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Vol. 13(43), pp. 4176-4182, 22 October, 2014
DOI: 10.5897/AJB12.641
Article Number: B28614AC95BA90D
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Pathogenicity and proteome production of *Isaria fumosorosea* (=*Paecilomyces Fumosoroseus*) (WISE) isolates against lemon butterfly, *Papilio demoleus* (Papilionidae: lepidoptera)

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Received 11 February, 2012; Accepted 1 June, 2012

The pathogenic potential and catalytic triad conserved amino acids of the isolates *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) (*Ifr*$_1$ and *Ifr*$_2$) in response to *Papilio demoleus* was analysed. The isolates showed its potential in killing *P. demoleus* causing mortality of 72.23 and 61.90% at the end of 8 days with 10^8 spores ml^{-1} concentrations. The enzyme assays (higher proteolytic and chitinolytic activity) also showed that the *Ifr*$_2$ was more efficient than *Ifr*$_1$. The predictions of catalytic triads (serine, histidine and asparagine) were also visualized in the peak level obtained in infra-red (IR) and H_1 nuclear magnetic resonance (NMR) spectra. With this information it was suggested that, partial characterization of catalytic domain was predicted in the fungal isolates *Ifr.*

Key words: Entomopathogenic fungi, *Isaria fumosorosea, Papilio demoleus,* biological control.

INTRODUCTION

The insect pest management programs heavily rely on the use of synthetic chemical based insecticides or pesticides and herbicides, which is a multibillion dollar industry. The main driving force behind chemical insecticides is the fast speed of kill, high efficacy and political influence of the companies involved in this business. Microbial control is another approach for biological means of plant protection. The use of pathogens in biological control can be integrated with other natural enemies and the immediate effect of a microbial control agent can protect the crops, when parasitoids and predators are unable to maintain the pest population below the economic threshold level.

Fungi are particularly important for controlling sap

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sucking insects (white flies) for which there is no alternative available in the present biocontrol agents. Among them, *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) strains occur in soils and insects world-wide. Their efficacy against *Bemisia argentifolii* and *Trialeurodes vaporariorum* has been described by Wraight et al. (1998) and Fang et al. (1985). Nevertheless, different strains of the same species do not have equal potentials for the control of the same arthropod species (Altre et al., 1999; Vey et al., 1982).

Pathogenicity of an antagonist towards an insect species is related to the ability of the fungus to germinate on the insects' cuticle and to penetrate it, to its production of secondary metabolites and to the defense mechanisms of the host to prevent fungal infection and growth (Kaijiang and Roberts, 1986; Rath et al., 1996; Clarkson et al., 1998; St. Leger et al., 1989). The synthesis of extracellular enzymes is crucial for the infection process of this fungus. Successful infection primarily relies on the synthesis of molecular scissors such as extracellular proteases, chitinases and esterases (Clarkson and Charnley, 1996). The insect infection relies on protease(s) action because 75% of the cuticle is made up of proteins. Furthermore, chitinase(s) help degradation of *N*-acetyl-D-glucosamine moieties present in the cuticle (Charnley, 1997).

Due to the significance of proteases in breaching the insect cuticle, they have received more attention from researcher's worldwide. During more than three decades of research on Entomo-Pathogenic Fungi (EPF), several investigators have established two proteases, namely subtilisins and trypsins, as important virulence factors (St. Leger et al., 1986, 1988). Although the overall fold of various serine proteases may differ, they all follow the same mechanism of action through an identical stereochemistry of the catalytic triad and oxyanion hole. In this mechanism, the serine functions as the primary nucleophile and the histidine plays a dual role as the proton donor and acceptor at different steps in the reaction. The role of asparagine is believed to bring the histidine into the correct orientation to facilitate the nucleophilic attack by the serine. The role of the oxyanion hole is to stabilize the developing negative charge on the oxygen atom of the substrate during the formation of the tetrahedral intermediate (Russell and Fersht, 1987; Dodson and Wlodawer, 1998; Birktoft and Blow, 1972).

