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Original Research Article

Effect of Cuticular and Non Cuticular Substrates on Protease Production in Submerged Cultures of High and Low Virulent Isolates of *Metarhizium anisopliae* Over Ten Days Growth and Autolysis Phases

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Abstract: Protease production pattern, the key factor governing virulence of entomopathogenic fungi was studied in the more virulent and less virulent isolates of the entomopathogenic fungus *Metarhizium anisopliae* using cuticular and non cuticle substrates like Cockroach, Prawn, Casein and Glucose as substrates. Protease production, efficiency of growth and degree of autolysis were recorded during growth and autolysis phases. The noncuticular substrate casein demonstrated higher value of protease when compared to the samples with cuticular substrates of cockroach, prawn. Effective utilization of exogenous carbon source from casein, glucose and cockroach supplements and the consequent increase in proteolytic activity detected in the high virulent isolate may be due to induction of the virulence related factors by the supplements. On the other hand, the low virulent isolate M10 showed marginal increase in proteolytic activity.

Key Words: Autolysis, carbohydrate utilized, casein, cockroach cuticle, prawn cuticle, and *M. anisopliae* Protease activity

Introduction

Entomopathogenic fungi infect the insect hosts primarily breaking through the cuticle comprised of protein fibrils embedded in chitin which forms a protective sheath. Protein (75–80%) constitutes major portion of the cuticle which could be degraded by extracellular enzymes of mycelial or spore origin and these are the hallmark of fungal infectious process. 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 resources. Entomopathogenic fungi produce various extracellular enzymes viz., Proteases, Chitinases, Lipases and toxins, which facilitates their penetration through the host cuticle by enzymatic degradation and mechanical pressure [1]. The insect pathogen, *Metarhizium anisopliae*, has been the focus of studies for host cuticle penetration and biocontrol of insect pests. *M. anisopliae* produces families of catalytically variant extracellular substilisin like Proteases (Pr1) [2, 3], Trypsin like proteases (Pr2) and metalloproteases [4] as well as several families of exo-acting peptides that are believed to be important in insect cuticle degradation.

It is well known that *in vitro* extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, physical factors, such as pH, temperature, inoculum density, dissolved oxygen and incubation time [5]. Rao *et al.,* [6] reported that mixtures of different carbon (shrimp shell powder + sucrose) and nitrogen (soy powder + yeast extract) sources in the culture medium significantly improved the production of protease from *B. bassiana*. Pinto *et al.,* [7] examined the production of cuticle-degrading extracellular proteases chymoelastase (Pr1) and trypsin (Pr2) in the isolates of *Metarhizium flavoviride*. Gillespie *et al.,* [8] reported that virulence of the fungal isolates depends on the quantitative difference of the protease production under *in vivo* conditions. Campos *et al.,* [9] reported the role of proteases and chitinases secreted by *Beauveria amorpha* and *Beauveria bassiana* during infection in the tick *Boophilus microplus*, and also on chitin amended synthetic medium and concluded that both chitin and tick cuticle induced chitinase, while protease was induced only by tick cuticle. Shah and Butt [10] reported that two strains of *M. anisopliae* differed in their stability when grown on synthetic nutrient medium and medium supplemented with cockroach cuticle (*Blaberus discoidalis*).

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In an attempt to understand the enzymatic factors governing virulence, *M. anisopliae* isolates M19 and M10 displaying high and low virulence respectively were investigated for understanding the dynamics of protease production during culture, growth and autolysis. The experiments were designed to understand the effect of carbon and nitrogen source from non-cuticular/cuticular substrates on protease production of *M. anisopliae* isolates in relation to their virulence status and to analyze the associated changes like pH, carbon source utilized, growth efficacy and autolysis during 10-day growth period.

Materials and Methods

Isolates of *M. anisopliae* obtained from ARSEF and EMBRAPA were maintained on SDAY (Sabouraud's Dextrose Yeast extract Agar) medium by subculturing at two-month intervals. Evaluation of *M. anisopliae* isolates for virulence against two lepidopteran insect pests, *Spodoptera litura* and *Helicoverpa armigera* from laboratory bioassays revealed M19 (ARSEF 1080) and M10 (EMBRAPA CG 47) isolates to be high and low virulent respectively (Bhanu prakesh 2008) [11] and these two isolates were selected for the present study.

