BIOCHEMICAL AND MOLECULAR STUDIES OF CHITIN DEACETYLASE FROM *METARHIZIUM* SPECIES

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BY

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UNDER THE GUIDANCE OF

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Affectionately dedicated to

my parents, husband and son

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled, **"Biochemical and molecular studies of chitin deacetylase from *Metarhizium* species"** submitted by Ms. Shuklangi A. Kulkarni was carried out under my supervision at the Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

M. V. Deshpande
Research Guide

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, **"Biochemical and molecular studies of chitin deacetylase from *Metarhizium* species"** submitted for the degree of Doctor of Philosophy in Microbiology to the Savitribai Phule Pune University, is the record of work carried out by me at Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune-411008, India, under the supervision of Dr. M. V. Deshpande. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Shuklangi Kulkarni

Research Scholar

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Shuklangi Kulkarni

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ABBREVIATIONS

Abbreviation	Full form
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
DNase	Deoxyribo nuclease
dNTP	Deoxynucleotide triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
kD	Kilo Dalton
NCBI	National Centre for Biotechnology Information
RAPD	Rapid amplification of polymorphic DNA
RH	Relative humidity
RNA	Ribonucleic acid
RNase	Ribo nuclease
rpm	Revolutions per minute
RT	Room temperature
U	Unit of enzyme activity
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside

ABSTRACT

The entomopathogenic fungi have excellent promise for use as selective bio-control agents as compared to bacteria and viruses as they are effective by contact, host specific, least effective against non-target and beneficial organisms and their mass production is easy. However, the entomopathogenic fungus to be used as a biocontrol agent should be virulent against the target host and the biochemical and molecular aspects of fungus-insect interaction should be known for the development of effective biocontrol agents. Understanding the mechanism of fungal pathogenesis in insects will help in the production of more efficient mycoinsecticides, either by identifying fungal virulence determinants or by identifying genes that could be upregulated or otherwise manipulated to enhance virulence. The entomopathogenic fungi enter through the host cuticle both by enzymatic degradation and mechanical pressure. The cuticle-degrading enzymes (CDEs) like chitinase (EC 3.2.1.14), protease (EC 3.4.21.62), lipase (EC 3.1.1.3), chitin deacetylase (CDA) (EC 3.5.1.41) and chitosanase (EC 3.2.1.132) actively destroy or modify the structural integrity of the host cuticle. Many studies regarding the role of CDEs viz. protease, chitinase and lipase in virulence of entomopathogenic fungi has been previously carried out. The possible role of CDA in the penetration process and self defense of the entomopathogenic fungus *Metarhizium* against insect chitinases was proposed by Nahar et al. (2004). Therefore, the present study was initiated to determine the importance of CDEs in the virulence of *Metarhizium* isolates against *Helicoverpa armigera* and further biochemical and molecular studies of CDA to understand its role in fungus-insect interaction.

Chapter 1: Introduction

This chapter is comprised of the literature survey on the mechanism of entomopathogenesis and the killing components responsible for virulence of the entomopathogenic fungi giving special emphasis on CDEs. Further, a comprehensive account of sources of CDAs, biochemical and molecular studies of CDAs as well as significance and applications of CDAs in different fields has been discussed. (The part of the literature survey has been published in Ghormade et al. (2010).

Chapter 2: Materials and methods

This chapter describes the sources of chemicals and kits used as well as the media used for the growth and enzyme production in the present study. The details of isolation, maintenance, growth, conidial and vegetative transfers of *Metarhizium* isolates and; preparation and maintenance of *Escherichia coli* JM109 competent cells have been described. The microbial techniques like germ tube formation and appressorium formation are outlined. Further, the methods used for the estimation of CDEs viz. chitinase, protease, lipase, CDA and chitosanase have been described. The rearing of *H. armigera* along with the procedure of bioassay is briefly described. The production of conidia of *Metarhizium* isolates using solid state fermentation has been included. The details of the molecular methods used including DNA extraction, RNA extraction, cDNA synthesis, gene amplification by PCR, qRT-PCR, cloning and transformation of desired amplicons, plasmid extraction, DNA sequencing and phylogenetic analysis have been described. The statistical methods used for data analysis have been mentioned.

Chapter 3: Screening of *Metarhizium* isolates for the control of *Helicoverpa armigera*

In this chapter, screening of sixty eight *Metarhizium* isolates obtained from the soil samples of different crop fields and the insect hosts was carried out based on the extracellular *in vitro* production of CDEs and virulence against 3rd instar larvae of *H. armigera*. The twelve out of sixty eight *Metarhizium* isolates exhibiting >90% mortality and higher CDE activities were used for the determination of LT_{50}. Further, on the basis of LT_{50} values, the five isolates (M34311, M34412, M81123, M91427 and M91629) with lowest LT_{50} values (3.3-4.1 d) were selected for the evaluation of LC_{50}. As the LC_{50} values of the three isolates (M34311, M34412, M81123) were lower (1.4×10^3-5.7×10^3 conidia/ml), they were further studied for conidia production on a solid substrate, viability and settling time of conidia. The *Metarhizium* isolate M34412 produced 67 g/kg rice conidia, exhibited higher conidial germination (97%) and faster sedimentation time (ST_{50}-2.3 h) in 0.1% (w/v) Tween 80 than the other two isolates; M34311 and M81123. These three isolates were identified as *Metarhizium anisopliae* based on ITS1-5.8S-ITS4 sequencing. On the basis of above results, *M. anisopliae* M34412 was considered as the most effective isolate among sixty eight *Metarhizium* isolates.

Chapter 4: Cuticle degrading enzymes as biochemical and molecular markers of *Metarhizium* isolates

A. Molecular characterization of *Metarhizium* isolates based on polymorphism in protease (Pr1A) gene

The present chapter describes the correlation between the polymorphism in Pr1A gene of sixty eight *Metarhizium* isolates, *in vitro* protease activity and mortality of *H. armigera*. Initially, a positive correlation was observed between the protease activities and mortality of *H. armigera* as the protease activities for the highly pathogenic (>85.6%), moderately pathogenic (67.7-85.6%) and less pathogenic (<67.7%) groups ranged between 2.1-3.38 U/ml, 1-2.4 U/ml and <1 U/ml, respectively. The Pr1A gene (1.2 kb) of *Metarhizium* isolates was amplified. The digestion of Pr1A amplicons using restriction endonucleases namely RsaI, MspI and DdeI showed multiple polymorphisms with 7, 2 and 5 restriction patterns designated as A-G, H-I and J-N, respectively. After combining the restriction digestion patterns of sixty six *Metarhizium* isolates, 15 cumulative profile types were produced. The cumulative profile type I (D-I-M pattern) was most prevalent as out of sixty six *Metarhizium* isolates, thirty three (50%) isolates exhibited this profile and eleven isolates among these were from highly pathogenic group possessing high protease activity (>2.0 U/ml) and >90% mortality. These results supported the selection of *M. anisopliae* M34412 as the most effective isolate among sixty eight *Metarhizium* isolates.

B. Effect of repeated *in vitro* sub-culturing and *in vivo* passage on cuticle degrading enzyme production and virulence of *Metarhizium anisopliae* against *Helicoverpa armigera*

The effect of repeated *in vitro* sub-culturing and *in vivo* passage of the most effective isolate *M. anisopliae* M34412 on different aspects considered as parameters of virulence has been described in this chapter. The morphological, cultural characteristics and germination efficiency of *M. anisopliae* was not significantly affected whereas ~20% reduction in the appressorium formation from 1st to 40th sub-culture due to repeated *in vitro* sub-culturing on PDA was observed which subsequently increased after passage in *H. armigera*. Further, a gradual decrease in the constitutive and induced production of chitinase, chitosanase, CDA and protease was observed due to repeated *in vitro* sub-culturing when conidial inoculum was used and these activities increased subsequently after passage in *H. armigera*. However,

there was no significant effect on lipase production. When mycelial inoculum from the 10th serial vegetative transfer was used, significant decrease in CDA activity was observed in both YPG (19.8%) and chitin containing (23.2%) medium as compared to the 1st vegetatively transferred mycelium inoculum. However, chitinase, protease, lipase and chitosanase activities in YPG and chitin containing medium were not significantly affected. Similar trend for the effect of repeated *in vitro* sub-culturing on mortality of *H. armigera* and LT_{50} and LC_{50} values was observed. The genetic analysis of *in vitro* and *in vivo* sub-cultures of *M. anisopliae* showed correlation with the biochemical data.

Chapter 5: Biochemical and molecular studies of chitin deacetylase

A. Evaluation of *Metarhizium* isolates based on chitin deacetylase activity

The chapter describes the screening of sixty eight *Metarhizium* isolates based on cluster analysis using *in vitro* extracellular CDA activity in YPG medium and corrected mortality against 3rd instar larvae of *H. armigera*. The *Metarhizium* isolate M161063 showing 82% mortality, high CDA (2.26 U/ml) and chitosanase (13.22 U/ml) activity and; low chitinase activity (1.01 U/ml) was selected for further studies. The *Metarhizium* isolate M161063 was identified to be *M. anisopliae* based on ITS1-5.8S-ITS4 sequencing. The intracellular CDA activity in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h) and blastospores (24 h) of *M. anisopliae* M161063 was determined and the trend of intracellular CDA activity was observed to be differentiation specific.

B. Sequencing of chitin deacetylase gene from *Metarhizium anisopliae* M161063

In this chapter, the molecular characterization of CDA gene from *M. anisopliae* M161063 was carried out for its further use in confirming the role of CDA in fungus-insect interaction. The CDA gene from *M. anisopliae* M161063 was amplified using degenerate primers (CDAF1-CDAR2) designed towards the conserved polysaccharide deacetylase domain of previously reported fungl CDA genes from the NCBI database. The partial CDA gene sequence (594 bp) was obtained that shared maximum identity with deacetylases of ascomycetous fungi. The deduced amino acid sequence of CDA displayed the presence of five conserved catalytic domains. Further, the phylogenetic tree constructed using the deduced amino acid sequence of *M. anisopliae* CDA with amino acid sequences of other fungal and bacterial deacetylases coincided with the

taxonomic classification of fungi. The partial sequence of CDA gene from *M. anisopliae* M161063 was further used for studying the differential expression of CDA.

C. Expression studies of chitin deacetylase from *Metarhizium anisopliae* M161063

The present chapter aimed at analyzing the change in expression of CDA gene in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h, 48 h, 72 h) and blastospores (24 h, 48 h, 72 h) of *M. anisopliae* M161063 using qRT-PCR. Initially, the housekeeping gene showing constant expression in above mentioned morphological forms was identified for normalization of CDA gene expression data obtained after qRT-PCR. The five housekeeping genes namely *18S RNA*, *GAPDH*, *Ubc*, *Tub-a* and *Tub-b* were evaluated by qRT-PCR for the expression stability. Out of five housekeeping genes studied, genes encoding *18S RNA* displayed relatively low CP values, indicating high expression of this gene in *M. anisopliae* as compared to other studied housekeeping genes. On the basis of gene expression stability of housekeeping genes determined using the software; *Bestkeeper*, *18S RNA* was identified as the most consistently expressed housekeeping gene as it exhibited SD (±CP) values <1. Subsequently, *18S RNA* was used as a reference gene for studying the expression of CDA gene in different morphological forms of *M. anisopliae* M161063. The primers for qRT-PCR studies of CDA gene were designed using the partial CDA sequence of *M. anisopliae* M161063. The expression of CDA increased in germinating conidia (12 h), appressoria (24 h) and mycelia (24 h) by 0.3-fold, 0.4-fold and 1.3 fold, respectively whereas the mycelia (48 and 72 h) and blastospores (24 h) showed a lower expression level of CDA. Further, the CDA gene was repressed in conidia from PDA slants (7 d) and blastospores (48 h and 72 h). Thus, the results of the qRT-PCR studies supported the trend of the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 suggesting the differentiation specific regulation of CDA in *Metarhizium* and its role in fungus-insect interaction.

Chapter 6: Summary and conclusions

All the results and findings of the present investigation are summarized in this chapter.

CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

Entomopathogenic fungi are considered as key regulatory factors of insect populations in nature. They infect a broader range of insects compared to bacteria and viruses. The fungal pathogens were recognized as potential insect pest control agents since the later part of the 19th century and they were used for the first time for the biological control of pests. The most prominently used entomopathogens with broad host range such as *Metarhizium*, *Beauveria*, *Nomuraea, Verticillium, Entomophaga, Paecilomyces* and *Hirsutella* living in diverse habitats have been extensively studied for their efficacy as biocontrol agents (Butt et al., 1995; Charnley, 1989). However, for development of effective biocontrol agents, a sound knowledge of biochemical and molecular aspects of fungus-insect interaction is essential. Further, the entomopathogenic fungus to be used as a biocontrol agent should be virulent against the target host. Understanding the mechanism of entomopathogenesis will help in the production of more efficient mycoinsecticides, either by identifying fungal virulence determinants or by identifying genes that could be upregulated or otherwise manipulated to enhance virulence.

1.1 Mechanism of entomopathogenesis

Understanding the mechanism of entomopathogenesis is important as the virulence of the entomopathogenic fungi depends on multiple factors. Fungi have a unique mode of infection; they reach the haemocoel through the cuticle. Ingested fungal conidia do not germinate in the gut and are voided in the faeces (Sandhu et al., 2012).

Entomopathogenic fungi invade their hosts by direct penetration through the host exoskeleton or cuticle. The cuticle is the outermost protective cover in insects composed of wax, lipids, protein and chitin (Figure 1.1). It is made up of two layers, the outer epicuticle which covers the bulky procuticle. The epicuticle is a very thin, dark pigmented multi-layered membrane lacking chitin. It is a complex structure containing phenol-stabilized proteins and is covered in a waxy layer containing fatty acids, lipids and sterols. The procuticle forms the major portion of the cuticle and is further composed of the exo- and endocuticle comprising of chitin and protein, wherein the exo-cuticle is generally melanised (Andersen, 2002). The protein accounts for up to 70% of the cuticle whereas the chitin share is 25-50% of dry weight of the cuticle.

The pre-infection requirements are adhesion and germination of conidia to form germ tubes or an infection structure, appressoria. A combination of mechanical

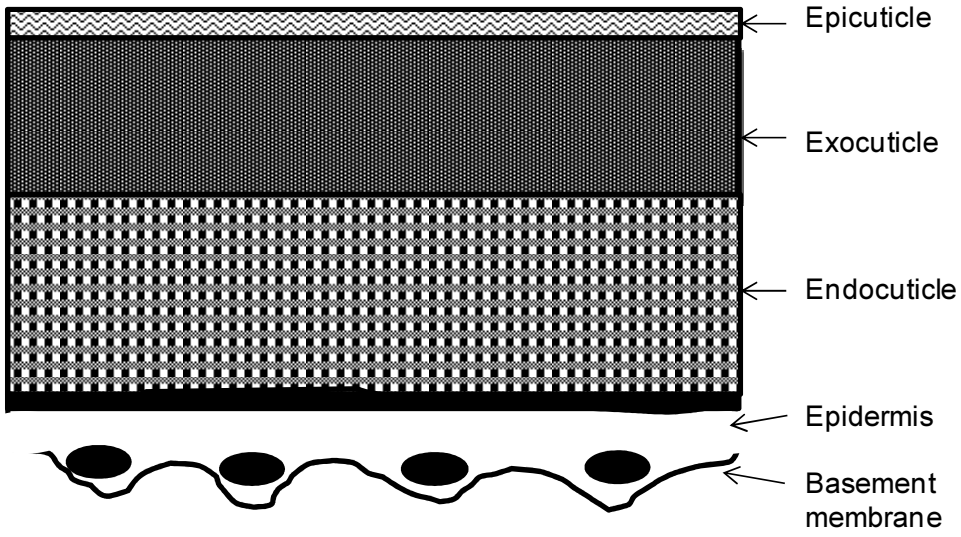


Figure 1.1 Structure of insect cuticle

pressure (created by appressoria) and production of cuticle-degrading enzymes (CDEs) helps the fungus to enter through the cuticle. For passing through the cuticle, the fungus undergoes differentiation to form infection pegs and penetrant plates/ hyphae. In the haemocoel, it again undergoes differentiation to form blastospores/ hyphal bodies. The death of the insect results from a combination of factors viz. mechanical damage resulting from tissue invasion, depletion of nutrient resources and production of toxins in the body of the insect. Under favourable conditions, particularly, high relative humidity, the fungus emerges back on the insect cuticle and grows in the typical hyphal form (Figure 1.2**)**. The mechanism of entomopathogenesis is discussed further.