In order to increase their utilization, research needs to concentrate on: (a) pathogen virulence and speed of kill, (b) pathogen performance under challenging environmental conditions (cool weather, dry conditions etc.), (c) efficiency in the production process, (d) formulations that enable ease of application, increased environmental persistence and longer shelf-life, (e) integration into managed ecosystems and interaction with the environment and other integrated pest management (IPM) components (Lacey et al., 2001). The three proteases PR1 (*Metarhizium anisopliae*), VCP1 (*Verticillium chladosporium*) and Ver112 (*Verticillium licanii*) have shown that they have a high degree of sequence similarity with each other and belong to the proteinase K family of subtilisin-like serine proteases (subtilases) which is a large family of endopeptidases found only in fungi and Gram-negative bacteria (Siezen and Leunissen, 1997). These enzymes show conservation of the Asp- His-Ser catalytic triad and catalytic domain.

However, the three-dimensional (3D) structures have not been resolved for any of the cuticle-degrading proteases so far by either X-ray crystallography or nuclear magnetic resonance (NMR) techniques. The lacuna of cuticle degrading protease, in the present study reveals that the enzymatic catalytic domain is analyzed through IR and NMR, which provide concrete idea of a particular domain involved in cuticle degradation.

MATERIALS AND METHODS

Preparation of the test insects and bioassay

The test lepidopteran insect *Papilio demoleus* (L.) (Papilionidae: Lepidoptera) was maintained on citrus fresh leaves at 27±2□C, 70±5% relative humidity (RH) and 14 h photoperiod under laboratory conditions. Citrus leaves were washed with diluted potassium permanganate solution (0.001%) followed by distilled water to prevent microbial contamination.

Leaves were kept in shade at room temperature until the distilled water evaporates. All the glassware used in the experiments were washed thoroughly in detergent, treated with 2% formalin and then dried in an oven at 70□C for 4 h to check microbial contaminations. The 2nd instar larvae of *P. demoleus* was collected from citrus field near Madurai, Tamil Nadu, India and brought into the laboratory, reared in a wooden cage (60 X 60 cm) providing adequate citrus leaves as a stock culture. The 3rd instar larvae were from stock in this study.

Isolation protocol of *I. fumosorosea*

Isolation protocol of *Ifr* isolates followed the method of Haraprasad et al. (2001). *Ifr* was isolated from the soil in different locations of Madurai and Theni district, Tamil Nadu. One gram of soil was diluted with 100 ml of distilled water and was serially diluted. From each dilution, 100 µl was placed on PDA medium and it was fortified with streptomycin (10 mg/100 ml). It was allowed to grow for 7 days at 27±2°C (Haraprasad et al*.,* 2001) in the respective media. After 7 days of incubation the fungal colony was identified. The identified fungal colony was sub-cultured in Saboraud Dextrose Agar (SDA) (Hi-Media). The sterilized medium was transferred into sterile Petri dishes (Borosil®) and test tubes (Borosil®) that were then inoculated with conidia by streaking. The isolated fungus *Ifr* was used for the pathogenicity and enzyme studies against *P. demoleus.*

Efficiency of *I. fumosorosea towards P. demoleus*

The isolates of *I. fumosorosea* were used to determine the pathogenicity of *P. demoleus*. Pure culture of the test fungal species, *I. fumosorosea* isolates was grown on SDA at 27±2°C until a dense sporulating mat was produced (14 days). The conidial suspension of 10^8 conidia per milliliter was prepared by counting the

Table 1. Cumulative mortality (%) of the third instar larvae of *Papilio demoleus* inoculated with various conidia concentrations of the *Irf*$_1$ isolate of *Isaria fumosorosea* .