Culture filtrate preparation

150 ml Erlenmeyer flasks containing 50 ml of YMP broth at pH 7 were inoculated with 1 ml of 10^6 conidia ml^{-1} in an aqueous solution of Tween- 80® and incubated at 25°C in an orbital incubator shaker at 200 rpm for 24–48 hrs. To determine the protease production and autolysis of the isolates, casein/glucose as non-cuticular substrates and cockroach/prawn as cuticular substrates were used at 1% (w/v) concentration in minimal basal salt medium ($(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4 and $MgSO_4$) at pH 7. Minimal medium(50 ml) amended with 1% (w/v) of cuticular/non-cuticular substrates was autoclaved at 121°C for 20 min was inoculated with 5 ml of YMP broth and incubated at 25°C ± 2°C with 200 rpm for 10 days. Three replicates for each substrate along with controls (with no carbon source) were maintained. Samples were collected at every 24 hr interval, filtered using Whatman no.1 filter paper and data of wet weight of the mycelial biomass, pH of the broth were recorded for dry weight, mycelium was dried in an oven at 70°C for 72 hrs.

Determination of proteolytic activity against casein

Caseinolytic activity was studied using Söderhäl and Unestam [12] method with slight modifications. 1ml of dilute enzyme (culture filtrate diluted with 0.1M phosphate buffer pH 7.6) was incubated with 1% (w/v) casein (0.1M phosphate buffer) in water bath at 40°C for 20min. The reaction was culminated by adding 3 ml of 5% (w/v) TCA and samples were allowed to stand at room temperature for 1hr. After centrifugation for 10 min at 10000 rpm, the supernatant was filtered through Whatman no.1 filter paper and absorbance was taken at 280nm in an UV spectrophotometer. Blank was prepared with phosphate buffer; crude enzyme was incubated for 2h in boiling water bath for inactivation and used as control. Enzyme activity was calculated as absorbance at 280nm enzyme dilution and expressed as µg min^{-1} ml^{-1}. One unit of enzyme activity (U) was defined as the amount of enzyme that liberates 1µg ml^{-1} of tyrosine per min under the experimental conditions and all the enzyme assays were carried in triplicate.

Determination of residual carbon substrate

Residual carbon substrate present in the medium throughout the growth period was determined using the method followed by Nelson [13]. The degree of autolysis was calculated as percent loss of mycelial dry mass from the day of maximum growth till 10^{th} day. Economic coefficient (EC) was calculated for understanding growth efficiency by using the formula; EC = Mycelial dry weight / Quantity of carbon source consumed.

The data was subjected to statistical analysis for calculation of ANOVA using SPSS version 15 software.

Results

Protease activity on non-cuticular and cuticular substrates

Proteolytic activity was recorded by monitoring protease production through growth and autolysis phases of the culture. Protease production gradually increased in a discrete manner and recorded maximum value on 5^{th} day and a decrease there after in both the isolates when casein was used as carbon and nitrogen source. The same trend was recorded with glucose supplement, as well as Prawn cuticle supplemented media through the range of protease activity was

different (Fig.1). On the other hand, Cockroach cuticle supplemented medium displayed gradual increase in protease production up to 6th day with respect to high virulent as well as low virulent isolates (Fig. 2).

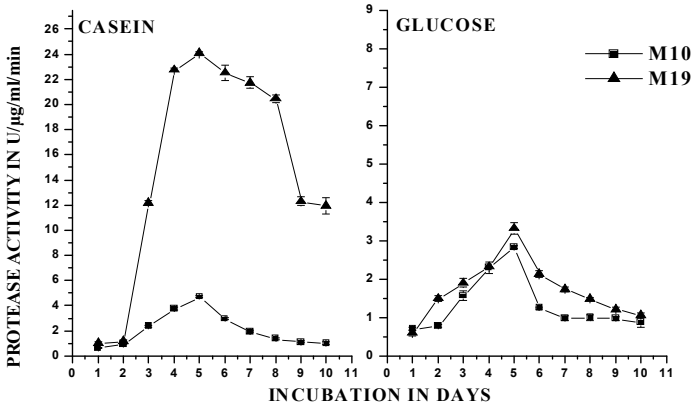

Figure 1: Protease activity of two isolates of *Metarhizium anisopliae* on non-cuticular substrates.