1.1.1 Adhesion of conidia to the host cuticle

The pathogenesis is initiated by adhesion of conidia to the insect cuticle. The entomopathogenic fungi use different strategies for their attachment to the insect cuticle. The conidia are either dry hydrophobic as in case of *Beauveria bassiana*, *Metarhizium anisopliae* and *Nomuraea rileyi*, or sticky hydrophilic as seen in *Verticillium lecanii* and *Hirsutella thompsonii* (Sosa-Gomez et al., 1997). However, if the factors essential for a phase of adhesion, microbial development, or pathogenesis

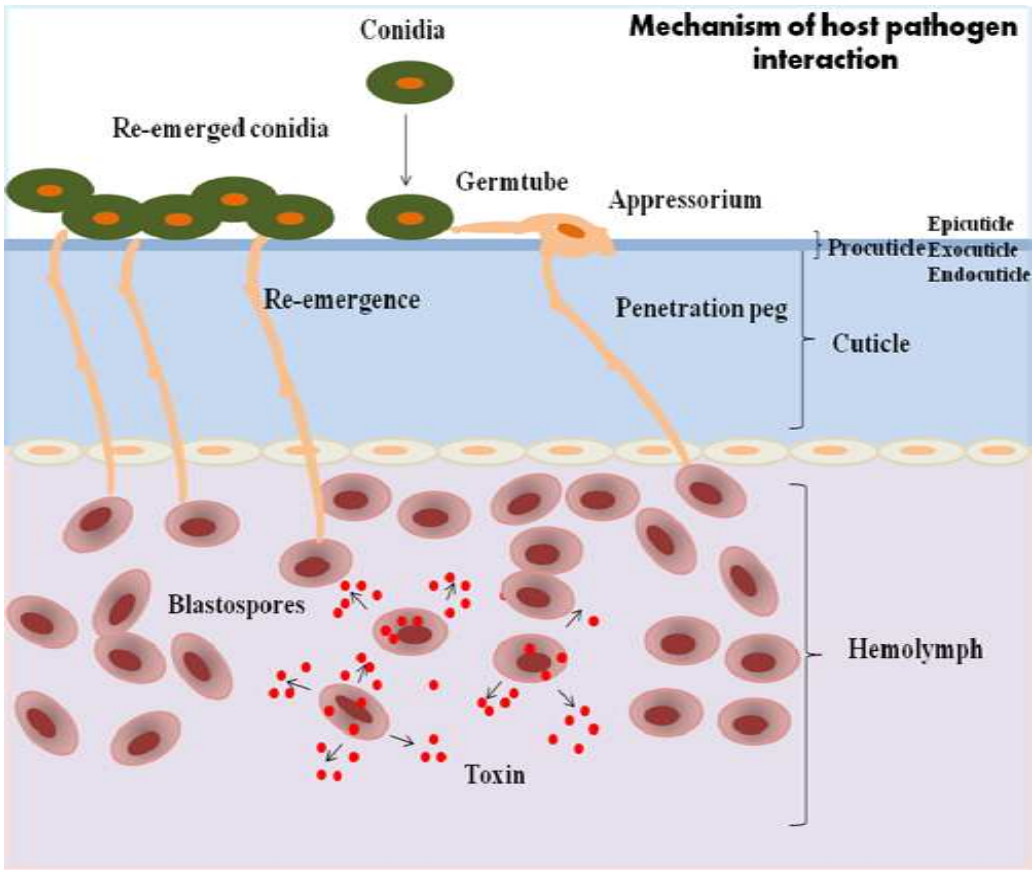


Figure 1.2 Mechanism of entomopathogenesis (Kapoor, 2012)

are absent, the infection can be aborted on the epicuticle. For instance, low humidity, inability to utilize available nutrients on the cuticle surface, or absence of factors necessary for recognition of a susceptible host or penetrable infection site prevents infection (Hajek and St. Leger, 1994). The presence of fatty amides on the cuticle of a book lice *Liposcelis bostrychophila* decreased the hydrophobicity and static charge which in turn hampered the conidial adhesion of *B. bassiana* and *M. anisopliae* (Lord and Howard, 2004). The adhesion is either passive (adsorption with the aid of wind or water) or active host-specific (germination and penetration) phenomenon.

In passive adhesion, the hydrophobic interactions between the conidial walls and the insect epicuticle are important. The conidia of the entomopathogens *B. bassiana*, *M. anisopliae* and *N. rileyi* possessed hydrophobic rodlet layer that helped in the attachment to the insect cuticle (Boucias et al., 1988). According to Boucias and Pendland (1991), the initial hydrophobic interactions between conidia and the cuticle surface were supplemented by the secretion of adhesive mucus as the conidium swelled during pre-germination development. For instance, the conidia of *V. lecanii* adhered more efficiently to larvae of the horticultural pest *Frankliniella*

occidentalis as compared to blastospores. The efficient adhesion of conidia was probably due to their mucilaginous coating (Meyer and Sermann, 2003). Further, the differences in surface characteristics among aerial conidia, blastospores and submerged conidia of *B. bassiana* were studied using atomic force microscopy (AFM), contact angle measurements and zeta potential determinations. The surface of aerial conidia was found to be more hydrophobic than the blastospores and submerged conidia (Holder et al., 2007). However, according to Ment et al. (2010), the correlation between adhesion and mortality was dependent on number of conidia and not on the host specificity, as the hydrophobic conidia of entomopathogenic fungi bind in a non-specific manner to the epicuticular surfaces of both susceptible and resistant hosts.

The active adhesion was considered to be because of the association of the lectins from spore surface with the insect cuticle and was host-specific (Barranco-Florido et al., 2002). The involvement of lectins in the attachment of the entomopathogenic fungus *Conidiobolus obscurus* to pea aphid *Acyrthosiphon pisum* was studied. However, the role of glucose and *N*-acetylglucosamine (GlcNAc) binding proteins in the pathogenicity of *C. obscurus*, or at least in initial steps of the adhesion process of the blastospore to the aphid cuticle was not reported (Latge et al., 1988). Further, the carbohydrate epitopes from the surface of aerial conidia, blastospores and submerged conidia of *B. bassiana* were studied to understand the role of lectins in the infection process and the aerial conidia were found to exhibit diverse lectin binding characteristics than submerged conidia and blastospores (Wanchoo et al., 2009).

1.1.2 Germination of conidia

Once the pathogen adheres to the host surface, it undergoes rapid germination and growth which are profoundly influenced by the availability of water, nutrients (carbon and energy source), oxygen as well as pH, temperature, and by the effects of toxic host-surface compounds. Fungi with a broad host range requires a wide range of nonspecific carbon and nitrogen sources for germination (Sandhu, 1995) whereas those with restricted host range possess more specific requirements for germination (St. Leger et al., 1989). The relationship between the nitrogen source, lipid/carbohydrate reserves and spore adhesion, germination, and virulence was determined by Khachatourians and Qazi (2008). Alterations in the epicuticular hydrocarbons of European corn borer *Ostrinia nubilalis* and European beetle

Melolontha melolontha larval integument were observed after cuticular application of conidia of *B. bassiana* or *Beauveria brongniartii* (Lecuona et al., 1991).

1.1.3 Formation of infection structures

During cuticular penetration, as the entomopathogenic fungi move continually through different environments, they undergo cellular differentiation and form series of specific morphological structures in response to the host environment. For instance, the germ tubes of *M. anisopliae* undergo differentiation to form infection structures like appressoria (located at the cuticle surface), infection pegs (in the epicuticle), penetrant hyphae and penetrant plates (in the procuticle), and yeast-like hyphal bodies/blastospores (in the haemocoel) (Hajek and St. Leger, 1994) (Figure 1.2**)**. The entomopathogenic fungi overcome the host barriers using these infection structures.

1.1.3.1 Appressorium

After initiation of germination, the germinating conidia get differentiated to form a peg like structure, known as appressorium (Figure 1.2). The appressorium represents an adaptation for concentrating physical and chemical energy over a very small area to make the entry process more efficient. Thus, the formation of appressorium is essential in establishing a pathogenic interaction with the host.

The appressorium can be produced *in vitro* on hard hydrophobic surfaces and the differentiation is stimulated by low levels of complex nitrogenous compounds whereas the appressorium formation *in vivo* is influenced by surface topography. In *M. anisopliae*, the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) were found to be involved in appressorium formation. The differentiation was initiated by contact-induced change in membrane potential, possibly because of the activation of a mechanosensitive ion channel that resulted in disruption of the apical Ca^{2+} gradient required for polar growth (St. Leger et al., 1991b). Similarly, in the rice blast fungus *Magnaporthe grisea*, the addition of cAMP to germinating conidia or vegetative hyphae on noninductive hydrophilic surface induced appressorium formation (Lee and Dean, 1993).

1.1.3.2 Penetrant hyphae/plates

During penetration of *M. anisopliae* through the insect cuticle, the epicuticle is initially breached by the appressorium. For further penetration through the cuticle, penetrant hyphae are formed which extend laterally and produce penetrant plates. The dispersal of CDEs produced by *Metarhizium* is facilitated by the fractures caused by

penetrant plates. Further, the infection hyphae penetrate down through the host cuticle and eventually emerge into the haemocoel of the insect (Hajek and St. Leger, 1994).

1.1.3.3 Yeast-like hyphal bodies/blastospores

After the successful penetration through the cuticle, the fungus is differentiated into unicellular yeast-like hyphal bodies/blastospores and gets distributed in the haemolymph (Bhattacharyya et al., 2004). The formation of blastospores is considered as a prerequisite for pathogenicity. Further, the insecticidal secondary metabolites produced by the fungi cause starvation or physiological disruption which ultimately leads to the death of the insect.

1.1.4 Killing components in fungus-insect interaction

The nutrients for the growth and reproduction of the entomopathogenic fungi are obtained by breaking down and assimilating the host material. The host-resistance mechanisms are overcomed by array of mechanisms. The killing components viz. low-molecular-weight secondary metabolites (toxins) and CDEs produced by the entomopathogenic fungi destroy or modify the structural integrity of the host, inhibit the selective processes or enzymes of the host and also interfere with the regulatory systems of the host.

1.1.4.1 Secondary metabolites

The entomopathogenic fungi secrete a wide range of compounds in the haemocoel. For example, *B. bassiana, B. brongniartii, H. thompsonii, M. anisopliae, Paecilomyces fumosoroseus, Tolypocladium sp*. and *V. lecanii* produce different toxins such as, bassianin, beuvericin, cytochalasin C, destruxin, dipicolonic acid, enfrapeptin, hirsutellin A, B, oosporein, swainsinone etc. (Amiri-Besheli et al., 2000; Vey et al., 2001). The different insects were found to show variation in their susceptibility to the toxins and these toxins also possessed diverse effects on various insect tissues (St. Leger et al., 1987b).

The *M. anisopliae* effective against lepidopterean insects produced destruxin which were observed to activate calcium channels directly or indirectly leading to flaccid paralysis. Furthermore, the effect of destruxins on insect midgut, malphigian tubules, haemocytes and muscle tissues was also reported (Clarkson and Charnley, 1996; Sandhu et al., 2012). The efrapeptin produced by *Tolypocladium* showed miticidal effects against potato beetle, mites, bud-worm and diamondback moth whereas the oosporein produced by *B. brongniartii* was effective against cockchafer

larvae and hirsutellin A produced by *H. thompsonii* was reported to be effective against citrus rust mite and mosquito larvae (Vey et al., 2001).

1.1.4.2 Cuticle degrading enzymes

The fungus-insect interaction is a multifactorial process and hydrolytic enzymes are key factors for the penetration step (Krieger de Moraes et al., 2003). The entomopathogenic fungi produce various cuticle degrading enzymes (CDEs) like chitinase (EC 3.2.1.14), protease (EC 3.4.21.62), lipase (EC 3.1.1.3), chitin deacetylase (CDA) (EC 3.5.1.41) and chitosanase (EC 3.2.1.132) that can degrade the major components of the insect cuticle. Due to the complex structure of insect cuticle, synergistic action of all these enzymes is required for penetration through the insect cuticle. The *M. anisopliae* when grown *in vitro*, produces a range of extracellular enzymes capable of degrading the major components of insect cuticle (Sandhu et al., 2012).

In *Metarhizium* and *Beauveria* cultures, the extracellular enzymes appear sequentially. During the *in vitro* submerged fermentation, the entomopathogenic fungi produced extracellular CDEs mainly chitinases, proteases and lipases when locust cuticle was used as a sole source of carbon (St. Leger et al., 1986b). It was reported that the sequence of appearance of CDEs was in accordance with cuticular structure. The production of proteolytic enzymes such as esterase, endopeptidase, aminopeptidase and carboxypeptidase was observed within first 24 h of growth whereas *N*-acetylglucosaminidase appeared later. Though chitin forms the main structural mesh, endochitinase, that hydrolyzes the chitin polymer randomly, was produced in significant quantities after 4 d whereas lipases were detectable after 5 d (Charnley, 1989). Further, the significance of any enzyme(s) was suggested to be dependent upon the cuticle characteristics and physiological state of the insect as well as the mechanism of invasion by the fungus. For instance, the lipase activity contributed to the initial phase of infection when the germinating conidium had to break the epicuticle layer to gain entry into the insect (Hegedus and Khachatourians, 1995). The protein degrading enzymes such as proteases, collagenases and chymoelastases were identified and characterized in the entomopathogenic fungi viz. *Aschersonia aleyrodis, B. bassiana, B. brongniartii, N. rileyi, M. anisopliae*, and *V. lecanii* (Charnley and St. Leger, 1991; Khachatourians, 1991; Khachatourians, 1996; Sheng et al., 2006). The strategies and processes that could be used for extraction, purification and characterization of different enzymes related to penetration, toxic

effect and pathogenesis of entomopathogenic fungi were explained by Ali et al. (2010).

1.2 Cuticle degrading enzymes as virulence factors

Various studies are going on all over the world to determine the enzymes involved in virulence of the entomopathogenic fungi. Many enzymes produced by the entomopathogenic fungi could be considered as potential virulence factors as these were similar to the toxic components found in bacteria and animal venoms (Ali et al., 2010). The molecular biology techniques provide the necessary tool for knowing the mechanisms of pathogenesis and for producing recombinant organisms with new and relevant characteristics. It has been suggested that investigation of virulence determinants of entomopathogenic fungi and isolation of pathogenic genes using genetic transformation can produce a strain with enhanced virulence (Sandhu et al., 2012). The role of different CDEs in virulence of the entomopathogenic fungi as well as their biochemical and molecular studies are discussed further.

1.2.1 Chitinase

Chitin being the major component of the insect cuticle, both endo and exo-chitinases play critical roles in the cleavage of *N*-acetylglucosamine polymer of the insect cuticle into smaller units or monomers. The extracellular chitinases were demonstrated to be virulence determinant factors by Khachatourians (1991). The virulent isolates of *N. rileyi* exhibited substantially higher levels of chitinase than avirulent strains (El-Sayed et al., 1989). The chitinolytic enzymes (*N*-acetyl-β-D-glucosaminidases and endochitinases) were reported to be present in the broth culture supplemented with the insect cuticles from *M. anisopliae*, *Metarhizium flavoviridae* and *B. bassiana* (St. Leger et al., 1996a). Further, the transformants of *B. bassiana* (*gpd-Bbchit1*) overproducing *Bbchit1* were constructed which showed enhanced virulence against the aphid, *Myzus persicae* (Fang et al., 2005).

The *M. anisopliae* were reported to produce multiple chitinase isozymes including both, endochitinases (CHI1, CHI2 and CHI3) and exochitinase under *in vitro* conditions (St. Leger et al., 1993). It was reported that the chitinase from *M. anisopliae* consisted of acidic proteins (pI 4.8) with molecular masses 43.5 kDa and 45 kDa (St. Leger et al., 1996a). Further, a chitinase with molecular mass of 60 kDa from *M. anisopliae* grown in a medium containing chitin as the sole carbon source with an optimum pH 5.0 was reported by Kang et al. (1998, 1999). An endochitinase

(*Bbchit1*) possessing molecular mass 33 kDa and pI 5.4 was purified from *B. bassiana* liquid cultures supplemented with chitin (Fang et al., 2005). Similarly, two extracellular chitinases with an apparent molecular mass 32 kDa (Chi32) and 46 kDa (Chi46) from the fungus *Paecilomyces variotii* were purified (Nguyen et al., 2009). The chitinase gene *Vlchit1* from the entomopathogenic fungus *V. lecanii* was cloned and overexpressed in *Escherichia coli* and the recombinant protein of molecular mass 88.4 kDa was obtained (Zhu et al., 2008).

1.2.2 Protease

The proteases and peptidases of entomopathogenic fungi possess multiple roles viz. degradation of the insect cuticle, saprophytic growth of the fungi, activation of the prophenol oxidase in the hemolymph and they also act as virulence factor. The proteases were assigned a major role in cuticle degradation, as over expression of the subtilisin-like protease gene (Pr1A) significantly enhanced the virulence of *M. anisopliae* (St. Leger et al., 1996b). Out of seven conidiation associated genes (*cag*) in *M. anisopliae*, the gene cag7, encoding an extracellular subtilisin-like proteinase (Pr1) was found to be essential for cuticle degradation (Small and Bidochka, 2005). An engineered mycoinsecticide of *M. anisopliae* over-expressed the toxic protease Pr1 in the haemolymph of *Manduca sexta* which ultimately activated the phenoloxidase system causing 25% reduction in the time of death and 40% reduction in food consumption (St. Leger et al., 1996b). The production of Pr1 and Pr2 proteases by *B. bassiana* was induced in presence of the cuticle of coffee berry borer (CBB) *Hypothenemus hampei* and alkaline pH (Dias et al., 2008).

The two extracellular proteases, BBP and Pr1 were purified and characterized from *B. bassiana* and it was observed that the PI of BBP (7.5) was lower than Pr1 (10.0) and it was 0.5 kDa smaller than Pr1 (Urtz and Rice, 2000). The synthesis of chymotrypsin (CHY1) of 374 amino acids with pI 5.07 and molecular mass 38.27 kDa with 186 amino acid *N*-terminal fragment from *M. anisopliae* has been reported by Screen and St. Leger (2000). The *B. bassiana* grown on cuticle/chitin cultures produced an extracellular subtilisin-like serine endoprotease (Pr1) which was synthesized as a large precursor with molecular mass 37.46 kDa containing a signal peptide for translocation, a propeptide, and the mature protein with molecular mass 26.83 kDa (Joshi et al., 1995).

1.2.3 Lipase

The insect surface is composed of various amounts of hydrocarbons (alkanes, alkenes, and their methyl-branched derivatives), fatty acids and esters, alcohols, ketones, and aldehydes, with minor components including triacylglycerols, epoxides, and ethers, as well as tanned (cross-linked) proteins (Ortiz-Urquiza and Keyhani, 2013). The variability in the production of extracellular enzymes of entomopathogenic fungi viz. *M. anisopliae*, *B. bassiana* and *Paecilomyces* species grown on different substrates was investigated and it was observed that lipase activity was produced by all the strains (Fernandes et al., 2012). The *M. anisopliae* cultivated in different carbon sources produced different amounts of lipase (Silva et al., 2005). Further, an extracellular lipase with molecular mass 31 kDa was purified from *Isaria fumosorosea*. The topical co-application of this purified lipase combined with fungal conidial suspensions increased the mortality of 2nd instar nymphs of *Dysmicoccus neobrevipes* as compared to the fungus alone. This suggested the degradation of parts of the insect cuticle by hydrolysing the ester bonds of lipoproteins, fats and/or wax layers by purified lipase (Ali et al., 2014).

