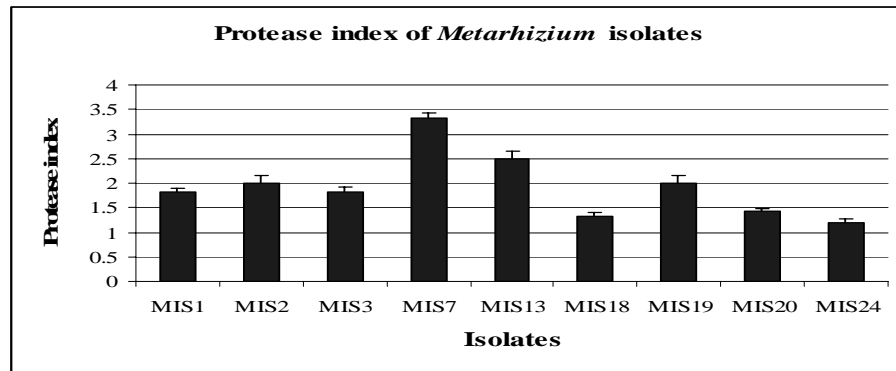


Fig. 2. Protease index of *Metarhizium* isolates.

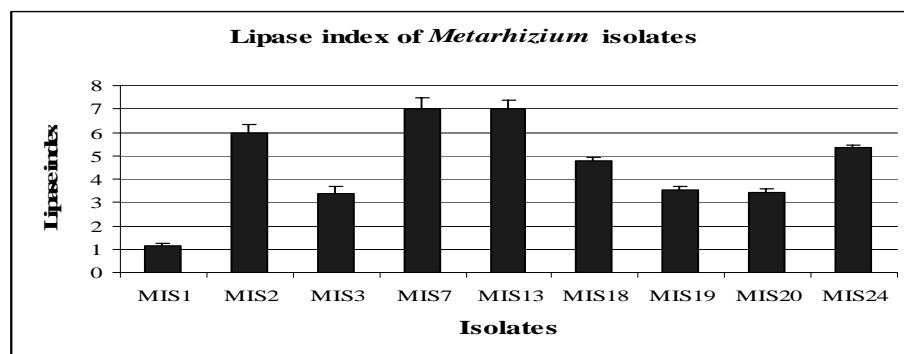


Fig. 3. Lipase index of *Metarhizium* isolates.

Quantitative Assay

All the isolates tested for chitinase production in submerged culture and the chitinolytic activity were ranged from 0.525 to 1.560 U/ml (Table 1). The highest activity of 1.560 U/ml was shown by the isolate MIS2 followed by MIS7. Moderate activity was in the range of 1.001 U/ml to 1.152 U/ml for isolates MIS13, MIS1 and MIS18. and the lowest activity was exhibited in MIS20.

Table 1. Chitinase activity of *Metarhizium* isolates.

Isolates	Chitinase Activity (U/ml)		
MIS1	1.082 ^d		
MIS2	1.560 ^a		
MIS3	0.662 ^g		
MIS7	1.430 ^b		
MIS13	1.152 ^c		
MIS18	1.001 ^e		
MIS19	0.603 ^h		
MIS20	0.525 ⁱ		
MIS24	0.780 ^f		
	SED	CD(.05)	CD(.01)
	0.0252	0.0535	0.0737

Protease activity ranged from 0.020 to 0.114 U/ml (Table 2). The highest protease activity was recorded in MIS7 (1.560 U/ml) followed by MIS20 (0.107 U/ml), MIS18(0.092 U/ml) MIS2(0.096 U/ml) MIS13(0.095U/ml). The isolate MIS20 showed the lowest protease activity.

Table 2. Protease activity of *Metarhizium* isolates.

Isolates	Protease Activity (U/ml)		
MIS1	0.075 ^e		
MIS2	0.096 ^c		
MIS3	0.020 ^f		
MIS7	0.114 ^a		
MIS13	0.095 ^c		
MIS18	0.092 ^c		
MIS19	0.083 ^d		
MIS20	0.107 ^b		
MIS24	0.079 ^{de}		
	SED	CD(.05)	CD(.01)
	0.0027	0.0058	0.0080

The lipolytic activity for the nine isolates were recorded and ranged from 0.153 to 0.500 U/ml (Table 3). Maximum lipase production was recorded for MIS7 with an activity of 0.50 U/ml followed by isolates MIS2 and MIS13 with activity of 0.492 U/ml. Moderate activity was observed in isolates MIS24 and MIS18. The lowest lipase activity of 0.153 U/ml was recorded in MIS1.