Differential response was recorded for the M19 and M10 isolates to the four nutrient supplements tested. Casein supplement displayed maximum protease activity of 24.07 for M19 followed by activity of 18.50 in cockroach cuticle added medium. M19 isolate utilized cockroach cuticle as carbon and nitrogen source more efficiently over M10 isolate and recorded enhanced proteolytic, k, activity. On the other hand, glucose and prawn cuticle supplements recorded a meager response for protease production and only marginal differences prevailed among two isolates M19 and M10 on 5th day of incubation. M19 isolate appeared to be highly responsive to casein and cockroach cuticle supplements rather than glucose and prawn cuticle supplements and recorded protease activity ranging from 3.32 to 24.07 in the four media tested, while the corresponding values for M10 isolate were 2.83 to 5.99.

pH of broth recorded continuous change during growth and autolysis phases of the culture in non cuticular as well as cuticular substrates and recorded an increase upto 5th day and decrease there after till 10th day of incubation. pH increased at a faster rate in M19 isolate compared to that of M10 isolate in casein supplement, whereas in glucose supplement pH decreased gradually till 10th day of incubation (Fig.3). pH value of the culture was proportionate with maximum protease activity. In case of cockroach cuticle, pH of the medium increased upto 6th day and a maximum pH value of 8.7 was recorded in M19 isolate (Fig. 4). On the other hand, low pH value of 2.1 was recorded in isolate M10 on 6th day in glucose-supplemented medium. There was gradual increase in the carbon source utilization by both the isolates during the 10 days incubation period. M19 isolate recorded efficient utilization of all the substrates tested except glucose more efficiently compared to M10 isolate. (Fig. 5 & 6).

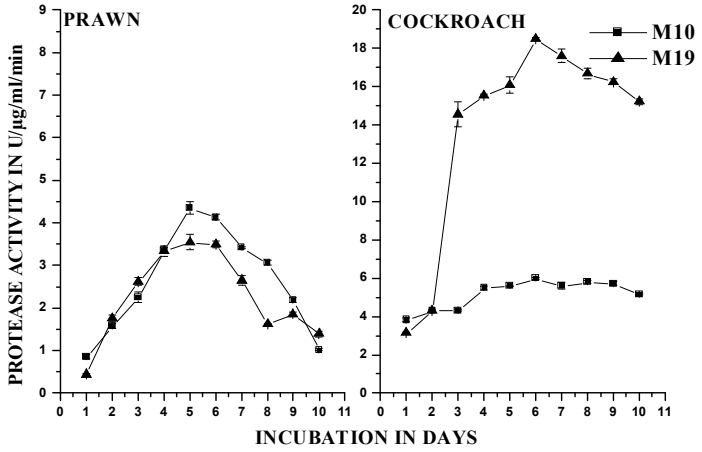

Figure 2: Protease activity of two isolates of *Metarhizium anisopliae* on cuticular substrates

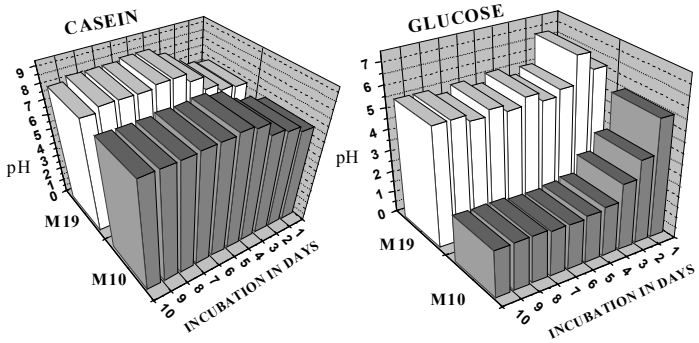

Figure 3: Change in pH of the broth during growth and autolysis phases of *Metarhizium anisopliae* isolates on non- cuticular substrates

