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Original Research Article

Alterations in electrophoresis patterns of Proteins, Isozymes and in vitro Protease activity under Thermal Stress in two isolates of *Metarhizium anisopliae*

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Abstract: Two isolates of *Metarhizium anisopliae* (Metsch.) M33 (thermal tolerant) and M26 (thermal sensitive) were tested for biochemical alterations under thermal stress 37°C (M33T) to optimum temperature 25°C (M33T, M26) growth conditions. SDS-PAGE gel electrophoresis revealed variation in protein production resulting in the emergence of 9 heat shock proteins of different molecular mass by M33T under stress condition, those missing under optimum condition in M33C and M26. There was considerable trek in antioxidant enzymes, catalase and peroxidase by producing increased number of isoforms (2 and 3 isoforms respectively) on native PAGE gel with thermal stress (M33T) in comparison to optimum conditions (M33C and M26). Other intra cellular enzymes, esterase and acid phosphatase depicted in loss of isoforms at 37°C when compared to M33C isolate. *In vitro* protease activity was in negative correlation with temperature stress, M33T isolate expressed the lowest protease activity throughout the incubation period of 6 days.

Key words: acid phosphatase, catalase, esterase, Metarhizium anisopliae, peroxidase, protease activity, thermal stress

Introduction

Fungal biopesticides used in biological control covers a wide range of fungal genera with applications including use as antagonists to fungi and agents to control insect pest. *Metarhizium anisopliae* have been extensively studied as key regulatory factor in insect populations and as agents of biocontrol (13). This ascomycetes fungus is a widespread, soil borne pathogen of insects (27) and strains of this fungus are being developed for the control of a number of pest species (3). The mechanism of infection of the fungus includes both mechanical pressure and enzymatic degradation. Growth of the fungus depends on adverse factors that can be either biotic or abiotic. Biotic include bacteria, fungi, insects, or disease causing organisms and abiotic include temperature, heavy metals, ultraviolet irradiation, metabolic repressors, famine (5). Environmental stresses or stimuli will change the structure or metabolism of an organism by acting as elicitors that effect gene expression and results in the synthesis of stress specific compounds (5). Among the environmental stress factors listed above, temperature is one of the largely influencing abiotic factors in the growth of the entomopathogenic fungi. Some can survive at the elevated temperatures of hot springs, in the high salinity of seawater, or in various other adverse environments. Exposure to heat by conduction in soil or by direct radiation is one of the most important stress factors encountered in the field use of entomopathogenic fungi which influence the effectiveness of the fungal biopesticide used (25). In nature there are several scenarios where *M. anisopliae* is exposed to elevated temperatures as hyphae are primarily found in the top 1-5 cm of soil (12). For *M. anisopliae*, the upper limit temperature for mycelial growth is 37°C-40°C (11).

Sudden changes in temperatures trigger a physiological response that leads to the synthesis of special proteins known as Heat Shock Proteins (HSP). In fungi, the synthesis of HSPs is a rapid process and in general, a rise of 5°C above the normal physiological temperature will induce synthesis of HSPs accompanied by several other biochemical and physiological changes one of which being oxidative stress. (5).

Biological systems have evolved several defence mechanisms that enable cells to cope with lethal oxidative environments (20). These antioxidant defense systems

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include enzymatic activities such as peroxidases (POX; EC 1.11.1.7), catalases (CAT; EC 1.11.1.6) and superoxide desmutases (SOD) that detoxify H_2O_2 and O_2^- respectively with both peroxidases and catalases involving in detoxification of H_2O_2. Extra cellular enzymes, proteases (23) and intra cellular enzymes esterases, acid phosphatases (14), the hall mark of fungal infectious process.are also influenced by the thermal stress. These when subjected to thermal stress conditions get denatured and also differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile (32) as also studied by many investigators (34;15).

The present study was undertaken to understand the influence of thermal stress on various biochemical processes involved during the infection procedure of the fungus which include extra cellular protease activity, intra cellular isoenzymes, esterase, acid phosphatase with protein production and two antioxidant enzymes catalase, peroxidase generated due to the stress. The study involves preset thermal tolerant isolate of *Metarhizium anisopliae* in comparison to a positive control (tolerant isolate grown under optimum condition) and a thermal sensitive isolate.