Conidial concentration (conidia ml^{-1})	Period (days) after treatment			
	2	4	6	8
1×10^5	0.00 (1.16)^{cd}	5.56 (13.63)^{d}	5.56 (13.63)^{d}	11.11 (19.47)^{d}
1×10^6	5.56 (13.63^{bd}	5.56 (13.63)^{cd}	11.11 (19.47)^{cd}	11.11 (19.47)^{cd}
1×10^7	5.56 (13.63)^{bd}	33.33 (35.26)^{b}	50.50 (45.00)^{b}	55.56 (48.19)^{b}
1×10^8	27.78 (31.80)^{a}	38.89 (38.58)^{a}	55.36 (48.19)^{a}	72.23 (58.20)^{a}
Control	0.00 (2.86)^{c}	0.33 (3.29)^{c}	0.67 (4.69)^{e}	4.67 (12.48)^{d}

Each value is mean of three replicates. Values in parenthesis are arc sine transformed values; a - d represents the levels of treatments: 'a' = best treatment and 'd'= pooor treatment.

spores in improved Neubauer counting chamber (Superior Marienfeld, Germany). The conidial suspension per milliliter (10^5 - 10^8 conidia per ml) was prepared for the experimental studies.

Bioassays with different *Ifr* fungal isolates were carried out by dipping 15 third instar larvae of *P. demoleus* in conidial suspensions plus 0.02% Tween 20 at each concentration for 30 s. After 30 s, the larvae was transferred to sterile filter paper and then placed in individual sterilized containers having single citrus leaf previously surface sterilized and was cleaned with sterilized paper towels to eliminate excess water. The bioassay setup was conducted in room at 27±2 °C at 70±5% RH. Each bioassay per concentration was performed in triplicates. A group of larvae (10 in each replicate) was maintained as control treatment; only distilled water plus 0.02% Tween 20. The larvae of *P. demoleus* were observed 2, 4, 6 and 8 days after inoculation with each conidial suspension. The dead larvae were placed in a controlled growth chamber to stimulate the development of fungal mycelia and confirm that the death was by infection of the *Ifr* isolates.

Fungal hydrolytic enzymes quantification and separation

The fungal hydrolytic enzyme activities such as α-amylase, proteolytic and chitinolytic activites were determined using Bernfeld (1956), St. Leger et al. (1987) and Ulhoa and Peberdy (1992) methods accordingly.

Prediction of catalytic triad of cuticle degrading protease

Ammonium sulphate precipitated culture supernatants were centrifuged at 5000 rpm for 10 min using refrigerated centrifuge. The precipitate was dissolved in the solvent (Butanol: Glacial acetic acid: Water in the ratio of 4:1:5) and then amino acid separations was carried out using Thin Layer Chromatography (TLC). The collected colored fractions were then used for the analysis in the Fourier Transform Infra-Red (FTIR, NEXUS-672 model) and the spectrum was taken in the mid Infra-Red (IR) region of 400-4000 cm^1. Nuclear Magnetic Resonance (NMR, Bruker (300 MHz) spectroscopy was also used to to predict the catalytic triad amino acids (asparagine, histidine and serine) of cuticle degrading protease of *I. fumosorosea* secretome. The samples were dissolved by using deuterated chloroform ($CDCL_3$) as solvent.

Statistical analysis

Analysis of variance and Duncan's multiple range test (DMRT) was performed to determine the best treatment using SPSS 10 and AGRESS softwares.

RESULTS

The efficacy of *I. fumosorosea* on *P. demoleus*

The present study reveals the efficiency of *I. fumosorosea* isolates against *P. demoleus* at various spore concentration of 10^5 to 10^8 at different days (2^{nd}, 4^{th}, 6^{th} and 8^{th} days) of post treatment (Tables 1 and 2). It was observed that, 72.23% (75%) mortality was found in *Ifr*$_1$ (Azhagar kovil) isolate on 8 days at 10^8 spore/ml whereas only 61.90% mortality was verified by *Ifr*$_2$ isolate. The control treatment unveiled the least mortality of *P. demoleus* by the tested isolates. The mortality due to *Ifr*$_2$ isolate at the initial spore concentration (10^5) was at the average of 4.76% only. The mean mortality of the *Ifr*$_2$ isolate ranged from 3.57 to 36.90%. Furthermore, by the 6 days at 10^8 concentrations only the *Ifr*$_1$ isolate promoted 50% mortality towards *P. demoleus.*