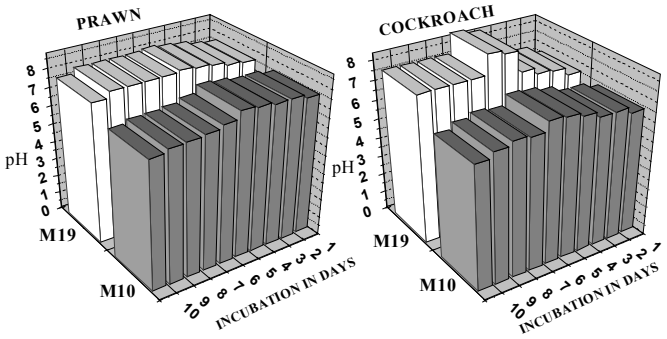

Figure 4: Change in pH of the broth during growth and autolysis phases of *Metarhizium anisopliae* isolates on cuticular substrates

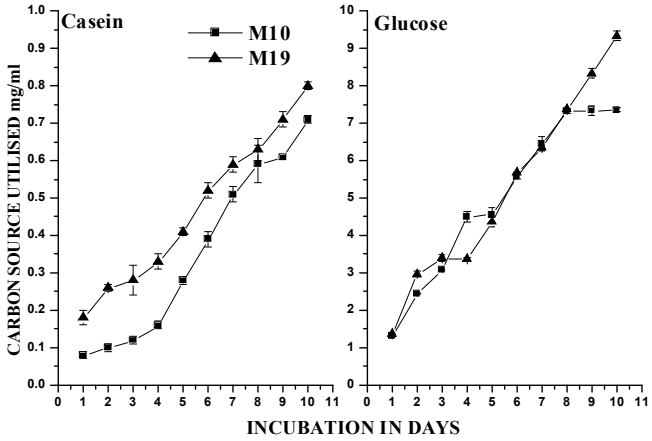

Figure 5: Carbon source utilized by two isolates of *Metarhizium anisopliae* on non-cuticular substrates

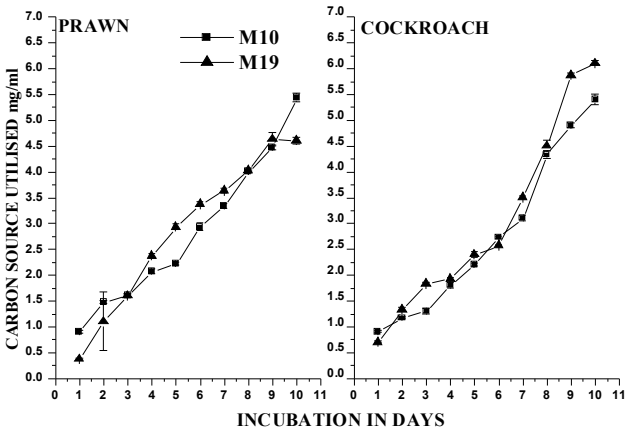

Figure 6: Carbon source utilized by two isolates of *Metarhizium anisopliae* on non-cuticular substrates

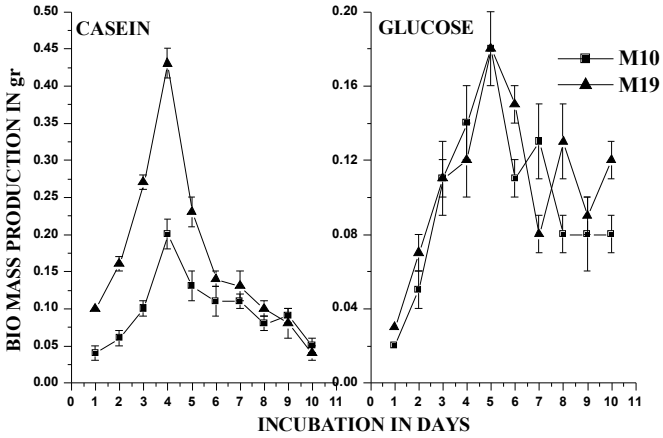

Figure 7: Biomass production in two isolates of *Metarhizium anisopliae* isolates on noncuticlular substrates.