Materials and Methods

Fungal isolates, growth conditions

Metarhizium anisopliae cultures were obtained from United States Department of Agriculture (USDA), Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF), Ithaca, New York, USA and maintained in the laboratory at optimum temperature 25°C on Sabouraud's Dextrose Agar (SDA) medium slants containing 4% Dextrose, 1% peptone, 1% yeast extract and 2% agar prepared at pH 7.0. Subculturing was done at regular intervals of 4 months and stocks were maintained as 20% glycerol cultures under refrigerated conditions. Two isolates M26 (ARSEF 2424) originally isolated from a larva of unknown host of Lepidoptera (Java) and M33 (ARSEF 3196) isolated originally from soil using *Tenebrio molitor* of Coleoptera as trap (Philippines) were considered for the present work

Thermal stress condition

For the study, the test isolate (M33T) was initiated in Sabourauds Dextrose (SD) broth by inoculating with 1 x 10^8 conidia ml^{-1} and set to heat shock at 37°C for 6 days and subsequently transferred to optimum temperature whereas positive (M33C) and negative (M26) controls were raised completely at optimum growth conditions, 25°C in an orbital shaker at 200 rpm for 6 days. For protein and isozyme studies, the mycelium was harvested, washed thoroughly with sterilized distilled water, collected and stored under refrigerated conditions till use, on the other hand, for extracellular protease assay, the filtrate was used.

Extraction of proteins

The culture preparation of the test (M33T) and controls (M33C), (M26) was similar to the above. The proteins were extracted using 0.1M phosphate buffer at pH 7.2 with 0.25M sucrose in it by grinding the mycelium using liquid nitrogen. The mixture was centrifuged at 10000g for 10 min in a refrigerated centrifuge at 4°C. Glass wool was used for uniform breakage of the hyphal cell wall for liberating the intracellular proteins during grinding into extraction buffer. The supernatant was used as protein source.

Extraction of isozymes

Two isozymes, esterases (EST; EC 3.1.1.1), acid phosphatases (AP; EC 3.1.3.2), with two antioxidant enzymes, peroxidases (POX; EC 1.11.1.7), catalases (CAT; EC 1.11.1.6) were evaluated. The enzyme extractions and staining procedures were done according to Sadasivam and Manickam (28) with slight modifications. Mycelium for the isozyme study was prepared as described above in thermal stress condition section. For EST, 500 mg of mycelium was ground to powder using liquid nitrogen in 10mM sodium phosphate buffer (pH 9.5), 1 mM EDTA Na+, 1 mM β mercaptoethanol. The homogenate was centrifuged at 10000g for 15min and supernatant was used as enzyme source. All the operations were done at 0°C - 4°C. For AP, mycelium (500 mg) was homogenized in 10 ml of ice-cold 50 mM citrate buffer (pH 5.0) in a prechilled motor and pestle using liquid nitrogen. The homogenate was centrifuged at 10000g for 15min at 4°C and supernatant was used as enzyme source. For POX, 1 g mycelium was ground in 0.1 M phosphate buffer pH 7.0 with precooled mortar and pestle. The homogenate centrifuged at 18000g at 4°C for 15 min and supernatant was used as enzyme source within 2-4 hrs. For, CAT, 500 mg of mycelium

was ground to powder in precooled mortar and pestle in 0.067 M phosphate buffer pH 7.0 and centrifuged at 4°C at 10000g and the supernatant was immediately used for enzyme study.

The concentration of each extract was determined using Lowry's protein estimation method using UV light at 280 nm against standard BSA prior to electrophoresis. All the samples were loaded at uniform concentration of 50µg ml^{-1} into the gel.

Electrophoresis

For protein analysis, SDS-PAGE was prepared as 5% stacking and 12% separating gel. Fifty µl of the protein sample mixed with one volume of tracking dye (0.125 M stacking buffer pH 6.8, 10% SDS, 4% β mercaptoethanol, 20% glycerol and a pinch of bromophenol blue) was heated at 100°C for 3 min to denature the protein and loaded into wells on the gel and was electrophoresed at 50 V/cm in Tris- Glycine (Tris 0.3%, 1.4% Glycine, 0.1% SDS, pH 8.3) electrophoretic buffer for 5-6 hrs. The gel was transferred to staining solution after completion of electrophoresis.