Furthermore, apart from the above discussed enzymes, the importance of CDA in virulence of many plant pathogenic and human pathogenic fungi as well as bacteria has been previously reported in many studies. For the first time, the involvement of constitutively produced CDA in combination with chitosanase as an alternate mechanism of cuticle degradation in *M. anisopliae* has been demonstrated by Nahar et al. (2004a). Considering the possible role of CDA in the entomopathogenesis of *Metarhizium*, the detailed biochemical, molecular studies of CDA and its biological role in other systems as well as the sources and applications of CDA has been discussed in following section.

1.3 Chitin deacetylase: A comprehensive account on host-pathogen interaction and other roles in nature

CDA is an enzyme that catalyzes the hydrolysis of acetamido groups of GlcNAc residues in chitin, converting it to chitosan, a glucosamine polymer (Figure 1.3). CDA is a polysaccharide deacetylase from Carbohydrate Esterase family 4 (CE4). On the basis of sequence homology, CEs are classified into 14 families. The family 4

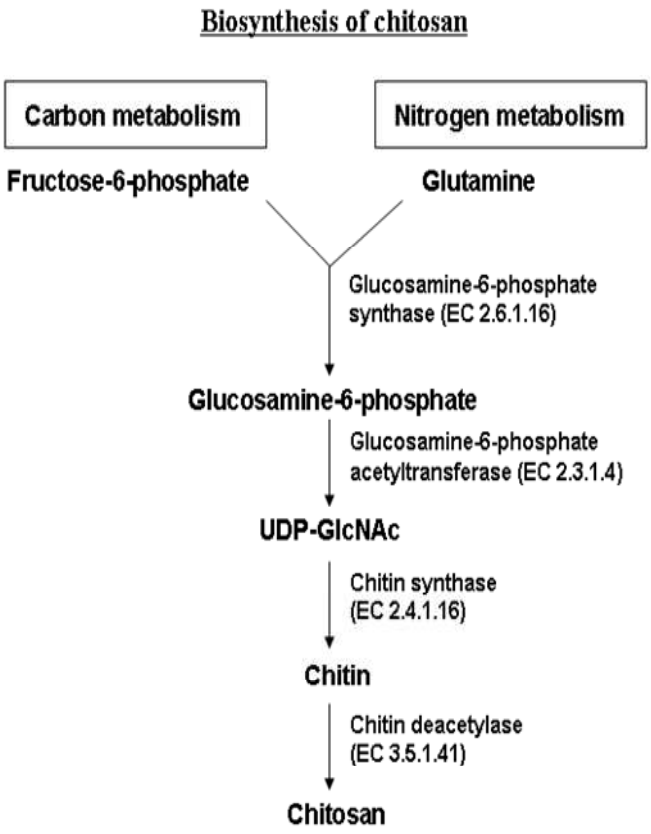


Figure 1.3 Synthesis of chitosan in nature

is by far the largest of the CE families, with over 1000 open reading frames. According to Henrissat classification (Coutinho and Henrissat, 2002), CE 4 family includes five members namely, CDA, rhizobial Nod B chitooligosaccharide deacetylases, peptidoglycan *N*-acetylglucosamine deacetylases (pgdA), acetyl-xylan esterases and xylanases. All 5 members of this family catalyze the hydrolysis of either *N*-linked acetyl groups from GlcNAc residues (CDA, rhizobial Nod B chitooligosaccharide deacetylases and pgdA), or O-linked acetyl groups from O-acetylxylose residues (acetyl xylan esterases and xylanases) of their substrates namely chitin, Nod B factors, peptidoglycan and acetyl xylan, respectively (Figure 1.4). The substrate specificity of CE4 family enzymes was studied considering the sequence similarities of the enzymes and the structural similarities of their respective substrates (Caufrier et al., 2003). The members of the CE4 family are metal-dependent enzymes (Blair and van Aalten, 2004).

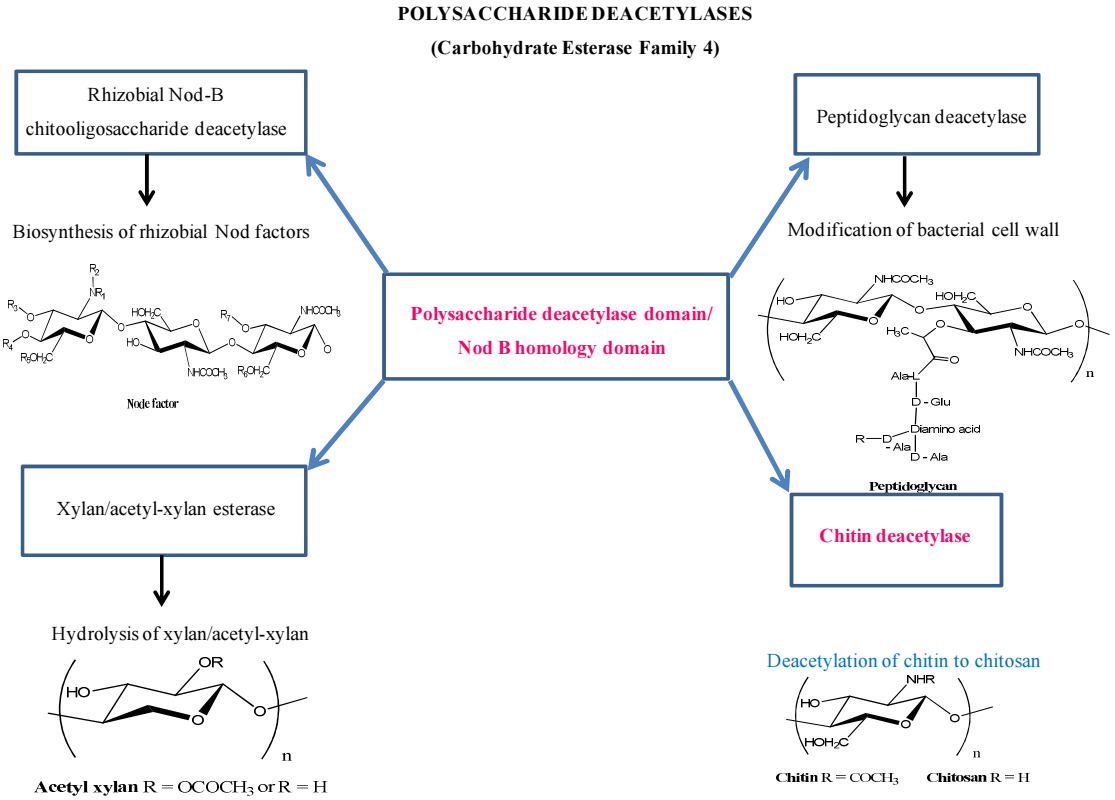


Figure 1.4 Types of polysaccharide deacetylases and their substrates

1.3.1 Sources of deacetylases

1.3.1.1 Fungal chitin deacetylase

The CDAs have been identified in several fungi. The partial purification and characterization of CDA from *Mucor rouxii* was carried out by Araki and Ito (1975) and Kafetzopoulos et al. (1993a). Martinou et al (2002) purified and characterized a cobalt-activated CDA (Cda2p) from *Saccharomyces cerevisiae* and observed that deglycosylation of the enzyme resulted in complete loss of enzyme activity which was restored by addition of 1 mM $CoCl_2$. Further, the role of a heat stable CDA in *Aspergillus nidulans* was studied by Alfonso et al. (1995). Gao et al. (1995) purified and characterized the CDA from the zygomycetous fungus *Absidia coerulea* whereas Tsigos and Bouriotis (1995) purified and characterized CDA from the culture filtrate of the plant pathogenic fungus *Colletotrichum lindemuthianum*. The significance of fungal CDAs is discussed further in section 1.3.5.

1.3.1.2 Bacterial chitin deacetylase

The nitrogen present in chitinous debris is mainly recycled by the marine bacteria present in oceanic and estuarine water. The chitin hydrolysis was shown to be carried

out by two enzymes viz. chitinase and beta-*N*-acetylglucosaminidase giving the final product $(GlcNAc)_2$ and GlcNAc, respectively (Pruzzo et al., 2008). The CDA gene was found to be involved in the chitin catabolic cascade of *Vibrio*s (Jung et al., 2008). In *Vibrio cholera*, the chitobiose induced genes were required for the transport and catabolism of non-acetylated chitin residues whereas the CDA was found to be active with chitin oligosaccharides (Meibom et al., 2004). Further, it was suggested that the oligomers generated by deacetylases could play a role in cellular communications (Li et al., 2007). From the gene bank sequence data, the CDAs from *Vibrio*s and photobacteria were found to be highly conserved.

1.3.1.3 Insect chitin deacetylase

Though most of the previously reported CDAs were from fungi and bacteria, recent studies have shown their presence in arthropods as well (Dixit et al., 2008; Luschnig et al., 2006). In *Drosophila melanogaster*, the elongation of tracheal tubes was restricted by proteins containing CDA domains, presumably by modification of terminal GlcNAc of the elongating chitin chain (Luschnig et al., 2006; Wang et al., 2006). The 5 major classes of CDA like proteins were reported in *Tribolium castaneum* and one of them was found to be specifically expressed in the gut (Dixit et al., 2008). Similar type of proteins were also reported in other insect species such as *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *D. melanogaster*, *Epiphyas postvittana*, *Helicoverpa armigera*, *Mamestra configurata*, *Spodoptera frugiperda* and *Trichoplusia ni* (Dixit et al., 2008; Guo et al., 2005; Hegedus et al., 2009; Luschnig et al., 2006; Negre et al., 2006; Pauchet et al., 2008; Paulino et al., 2006; Simpson et al., 2007; Toprak et al., 2008; Wang et al., 2006). Most of the reported insect CDAs and/or CDA like proteins were associated with the midgut peritrophic membrane (PM) and evenly distributed throughout the entire length of PM. These enzymes were detected in larval midgut tissue during the feeding period and the presence of these enzymes may be essential for increased absorption of nutrients. In *M. configurata*, the CDA activity was detected from gut proteins expressed in *E. coli* (Toprak et al., 2008). A novel midgut peritrophic membrane protein (Tn PM-P42) from the cabbage looper, *T. ni* was identified that possessed a putative polysaccharide deacetylase domain with sequence similarities to the CDA domains from fungi like *Amylomyces rouxii*, *S. cerevisiae* CDA1 and CDA2 and nodulation protein B from chitooligosaccharide deacetylase of *Sinorhizobium meliloti* (Guo et al., 2005).

1.3.2 Evolutionary relatedness between fungal, bacterial and insect deacetylases

The evolutionary relatedness among the different types of deacetylases was determined by carrying out the phylogenetic analysis using the amino acid or nucleotide sequences of deacetylases. The protein sequence comparisons between the fungal CDAs and bacterial deacetylases revealed significant similarities between the CDA from *M. rouxii*, rhizobial nod B proteins and an uncharacterized protein encoded by a *Bacillus stearothermophilus* open reading frame. Thus, these deacetylases defined a group of proteins with structural and functional homology although evolutionarily distant (Kafetzopoulos et al., 1993b). The amino acid sequence of a pgdA from *Streptococcus pneumoniae* also showed similarity to *S. cerevisiae* CDA and *S. meliloti* nodulation protein B (Vollmer and Tomasz, 2000). For the first time, a polysaccharide deacetylase homologue, PdaA from *Bacillus subtilis* was reported that exhibited *N*-acetyl-muramic acid deacetylase activity eventhough it was homologous to several polysaccharide deacetylases (Fukushima et al., 2002). Similarly, the close similarity of acetyl-xylan esterase from *Streptomyces lividans* and *Clostridium thermocellum* to pgdA was reported by Taylor et al. (2006).

The evolutionary relationship in fungi was reported to be class specific. The phylogenetic tree derived using the nucleotide sequence of CDA from different fungi formed three clusters containing basidiomycota, zygomycota, and ascomycota (Maw et al., 2002). Similarly, the phylogenetic analysis using CDAs from four different insect species belonging to different insect orders such as *T. castaneum* (coleopteran), *D. melanogaster* and *A. gambiae* (dipterans); and *A. mellifera* (hymenopteran) formed four major classes, each corresponding to a unique gene or gene cluster. This suggested the evolution of CDA isoforms long before the divergence of insect orders (Dixit et al., 2008). It has been suggested that the scattered distribution of the deacetylases might be due to extensive secondary loss, if a common ancestor contained the gene encoding the enzyme. Alternately, this distribution might be resulted from a series of lateral gene transfers (LGTs) (Das et al., 2006). All these reports suggested the evolutionary relatedness of fungal, bacterial and insect deacetylases with respect to the structure and function inspite of the differences in substrate specificity.

1.3.3 Biochemical studies of chitin deacetylase

As discussed in section 1.3.1.1, the CDAs have been identified in several fungi. It has been isolated and purified from mycelial extracts of fungi, such as *M. rouxii* (Araki

and Ito, 1975; Kafetzopoulos et al., 1993a), *A. coerulea* (Gao et al., 1995), and *A. nidulans* (Alfonso et al., 1995) and from the culture filtrate of the fungus *C. lindemuthianum* (Kauss et al., 1982; Tokuyasu et al., 1996; Tsigos and Bouriotis, 1995). Similarly, the extracellular CDA from *Scopulariopsis brevicaulis* has been purified and characterized by Cai et al. (2006) whereas the intracellular CDA from mycelial extracts of *Cunninghamella bertholletiae* has been partially purified by Amorim et al. (2005) (Table 1.1).

All CDAs are glycoproteins and are secreted by fungi either into the periplasmic space or into the culture medium depending on their function (Table 1.1). The intracellular CDAs were mostly found in the zygomycetous fungi which contain chitosan as the major component of their cell wall. The CDA from *M. rouxii*, *A. coerulea*, *A. nidulans* and *C. bertholletiae* were found to be secreted into the periplasmic space and were generally associated with cell wall modification. The presence of extracellular CDAs was reported in plant and insect pathogenic fungi namely *C. lindemuthianum* and *M. anisopliae* respectively (Kauss et al., 1982; Nahar et al., 2004a) and was reported to be associated with their protection against plant and insect chitinases elicited by the chitin oligomers. The extracellular CDA from *A. nidulans* was found to be secreted into the culture medium during the phase of autolysis (Alfonso et al., 1995). Further, the CDA activity of *C. lindemuthianum* was detected once the mycelial growth was completed after 8 d incubation in the medium. The secretion of CDA was correlated to the induction of black spore formation in *C. lindemuthianum* apart from its role in self defense (Tokuyasu et al., 1996).

The level of deacetylation depends on the subsite affinity of CDA. The endo-type CDA from *C. lindemuthianum* secreted during penetration of plant cells was reported to modify its own cell wall chitin for protection against plant endo-chitinases. The enzyme was suggested to possess four enzyme subsites (-2, -1, 0, and +1) in the catalytic domain that recognize and interact with GlcNAc residues of the substrate. The chitin was only partially deacetylated if the *N*-acetyl groups were recognized by subsites -2 and 0 and the partial deacetylation of fungal cell wall was necessary to provide effective resistance against plant endo-chitinases (Hekmat et al., 2003). Similarly, the structure of CDA from *C. lindemuthianum* was determined

Table 1.1 Biochemical characteristics of fungal chitin deacetylases

Organism	Optimum		pI	Acetate inhibition	Metal ion effect	Isozymes	Mol. Mass (kDa)	Reference
	Temp.(°C)	pH						
*A. coerulea**	50	5.0	NA	Yes	+ (Mn^{2+})/ - (Fe^{2+})	NA	75	Gao et al. (1995)
*A. nidulans**	50	7.0	2.75	No	- (Cd^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Sn^{2+}, Mg^{2+}, Mn^{2+}, Ag^{2+}, Pb^{2+})	2	27.5 and 27.3	Alfonso et al. (1995)
*C. lindemuthianum***	60	11.5	3.7	No	+ (Co^{2+})/ - (Mn^{2+}, Ni^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+})	NA	31.5-33	Tokuyasu et al. (1996)
*C. lindemuthianum***	NA	8.5	NA	No	NA	NA	150	Kauss et al. (1982)
*C. lindemuthianum***	50	8.5	3-5	No	+ (Co^{2+})/ - (Zn^{2+}, Mn^{2+}, Na^{2+})	NA	<150	Tsigos et al. (1999)
*C. lindemuthianum***	60	8.0	NA	NA	+ (Co^{2+})/ - (Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+})	NA	25	Shrestha et al. (2004)

Organism	Optimum		pI	Acetate inhibition	Metal ion effect	Isozymes	Mol. Mass (kDa)	Reference
	Temp. (°C)	pH						
*C. berthollitae**	50	4.5	NA	NA	NA	NA	NA	Amorim et al. (2005)
*F. velutipes**	60	7.0	NA	No	+ (Co^{2+}, Ca^{2+}, Zn^{2+})/ - (Cu^{2+}, Ni^{2+})	NA	31	Yamada et al. (2008)
*G. butleri**	NA	NA	NA	NA	NA	NA	70	Maw et al. (2002)
*M. anisopliae***	37	8.5	2.65, 3.8, 4.11	No	NA	3	70, 37, 26	Nahar et al. (2004a)
*Mortierella sp.**	60	5.5	NA	Yes	+ (Ca^{2+}, Co^{2+})/ - (Hg^{2+}, Zn^{2+}, Ag^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Sn^{2+})	2	50, 59	Kim et al. (2008)
*M. rouxii**	NA	5.5	3.0	Yes	+ (Zn^{2+})/- (Co^{2+}, Mn^{2+}, Na^{2+})	NA	75	Araki and Ito (1975)
*M. rouxii**	50	4.5	3.0	NA	NA	NA	75-80	Hunt et al. (2008)

Organism	Optimum		pI	Acetate inhibition	Metal ion effect	Isozymes	Mol. Mass (kDa)	Reference
	Temp. (°C)	pH						
*M. racemosus**	NA	7.0	NA	NA	NA	3	26-64	Trudel and Asselin (1990)
*R. circinans**	37	5.5-6.0	NA	NA	+ (Mn^{2+}, Mg^{2+})/ - (Cu^{2+})	NA	75	Gauthier et al. (2008)
*R. nigricans**	NA	7.0	NA	NA	NA	4	26-64	Trudel and Asselin (1990)
*R. nigricans**	NA	NA	NA	NA	NA	NA	100	Jeraj et al. (2006)
*S. cerevisiae**	50	8.0	NA	Yes	+ (Co^{2+})/ - (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+})	NA	43	Martinou et al. (2002)
*S. brevicaulis***	55	7.5	NA	NA	NA	NA	55	Cai et al. (2006)
*U. viciae-fabae***	NA	5.5-6.0	NA	NA	NA	5	48.1, 30.7, 25.2, 15.2, 12.7	Deising and Siegrist (1995)

*Periplasmic CDA; ** Extracellular CDA

by mass spectroscopy and it was suggested that *Cl*CDA possessed a highly conserved substrate binding groove, with subtle alterations that influenced the substrate specificity and substrate affinity. For the enzyme to be active, atleast 0 and +1 subsites should be occupied by $(GlcNAc)_2$ (Blair et al., 2006).