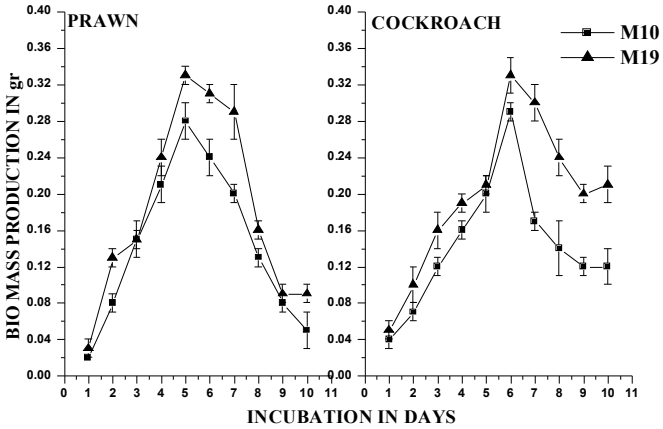

Figure 8: Biomass production in two isolates of *Metarhizium anisopliae* isolates on cuticular substrates

Efficiency of growth and percentage of autolysis

Casein recorded a conspicuous increase in the biomass production till 4th day of incubation for both the isolates. However maximum biomass produced by isolate M10 was 0.20 g/flask, compared to 47% of the corresponding value (0.43 g/flask) shown by isolate M19 (Fig. 7). For both the isolates, exogenous carbon depletion started from the 5th day of incubation, culminating in autolysis. After 10 days of incubation, M10 isolate showed 75% and M19 isolate 90% autolysis. Regarding efficiency of growth, economic coefficient value gradually increased and maximum values were recorded on 4th day for both the isolates (Table 1). Exogenous substrate utilization for M10 isolate was 22% of the total input and for M19 isolate it was

41% of input on 4th day of incubation. When glucose was supplemented as carbon and nitrogen source in minimal basal salt medium, conspicuous increase in the biomass production could be observed until 4th day of the experiment. However the isolates displayed highest biomass production of 0.18 g/flask while exogenous carbon depletion and autolysis started from 6th day onwards. The efficiency of growth for both the isolates gradually increased and maximum value was recorded on 5th day.

Table 1: Efficiency of growth and percentage of autolysis during incubation of *Metarhizium anisopliae* in medium containing non-cuticular substrates

Incubation in days	Casein				Glucose			
	Efficiency of growth		Percentage of autolysis[a]		Efficiency of growth		Percentage of autolysis[a]	
	M10	M19	M10	M19	M10	M19	M10	M19
1	0.50	0.55	--	--	0.01	0.02	--	--
2	0.60	0.61	--	--	0.02	0.02	--	--
3	0.83	0.96	--	--	0.03	0.03	--	--
4	1.25	1.30	--	--	0.03	0.03	--	--
5	0.40	0.56	35%	47%	0.03	0.04	--	--
6	0.28	0.26	45%	67%	0.01	0.02	38%	16%
7	0.21	0.22	45%	69%	0.02	0.02	27%	25%
8	0.13	0.15	60%	77%	0.01	0.01	55%	27%
9	0.14	0.11	55%	81%	0.01	0.01	55%	50%
10	0.07	0.05	75%	90%	0.01	0.01	55%	33%

[a]autolysis was calculated with respect to the highest dry mass production

Table 2: Efficiency of growth and percentage of autolysis during incubation of *Metarhizium anisopliae* in medium containing cuticular substrates

Incubation in days	Prawn				Cockroach			
	Efficiency of growth		Percentage of autolysis[a]		Efficiency of growth		Percentage of autolysis[a]	
	M10	M19	M10	M19	M10	M19	M10	M19
1	0.02	0.08	--	--	0.04	0.07	--	--
2	0.05	0.11	--	--	0.05	0.08	--	--
3	0.09	0.09	--	--	0.09	0.08	--	--
4	0.10	0.10	--	--	0.08	0.09	--	--
5	0.12	0.11	--	--	0.09	0.08	--	--
6	0.08	0.09	14%	06%	0.10	0.12	--	--
7	0.06	0.07	28%	12%	0.05	0.08	41%	9%
8	0.03	0.03	53%	51%	0.03	0.05	51%	27%
9	0.01	0.01	71%	72%	0.02	0.03	59%	39%
10	0.01	0.01	82%	72%	0.02	0.03	59%	36%