For isozyme analysis, native PAGE without SDS was prepared and the given sample were analyzed by the native charge they carry at pH of the separating gel (pH 8.8) which can separate according to their differential electrophoretic mobilities and sieving effects of the gel. Separating gel was prepared as 8% and stacking as 3% acrylamide gels and 30 µl of the enzyme sample mixed with one volume of tracking dye (0.125% bromophenol blue in 30% sucrose solution) and gel was electrophoresed at 15 V/cm. Electrophoresis was carried out at 4°C and on completion electrophoresis the gels were recovered and transferred to appropriate staining solution for visualization of the isozymes.

Staining of gels

Protein gels were stained by incubating the gel in solution containing Commassie brilliant blue R 250 (0.1%) in 40 ml methanol, 10ml acetic acid and 50ml double distilled water for 3-4 hrs and later destaining the gels with above solution without dye on a rocking platform for 2-3 hrs, repeated for 2-3 times and then transferring the gel to other destaining solution of acetic acid (7ml), methanol (5ml) and double distilled water (88 ml) and repeated washings until the solution is no longer blue.

For detecting activity of EST, gels were incubated at 37°C in dark for 30 min – 1 hr in a staining solution containing sodium dihydrogen phosphate (2.8 g), Disodium hydrogen phosphate (1.1 g), Fast blue RR salt (0.2 g), α napthyl acetate (0.03 g) made upto 200 ml with distilled water. After appropriate staining of the bands, the enzyme reaction was ceased by adding a mixture of methanol: water: acetic acid: ethyl alcohol (10:10:2:1).

For detecting activity of AP, gels were washed for 3-4 times at 15 min interval with 0.1 M acetate buffer (pH 5) prior to the addition of staining solution in order to lower pH of the gel to pH 5. The gels were then incubated at 37°C over night in the solution of α napthyl phosphate (0.05 g), Fast blue RR (0.05 g) NaCl (1 g), 10% $MgCl_2$ (0.5 ml), 0.1 M acetate buffer (pH5) (50 ml). The gels were fixed in 50% ethanol after complete staining of the bands.

For detecting activity of POX, gels were incubated in solution of Benzidine (2.08 g), acetic acid (18 ml), Hydrogen peroxide 3% (100 ml) and distilled water (80 ml), when the bands were stained sufficiently, the reaction was arrested by immersing the gel into a large volume of 7% acetic acid solution for 10 min.

For detecting CAT activity, the gels were soaked in 25 mM hydrogen peroxide for 5 min with gentle shaking and then transferred to freshly prepared solution containing 1% potassium ferricyanide and 1% ferric chloride. The reaction was stopped by adding 7.5% acetic acid after appropriate staining.

Proteolytic activity against casein substrate

The fungal culture was generated by inoculating 50 ml of YMP (yeast extract 0.3%, malt extract 0.3%, peptone 0.2% with pH 7.0) broth with 1 x 10^8 conidia ml^{-1} in Tween 80 and incubating at 25°C in an orbital shaker at 200 rpm for 2-3 days. After incubation, 5 ml of the germinating mycelium was transferred to 50 ml of pre sterilized minimal medium ($(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4 and $MgSO_4$) supplemented with 1% casein. The experiment was conducted in triplicate. Three flasks inoculated with M33 were incubated at

37°C and other three were incubated at 25°C along with sensitive isolate M26 in an orbital shaker at 200 rpm for 4 days. The sample filtrate for enzyme assay was collected at every 24 hrs interval by sieving through Whatman no. 1 filter paper.

The caseinolytic activity was estimated according to 30 with few modifications. One ml of the dilute enzyme (filtrate diluted with 0.1 M phosphate buffer pH 7.6) of each isolate in test tube was incubated with 1% casein (prepared in 0.1 M phosphate buffer) in water bath at 40°C for 20 min. The reaction was ceased by adding 3 ml of 5% TCA (Trichloro acetic acid) and incubating for 1 hour at room temperature. The content was then centrifuged at 10000g for 10 min and then filtered through Whatman no. 1 filter paper. The absorbance of the filtrate was tabulated at 280 nm under UV spectrophotometer against phosphate buffer without enzyme as blank. The enzyme activity was expressed as µg tyrosine equivalents min^{-1} ml^{-1}. One unit of enzyme activity (U) was defined as the amount of enzyme that liberates 1 µg ml^{-1} of tyrosine/min under the experimental conditions.