Many studies have revealed the specificity of CDA using substrates with varying degree of deacetylation. The soluble or colloidal forms of these polymers were found to be faster deacetylated than crystalline forms which were less accessible to the enzyme. In case of *M. anisopliae*, maximum CDA activity was detected on a soluble form of chitin, ethylene glycol chitin (EGC) (Nahar et al., 2004a). Further, the specificity of CDA also varies with the length of the polymer i.e. degree of polymerization (Nahar et al.). The chitin oligomers with DP higher than two are required for the enzyme to act on the substrate. For instance, *M. rouxii* and *C. lindemuthianum* had maximum affinity towards the substrates with at least four GlcNAc residues (Kafetzopoulos et al., 1993a; Tokuyasu et al., 1996). It was suggested that minimum 3-4 consecutive GlcNAc residues were needed for the CDA activity (Alfonso et al., 1995; Kafetzopoulos et al., 1993a; Tokuyasu et al., 1997). Further it was observed that the enzyme from *M. rouxii* could not effectively deacetylate chitin oligomers with a DP lower than three. The tetra-*N*-acetylchitotetraose $(GlcNAc)_4$ and penta-*N*-acetyl chitopentaose $(GlcNAc)_5$ were fully deacetylated by the enzyme due to the formation of the enzyme-substrate complex that further induced the deacetylation. However, in tri-*N*-acetylchitotriose $(GlcNAc)_3$, hexa-*N*-acetylchitohexaose $(GlcNAc)_6$ and hepta-*N*-acetylchitoheptaose $(GlcNAc)_7$, the reducing end residue remained intact (Tsigos et al., 1999). Similarly, the CDA from *S. brevicaulis* possessed the deacetylating activity for the *N*-acetyl-chitooligosaccharides with DP 2-6 (Cai et al., 2006).

From the previously reported studies, it could be observed that CDAs of periplasmic origin possessed low optimum pH whereas the extracellular CDAs were active at high pH. For instance, the extracellular CDA from *M. anisopliae* showed activity in the pH range 7.5-9.2 and the optimum pH for the enzyme activity was found to be 8.5 (Nahar et al., 2004a). Similarly, the extracellular CDA from *C. lindemuthianum* and *S. brevicaulis* also exhibited pH 11.5 and 7.5, respectively as optimum for CDA activity (Cai et al., 2006; Tokuyasu et al., 1996). On the contrary, lower pH was reported to be optimum for periplasmic CDA from *M. rouxii*, *A. coerulea*, *A. nidulans*, *S. cerevisiae*, *Rhizopus nigricans* and *C. bertholletiae* (Table

1.1). In the dimorphic fungus *Benjaminiella poitrasii*, the proportion of chitosan in yeast form was found to be more than that of mycelial form and acidic pH favoured the yeast form. Thus, lower pH may be favouring the maximum production of CDA which in turn is responsible for triggering the yeast form of this fungus (Doiphode, 2007).

The *M. anisopliae* culture filtrate fraction precipitated using ammonium sulphate (65–80%) showed activity at pH 2.65, 3.8, and 4.11 (Nahar et al., 2004a). Further, the pI values of 3.7, 3.0 and 2.75 were reported for CDA from *C. lindemuthianum*, *M. rouxii* and *A. nidulans* respectively (Table 1.1).

The optimum temperature above 50°C were reported for CDA from *C. lindemuthianum*, *Flammulina velutipes*, *S. brevicaulis* and *Mortierella sp.* whereas for *A. coerulea, A. nidulans*, *C. Berthollitae*, *M. rouxii* and *S. cerevisiae* the optimum temperature of the deacetylation reaction was 50°C. In case of *M. anisopliae* and *R. circinans*, the CDA activity was maximum at 37°C (Table 1.1).

The CDA of periplasmic origin was observed to be inhibited by acetate (Table 1.1) and this inhibition was supposed to be due to the localization of the periplasmic enzymes. The intracellular CDA of dimorphic fungus *M. rouxii* was shown to be inhibited by sodium acetate (Kafetzopoulos et al., 1993a). On the contrary, the extracellular CDAs did not show any change in activity in presence of acetate viz. the extracellular CDA from *C. lindemuthianum* and *M. anisopliae* were not inhibited in the presence of sodium acetate. The CDA from *M. anisopliae* exhibited activity in presence of sodium acetate (1-5 mM) (Nahar et al., 2004a) whereas 96% and 70% CDA activity of *C. lindemuthianum* was retained in presence of 100 mM and 800 mM sodium acetate, respectively (Tokuyasu et al., 1996). Further, the extracellular CDA from *A. nidulans* showed increase in CDA activity in the presence of lower concentrations of acetate (0.4-4mM) but not at higher concentrations (40mM) (Alfonso et al., 1995).

Furthermore, the metal ions are known to act as activators or inhibitors of enzyme activity. The CDA activity was reported to be either activated or inhibited by metal ions depending on their concentration. For instance, Zn^{2+} (1mM) slightly promoted the CDA activity in *C. lindemuthianum* but increase in the concentration to 10 mM strongly inhibited the activity of the enzyme (Tokuyasu et al., 1996). Further, the CDA of *S. cerevisiae*, *C. lindemuthianum* and *F. velutipes* was activated in presence of Co^{2+} but of *A. nidulans* and *M. rouxii* was inhibited (Table 1.1). The

possible reason for the different responses of CDA to the presence of metal ions could be the sources of CDA and the varying concentration of metal ions used. The CDA of *C. lindemuthianum* was suggested to be a metalloenzyme by Blair et al. (2006)

In several fungi, the presence of isozymes of CDA was reported (Table 1.1). In case of the broad bean rust fungus, *Uromyces viciae-fabae*, five isozymes of CDA were produced in structures from substomatal vesicles in coordination with the penetration of the fungus through the leaf stomata. The molecular masses of the five CDA isozymes apparently ranged from 12.7-48.1 kDa (Deising and Siegrist, 1995). The three isozymes of CDA in *Mucor racemosus* and four isozymes in *R. nigricans* with molecular masses ranging from 26-65 kDa were reported by Trudel and Asselin (1990). Similarly, *M. anisopliae* produced three isozymes with apparent molecular masses of 70, 37, and 26 kDa (Nahar et al., 2004a). The molecular mass of purified *C. lindemuthianum* CDA was between 31.5 and 33 kDa whereas of *M. rouxii* CDA was 75 kDa (Kafetzopoulos et al., 1993a; Tokuyasu et al., 1996). Recently, the CDA from *A. nidulans* was amplified, cloned and expressed in *E. coli* and the molecular mass of the expressed protein was found to be 24.2 kDa (Wang et al., 2010) unlike previously reported CDA from *A. nidulans* (19.5 kDa) (Alfonso et al., 1995). The two CDAs had different Km and optimal pH suggesting the presence of isozymes in *A. nidulans*.

1.3.4 Molecular studies of chitin deacetylase

In several fungi viz. *M. rouxii* (Kafetzopoulos et al., 1993b), *C. lindemuthianum* (Shrestha et al., 2004; Tokuyasu et al., 1999b), *S. cerevisiae* (Mishra et al., 1997), *Schizosaccharomyces pombe* (Matsuo et al., 2005), *R. circinans* (Gauthier et al., 2008) and *F. velutipes* (Yamada et al., 2008) and; insects viz. *D. melanogaster* (Luschnig et al., 2006), *M. configurata* (Toprak et al., 2008), *T. castaneum* (Dixit et al., 2008) and *T. ni* (Guo et al., 2005), the CDA genes have been cloned and characterized. For the first time, the CDA gene was isolated, characterized and sequenced from *M. rouxii* by Kafetzopoulos et al. (1993b). The CDA gene from *C. lindemuthianum* was isolated and was overexpressed in *E. coli* using a signal sequence for chitinase gene from *S. lividans* (Tokuyasu et al., 1999a; Tokuyasu et al., 1999b). Further, the CDA gene from the dimorphic human pathogenic fungus *Cryptococcus neoformans* was isolated by screening the genomic database library with a primer constructed towards the *N*-terminal region of the purified protein (Biondo et al., 2002).

In several fungi presence of multiple CDA genes has been reported. In *S. cerevisiae*, two genes CDA1 and CDA2 were identified that showed protein sequence homology with *M. rouxii* CDA gene (Christodoulidou et al., 1996; Mishra et al., 1997). Similarly, three putative CDA genes (RC, D2 and I3/2) were isolated and sequenced from the cDNA library of *Rhizopus circinans* by Gauthier et al. (2008). Furthermore, more than one CDA transcripts were also observed in insects. The presence of nine transcripts of CDA was reported in *T. castaneum* (Arakane et al., 2009) whereas two transcripts of CDA (CfCDA2a CfCDA2b) were estimated in *Choristoneura fumiferana* (Quan et al., 2013).

The members of the CE4 family share a universal conserved region known as polysaccharide deacetylase domain/NodB homology domain because of its similarity to NodB proteins (Figure 1.4). The NodB domain also possess certain regions from acetyl xylan esterases and xylanases of bacteria and uncharacterized open reading frames (ORFs) in *Bacillus* species (Coutinho and Henrissat, 2002; Tsigos et al., 2000). Thus, the CDA genes from different organisms were observed to share the conserved polysaccharide deacetylase domain lying in the middle of the genes and did not share a common amino or carboxyl terminal (Jeraj et al., 2006; Kafetzopoulos et al., 1993b; Maw et al., 2002; Tokuyasu et al., 1999a).

1.3.5 Role of chitin deacetylase in host-pathogen interaction

1.3.5.1 Plant-pathogen interaction

The CDA was reported to play an important role in plant-pathogen interactions. It was found to be important during the penetration process of the fungal hypha in plant tissues. The CDA activity modified the chitin molecule on the surface of the penetrating hypha which ultimately resulted in less efficient cleavage of fungal chitin by plant endochitinases due to decreasing degrees of acetylation. Thus, the penetrating hyphae were protected by enzymatic deacetylation (Kauss et al., 1982). In *U. viciae-fabae* an increase in CDA activity was observed during appressorium development (Deising and Siegrist, 1995). Recently, it was also reported that *M. grisea* CDA could possibly be involved in sensing the factors that induced appresorium formation. For instance, CDA deficient mutants were unable to form appresoria on artificial surface such as polycarbonate (Kamakura et al., 2002).

1.3.5.2 Fungus-insect interaction

It is well known that the entomopathogenic fungi act by contact to initiate killing process of the insect (Deshpande, 1999). They generally invade the insect host by a

combination of mechanical pressure and secretion of CDEs. It has been reported that the insect hosts are comparatively resistant to proteolytic and chitinolytic enzyme attack due to melanization of the cuticle (Nahar et al., 2001; St. Leger et al., 1986b). However, *Metarhizium* isolates constitutively produced extracellular CDA that was not affected by the melanin in insect cuticle and converted insect cuticular chitin into chitosan facilitating the entry of the fungus through the insect cuticle (Kulkarni et al., 2008; Nahar et al., 2004a).

1.3.5.3 Human-pathogen interaction

In some human pathogenic fungi also, the role of CDA has been investigated. In opportunistic human pathogen, *C. neoformans*, causal agent of cryptococcal meningoencephalitis, the structure and integrity of the cell wall is needed for the localization or attachment of virulence factors during host-pathogen interactions. The cell wall integrity of this fungus was maintained by CDA (Baker et al., 2007). For the first time, in *C. neoformans*, a cryptococcal gene product of 25 kDa showing homology to polysaccharide deacetylases was shown to induce protective immune response after infection (Biondo et al., 2002). Further, the transcriptional profiling of yeast and mycelial forms of *Paracoccidioides brasiliensis*, the causative agent of paracoccidioidomycoses was carried out to identify possible targets for the control of disease. The transcriptional analysis identified a CDA gene that was upregulated in the pathogenic yeast form of the fungus (Felipe et al., 2005). The human pathogen *Encephalitozoon cuniculi* is a unicellular obligate intracellular parasite, resistant to chitinase treatments due to presence of chitosan in the microsporidian spore wall which was produced due to accumulation of CDA (Brosson et al., 2005).

1.3.6 Role of chitin deacetylase in growth and sporulation

1.3.6.1 Cell wall formation

The deacetylated form of chitin i.e. chitosan though less prevalent in nature is a useful and easily accessible derivative of chitin. It is necessary for maintaining the cell wall integrity of several fungi. It is found in the cell wall of certain groups of fungi, particularly zygomycetous fungi. The chitosan contents of the zygomycetous fungi like *A. coerulea*, *Rhizopus delemar*, *Cunninghamella blackesleeana*, *M. rouxii*, *B. poitrasii* and *Mortierella isabelina* were reported to range from 1-10% of the dry weight of the cells (Miyoshi et al., 1992) (Deshpande, 2005; Khale et al., 1992). In case of other zygomycetous fungi like *Mucor circinelloides*, *Syncephalastrum racemosum* and *C. bertholletiae*, the activity of CDA was found to be related to

growth phase which in turn was related to the chitosan content of the cell wall (Amorim et al., 2005).

It was proposed that the CDA was involved in cell wall chitosan biosynthesis. The chitin was synthesized by chitin synthase by the polymerization of GlcNAc residues from uridine 5-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc) and the *N*-acetamido bonds in the nascent chitin chain were further hydrolyzed by CDA. The synergistic and consecutive action of chitin synthase and CDA resulted in formation of chitin-chitosan containing fibrils that get crystallized to form the main structural mesh of the cell walls (Davis and Bartnicki-Garcia, 1984). Similarly, the CDA from *A. coerulea* was found to convert the nascent chitin synthesized by chitin synthase to chitosan *in vitro* and was active on chitooligosaccharides with more than two GlcNAc residues (chitobiose) (Gao et al., 1995). The filamentous fungi were reported to synthesize lytic enzymes during autolysis. For instance, the production of extracellular CDA was detected during the natural autolysis of *A. nidulans* and was suggested to be involved in deacetylation of chitin oligosaccharides after the action of endo-chitinases on the cell wall (Alfonso et al., 1995).

1.3.6.2 Ascospore wall formation

As reported earlier, the ascospores of *S. cerevisiae* contained chitosan layer that probably contributed to the resistance of the spores against the enzymes like chitinase (Briza et al., 1988). The importance of two sporulation specific CDA encoding genes (Cda1 and Cda2) for maintaining the structural rigidity of ascospore wall of *S. cerevisiae* and resistance to various stresses was shown by Christodoulidou et al. (1996). Further it was proposed that the spore wall of *S. cerevisiae* was made up of four layers; the two inner layers formed by glucan and mannan whereas the outermost layer consisted of a dityrosine-rich polymer closely associated with the underlying chitosan layer. Because of this layer the spore wall was more resistant to stress conditions as compared to the wall of vegetative cells. The expression of CDA1 and CDA2 was demonstrated to be exclusively during sporulation (Mishra et al., 1997). Similarly, the ascospores of *S. pombe* were also reported to possess chitosan (Matsuo et al., 2005).

The role of CDA in the formation of *S. cerevisiae* spore walls was investigated by comparison of the dityrosine and glucosamine contents of spore walls from a wild type strain, Cda1 and Cda2 disruption strains and a double disruption strain. It was observed that in the absence of Cda2 protein (Cda2p), the remaining chitin could not

form a distinct polymer whereas elimination of all the CDA activity prevented any addition to this layer. Consequently, it was demonstrated that not only chitosan but chitin synthesis was also dependent on chitin deacetylation as decrease in deacetylation affected the quantity of chitin produced (Christodoulidou et al., 1999).

1.3.7 Role of chitin deacetylase in self defense

The CDA has been reported to deacetylate the cell wall chitin of the plant pathogenic fungi for self-defense against plant endochitinases. In *M. grisea*, *C. lindemuthianum* and several rust fungi, the cell walls showed decreased affinity to wheat germ agglutinin (WGA), suggesting either lowered chitin content or modification of chitin (Freytag and Mendgen, 1991; Howard et al., 1991; O'Connell et al., 1996).

In case of *C. lindemuthianum*, the chitin oligomers (tetramer or hexamer) were supposed to elicit the plant-defense mechanisms. However, it was proposed that CDA might deacetylate the chitin oligomers from the fungal cell wall before the activity of plant chitinases and these deacetylated chitin oligomers were not found to elicit the plant-defense mechanisms protecting the fungus against plant endochitinases (Kauss et al., 1982). Similarly, insects also produce chitinase as one of the defense mechanisms against fungal attack and also to degrade old cuticle during moulting. It has been observed that the CDA produced by *M. anisopliae* converted its own cell wall chitin into chitosan for protection against insect chitinases (Nahar et al., 2004a).