Enzymatic role of *I. fumosorosea* in the pathogenesis of *P. demoleus* was analysed quantitatively by different hydrolytic enzyme assays such as α-amylase activity, proteolytic activity and chitinolytic activity and are represented in Figures 1 and 2. Supernatant obtained from minimal medium in the presence and absence of *P. demoleus* exoskeleton were double filtered after 3 days of incubation and comparatively higher protein secretion was found in the Minimal Medium + Cuticle (MMC) by the *Ifr*$_1$ than MM and *Ifr*$_2$. This may also have helped the *Ifr*$_1$ isolate to contribute higher percent mortality than *Ifr*$_2$.

Structural elucidation of catalytic triad

Prediction of catalytic triad conserved amino acids of cuticle degrading protease such as serine, histidine and asparagine in the fungal secretome was carried out using FTIR and ^{1}H NMR with their basic structures (Figures 3 and 4).

DISCUSSION

Naturally occurring entomopathogens play an important role in our ecosystem. Invertebrates, viruses, bacteria and fungi can be found as regulatory factors in insect populations. Hence, many species are used as biological control agents of insect pests in row and glasshouse crops, orchards, turf, stored products and forestry and for abatement of vector insects of veterinary and medical

Table 2. Cumulative mortality (%) of the third instar larvae of *Papilio demoleus* inoculated with various conidia concentrations of the *Irf*$_2$ isolate of *Isaria fumosorosea*

Conidial concentration (conidia ml^{-1})	Period (days) after treatment			
	2	4	6	8
1×10^5	0.00 (1.08) ^{c}	4.76 (12.60)^d	4.76 (12.60)^d	4.76 (12.60)^d
1×10^6	4.76 (12.60)^{bd}	14.26 (22.20)^c	19.04 (25.87)^c	23.80 (29.20)^c
1×10^7	4.76 (12.60)^{bd}	19.04 (25.87)^{bc}	23.80 (29.20)^{bc}	48.61 (43.63)^b
1×10^8	19.04 (25.87)^a	28.57 (32.31)^a	38.09 (38.11)^a	61.90 (51.88)^a
Control	0.00 (2.86) ^{c}	0.00 (2.86)^c	0.67 (4.69)^c	3.00 (9.97)^c

Each value is mean of three replicates; Values in parenthesis are arc sine transformed values; a - d represents the levels of treatments. That is 'a' = best treatment and 'd'= poor treatment.

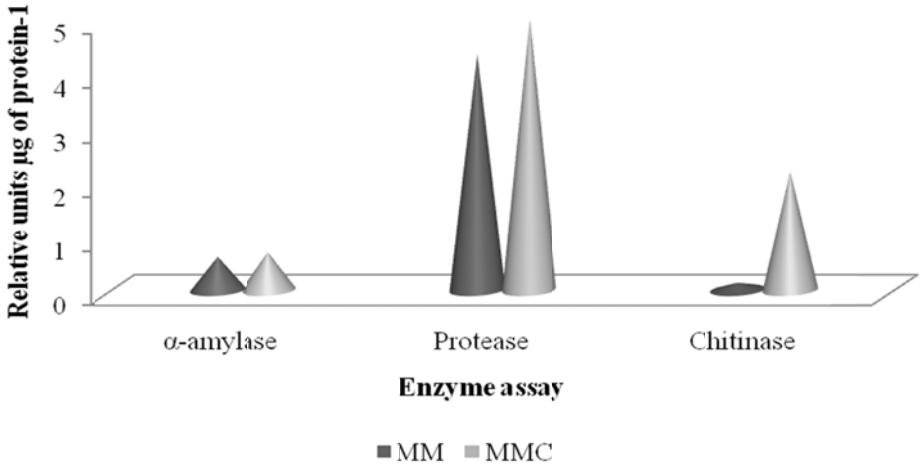

Figure 1. Secretome production of *Ifr*$_1$ isolate of *Isaria fumosorosea* response to *Papilio demoleus* exoskeleton.