Table 3. Lipase activity of *Metarhizium* isolates.

Isolates	Lipase Activity (U/ml)		
MIS1	0.153 ^f		
MIS2	0.492 ^a		
MIS3	0.200 ^d		
MIS7	0.500 ^a		
MIS13	0.492 ^a		
MIS18	0.364 ^c		
MIS19	0.196 ^d ^e		
MIS20	0.190 ^e		
MIS24	0.400 ^b		
	SED	CD(.05)	CD(.01)
	0.0045	0.0096	0.0132

Discussion

From the result it was observed that all the nine isolates showed chitinase index ranged from 1.5 to 2.2 and the chitinolytic activity ranged from 0.525 to 1.560 U/ml. In early works chitinase activity 0.01-0.0398U/ml was recorded for various *M. anisopliae* isolates studied by Nahar *et al.* (2004). Braga *et al.* (1998) evaluated chitinolytic activity of seventeen isolates of *M. anisopliae* isolates and reported the activity to vary from 0.0261 to 0.1340U/ml. Similarly St. Leger *et al.* (1986) reported chitinase activity of 0.027U/ml for *M. anisopliae*. Wu (2010) evaluated the chitinase activity of *M. anisopliae* and recorded chitinase yield of 105.32mU/ml. Markedly higher chitinolytic activity (8.66mU/ml) was detected in the culture fluid when *M. anisopliae* were grown in a medium containing colloidal chitin as a sole carbon source (Kang *et al.* 1999).

The nine isolates showed protease index in the range of 1.2 to 3.3 and protease activity ranged from 0.020 to 0.114 U/ml. This result support the study of Nahar *et al.* (2004) who reported protease activity of 0.01U/ml for *M. anisopliae* in both YPG and chitin medium. Protease index of forty segregants of *M. anisopliae* were ranged from 1.357-1.923 (Valadares-Ingliš and Azevedo 1997). Protease index on mineral agar medium amended with gelatin was 3.56 and 3.25 and in mineral agar medium amended with casein was 1.87 and 2.2 respectively at pH 6.8 and 8.5 for *B. bassiana* (Dias *et al.* 2008). *M. anisopliae* is the mainly targeted entomopathogenic fungi for the study of cuticle-degrading protease as they are reported to produce a variety of fungal proteases (Cole *et al.* 1993).

In the present study index was recorded 1.15 to 7.0 were recorded by the nine isolates in medium containing tributyrin containing medium. Screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas *et al.* 2001) and clear zones around the colonies indicate production of lipase (Sharma *et al.* 2001). The lipase activities of the isolates were ranged from 0.153 to 0.500 U/ml. Nahar *et al.* (2004) showed lipolytic activity of 0.312 and 0.015U/ml in YPG and chitin medium respectively. Fungi are widely recognized as preferable lipase sources.

Braga *et al.* (1998) carried out chitinolytic activity together with estimates of the genetic parameters of such activities and suggested that these parameters help in improving these traits in *M. anisopliae*. Mustafa and Kaur (2009) studied *in-vitro* production of cuticle-degrading enzymes, such as chitinase, proteinase, caseinase, lipase and amylase in fourteen isolates of *M. anisopliae* and suggested that the enzyme production exhibited significant natural isolate variability. Two chymoelastases and three trypsinlike proteases were separated from culture filtrates of the entomopathogen *M. anisopliae* by St. Leger *et al.* (1987) who reported rapid production of proteases (Pr1 and Pr2) by *Metarhizium anisopliae* in culture media and in situ on insect cuticle (St. Leger *et al.* 1987a). The production of the cuticle-degrading extracellular proteases, chymoelastase (Pr1) and trypsin (Pr2) were reported by Pinto *et al.* (2002) by the isolates of *M. flavoviride*.

In present study three isolates such as (MIS2, MIS7 and MIS13) demonstrated higher CDE production to have higher virulence based on the bioassay conducted earlier by the authors (Remadevi *et al.* 2010). This strongly substantiates the role of CDEs in deciding the virulence of *Metarhizium* isolates.

Conclusion

Variation in the overall enzyme production of each isolate parallels the differing virulence among isolates, indicating that the whole cuticle-degrading enzyme machinery, rather than the individual enzymes, determines virulence (Santiago and Gabriel 2000). The present investigation suggests that the isolates of *M. anisopliae* are virulent as produced increased amount of chitinase, protease and lipase enzyme. Attempts to enhance the enzyme production of the isolates by strain improvement, modification of culture conditions or genetic manipulation may facilitate the development of a much proficient pest control strategy.

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