1.3.8 Roles of other deacetylases

1.3.8.1 Rhizobial Nod B chitooligosaccharide deacetylase

The symbiotic association between Rhizobia and leguminous plants depends on the specific recognition of signal molecules produced by each partner, resulting in formation of root nodules in which the bacteria fix nitrogen. The flavonoids secreted by the plants induce the synthesis of lipo-chitooligosaccharides (LCOs) by the Rhizobia which are specific nodulation signals called Nod factors (NFs). It was reported that the NFs are modified LCOs i.e. ß-1, 4-linked oligomers of GlcNAc, with a fatty acid replacing the *N*-acetyl group on their non-reducing end. The deacetylation at the non-reducing end of the oligosaccharide backbone was observed to be necessary for attachment of the fatty acyl chain during the synthesis of NFs (Lerouge et al., 1990; Spaink et al., 1991).

1.3.8.2 Acetyl-xylan esterase/Xylanase

Xylan, the most abundant hemicellulosic polysaccharide in plants is predominantly a 1,4-ß-D-xylose polymer containing various substituted side groups like acetyl, L-

arabinofuranosyl, and 4-o-methylglucuronyl residues (Degrassi et al., 2000). Several bacteria and fungi were reported to grow on xylan as a carbon source by using an array of enzymes. The acetyl xylan esterases were observed to hydrolyse the ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of acetylated xylan (Biely et al., 1985).

1.3.8.3 Peptidoglycan *N*-acetylglucosamine deacetylase

Peptidoglycan, the ß, 1-4 linked heteropolymer of GlcNAc and *N*-acetyl muramic acid is one of the main constituents of the bacterial cell wall and is responsible for stability and viability of bacterial cells. The deacetylation of GlcNAc in the peptidoglycan of bacteria play important role in number of functions such as bacterial growth, division and autolysis (Boneca, 2005). The enzyme pgdA is responsible for deacetylation of the peptidoglycan and has been reported in several human pathogenic bacteria. The different hydrolytic enzymes like lysozyme produced by the mammalian immune system break down the peptidoglycan layer. However, the cell wall peptidoglycan of invading bacteria is modified by their own pgdA as a defense mechanism against mammalian hydrolytic enzymes. The pgdA was reported to be virulence determinant of the human pathogen *S. pneumoniae*. The deacetylation of the peptidoglycan by the activity of pgdA was responsible for the relative resistance of the pneumococcal peptidoglycan against the lysozyme of the human host thereby imparting the virulence whereas the pgdA mutants became hypersensitive to exogenous lysozyme and showed decreased virulence (Vollmer and Tomasz, 2000). The free amino group contents between the lysozyme-resistant and lysozyme-sensitive *Bacillus cereus* cells were compared and the lysozyme resistance of *B. cereus* was accounted for the presence of more amounts of *N*-nonsubstituted glucosamine residues in the cell walls than the lysozyme-sensitive cells (Araki et al., 1972).

1.3.9 Applications of chitin deacetylase

1.3.9.1 Chitin deacetylase in chitosan production

Chitin is an abundant renewable natural resource obtained from marine invertebrates, insects, fungi and algae. The chitin polymer was reported to display 0.9% degree of acetylation, 7% nitrogen content and nitrogen/carbon ratio of 0.146 (Deshpande, 2005). As chitin is not readily soluble, it has limited industrial applications (Kurita, 1986). On the contrary, the chitosan which is the deacetylated form of chitin is relatively soluble and the degree of acetylation was found to be less than 0.4% and

nitrogen content >7% (Patil et al., 2000). The natural synthesis of chitosan occurs as an abundant wall component of zygomycetous fungi (Figure 1.3).

Chitosan has a great potential in biotechnology and in the biomedical and pharmaceutical industries. The various uses of chitosan are illustrated in Table 1.2.

Table 1.2 Applications of chitosan

Application	**Reference**
Food industry	
Animal feed additives	Austin et al. (1981)
Food preservation	Roller (2002)
Biomedical applications	
Wound dressing	Mi et al. (2002)
Dietary supplement	Rodriguez et al. (2000); Wadstein et al. (2000)
Drug delivery and therapies	Lubben et al. (2000); Onishi et al. (1997); Rubin et al. (2000); Thanou et al. (2000)
Dentistry	Kochanska and Sramkiewicz (2000)
Cosmetic applications	Pittermann et al. (1997)
Industrial applications	
Immobilization of enzymes	Hisamatsu (1997)
Use in ethanol sensor	Karauchi and Ohga (1997)
Membranes	Sakurai (1997)
Water engineering	
Metal capture from wastewater	Jha et al. (1988); Peniche-covas et al. (1992)
Colour removal from textile mill effluents	Knorr (1983)

The zygomycetous fungi can be readily grown in the laboratory on cheap nutrients and the wall material can be recovered by simple chemical procedures (McGahren et al., 1984). Some fungi possess a low molecular weight chitosan that is useful for medical applications and in agriculture (Doiphode et al., 2009). Commercially, it is produced from shrimp/crab/squid chitin via a harsh thermochemical process which is environmentally unsafe and not easily controlled, leading to a broad and heterogenous range of products. The enzymatic deacetylation of chitin by CDA could be potentially employed as an alternative procedure. The more homogenous chitosan oligomers with

range of molecular weight and content of *N*-acetylated residues can be produced using enzymatic deacetylation which is environmentally safe and not a random process like chemical deacetylation.

The CDA from *C. lindemuthianum* was used to synthesize a novel substrate, p-nitrophenyl-2-amido-4-O-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (GlcNGlcNAc-pNP) which was used to differentiate the action of *N*-acetylglucoaminidases hydrolysing the substrate in a stepwise manner or by recognizing the terminal residue (Tokuyasu et al., 1999c). Further, the CDA from *A. coerulea* was reported to produce chitosan polymers with a lower degree of acetylation. For the first time, the CDA from *A. coerulea* was immobilized which offered the highest activity and stability by covalent binding of CDA (Jaworska et al., 2009). The waste mycelium of *Aspergillus niger* from citric acid production plant was efficiently converted to chitosan employing the extracellular CDA from *S. brevicaulis* (Cai et al., 2006). The lethal sepsis and destructive wound infections caused by marine bacterium *Vibrio vulnificus* could be treated using partially depolymerized chitosan (Lee et al., 2009).

Furthermore, with the help of molecular biology techniques, the CDAs can be manipulated to get enzymes with novel properties which can be used for the preparation of chitosan polymers and oligomers. The cloning and over-expression of CDA gene has application in large-scale production of the enzyme for its commercial use. The expression of *A. nidulans* CDA in *E. coli* was not convincing for industrial application. On the contrary, the recombinant CDA from *C. lindemuthianum* expressed in *Pichia pastoris* was significantly activated by Co^{2+} ions (Wang et al., 2010).

1.3.9.2 Chitin deacetylase in transgenics

One of the interesting applications of CDA could be in transgenics. Recently, there has been interest in the paper and textile industries in developing chitin: chitosan and chitosan: cellulose blends. It was proposed that the genetic manipulation of plants can be carried out by expression of chitin synthase and CDA genes. Such transgenic plants with altered cell wall can be used as a single source of cellulose, chitin and chitosan for industrial uses and improved disease resistance (Dhugga et al., 2000).

1.3.9.3 Chitin deacetylase in biocontrol

The CDA could also be a versatile tool in the biological control of insect pests. As in *M. anisopliae*, the CDA was reported to play a dual role in modifying the insect

cuticular chitin as well as in altering its own cell wall for defense from insect chitinases, it could be used in integrated pest management i.e. in combination with other hydrolytic enzymes for the control of pests and pathogens (Nahar et al., 2004a). In *C. lindemuthianum*, the Cu^{2+} was found to be inhibitory to CDA (Tokuyasu et al., 1996). Therefore, the fungicides containing copper could be effectively used for the pathogen control. Further, it was observed that recombinant baculovirus expressing a midgut CDA like protein from *H. armigera* showed significant increase in the pathogenicity against *Spodoptera* species. It was suggested that the insecticidal properties of baculovirus can be improved and applied for better pest control (Jakubowska, 2010 #223). As CDA is a major protein secreted in the peritrophic matrix of the arthropod gut during feeding, the chitin from the gut is modified for protection against parasite invasion. The inhibition of CDA could be used to control the insect pests (Hegedus et al., 2009).

The part of this work has been published in Ghormade et al. (2010).

1.4 Objectives of the present investigations

Based on the above background, the aim of the present study was to study the importance of CDEs with special reference to CDA in the virulence of *Metarhizium* isolates against *H. armigera*. The biochemical and molecular investigations on CDA are necessary to know its role in fungus-insect interaction which will be further helpful in the better insect control. The objectives of the present investigation were –

1. Screening of *Metarhizium* isolates for the control of *Helicoverpa armigera*
2. Cuticle degrading enzymes as biochemical and molecular markers of *Metarhizium* isolates
   A. Molecular characterization of *Metarhizium* isolates based on polymorphism in protease (Pr1A) gene
   B. Effect of repeated *in vitro* sub-culturing and *in vivo* passage on cuticle degrading enzyme production and virulence of *Metarhizium anisopliae* against *Helicoverpa armigera*
3. Biochemical and molecular studies of chitin deacetylase
   A. Evaluation of *Metarhizium* isolates based on chitin deacetylase activity
   B. Sequencing of chitin deacetylase gene from *Metarhizium anisopliae* M161063
   C. Expression studies of chitin deacetylase from *Metarhizium anisopliae* M161063

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

The chemicals, media constituents and kits used in the present study are listed in Table 2.1

Table 2.1 List of chemicals, media constituents and kits

Name	Source
N-acetylglucosamine (GlcNAc), ampicillin, β-mercaptoethanol, bovine serum albumin (BSA), bromophenol blue, chitin, chitosan, cyclohexamide, dialysis tubings, diethylpyrocarbonate (DEPC), *N*-dodecylguanidine monoacetate (dodine), ethylene glycol chitosan, ethylene diamine tetra acetic acid (EDTA), glucosamine, gum arabic, 3-methyl-2-benzothiazoline hydrazone (MBTH), piperazine-1, 2-bis [2-ethane sulfonic acid] (PIPES), streptomycin, tetracycline, triton X-100, tyrosine, xylene cyanol	Sigma-Aldrich, Germany
Ammonium chloride, ammonium nitrate, ammonium sulphate, ammonium sulfamate, boric acid, calcium chloride, cobalt chloride, p-dimethyl aminobenzaldehyde (DMAB), ferric chloride, ferrous sulphate, glycerol, magnesium chloride, magnesium sulphate, manganous chloride, manganous sulphate, dipotassium hydrogen orthophosphate, potassium acetate, potassium chloride, potassium dihydrogen orthophosphate, potassium hydrogen sulphate, potassium hydroxide, disodium hydrogen orthophosphate, sodium acetate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydroxide, sodium nitrate, tris (hydroxymethyl) aminomethane, urea, zinc sulphate	S D fine-chemicals, India

Name	Source
Agar powder, beef extract, bacteriological peptone, glucose, mycological peptone, olive oil, potato dextrose agar (PDA) powder, tryptone, yeast extract	Hi-media, India
Agarose, Hammerstein casein, sodium tetraborate, sodium dodecyl sulphate	Sisco Research Laboratoies, India
Oxgall	Loba Chemie, India
Tri-chloro acetic acid (TCA)	Fischer Scientific, UK
Acetic acid glacial, acetic anhydride, chloroform, ortho-phosphoric acid	Thomas Baker, UK
Ethanol, isopropanol	MP Biomedicals, USA
Acetic acid (ACS manual format) assay kit	Megazyme International Ireland Limited, UK
Unicorn bags	Unicorn Imp and Mfg. Corp., USA
Oligonucleotides	IDT, USA
Taq polymerase	Bangalore Genei, India
Phusion DNA polymerase	Finnzymes, Finland
Restriction enzymes	New England Bio Labs, UK
DNeasy plant mini kit, RNeasy mini kit, QIAquick PCR purification kit	Qiagen, USA
pGEM-Teasy vector, RNAase, RNase free DNase, T4-DNA ligase	Promega, USA
Gene ruler DNA ladder mix	Fermentas, USA
Verso cDNA synthesis kit	Thermo Scientific, USA
Real Master Mix SYBR ROX	5 Prime, Germany

2.2 Buffers and solutions

The buffers and solutions used in the present study were prepared in deionized water and are listed in Table 2.2. All percentages are weight by volume (w/v) unless otherwise mentioned.

Table 2.2 Compositions of buffers and solutions

Name	Composition
Acetate buffer (50 mM)	0.1 M acetic acid, 0.1 M sodium acetate, pH 5.0
Borate buffer (20 mM)	0.56 M potassium hydroxide, 1.12 M boric acid, pH 9.2
Carbonate-bicarbonate buffer (50 mM)	0.2 M sodium carbonate, 0.2 M sodium bicarbonate, pH 9.7
Phosphate buffer (50 mM)	0.05 M disodium hydrogen orthophosphate, 0.05 M potassium dihydrogen orthophosphate, pH 6.8
Sodium tetraborate buffer (50 mM)	0.05 M sodium tetraborate, pH 8.5
6X DNA loading dye	0.25% bromophenol blue, 0.25% xylene cyanol, 40% glycerol
Alkaline lysis solution I/ GTE solution	0.05 M glucose, 0.025 M Tris-Cl, 0.01 M EDTA, pH 8.0
Alkaline lysis solution II	0.2 N sodium hydroxide, 1% sodium dodecyl sulphate
Alkaline lysis solution III	3 M potassium acetate, pH 6.0
Tris-acetate-EDTA (TAE) buffer	0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0
Tris-EDTA (TE) buffer	0.01 M Tris-Cl, 0.001 M EDTA, pH 8.0
PIPES	0.5 M PIPES, 5 M potassium hydroxide, pH 6.7
Inoue transformation buffer	0.055 M manganous chloride, 0.015 M calcium chloride, 0.25 M potassium chloride, 0.5 M PIPES

2.3 Culture media

The culture media used in the present study were prepared using glass distilled water. The compositions of the culture media used are given in Table 2.3.

Table 2.3 Compositions of culture media

Name	Constituents (g or ml/l)
PDA	PDA powder, 39.0; pH 6.0
Selective medium	Bacteriological peptone, 10.0; glucose, 20.0; agar, 18.0; streptomycin, 0.6; tetracycline, 0.05; cyclohexamide, 0.05; dodine, 0.1 ml
Chitin containing medium	Potassium dihydrogen orthophosphate, 3.0; dipotassium hydrogen orthophosphate, 1.0; magnesium sulphate, 0.7; ammonium sulphate, 1.4; sodium chloride, 0.5; calcium chloride, 0.5; yeast extract; 0.5; bacteriological peptone, 0.5; urea, 0.3; oxgall, 1.0; chitin, 5.0; trace metal solution, 1.0 ml containing ferrous sulphate, 0.005; manganous sulphate, 0.0015; zinc sulphate, 0.0033; cobalt chloride, 0.002; pH 6.0
Yeast extract peptone glucose (YPG) medium (1%)	Yeast extract, 3.0; mycological peptone, 5.0; glucose, 10.0; pH 5.5
Corn steep liquor containing medium	Corn steep liquor, 30.0; yeast extract, 40.0; bacteriological peptone, 40.0; pH 5.0
Semi-synthetic complete medium	Potassium dihydrogen orthophosphate, 0.36; disodium hydrogen orthophosphate, 1.6; potassium chloride, 1.0; magnesium sulphate, 0.6; ammonium nitrate, 0.7; yeast extract, 5.0; glucose, 10.0; pH 7.0
Luria Bertani agar	Yeast extract, 5.0; tryptone, 10.0; sodium chloride, 10.0; agar, 20.0; pH 7.0
Super optimal broth with catabolite repression (SOC) medium	Tryptone, 20.0; yeast extract, 5.0; sodium chloride, 0.5; 0.25 M potassium chloride, 1.0 ml; pH 7.0

2.4 Organisms and maintenance

2.4.1 Isolation of *Metarhizium*

The soil samples and infected insects cadavers were collected from Pune (18º31'13"N 73º51'24"E) and Buldhana (19º58'36"N 76º30'30"E) district, Maharashtra, India. The *Metarhizium* isolates were obtained from soil samples by soil dilution plating method (Goettel and Inglis, 1996). Initially, 10 g of each soil sample was placed separately into 90 ml sterile 0.1% (w/v) Tween 80. The samples were then homogenized by stirring the slurry with a magnetic stir bar for 60 min to release the conidia from the soil matrix. After homogenization, 100-200 µl aliquots from each sample were spread on to selective medium plates (Keller et al., 2003) and incubated at 28ºC for 3-7 d. The individual sporulating colonies were then sub-cultured on the same medium to obtain pure cultures. For isolation of *Metarhizium* from infected insect cadavers, the insects showing abnormal behaviour with poor coordination were collected from fields, kept at 28ºC until death and transferred to moist chambers for further mycosis and sporulation, if any. The conidia from sporulating cadavers were then streaked on selective medium and the pure cultures were obtained by further sub-culturing for 2-3 times on the same medium.

2.4.2 Maintenance of *Metarhizium* isolates

The pure cultures of all the *Metarhizium* isolates were streaked on PDA slants and incubated at 28ºC and 70-80% RH for 7 d. The sporulated mother cultures were maintained at 8ºC until further use. The sub-culturing of isolates was done every 15 d and *in vivo* passage through the insect was done every 3 months. After passage, the culture was re-isolated, maintained on PDA and used as a stock for all the experiments.

2.4.3 *Escherichia coli* (JM109 strain) competent cells

All the cloning experiments were carried out using *Escherichia coli* (JM109 strain) competent cells as a host strain. The competent cells were stored at -80°C until further use.

2.5 Conidial suspension

For preparation of the conidial suspension of *Metarhizium* isolates, conidia were scrapped from sporulating PDA slants (7 d old) and suspended in 0.1% (w/v) Tween 80 so as to get the desired conidial count. For all the experiments, the conidial suspensions containing 1×10^7 conidia/ml were used unless otherwise mentioned.