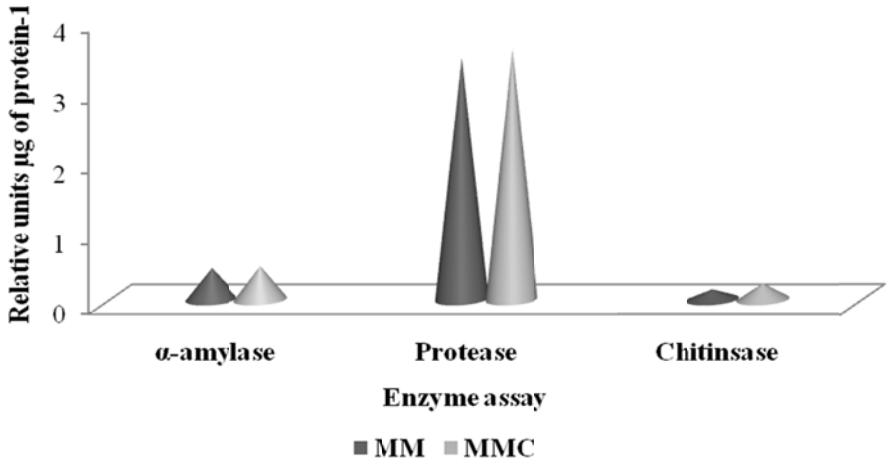

Figure 2. Secretome production of *Ifr*$_2$ isolate of *Isaria fumosorosea* response to *Papilio demoleus* exoskeleton.