Statistics

In vitro protease activity was statistically analyzed for differences by one way anova and students newman keul test using statistica 6.0 software for windows with a significance level $p \leq 0.05$. Mean values and standard error of all the three replicates and of each isolate was tabulated. Graph was created using mean values.

Results

Selection of isolates for present work

The isolates for the study were considered on the basis of the observations of the temperature compatibility under variable temperature profiles (13°C - 37°C) on 12 *Metarhizium anisopliae* isolates (31). The upshot of the experiment was, M26 isolate was sensitive to temperatures at both the extremes and hence elected as negative control, on the other hand M33 was the only isolate that exhibited growth under 37°C thermal stress and so preferred as test isolate. M33 grown at optimum temperature 25°C was premeditated as positive control.

Heat shock proteins

The production of heat shock proteins was evident on SDS-PAGE gel. Proteins with a range of molecular weights deficient in either of the controls were prominent and nine HSPs with a molecular weight as high as 139.24 kDa to a low of 26.72 kDa were perceptible on the gel in isolate M33T (Fig.1). Proteins with much lower molecular weights were difficult to resolve even after several repetitions of the experiment and hence were not considered for compiling the observations.

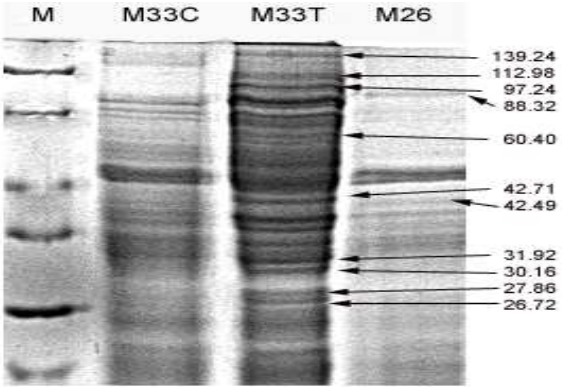
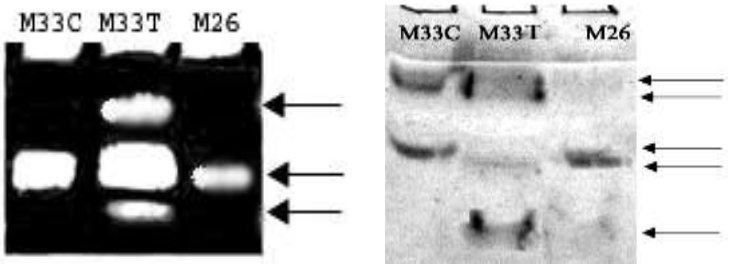


Figure 1: SDS-PAGE gel with arrows depicting the developed heat shock proteins under 37°C thermal stress M33T in comparison to positive (M33C) and negative (M26) controls.
M- Standard molecular weight marker from MBI Fermentas ranging from 14.4KDa to 116.0KDa

Antioxidant enzymes

The isozyme patterns of CAT and POX enzymes on native PAGE gels of isolates M33 and M26 under thermal stress (M33T) and optimum conditions (M33C- positive control; M26- negative control) delivered substantiation of increased number of isoforms under stress condition when compared to optimum conditions (Fig. 2). In case of CAT there was induction of two isoforms with one high molecular weight and other with low molecular weight when compared to both the controls. The intensity of the isoforms developed under stress condition was superior in comparison to controls (Fig. 2A). POX depicting native PAGE posed four isoforms under stress induced condition with two in positive control and one in negative control. The molecular weights of the specific isoforms were low in comparison to the controls (Fig 2B).

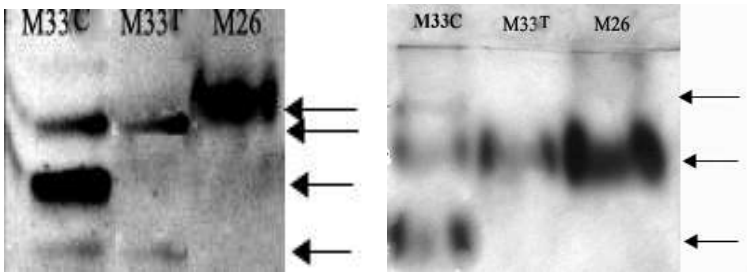


(A) (B)

Fig 2: Antioxidant enzyme patterns on native PAGE gel under thermal stress (37°C), M33T and at optimum temperature (25°C), M26 (negative control) and M33C (positive control) with figure (A) Catalase and (B) Peroxidase and arrows indicating the positions of the isoforms.