2.6 *In vitro* conidial sub-culturing and *in vivo* passage of *Metarhizium*

For repeated *in vitro* conidial transfers, one of the *Metarhizium* isolate was streaked on PDA slants and incubated at 28ºC until sporulation was observed (~7 d). The conidia from PDA slants were harvested by scrapping with a loop and sub-cultured on fresh PDA slants. This multi-spore *in vitro* transfer was repeated up to 40th sub-culture. The 40th sub-culture was further used for *in vivo* passage through the insect host. The *in vivo* passage was carried out using 3rd instar larvae of *H. armigera*. The larvae were dipped in the conidial suspension of 40th sub-culture for 5 sec and then placed individually in sterile vials containing moist Whatmann filter paper No. 1 and a piece of disinfected okra (*Abelmoschus esculentus*). The diet was changed every other d and the larvae were kept at 25±2°C, 65±5% RH and 16:8 (L: D) until they died. The dead larvae were transferred to sterile petri plates containing moist cotton swabs and kept at 28ºC and 70-80% RH for at least 3-7 d to allow mycelia and conidia formation over the cadavers. The conidia from the cadavers were scrapped with a pointed metal needle, suspended in 0.1% (w/v) Tween 80 and were further used to inoculate additional insects. The *in vivo* passage through the *H. armigera* was repeated 5 times. The repeated *in vitro* sub-cultures and *in vivo* passaged cultures were also maintained as described in section 2.4.2.

2.7 *In vitro* vegetative transfers of *Metarhizium*

For repeated *in vitro* vegetative transfers, the conidial suspension (0.5 ml) of 1st sub-culture of one of the *Metarhizium* isolate was inoculated in 50 ml YPG medium and the flask was incubated at 28ºC under shaking condition (180 rpm) for 24 h. The mycelium biomass (24 h) (10% w/v) was re-inoculated in 50 ml fresh YPG medium repeatedly 10 times. The mycelium was harvested aseptically by filtration and stored in sterile vials at -20ºC until further use.

2.8 Growth, conidial germination and appressorium formation

The conidial suspensions of 1st and 40th sub-cultures (section 2.6) of *Metarhizium* isolate were streaked on PDA plates and incubated at 28°C for 48 h. The single mycelium colony (approximately 2 mm diameter) was cut and placed at the centre of fresh PDA plate and incubated at 28°C for 8 d. The PDA plates were then observed for the vegetative growth and sporulation.

For conidial germination, the conidial suspensions of 1st and 40th sub-cultures of *Metarhizium* isolate were streaked on PDA agar on slides (Nahar et al., 2003) and incubated at 28°C and 70-80% RH for 24 h. The germination was observed under light microscope every 2 h and the number of germinated and non-germinated conidia were counted randomly in 10 different fields, in triplicate.

The appressorium formation on artificial surface was checked as described earlier (Kulkarni et al., 2008; Xavier-Santos et al., 1999). The conidial suspensions (100 µl) of 1st and 40th sub-cultures of *Metarhizium* isolate were inoculated separately in 5 ml YPG medium and incubated at 28ºC under shaking condition (180 rpm). The initiation of germination was observed under light microscope after every 2 h. Once the germination started, the conidia were separated from the YPG medium by centrifugation at 10,000 g for 10 min. The germinated conidia were washed twice with sterile distilled water and re-suspended in 100 µl sterile distilled water. The suspensions of germinated conidia were further placed separately at the centre of polypropylene petri plates, sealed with parafilm and incubated at 28ºC and 70-80% RH. The plates were observed after every 2 h under the microscope during next 24 h for appressoria development. The number of conidia producing germ tubes and/ or appressoria was counted randomly in 10 different fields for each plate and each experiment was carried out in triplicate. In total, for each plate, not less than 300 conidia were recorded as germinated or non-germinated.

2.9 Cultivation conditions

2.9.1 Production of extracellular cuticle degrading enzymes

The extracellular production of CDEs viz. chitinase, protease, lipase, CDA and chitosanase was studied using the conidial inoculums of all *Metarhizium* isolates as well as *in vitro* sub-cultures and *in vivo* passaged cultures of *Metarhizium* isolate (section 2.6). The production of induced CDEs was studied in chitin containing medium as described by Vyas and Deshpande (1989) whereas the constitutive production of CDEs was checked in YPG medium. Similarly, the extracellular production of CDEs using mycelium inoculums of 1st and 10th vegetatively transfered *Metarhizium* isolate (section 2.7) was also studied in both chitin containing medium and YPG medium.

For production of CDE complex using conidial inoculums, the conidial suspensions (0.5 ml) of all *Metarhizium* isolates as well as *in vitro* sub-cultures and *in*

vivo passaged cultures whereas for production of CDE complex using mycelium inoculums, the mycelium of 1st and 10th vegetatively transferred *Metarhizium* isolate (10% w/v) were added individually in 50 ml YPG and chitin containing medium. The flasks were incubated at 28ºC under shaking condition (180 rpm) for 72 h and 96 h, respectively. The mycelium biomass was separated by centrifugation at 4,000 g for 15 min and the cell free supernatant obtained after centrifugation (culture filtrate) was used for the estimation of CDE activities.

2.9.2 Production of intracellular constitutive CDA in different morphological forms of *Metarhizium*

The intracellular constitutive CDA production was studied in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h) and blastospores (24 h) of one of the *Metarhizium* isolate. The biomass of these morphological forms was collected on Whatmann filter paper No.1 and washed with ice cold distilled water. The cells were suspended in sodium tetraborate buffer (50 mM; pH 8.5) containing 3 mM dithiothreitol (DTT), 1 mM phenyl methane sulphonyl fluoride (PMSF) and 1 mM EDTA. The cells were disrupted using glass beads in Braun's homogenizer for 5 cycles of 15 sec each and then centrifuged at 12,000 g for 15 min to obtain cell extract. The cell extract was used for the detection of CDA activity. The cultivation conditions used for obtaining the biomass of different morphological forms of *Metarhizium* are as follows.

2.9.2.1 Conidia

The *Metarhizium* conidia were streaked on PDA slants and incubated at 28ºC and 70-80% RH for 7 d to obtain fresh conidia.

2.9.2.2 Germinating conidia

The conidial suspension of *Metarhizium* isolate was inoculated in YPG medium and incubated at 28ºC under shaking condition (180 rpm) for 12 h to obtain germinating conidia.

2.9.2.3 Appressoria

The conidial suspension of *Metarhizium* isolate was inoculated in YPG medium and the germinating conidia were processed for appressorium development (section 2.8). The contents of the petri plates were centrifuged at 10,000 g for 20 min to obtain appressorium biomass (24 h).

2.9.2.4 Mycelia

The conidial suspension of *Metarhizium* isolate was inoculated in YPG medium and incubated at 28ºC under shaking condition (180 rpm) for 24 h to obtain mycelium biomass.

2.9.2.5 Blastospores

For preparation of inoculum for blastospore production, the conidial suspension of *Metarhizium* isolate was inoculated in YPG medium and incubated at 28ºC under shaking condition (180 rpm) for 48 h. The 48 h old mycelium inoculum (15% v/v) was added in corn steep liquor containing medium (Adamek, 1963) and incubated at 28ºC under shaking condition (180 rpm) for 24 h. The biomass collected was filtered through sterile muslin cloth to separate the mycelium and the filtrate was centrifuged at 8,000 g for 20 min to obtain blastospores.

2.10 Enzyme assays

2.10.1 Chitinase assay

The total chitinase activity was estimated using acid-swollen chitin as a substrate (Kulkarni et al., 2008; Vyas and Deshpande, 1989). For preparation of acid-swollen chitin, 10 g chitin (purified powder from crab shells) was suspended in 300 ml *O*-phosphoric acid (88% w/v) and kept at 4-8ºC for 1 h with occasional stirring. The mixture was poured into ice-cold distilled water (4 l) and kept for 30 min. After repeated washing with ice-cold distilled water, the pH of the mixture was adjusted 7.0 by a wash with 1% (w/v) sodium hydrogen carbonate ($NaHCO_3$). The swollen chitin was dialysed at 4-8ºC against distilled water and homogenised in a waring blender for 1 min. The concentration of acid swollen chitin was adjusted to 7 mg/ml by adding acetate buffer (50 mM; pH 5.0).

The reaction mixture containing 1.0 ml 0.7% acid swollen chitin, 1.0 ml acetate buffer (50 mM; pH 5.0) and 1.0 ml culture filtrate was incubated at 50ºC for 1 h. After incubation, the reaction mixture was centrifuged at 10,000 g for 10 min and the supernatant was used for the estimation of GlcNAc. The supernatant (0.5 ml) was added to 0.1 ml borate buffer (20 mM; pH 9.2) and the mixture was boiled for 3 min. After cooling, 3.0 ml DMAB was added to the mixture and incubated at 37°C for 20 min. The GlcNAc residues produced were estimated colorimetrically at 585 nm (Reissig et al., 1955). One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol GlcNAc per ml per min.

2.10.2 Protease assay

The protease activity was measured using Hammerstein casein as a substrate (Kulkarni et al., 2008; Vyas and Deshpande, 1989). The reaction mixture containing 1.0 ml Hammerstein casein (1%), 0.9 ml carbonate-bicarbonate buffer (50 mM; pH 9.7) and 0.1 ml culture filtrate was incubated at 35ºC for 20 min. The reaction was terminated by addition of 3.0 ml TCA (2.6 ml 5% TCA + 0.4 ml of 3.3 N HCl) and the reaction mixture was filtered through Whatmann filter paper No. 1. The tyrosine residues in the TCA soluble fraction were estimated colorimetrically at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol tyrosine per ml per min.

2.10.3 Lipase assay

The lipase activity was determined using olive oil emulsion as a substrate (Pignede et al., 2000). For preparation of olive oil emulsion, 16.5 g gum arabic was dissolved in 130 ml distilled water and the volume was adjusted to 165 ml. To this solution, 20 ml olive oil and 15 g crushed ice was added. The emulsion was mixed in the blender and stored at 4-8ºC.

The reaction mixture containing 5.0 ml olive oil emulsion, 2.0 ml phosphate buffer (50 mM; pH 6.8) and 1.0 ml culture filtrate was incubated at 37ºC for 1 h under shaking condition (80 rpm). The reaction was terminated by addition of 4.0 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. The fatty acids released were determined by titration with sodium hydroxide (NaOH) (50 mM). One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol fatty acids per ml per min.

2.10.4 Chitin deacetylase assay

The CDA activity was measured either by MBTH method (Kauss and Bauch, 1988; Nahar et al., 2004a) or by enzymatic method (Bergmeyer, 1974). The substrate, EGC used in both the methods was prepared as described by Araki and Ito (1975). To prepare EGC, 0.04 g ethylene glycol chitosan was added in 4.5 ml aqueous solution containing 0.4 g $NaHCO_3$ and acetic anhydride (200 µmol) and kept at 4-8ºC. After 24 h, 0.2 ml acetic anhydride was added and the mixture was kept at 4-8ºC for further 24 h. After thorough dialysis, the concentration of EGC was adjusted to 1 mg/ml by adding sodium tetraborate buffer (50 mM; pH 8.5).

The reaction mixture containing 0.1 ml 0.1% EGC, 0.1 ml sodium tetraborate buffer (50 mM; pH 8.5) and 0.05 ml culture filtrate was incubated at 37ºC for 30 min.

After terminating the reaction, the glucosamine or acetate released were determined either by MBTH or Bergmeyer's method, respectively.

2.10.4.1 Determination of glucosamine by MBTH method

The glucosamine residues were determined by MBTH method as described earlier by Kauss and Bauch (1988). In MBTH method, the reaction was terminated by addition of 0.25 ml 5% (w/v) potassium hydrogen sulphate ($KHSO_4$). To this mixture, 0.25 ml 5% (w/v) sodium nitrite ($NaNO_2$) was added and allowed to stand for 15 min at RT and then 0.25 ml 12.5% (w/v) ammonium sulfamate ($N_2H_6SO_3$) was added and kept for 5 min. For colour development, 0.25 ml freshly prepared 0.5% (w/v) MBTH was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled and 0.25 ml freshly prepared 0.5% (w/v) ferric chloride ($FeCl_3$) was added and allowed to stand at RT for 30 min. The glucosamine residues released were measured colorimetrically at 650 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of glucosamine per ml per min.

2.10.4.2 Determination of acetate by Bergmeyer's method

For determination of acetate molecules released during CDA activity, the reaction was terminated by heating at 100°C for 15 min. The acetate molecules released were determined using acetic acid (ACS manual format) assay kit according to manufacturer's protocol. In the Bergmeyer's enzymatic method, the acetate molecules released by the action of CDA on EGC were determined via three coupled enzyme reaction.

Enzyme reaction:

$$\text{Chitin} \xrightarrow{\textbf{CDA}} \text{Chitosan + Acetate}$$

Acetate determination:

1. $\text{Acetate + ATP + CoA} \xrightarrow{\textbf{ACS}} \text{acetyl-CoA + AMP + pyrophosphate}$
2. $\text{Acetyl-CoA + oxaloacetate + } H_2O \xrightarrow{\textbf{CS}} \text{citrate + CoA}$
3. $\text{L-Malate + } NAD^+ \xleftrightarrow{\textbf{MDH}} \text{oxaloacetate + NADH + } H^+$

Acetyl-coenzyme A synthetase (ACS) (EC 6.2.1.1) in the presence of adenosine-5'-triphosphate (ATP) and coenzyme A converted acetate into acetyl-CoA, with the formation of adenosine-5'-monophosphate (AMP) and pyrophosphate (1). Citrate synthase (CS) (EC 2.3.3.1), in the presence of acetyl-CoA converted oxaloacetate into citrate (2). The oxaloacetate required in reaction (2) was formed from L-malate and nicotinamide-adenine dinucleotide (NAD^+) in the presence of L-malate dehydrogenase (L-MDH) (EC 1.1.1.37) (3). In this reaction, NAD^+ was reduced to NADH. The acetate released was determined based on NADH formation that was measured by increase in the absorbance at 340 nm.

The protocol followed for the acetate determination was as follows:

Pipetted into cuvettes	Blank	Sample
Distilled water (at ~ 25°C)	2.10 ml	2.00 ml
Sample	-	0.10 ml
Solution 1 (buffer)	0.50 ml	0.50 ml
Solution 2 (NAD^+/ATP/CoA/PVP)	0.20 ml	0.20 ml
Mixed* and the absorbance of the solution (A_0) was measured after approximately 3 min and the reaction was started by addition of:		
Suspension 3 (L-MDH/CS)	0.02 ml	0.02 ml
Mixed* and the absorbance of the solution (A_1) was measured after approximately 4 min and the reaction was started by addition of:		
Suspension 4 (ACS)	0.02 ml	0.02 ml
Mixed* and the absorbance of the solution (A_2) was measured at the end of the reaction (approximately 12 min).		

* Mixed with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or parafilm.

The absorbance differences (A_1–A_0) and (A_2–A_0) for both blank and sample were determined. As there was no linear proportionality between the measured absorbance difference and the acetic acid concentration with the equilibrium of the preceding indicator reaction (3), ΔAacetic acid was calculated using the equation below.

$$\Delta A_{\text{acetic acid}} =$$

$$\left[(A2 - A0)_{\text{sample}} - \frac{(A1 - A0)^2_{\text{sample}}}{(A2 - A0)_{\text{sample}}}\right] - \left[(A2 - A0)_{\text{blank}} - \frac{(A1 - A0)^2_{\text{blank}}}{(A2 - A0)_{\text{blank}}}\right]$$

The concentration of acetic acid was calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{acetic acid}} \qquad [g/l]$$

where:

V = final volume [ml]

MW = molecular weight of acetic acid [g/mol]

ε = extinction coefficient of NADH at 340 nm = 6300 [$1 \times mol^{-1} \times cm^{-1}$]

d = light path [cm]

v = sample volume [ml]

For acetic acid the calculations were as follows:

$$c = \frac{2.84 \times 60.05}{6300 \times 1 \times 0.10} \times \Delta A_{\text{acetic acid}} \qquad [g/l]$$

$$= 0.2707 \times \Delta A_{\text{acetic acid}} \qquad [g/l]$$

One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of acetate per ml per min.

2.10.5 Chitosanase assay

The chitosanase activity was estimated using acid-swollen chitosan as a substrate (Kulkarni et al., 2008). For preparation of acid-swollen chitosan, 10 g crystalline chitosan was suspended in 10 N HCl and kept at 4-8ºC for 1 h with occasional stirring. The pH of the mixture was adjusted 7.0 with 1 N NaOH. The swollen chitosan was repeatedly washed with ice-cold distilled water by centrifugation at 10,000 g for 10 min and dialysed at 4-8ºC against distilled water. After homogenization in a waring blender for 1 min, the concentration of acid swollen chitosan was adjusted to 10 mg/ml by adding acetate buffer (50 mM; pH 5.0).

The reaction mixture containing 1.0 ml 1% acid-swollen chitosan, 1.0 ml acetate buffer (50 mM; pH 5.0) and 1.0 ml culture filtrate was incubated at 50ºC for 1

h. After incubation, the reaction mixture was centrifuged at 10,000 g for 10 min and the supernatant was used for the estimation of glucosamine. The supernatant (1.0 ml) was added to 0.2 ml acetic anhydride (5% in acetone). To this mixture, 1.0 ml of borate buffer (20 mM; pH 9.2) was added and boiled for 3 min. After cooling, 7.8 ml DMAB was added to the mixture and incubated at 37°C for 20 min. The glucosamine residues produced were estimated colorimetrically at 585 nm (Good and Bessman, 1964). One unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of glucosamine per ml per min.

2.11 Protein estimation

The protein concentration was estimated according to Lowry et al. (1951) using BSA as the standard.

2.12 Insect rearing

The initial culture of *H. armigera* was established by collecting larvae and pupae of the insect from the fields. The rearing was done by growing the larvae individually in sterile polypropylene vials (42×65 mm, 50 ml capacity; Laxbro Manufacturing Co., Pune, India) containing pieces of okra disinfected with 0.5% (v/v) sodium hypochlorite for 10 min (Ignoffo et al., 1975). The insect eggs laid during rearing were also surface-sterilised with 0.5% (v/v) sodium hypochlorite. The larvae were maintained at 25±2°C and 65±5% RH.