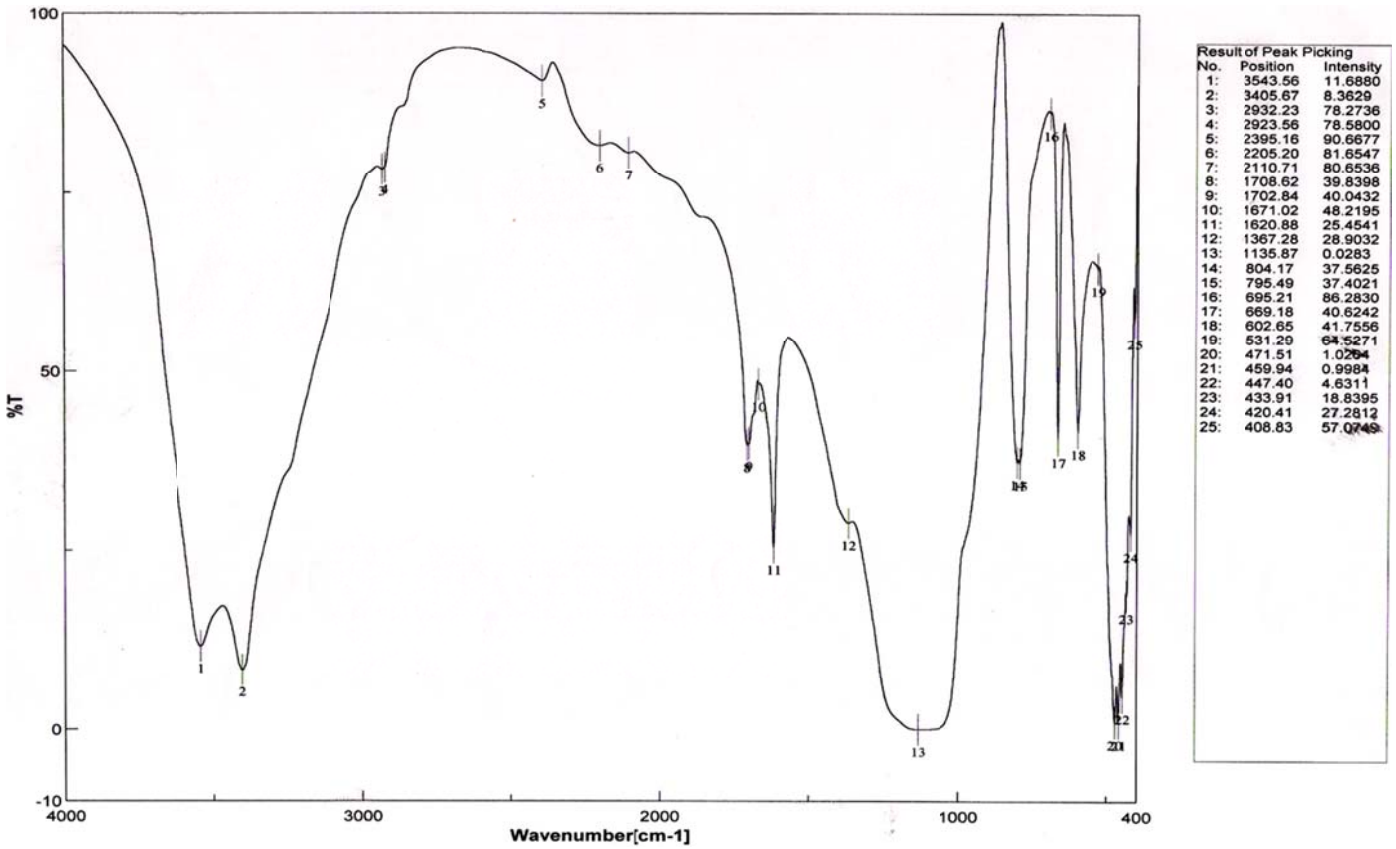

Figure 3. Fourier transform infra-red (FTIR) analysis of catalytic triad conserved amino acid of cuticle degrading proteases of *Isaria fumosorosae.*

importance. However, while fungal insecticides have been employed widely in China and to a lesser extent in Eastern Europe as well as in parts of South America, fungi have been little used elsewhere (Charnley, 1997). The virulence of the isolates towards *P. demoleus* was determined to identify the most promising candidate.

In the present study, the tested isolates differed in their pathogenicity towards *P. demoleus* that is the highest mortality caused by the Ifr_1 and Ifr_2 isolates was 72.23 and 61.90% respectively. The results of present investigations are in accordance with the findings of Nugroho and Ibrahim (2004).

The faster infectivity or shorter time to the broad mite mortality was caused by *P. fumosoroseus* with 2.783 days and gave 50% mortality while *Beauveria bassiana* took 3.349 days and *M. anisopliae* took 4.280 days to cause 50% mortality (Nugroho and Ibrahim, 2004). The reason behind the difference in mortality may be due to the larval susceptibility.

The insect cuticle acts as a barrier for fungal penetration and its thickness increases with every molting so that differences in the susceptibility of different larval instars to entomopathogenic fungi can be explained by their cuticle properties (Boucias and Pendland, 1991). Malsam (1999) reported no differences in the susceptibility of different larval stages to entomopathogens; an increased adult mortality and a decrease in the reproduction caused by *M. anisopliae*. Van De Veire et al. (1996) observed similar susceptibility to *P. fumosoroseus* in all larval stages and adults except for the 2nd stage, which was less susceptible.

The length of the inter-molt period depends upon the environmental conditions and the shorter it gets the less time remains for the fungus to germinate and penetrate. If molting occurs shortly after inoculation the entreating fungus may be removed prior to the colonisation of the insect (Vey and Fargues, 1977; Fargues and Rodriguez-Rueda, 1971).