[a]autolysis was calculated with respect to the highest dry mass production

However, biomass production for M10 isolate (0.28 g/flask) was low compared to the corresponding value of (0.33 g/flask) M19 isolate on prawn cuticle supplement (Fig. 8). The growth efficiency for both the isolates gradually increased till 5th day (Table 2) and economic coefficient calculated on that day, recorded maximum value for both the isolates. (Utilization of exogenous substrate for M10 isolate was 41% and 64% for M19 isolate).

Discussion

Proteases are key virulence determinants for the infection process of entomopathogenic fungi [14]. The high and low virulent isolates of M. anisopliae employed in the present study revealed significant differences in the protease activity between the two isolates and against the four substrates tested. In the medium supplemented with Casein / Glucose or Cockroach cuticle, the more virulent isolate M19 showed higher proteolytic activity than less virulent isolate M10. Protease production appears to be triggered in response to carbon and nitrogen, abundantly available in casein and cuticular substrates. Pinto *et al.,* [7] recorded an increase of about 2 to 8 times higher enzyme activity in minimal medium supplemented with casein and about 4 to 20 times higher in minimal medium supplemented with cuticle compared to minimal medium alone. The higher levels of Pr1 found in minimal medium supplemented with cuticle cultures were explained to be due to the induction of Pr1 by insect cuticle components.

In addition, Pr1 is indicated as one of the factors responsible for virulence of the fungus [15, 4]. Paterson *et al.,* [16] determined Pr1 activity of starved *M. anisopliae* mycelium, which is specifically induced by cuticle from *Schistocerca gregaria*. St. Leger *et al.,* [17] demonstrated significant variations in Pr1 sequences among strains of entomopathogenic fungi. It has also been described that *M. anisopliae* proteases comprise multiple isoforms.

Variability in the production of protease has been recorded in *M. anisopliae* isolates. Enhanced proteolytic activity in the high virulent isolate M19 appear to be due to genetic differences among the isolates and efficient utilization of exogenous carbon source from casein, glucose and cockroach supplements. Magalhães *et al.,* [18] reported enhancement in the activity of Pr1 after growth in *Rhammatocerus schistoceroides* and expressed the opinion that it lead to enhanced virulence against grasshopper species. Rao *et al.,* [6] observed altered

protease activity of *Beauveria bassiana* A1 on various media supplements.

The pH decreased in the period of enzyme synthesis in minimal medium supplemented with glucose, and stood constant up to the end of fermentation. The low pH values (3.9 and 2.2 in the two isolates) observed on the 10th day of incubation may be due to consequence of metabolite accumulations resulting from D-glucose degradation. There was a similar instance of progressive increase in the pH during growth and decrease in autolysis phases was recorded in *B. bassiana* with different carbon and nitrogen source [6]. St. Leger *et al.,* [19] reported de-repression of Pr1 production when the external pH is alkaline, even in the absence of cuticle; presence of cuticle enhanced Pr1 production threefold at pH 8, even though the inductive effects of cuticle do not override the negative effects on gene transcription of non optimum pH.

In the present study protease activities could be detected from 24-48 hrs of growth and increased after the exponential phase. Similar results were also observed by Moreira *et al.,* [20] in *Streptomyces clavuligerus* in minimal medium supplemented with soya bean filtrate and glycerol. According to St. Leger *et al.,* [21], analysis of end products in liquid culture revealed that ammonia was produced during growth on yeast extract at level sufficient to account for rise in pH of the medium. In the present work, it is apparent that due to the exogenous carbon depletion, onset of autolysis and the maximum protease activity was displayed on 5th day with respect to Casein, Glucose and Prawn cuticle substrates and on 6th day with Cockroach cuticle. Change in the protease production pattern during growth and autolysis phases of the isolates of *M.anisopliae* appera to be associated with change in the pH of the culture media.

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