Esterase and acid phosphatase

Decrease in the number of isoforms under stress conditions for both EST and AP was observable by the electrophoretic patterns on native PAGE gels (Fig 3). Positive control produced three isoforms of EST of which two were reproduced in the stress induced isolate but there was loss of one form that was more intensified (Fig 3A). The position and intensity of EST in negative control was predominantly different from both positive control and stress induced isolate. For AP, there was loss of two isoforms in the stress induced isolate in comparison to three in positive control. The two nonexistent isoforms are of high and low molecular weight. The intensity of all the AP isoforms produced in positive control and stress induced isolate was faint. The negative control (M26) produced a single isoform at position in correspondence with M33C and M33T but with high intensity (Fig. 3B).



(A) (B)

Fig 3: Isozyme patterns of (A) Esterase and (B) Acid phosphatase under thermal stress (37°C), M33T and at optimum temperature (25°C), M26 (negative control) and M33C (positive control) with arrows indicating the positions of the isoforms.

Extra cellular Protease activity

The activity was tabulated from 24hrs after inoculation into casein substrate medium for the test and two controls (Table 1). Protease activity in all the isolates was parabolic showing consistent raise up till day 5 and gradual downfall later in positive and negative controls and in test isolate the deterioration of the activity initiated before day 5 (Fig. 4). Activity was more in negative control at optimum temperature 25°C whereas completely absent at test temperature 37°C as the isolate failed to initiate growth. On the other hand, in test isolate M33T activity was lower than the negative control (M26) and positive control (M33T) at both 37°C and 25°C. There was negative correlation between protease activity and temperature. The statistical analysis revealed significance difference in protease activity among the isolates (f= 18.017 at $p \leq 0.05$).

Table 1: Protease activity of *Metarhizium anisopliae* isolates against casein substrate over an incubation period of 6 days

Incubation time (days	Proteolytic activity vs. casein substrate[a]		
	M33T[b]	M33C[c]	M26[d]
1	0.39±0.09	1.09±0.10	3.94±0.16
2	0.67±0.04	1.19±0.07	5.32±0.18
3	0.82±0.03	1.67±0.06	7.06±0.03
4	1.44±0.05	2.54±0.05	7.93±0.04
5	0.96±0.03	4.07±0.08	8.21±0.11
6	0.93±0.04	2.78±0.09	5.17±0.14

Each result represents the mean value of three replicates ± s.e.m

[a]Activity determined by the variation in absorbance at 280nm during the period of incubation

[b]Isolate M33 stress induced at temperature 37°C during the period of incubation

[c]Isolate M33 grown at optimum temperature 25°C treated as positive control

[d]Isolate M26 grown at optimum temperature 25°C treated as negative control

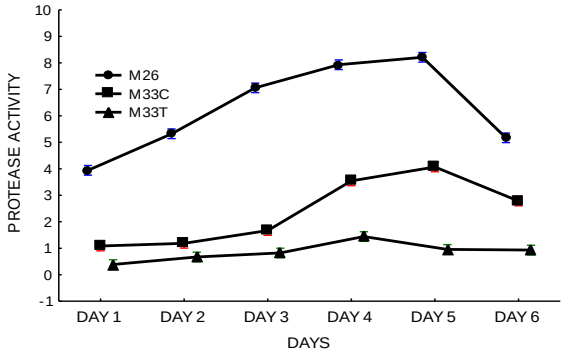


Fig 4: Protease activity of *Metarhizium anisopliae* isolates under thermal stress (37°C), M33T and at optimum temperature

(25°C), M26 (negative control) and M33C (positive control) through a period of 6days

Discussion

The two isolates in the study are from different geographical locations and with different levels of thermotolerance. Optimum temperature observed during the screening of 12 *Metarizium anisopliae* isolates was 25ºC (31) However, differences can be observed between isolates with respect to thermotolerance (9).This could be related with their geographical origin (6;7;24;25). Also, high variability in thermotolerance was observed among M. anisopliae isolates when conidial suspensions were exposed to high and low temperatures (25;10;16). Similar result was obtained and the test isolate M33 was selected based on this for the present work.