Hence, the differences in the mortality can be explained by the time of inoculation regarding the remaining period to ecdysis. Presumably, susceptibility of most insects to entomopathogens is related to spore dosage. The speed of kill is influenced by the number of infection propagules in contact with the cuticle.

For most insect/ pathogen combinations a positive correlation between the number of infective spores and mortality by mycosis has been established (Liu et al., 1989; Vestergaard et al. 1995). Additionally, not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi (Butt and Goettel,

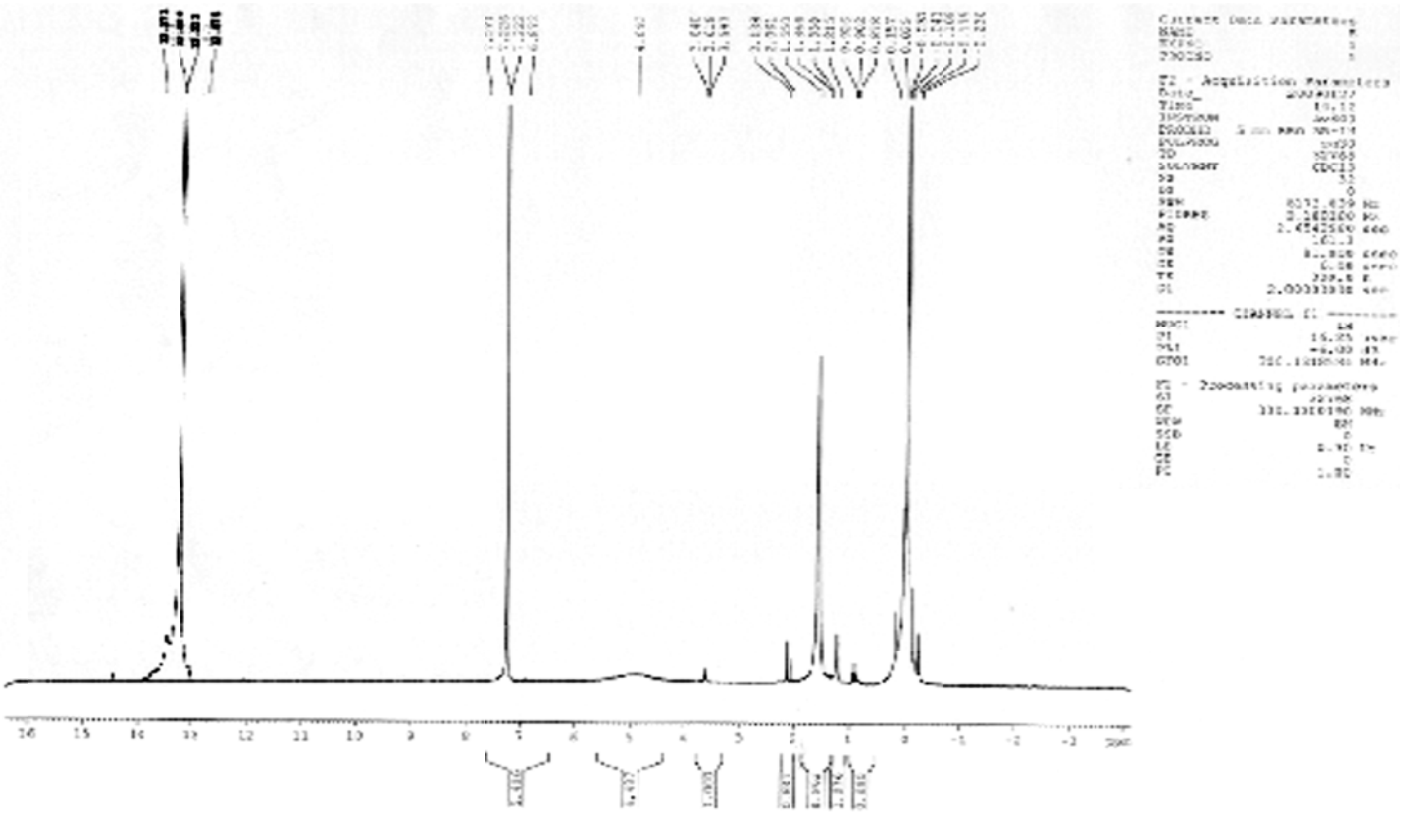

Figure 4. ^{1}H nuclear magnetic resonance (NMR) analysis of catalytic triad conserved amino acid of cuticle degrading proteases of *Isaria fumosorosea.*