2.13 Insect bioassay

The insect bioassays were carried out using 3rd instar larvae of *H. armigera*. The set of 30 larvae in triplicate were dipped individually in 10 ml conidial suspension of *Metarhizium* isolates for 5 sec. After treatment, each larva was individually transferred to a separate sterile vial containing moist Whatmann filter paper No. 1 and a piece of disinfected okra that was changed on alternate days. The larvae were kept at 25±2°C, 65±5% RH and 16:8 (L: D) for 14 d or until they died. To allow mycelia and conidia formation over the cadavers, the dead larvae were transferred to sterile petri plates containing moist cotton swabs and kept at 28°C and 70-80% RH for at least 3-7 d. A set of 30 larvae in triplicate treated with 0.1% (w/v) Tween 80 in sterile distilled water served as control. The experiment was conducted in triplicate using freshly prepared conidial suspensions. The data on percent mortality from three experiments

were pooled to get average values, which were corrected by Abbott's formula (Abbott, 1925).

The median lethal concentration (LC_{50}) of *Metarhizium* isolates against 3rd instar larvae of *H. armigera* was determined to increase the possibility of identifying the difference in virulence of isolates with high mortality values that might go undetected if only a single dose was used. The LC_{50} values were determined using four concentrations (1×10^3, 1×10^5, 1×10^7 and 1×10^9 conidia/ml) of conidial suspension. The experimental layout was a RCBD with each treatment containing a set of 30 larvae repeated 3 times.

2.14 Production of conidia

The conidia of *Metarhizium* isolates were produced by solid state fermentation. For preparation of inoculum, the conidial suspensions of *Metarhizium* isolates (2.0 ml) were inoculated in 200 ml YPG medium and the flasks were incubated at 28ºC under shaking condition (180 rpm) for 48 h. The mass production of conidia was carried out by solid state fermentation using rice as a substrate. The unicorn-bags (autoclavable, type/14 with single microvented filter of 0.2 µm, 2 kg capacity, 64×36 cm) were filled with 2 kg of rice. The rice in bags was soaked overnight in 1000 ml distilled water (Nahar, 2004) and autoclaved at 121ºC for 45 min (Kulkarni et al., 2008). The bags were inoculated with 48 h old mycelium inoculums (200 ml/35 g±3 g wet weight) and incubated at 28ºC and 70-80% RH for 14 d. The bags containing sporulated rice were dried at 37ºC for 2 d to reduce the moisture content (<20%). The conidia were harvested with a mycoharvester (CABI Bioscience, UK) and the yield (g/kg substrate) and percent viability of conidia were determined from three different bags.

For determining percent viability, the conidial suspensions were prepared in 0.1% (w/v) Tween 80 and the count was adjusted to 1×10^3 conidia/ml. The conidial suspensions (0.1 ml) were spread on PDA plates in triplicate and incubated at 28ºC and 70-80% RH for 72 h. The isolated colonies were counted and the total viable count was calculated. The experiment was conducted in triplicate.

2.15 Conidial sedimentation rate

The conidial sedimentation rates for *Metarhizium* isolates were determined as described by Jeffs and Khachatourians (1997). The sedimentation rates of conidia were studied in 0.2 M ammonium sulphate [$(NH_4)_2SO_4$] and 0.1% (w/v) Tween 80.

The count of conidial suspension was adjusted to ~7±0.3×10^7 conidia/ml so as to obtain an initial absorbance of 0.600 at 540 nm. The cuvettes were allowed to stand undisturbed for 6 h for settling of conidia. The absorbance was recorded up to 6 h with an interval of 1 h. The sedimentation rate was expressed in percent and the time required for 50% sedimentation (ST_{50}) was calculated. The experiment was repeated thrice using freshly prepared conidial suspensions.

2.16 Molecular methods

2.16.1 Extraction of genomic DNA from *Metarhizium* isolates

For extraction of genomic DNA, the conidial suspensions of all *Metarhizium* isolates were inoculated separately in semisynthetic complete medium and the flasks were incubated at 28ºC under shaking condition (180 rpm) for 48 h. The mycelium biomass was harvested separately by vacuum filtration through Whatmann filter paper No. 1 and washed with sterile distilled water.

The genomic DNA was extracted using DNeasy plant mini kit according to manufacturer's protocol. The mycelium biomass was ground to fine powder manually after freezing in liquid nitrogen and transferred immediately to pre-cooled 2.0 ml microfuge tube. To a maximum of 0.1 g (wet weight) ground biomass, 400 µl buffer AP1 and 4 µl RNaseA stock solution (100 mg/ml) was added immediately and vortexed vigorously. The mixture was incubated at 65ºC for 10 min with intermittent mixing. After incubation, 130 µl buffer AP2 was added, mixed and incubated on ice for 5 min. The mixture was centrifuged at 20,000 g for 5 min and the supernatant (lysate) was used in subsequent steps. The lysate was transferred to the QIAshredder mini spin column placed in a 2.0 ml collection tube and centrifuged at 20,000 g for 2 min. After collecting the flow-through in new microfuge tube, 1.5 volumes of buffer AP3/E was added and mixed immediately by pipetting. From this, 650 µl was transferred to the DNeasy mini spin column and centrifuged at ≥6,000 g for 1 min. After discarding the flow-through, the same step was repeated with remaining sample using the same collection tube. The flow-through and the collection tube were then discarded. The DNeasy mini spin column was placed in a new 2.0 ml collection tube and 500 µl buffer AW was added to the column and centrifuged at ≥6,000 g for 1 min. After discarding the flow-through, 500 µl buffer AW was added and centrifuged at 20,000 g for 2 min to dry the membrane. The DNeasy mini spin column was placed in a new 1.5 ml microfuge tube and 50 µl buffer AE was added directly onto the DNeasy

membrane. The column was incubated at RT for 5 min and centrifuged at ≥6,000 g for 1 min to elute DNA. The concentration and purity of the genomic DNA was determined by measuring the absorbance at 260 and 280 nm on Nanodrop 1000 UV-visible spectrophotometer. The DNA concentration was estimated assuming that A_{260}=1 corresponds to 50 µg/ml of double stranded DNA (Sambrook and Russell, 2001) whereas A_{260}/A_{280} ratio ≥1.8 was considered as pure DNA. The genomic DNA was analyzed on 0.8% agarose gel and stored at -20°C until further use.

2.16.2 Extraction of total RNA from *Metarhizium* isolate

The total RNA was extracted from different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h, 48 h, 72 h) and blastospores (24 h, 48 h, 72 h) of one of the *Metarhizium* isolates. The biomass of these morphological forms of *Metarhizium* was obtained as described in section 2.9.2. After harvesting, the biomass was washed with sterile 0.1% DEPC water. The glasswares and plasticwares used for RNA extraction were sterilized after overnight treatment with 0.1% DEPC water.

The total RNA was extracted using RNeasy mini kit according to manufacturer's protocol. The biomass was ground to fine powder manually after freezing in liquid nitrogen and transferred immediately to pre-cooled 2.0 ml microfuge tube. To a maximum of 0.1 g (wet weight) ground biomass, 600 µl buffer RLT containing β-mercaptoethanol (10 µl/ml) was added and vortexed properly. The mixture was centrifuged at 20,000 g for 2 min and the supernatant (lysate) was used in subsequent steps. To the homogenized lysate, one volume of 70% ethanol was added and mixed thoroughly by pipetting. From this, 700 µl was transferred to the RNeasy spin column placed in a 2.0 ml collection tube and centrifuged at ≥8,000 g for 15 sec. After discarding the flow-through, the same step was repeated with remaining sample using the same collection tube. The flow-through was discarded and 700 µl buffer RW1 was added to the RNeasy spin column and centrifuged at ≥8,000 g for 15 sec. After discarding the flow-through, 500 µl buffer RPE was added to the RNeasy spin column and centrifuged at ≥8,000 g for 15 sec. To the RNeasy spin column, again 500 µl buffer RPE was added after discarding the flow-through and centrifuged at ≥8,000 g for 2 min to wash the spin column membrane. The flow-through and the collection tube were then discarded. The RNeasy spin column was placed in a new 2.0 ml collection tube and centrifuged at 20,000 g for 1 min to dry the membrane. The RNeasy spin column was placed in a new 1.5 ml microfuge tube and 30 µl RNase free

water was added directly onto the spin column membrane. The column was centrifuged at ≥8,000 g for 1 min to elute RNA. The concentration and purity of the RNA was determined by measuring the absorbance at 260 and 280 nm on Nanodrop 1000 UV-visible spectrophotometer. The RNA concentration was estimated assuming that A_{260}=1 corresponds to 40 µg/ml of RNA (Sambrook and Russell, 2001) whereas A_{260}/A_{280} ratio ≥1.8 was considered as pure RNA. The RNA was analyzed on 1.0% agarose gel and immediately used for cDNA synthesis.

2.16.3 cDNA synthesis

The RNA of all the morphological forms of *Metarhizium* was treated separately with DNase according to manufacturer's protocol, to remove contaminant DNA, if any. The reaction mixture (10 µl) containing 8 µl 1 µg RNA, 1 µl 10X DNase buffer and 1 µl 1 U/µg DNase was incubated at 37ºC for 30 min and the reaction was stopped by adding 1 µl DNase stop solution. The DNase was denatured by incubating the reaction mixture at 65°C for 10 min.

The cDNA was synthesized from 1 µg of RNA using Verso cDNA synthesis kit according to manufacturer's protocol. The reaction mixture consisted of 5 µl 1 µg RNA template, 4 µl 5X cDNA synthesis buffer, 2 µl 5 mM dNTPs, 1 µl oligo dT, 1 µl RT enhancer and 1 µl verso enzyme mix in a final reaction volume of 20 µl. The cDNA cycling programme conditions were: cDNA synthesis at 42°C for 30 min followed by inactivation at 95°C for 2 min. The quality of cDNA was checked by carrying out PCR using cDNA as a template and ITS1 and ITS4 primers. The PCR products were analyzed on 1.5% agarose gel.

2.16.4 Amplification of ITS and RAPD region

The amplification of ITS1-5.8S-ITS2 and 26S rDNA region was carried out for the identification of *Metarhizium* isolates as well as for checking the quality of cDNA synthesized. The amplification was carried out using genomic DNA/cDNA as a template and ITS1 forward and ITS4 reverse primer (Table 2.4) (White et al., 1990). For RAPD analysis, random primers of ten nucleotides (Table 2.4) were used for the amplification of genomic DNA. The amplification reactions were carried out using thermal cycler (Mycycler, Biorad, USA). The reaction mixture for amplification of ITS region and RAPD consisted of 10-20 ng DNA template, 2 µl 10X Taq polymerase buffer, 1 µl 10 mM dNTPs, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer and 1 unit Taq DNA polymerase in a final reaction volume of 20

Table 2.4 List of primers used

Gene/region amplified	Primer sequence (5'-3')
ITS	ITS1 – TCCGTAGGTGAACCTGCGG
	ITS4 – TCCTCCGCTTATTGATATGC
RAPD	935G – GGGTTGTGGG
	947G – GGTTGGTGGG
	OPA03 – AGTCAGCCAC
	OPA07 – GAAACGGGTG
	OPA13 – CACCACCCAC
Protease	METPR2 – AGGTAGGCAGCCAGACCGGC
	METPR5 – TGCCACTATTGGCCGGCGCG
Chitin deacetylase	CDA F1 – GCCBTVACBTATGABGACGGTCC
	CDA R1 – GACHGTCTGCTBGTGCACGTCGTG
	CDA F2 – CCVAMBTABATGMGHSCRCCGTA
	CDA R2 – ATCGYCVADRCAHTCGCCVACKGT

µl. The PCR programme for ITS region amplification was: initial denaturation at 95°C for 2 min, 35 cycles of: denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min; followed by final extension at 72°C for 10 min. For RAPD, PCR programme was same except annealing temperature (50°C for 1 min). The products of the PCR reactions were analysed on 1.5% agarose gel to determine the size of the amplicons obtained.

2.16.5 Amplification of Pr1A and CDA gene

For amplification of the Pr1A gene from all the *Metarhizium* isolates, METPR2 and METPR5 primer pair (Table 2.4) was used as described by Ghormade et al. (2007) and Leal et al. (1997) whereas for amplification of CDA gene from *Metarhizium* isolate, degenerate primers directed towards conserved polysacharide deacetylase domains in CDA (Table 2.4) were used. The amplification of Pr1A and CDA gene were carried out using thermal cycler (Mycycler, Biorad, USA) using genomic DNA and cDNA as a template, respectively. The reaction mixture consisted of 50-100 ng DNA or cDNA template, 4 µl 5X HF buffer, 1 µl 10 mM dNTPs, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer and 0.4 unit phusion DNA polymerase in a final reaction volume of 20 µl. The amplification conditions for CDA gene were: initial

denaturation at 98°C for 2 min, 32 cycles of: denaturation at 98°C for 30 sec, annealing at 60°C for 45 sec, extension at 72°C for 1 min; followed by final extension at 72°C for 7 min. For Pr1A gene, the amplification was carried out in a final reaction volume of 30 µl and the amplification conditions were same except annealing temperature (68°C for 45 sec). The products of the PCR reactions were analysed on 1.5% agarose gel to determine the existence of the amplicons of the desired length.

2.16.6 Digestion of Pr1A amplicon using restriction enzymes

The Pr1A amplicons of all the *Metarhizium* isolates were digested using restriction endonucleases *viz*. RsaI, MspI and DdeI according to manufacturer's protocol. The reaction mixture consisted of 7.5 µl PCR product, 1.5 µl 10X NE buffer and 1 unit of RsaI and DdeI enzyme and 2 units of MspI enzyme separately in a final reaction volume of 15 µl. The reaction mixture was incubated at 37°C for 2 h. The products obtained after restriction digestion were analysed on 2.5% agarose gel.

2.16.7 Quantitative real-time PCR (qRT-PCR)

The primers for qRT-PCR were designed using ClustalW in the Bioedit program based on sequences of housekeeping genes for *Metarhizium* species viz. *Ubc*, *Tub-a* and *Tub-b* (U66493, DQ393576 and DQ522536 respectively) obtained from NCBI database whereas for *18S RNA* and *GAPDH* the primers used were as described by Fang and Bidochka (2006). The primer pair for qRT-PCR analysis of CDA gene was designed using ClustalW in the Bioedit program based on CDA gene sequence of *M. anisopliae* obtained in the present study. For testing the PCR efficiency of each primer pair, equal amounts of cDNA from all the morphological forms of *Metarhizium* (section 2.16.2 and 2.16.3) were mixed and ten-fold serial dilutions of the cDNA mixture (from 50 ng to 5 pg) were used as the template. The qRT-PCRs were performed using Real Master Mix SYBR ROX (5 Prime, Germany) and mastercycler epgradient realplex (Eppendorf, Germany) according to manufacturer's protocol. The reaction mixture consisted of 1 µl cDNA template, 4 µl 2.5X Real master mix, 0.5 µl 20X SYBR, 0.5 µl 10 µM forward primer and 0.5 µl 10 µM reverse primer in a final reaction volume of 10 µl. A non-template negative control was included for each primer set to check primer-dimer formation or contamination in the reaction. The qRT-PCR programme conditions were: denaturation at 95ºC for 1 min, 40 cycles of: denaturation at 95ºC for 15 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 45 sec. The specificity of amplification was verified with

melting curve analysis at the end of the qRT-PCR and the products were analyzed on 1.5% agarose gel. All qRT-PCR amplifications were conducted in triplicate.

2.16.8 Agarose gel electrophoresis

The agarose gels were prepared by melting agarose in 1X TAE buffer and adding 0.5 µg/ml ethidium bromide. To cast the gel, Bio-Rad mini, midi or maxi electrophoresis cells (Bio-Rad, USA) were used. The concentration of agarose varied according to the size of the molecules being separated. The gel in the electrophoresis cell was covered up to approximately 5 mm with 1X TAE buffer. The samples were mixed with 6X DNA loading dye and loaded into the wells. The gene ruler DNA ladder mix (100 bp-10 kb) was used as molecular weight marker. The electrophoresis was carried out at 5 V/ cm and the samples were visualized using UV transilluminator (Bio-Rad, USA).

2.16.9 Cloning of DNA

2.16.9.1 Purification of PCR products

The PCR products obtained after amplification of ITS region, protease and CDA gene and the qRT-PCR products of housekeeping genes were purified using QIAquick PCR purification kit according to manufacturer's protocol. One volume of PCR sample was mixed with five volumes of buffer PB. The mixture was transferred to QIAquick column placed in a 2.0 ml collection tube and centrifuged at 17,000 g for 60 sec. After discarding the flow-through, 750 µl buffer PE was added to the QIAquick column and centrifuged at 17,000 g for 60 sec. The QIAquick column was again centrifuged at 17,000 g for an additional 1 min for removing the residual wash buffer. The QIAquick column was then placed in a new 1.5 ml microfuge tube and 50 µl buffer EB was added to the centre of the QIAquick membrane. The column was centrifuged at 17,000 g for 1 min to elute DNA. The purified PCR products of ITS region were directly used for sequencing whereas purified PCR products of protease, CDA gene and housekeeping genes were used in Poly A tailing reaction, ligated and transformed in *E. coli* JM 109 competent cells and the plasmid DNA extracted from the transformants was used for sequencing.

2.16.9.2 Poly A tailing of purified PCR products

The reaction mixture for poly A tailing consisted of 7.5 µl purified PCR product, 1 µl 10X Taq polymerase buffer, 0.5 µl 10 µM dATP and 1 unit *Taq* DNA polymerase in a final reaction volume of 10 µl. The mixture was incubated at 70ºC for 30 min. The precipitation of A-tailed PCR product was carried out by adding two volumes of

chilled ethanol and 1/10th volume of sodium acetate (3M, pH 5.2). The tubes were inverted 3-4 times gently and centrifuged at 13,000 g at 4ºC for 10 min. The supernatant was decanted and the pellet was washed with 100 µl 70% ethanol by centrifugation at 13,000 g for 10 min. The pellet was air dried and dissolved in 10 µl TE buffer (pH 8.0).