The tolerable temperature of the *Metarhizium anisopliae* isolate interested in the present study was 37°C which was justified by the report of 35 which suggested 37°C to be the maximum temperature for germination of *M. anisopliae* var. *anisopliae*. The thermal stress (37°C) induced isolate M33T responded by synthesizing 9 species of heat shock proteins (HSPs) with molecular weights ranging from 26.72 to 139.24 that neither of the controls (M33C-positive control and M26-negative control) expressed. Such variation in HSP synthesis by different species or strains at various elevated temperatures (from 30°C to 50°C) was suggested by 5 which also suggest that optimum temperature for the production of HSPs varies from organism to organism.

Apart from the HSPs other proteins similar in molecular weights of proteins in positive control were also synthesized in the stress induced isolate of the present study. It was reported by 33 that during the entire period of elevated temperature, HSPs are the main proteins synthesized in the cell. So, the results of present study may be due to the transfer of the test isolate to the optimum temperature 25°C after stress at 37°C for a period of 6 days.

The antioxidant enzymes CAT, POX play a vital role in detoxifying H_2O_2 resulting from ROS. The present result of electrophoretic patterns demonstrates that our heat-shock conditions were disturbing cell function sufficiently to elicit an increased response of CAT and POX. There was increased number of isoforms of CAT in stress induced isolate M33T than in both the controls. Induced catalase activity with heat treatment at 50°C in *Aspergillus nidulans* was reported by 21 and it was suggested that increased aeration also increases catalase activity (18).

In the present study, the activity of POX was similar to that of CAT. Three isoforms different from those expressed in control were found in stress induced isolate. 17 reported a 6-fold increase in specific activity of POX in the heat-shocked cells of *N. crassa* and also suggested that peroxidase was virtually undetectable in untreated cells but was apparent within 10 minutes of stress treatment. But as per our observation in *M. anisopliae* isolates, POX activity was detectable in the positive control as well as negative control, and the heat-shocked cells appear to be associated with increament of the enzyme. POX induces thermotolerance under stress treatment was reported by 29 in *Neurospora crassa*.

Two other intracellular enzymes EST and AP involved in the present study were also altered with the stress treatment. There was loss of isoform after induction when compared to un-induced isolates in case both EST and AP. 8 reported influence of elevated temperatures on fungal methyl esterase activity as stimulated under increased temperature contrary to the present result obtained. 1 studied variation in esterase activity in cold shock conditions in psychrotropic *Acinitobacter* sps. and suggested requirement of esterase for growth at low temperatures.

AP is also one of the industrially important enzymes produced by fungi. In the present study induced thermal shock reduced the number of isoforms when compared to two controls. The band intensity was also low, which appear as faint. 36 reported denaturation of acid phosphatases of *Aspergillus fumigatus* and *A. niger* when treated at elevated temperatures of 50°C to 70°C, on the other hand, *A. niger* pH 2.5 acid phosphatase displayed considerably higher stability.

The other enzyme in the present study was extra cellular protease which was altered with thermal stress among the isolates

considered. The assay suggested negative correlation of activity with temperature stress. The activity was low in stress induced isolate when compared to both controls. But positive correlation was observed with incubation days upto 5 days in control samples and upto 4 days in stress induced sample and contrary later. 2 reported that protease production is reduced by about 50% at 4°C above the optimum growth temperature in *Pseudomonas fluorescens* as observed in the present study and is almost completely inhibited at 32°C (19). 26 stated that alkaline proteases were stable and active at high temperatures of 50°C-60°C and are more suitable for industrial applications. 22 studies on *P. aeruginosa* proteases K-187 suggested the influence of physical factors, such as pH, temperature, incubation time on protease production and reported decreased protease production with increased temperature providing similarity to the present result obtained. Similar work was reported by 4 on *Bacillus* species.

Finally, the results obtained in the present study suggest alteration under stress condition in all the aspects considered. The increased antioxidant enzyme activity suggests active defensive mechanism developed against ROS by the tolerant isolate under thermal stress conditions of 37°C. The shrink in intra cellular enzymes, esterase and acid phosphatase and extra celluar protease activity probably suggest that the virulence property of the isolate under stress induced condition has been reduced. The study conducted would light on the consequences of different metabolic processes under thermal stress condition in *Metarhizium anisopliae* isolate *in vitro* which would aid in isolate selection for field application.

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