2000). The preferential sites of invasion by fungi are often the buccal cavity, the area under the elytra, the inter-segmental folds or spiracles, where locally high humidity promotes germination and the cuticle is nonsclerotised and more easily penetrated (Charnley, 1989; Clarkson and Charnley, 1996; Hajek and St. Leger, 1994; Schabel, 1976). In the present study, it was evident that, spore concentration at 10^8 revealed higher mortality to *P. demoleus* and particularly, Ifr_1 isolate hed the higher control potential among the isolates and the spore concentration considered and reccommended for the effective mangement of *P. demoleus.*

The present investigation found relatively slight increase in the secretion of protease by the Ifr_1 in the presence of *P. demoleus* cuticle. The secretion of protease in the presence of cuticle in the present investigation was lower compared to the results found by Murad et al. (2006, 2007), whose reports contained higher hydrolytic enzyme secretion in the presence and absence of *Collasobruchus maculatus* exoskeleton treated with *Metarhizium anisopliae* and *Beauveria bassiana* accordingly.

However the present investigation indicates relatively lower protein secretion by the Ifr_1 in response to *P. demoleus* exoskeleton than Ifr_2. The secretions were expected since the unique carbon and nitrogen sources supplied to the fungus were derived from chitin-rich and protein-rich exoskeletons. The proteinases and chitinase clearly indicates the strong support played by the secretomes in insect killing efficiency of the isolate Ifr_1 than Ifr_2.

According to the basic structure of amino acids, and the presence of catalytic triad conserved amino acids, cuticle degrading proteases of the *I. fumosorosea* was confirmed with the report from FTIR and ^{1}H NMR. In the FTIR analysis, the presence OH, NH_2, COOH, CH_2 and CH groups of the catalytic triad amino acids (Serine, Histidine and Asparagine) were confirmed with peak positions. Presence of serine confirmed by obtaining the peak position at 3543, 3405 and 1135 (cm^{-1}) was shared by OH and NH_2 group.

The peak position for Carboxyl (COOH), CH_2 and CH group were obtained at 2923 (cm^{-1}) (OH), 2932 (cm^{-1}) (OH), 1708(C-O), 1702 (cm^{-1}) (C-O), 1367 (cm^{-1}) (O-C), 2932 and 2932 (cm^{-1}) accordingly. Besides, peak positions at 3459, 1011, 3405, 2715, 1216, 1224, and 2715 (cm^{-1}) was shared by the histidine and asparagine amino acids. Thus, in the present study, not only presence of catalytic triad amino acids was confirmed but also the enzyme activity of the isolate comes under the serine protease criteria. Similarly, the presence of serine was also verified clearly in the ^{1}H NMR. The peak in the

range 11-14 ppm confirmed the presence of OH group which was the main side chain of the amino acids serine besides, peak at 7.571 ppm (CH), 7.226, 7.222 and 6.873 ppm (CH), 4.893 and 3.640 ppm (CH), 3.619 and 3.597 ppm (CH2), 2.138 and 2.061 ppm (NH_2), represents the asparagine and its 2- Pyrrole imidazole ring (7.571 ppm (CH) and 6.873ppm (CH). The peak at the 550, 1.364, 1.338, 1.215, 0.926, 0.902 and 0.878 ppm has the histidine counterparts. Thus, the above information clearly confirm the presence of catalytic triad amino acids in the secreted proteome of *I .fumosorosea* towards *P. demoleus* exoskeleton.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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