2.16.9.3 Ligation

The reaction mixture for ligation consisted of 8 µl poly A tailed PCR product, 11 µl 2X ligation buffer, 0.5 µl 50 ng pGEM-Teasy vector and 0.5 unit T4 DNA ligase in a final reaction volume of 20 µl. The ligation reaction was carried out at 4^{o}C overnight.

2.16.9.4 Transformtion of ligated products

2.16.9.4.1 Preparation of *Escherichia coli* (JM109 strain) competent cells

The competent cells of *E. coli* JM 109 strain were prepared by Inoue method for preparation of ultra-competent cells (Sambrook and Russell, 2001). The suspension of *E. coli* JM 109 strain was streaked on LB agar and the plates were incubated at 37°C for 16-20 h. For preparation of starter culture, a single bacterial colony, 2-3 mm in diameter was inoculated into 50 ml LB broth and the flask was incubated at 37°C for 6-8 h at 180 rpm. Three flasks each containing 250 ml LB broth were inoculated with 5.0 ml, 3.0 ml and 1.0 ml starter culture and incubated at 18-22°C with moderate shaking. The growth of the culture was monitored by reading the absorbance at 600 nm. For efficient transformation, the number of viable cells should not be more than 10^8 cells/ml or A_{600} should be approximately 0.55. When A_{600} of the culture from one of the three flasks reached 0.55, the flask was transferred to an ice-water bath for 10 min to freeze the growth. The contents of the flask were centrifuged at 2,500 g at 4°C for 10 min. The medium was decanted and the cells were suspended in 80 ml ice cold Inoue transformation buffer and mixed gently by swirling. The bacterial cell suspension was centrifuged at 2,500 g at 4°C for 10 min. The buffer was decanted and the cells were suspended in 20 ml ice cold Inoue transformation buffer and mixed gently by swirling. To this bacterial cell suspension, 1.5 ml DMSO was added, mixed gently by swirling and allowed to stand on ice for 10 min. The suspension of competent cells was quickly dispensed as aliquots of 100 µl into pre-cooled sterile microfuge tubes, freezed immediately in liquid nitrogen and stored at -80°C until further use.

2.16.9.4.2 Transformation

The 100 µl aliquot of competent cells was thawed on ice for 5 min and added with 10 µl ligated product. The mixture was gently flicked and placed on ice for 30 min. It was incubated at 42°C for 90 sec for heat shock treatment and then placed on ice for 10 min. To this mixture, 700 µl SOC medium was added and incubated at 37°C under shaking condition (150 rpm) for 1 h. The contents of the tubes were centrifuged at 10,000 g for 1 min and 600 µl supernatant was decanted. The transformed cells were mixed gently in remaining medium. The 100 µl transformed cells were spread on LB-ampicillin (100 µg/ml) agar plates that were initially spread with 20 µl X-gal (50 mg/ml in DMSO) and 100 µl IPTG (24 mg/ml). The plates were incubated at 37°C for 24 h. The positive clones were selected by blue-white screening.

2.16.9.5 Colony PCR for selection of desired clone

The white colonies (assumed to be *E. coli* transformants possessing the fragment of desired size) were scrabbed with sterile tip and suspended in 50 µl sterile distilled water. The suspended cells were heated at 95ºC for 5 min and centrifuged at 10,000 g for 5 min. The supernatant was used as a template for amplification using SP6 and T7 promoter primers. The reaction mixture consisted of 5 µl template, 4 µl 5X HF buffer, 1 µl 10 mM dNTPs, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer and 0.4 unit phusion DNA polymerase in a final reaction volume of 20 µl. The amplification conditions for colony PCR were: initial denaturation at 98°C for 2 min, 32 cycles of: denaturation at 98°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 1 min; followed by final extension at 72°C for 7 min. The products of the PCR reaction were analysed on 1.5% agarose gel to determine the existence of the amplicons of the desired length.

2.16.9.6 Extraction of plasmid DNA from *Escherichia coli*

The *E. coli* transformants showing amplicons of the desired length after colony PCR were used for plasmid extraction. The plasmid DNA was extracted by the alkaline lysis method described by Sambrook and Russell (2001). A single white coloured colony was inoculated into 5.0 ml LB medium containing ampicillin (100 µg /ml) and incubated at 37°C under shaking condition (180 rpm) for 12-16 h. The transformants grown in LB medium were transferred to 2.0 ml microfuge tubes and centrifuged at 12,000 g for 5 min. The supernatant was decanted and the pellet was suspended in 200 µl ice-cold alkaline lysis solution I/GTE solution, mixed by vortexing and kept on ice for 10 min. To this mixture, 400 µl freshly prepared alkaline lysis solution II was

added, mixed by inverting the tubes and placed on ice for 10 min. To this mixture, 300 µl alkaline lysis solution III was added, vortexed for 10 sec and kept on ice for 10 min. The mixture was centrifuged at 15,000 g at 4°C for 10 min. The supernatant was transferred to fresh microfuge tube without disturbing the pellet and 0.8 volumes chilled isopropanol was added for precipitation of the plasmid DNA and incubated at -80ºC for 30 min. The plasmid DNA was pelleted by centrifugation at 15,000 g for 15 min and the supernatant was decanted. The plasmid pellet was washed with 0.5 ml 70% ethanol by centrifugation at 15,000 g for 5 min, dried at 37°C and dispensed in 50 µl TE buffer (pH 8.0). For RNase treatment, 5 µl RNaseA stock solution (100 mg/ml) was added to the plasmid DNA and incubated at 37°C for 30 min. The concentration and purity of the plasmid DNA was determined by measuring the absorbance at 260 and 280 nm on Nanodrop 1000 UV-visible spectrophotometer. The plasmid DNA was analyzed on 1.0% agarose gel and stored at -20°C until further use.

2.16.10 DNA Sequencing and data analysis

The sequencing of purified PCR products of ITS region and plasmid DNA extracted from *E. coli* transformants of protease, CDA and housekeeping genes was done using the ABI prism 3730 DNA analyzer (Applied Biosystems, Foster City, USA).

The sequences were read and edited using the Chromas 2.1 software. The BLAST search of the nucleotide or deduced amino acid sequences was performed at NCBI GeneBank data library (http://www.ncbi.nlm.nih.gov/BLAST) for close homology and for the presence of conserved domains. The alignments of nucleotide or deduced amino acid sequences with close homology were performed using ClustalW in the Bioedit program (Thompson et al., 1994) and the sequences were deposited in NCBI GeneBank database.

2.16.11 Phylogenetic analysis

Neighbor-joining phylogenetic trees of maximum-likelihood distances as calculated with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method were constructed (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) using Tree View 1.6.6 program. The trees constructed were analyzed with the prodist program using the default run options with bootstrapping (1000). The scale bar indicates 0.1 substitutions per site. Branch support was estimated by bootstrapping using both neighbor-joining trees of Jukes-Cantor distance and using parsimony.

2.17 Statistical analysis

The data for CDE activities, percent appressorium formation and percent viability was subjected to analysis of variance and the means were compared using F test at α=0.05 level of critical difference as described by Panse and Sukhatme (1989). The median lethal time (LT_{50}) as well as the median lethal concentration (LC_{50}) was determined using probit analysis (Finney, 1981; Throne et al., 1995).

The correlation between CDE activities and mortality against *H. armigera* was determined by performing regression analysis whereas the *Metarhizium* isolates were clustered based on the CDE activities, mortality data and the restriction digestion patterns. The regression and cluster analysis was performed using the programme SPSS 11.0.

The percent corrected mortality data of *Metarhizium* isolates was transformed by arcsine square root to normalize the mean percentage and used to obtain a nonlinear regression curve of frequency distribution using the software GraphPad Prism.

For determining the most stably expressed housekeeping gene(s), an Excel based software application named *Best-Keeper* (Pfaffl et al., 2004) was used. The raw data obtained in the form crossing point (CP) values were entered in the *Best-Keeper* program and the standard deviation (SD) and the coefficient of variation (CV) based on the CP values of all housekeeping genes were calculated. The change in expression of CDA gene in different morphological forms of *M. anisopliae* was presented as log to the scale 2 (Livak and Schmittgen, 2001) and was normalized to the most stably expressed housekeeping gene.

CHAPTER 3

SCREENING OF *Metarhizium* ISOLATES FOR THE CONTROL OF *Helicoverpa Armigera*

M. anisopliae has been regarded as a mycoinsecticide since the time of Metchnikoff (Lord, 2005). Because of high virulence, specificity and no adverse effects on the environment, it is considered as one of the promising mycoinsecticides (Ignacimuthu, 2008). *M. anisopliae* has been used for the control of *H. armigera* which is the most destructive pest of global importance. *H. armigera* was reported to attack several economically important crops like maize, sorghum, sunflower, cotton, soyabean, chickpea and pigeonpea (Fitt, 1989). The efficacy of *M. anisopliae* against *H. armigera* in chickpea was evaluated under field conditions (Rijal et al., 2008a) whereas different formulations of *M. anisopliae* were investigated for their effect against *H. armigera* in soyabean field (Agarwal et al., 2012).

A high level of variation exists among *M. anisopliae* isolates in relation to pathogenicity, optimal temperature and viability (Shahid et al., 2012). In addition, use of a single strain for biocontrol could perhaps lead to development of resistance. Therefore, for development of an effective mycoinsecticide, an isolate that is highly virulent for the target host and; genetically and biologically stable should be selected (Milner et al., 2002). The fungal virulence is determined by variety of factors, including host and fungal physiology in interactions with various environmental factors. It has been reported that different events such as germination of conidia, formation of appressoria for penetration and yeast-like cells for colonisation are important in fungus-insect interaction (Chavan et al., 2006; Hajek and St. Leger, 1994). Furthermore, several researchers have found correlation between the production of extracellular enzymes and pathogenicity. In *Trichoderma harzianum*, the enzymes such as serine protease, chitinase and the antibiotic peptaibol were suggested to be virulence factors that imparted pathogenicity towards the mealworm *Tenebrio molitor* (Shakeri and Foster, 2007). The effectiveness of *M. anisopliae* and *B. bassiana* strains was evaluated based on their virulence against *Spodoptera litura*, germination rate, conidia production, radial growth and protease and chitinase activity (Petlamul and Prasertsan, 2012).

In the present study, sixty eight *Metarhizium* isolates obtained from soils and insect cadavers from different fields were screened based on the CDE production, mortality of *H. armigera*, conidia production and; viability and sedimentation time of conidia. The part of this work has been published in Kulkarni et al. (2008).

Results and discussion

3.1 Isolation of *Metarhizium* isolates

In the present investigation, the sixty eight *Metarhizium* isolates were obtained from soil and insect cadavers using selective medium mentioned in Table 2.3. The colonies showing dark green sporulation on selective medium were identified as *Metarhizium* isolates (Bridge et al., 1993) and further confirmed based on conidial morphology (Barnett and Hunter, 1999). Out of sixty eight isolates, sixty three isolates were from Pune district and fifty three isolates of these were obtained from soil under different cultivated crops like tomato, custard apple, sugarcane, brinjal, okra, pigeon pea and chick pea using soil dilution method whereas ten isolates were obtained from insect hosts viz. pigeon pea-greasy cutworm, sugarcane-mealy bug, sugarcane-white grub, sugarcane-beetle and sugarcane-*Pyrilla perpussila*. The remaining five isolates out of sixty eight were obtained from Buldhana district from soil under different cultivated crops like custard apple, cotton, jawar and bajra using soil dilution method (Table 3.1). The isolates were numbered with respect to the field number, plot number and sample number (Table 3.2).

In one of the earlier reported studies twenty one *M. anisopliae* isolates were obtained from soil samples of different agricultural fields of Tamil Nadu, India and their biocontrol potential against the red cotton bug *Dysdercus cingulatus* was evaluated (Sahayaraj and Borgio, 2009). The genetic variability among eighty three *M. anisopliae* isolates was analyzed and it was observed that the distribution and abundance of *M. anisopliae* was influenced by habitat rather than insect host (Bidochka et al., 2001). Further, the rhizosphere competence of *M. anisopliae* in field studies with cabbage plants up to a period of one year was demonstrated (Hu and St. Leger, 2002). Presumably, *Metarhizium* species can very well survive and grow saprophytically in the soil, suggesting that all the sixty eight isolates in the present study may not be highly virulent.

3.2 Evaluation of *Metarhizium* isolates based on production of cuticle degrading enzymes and mortality of *Helicoverpa armigera*

The sixty eight *Metarhizium* isolates were evaluated based on the extracellular *in vitro* production of CDEs in YPG medium and chitin containing medium after 72 h and 96

Table 3.1 Origin of *Metarhizium* isolates

Source: Soil (58 isolates)		
Isolate No.	**Crop**	**Total isolates**
M1311, M1322, M1333, M2104, M2305, M2416, M2427, M2508, M42014, M45115, M45216, M45317, M79120, M79221, M79322	Tomato	15
M3419, M34210, M34311, M34412, M34513, M171264	Custard apple	06
M81123, M91124, M91225, M91326, M91528, M91427, M91629, M91730, M91831, M91932, M111145	Sugarcane	11
M101133, M101234, M101335, M101436, M101537, M101638, M101739, M101840, M101941, M102042, M102143, M102244	Brinjal	12
M51118, M51219	Okra	02
M131150, M141151, M141252, M151153	Pigeon pea	04
M121146, M121247, M121348, M121449	Chickpea	04
M183365	Cotton	01
M193166	Jawar	01
M202367, M214268	Bajra	02
Source: Insect hosts (10 isolates)		
Isolate No.	**Insect**	**Total isolates**
M16255, M16356, M16457, M16558, M16659	Pigeon pea-greasy cutworm	05
M16154, M16760	Sugarcane-mealy bug	02
M16861	Sugarcane-white grub	01
M16962	Sugarcane-beetle	01
M161063	Sugarcane-*Pyrilla perpussila*	01

Table 3.2 Nomenclature of *Metarhizium* isolates

Isolate No.	Crop/ Insect	Nomenclature			
		Field No.	Plot No.	Soil Sample No.	Isolate No.
M1311	Tomato	1	3	1	1
M34210	Custard apple	3	4	2	10
M101133	Brinjal	10	1	1	33
M16154	Sugarcane-mealybug	16	1	-	54
M161063	Sugarcane-*Pyrilla perpussila*	16	10	-	63

M – *Metarhizium*

h respectively. The enzyme activities of the sixty eight *Metarhizium* isolates in chitin containing medium ranged from 0.42-6.90 U/ml for chitinase, 0.30-3.38 U/ml for protease, 0.08-0.99 U/ml for lipase, 0.03-2.06 U/ml for CDA and 0.23-49.60 U/ml for chitosanase (Table 3.3) whereas the enzyme activities in YPG medium ranged from 0.17-2.66 U/ml for chitinase, 0.01-2.01 U/ml for protease, 0.06-0.31 U/ml for lipase, 0.20-6.48 U/ml for CDA and 0.12-11.50 U/ml for chitosanase (Table 3.4). The induced CDEs like chitinase, protease, lipase and chitosanase were higher in chitin containing medium than YPG medium. On the contrary, the constitutive CDA activity in YPG medium was higher than chitin containing medium.

It has been previously reported that large variations exists in the levels of enzyme production between and within species of entomopathogenic fungi (St. Leger et al., 1986a). The entomopathogenic fungi like *M. anisopliae*, *B. bassiana* and *Paecilomyces* sp. showed variation in the production of extracellular enzymes when grown on different substrates in the presence or absence of glucose (Fernandes et al., 2012). The nine isolates of *M. anisopliae* when grown in potato dextrose broth fortified with 1% yeast extract produced chitinase, protease and lipase activity in the range of 0.525-1.560 U/ml, 0.020-0.114 U/ml and 0.153-0.500 U/ml respectively within 7 d (Bai et al., 2012).

For further evaluation of *Metarhizium* isolates, chitinase, protease, lipase and chitosanase activities from chitin containing medium and CDA activity from YPG medium were used.

Table 3.3 Cuticle degrading enzymes produced by *Metarhizium* isolates in chitin containing medium

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M1311	1.71±0.02	1.32±0.06	0.49±0.04	0.32±0.03	13.05±0.08
M1322	2.39±0.05	1.87±0.02	0.41±0.04	0.57±0.04	3.11±0.08
M1333	2.65±0.04	2.27±0.08	0.49±0.04	2.06±0.03	4.04±0.26
M2104	3.23±0.13	3.24±0.06	0.49±0.08	0.60±0.01	49.60±0.17
M2305	1.73±0.03	1.40±0.03	0.24±0.04	0.20±0.01	13.08±0.90
M2416	2.73±0.02	1.60±0.03	0.24±0.07	0.36±0.03	14.28±0.62
M2427	2.82±0.07	2.03±0.02	0.41±0.04	0.84±0.03	42.60±0.17
M2508	0.49±0.02	0.70±0.03	0.24±0.04	0.68±0.03	16.64±0.10
M3419	0.75±0.04	1.43±0.11	0.16±0.04	0.10±0.01	12.63±1.41
M34210	3.50±0.18	1.24±0.08	0.66±0.11	1.25±0.02	10.20±0.85
M34311	3.53±0.19	3.28±0.21	0.74±0.08	1.87±0.03	35.70±2.11
M34412	3.96±0.12	3.38±0.16	0.99±0.04	0.41±0.01	32.40±1.13
M34513	2.90±0.04	2.45±0.04	0.49±0.04	0.46±0.03	6.20±0.12
M42014	1.67±0.05	0.70±0.02	0.33±0.04	0.42±0.03	3.29±0.09
M45115	0.88±0.04	0.47±0.02	0.16±0.04	0.28±0.03	4.15±0.12
M45216	1.58±0.04	0.68±0.03	0.24±0.04	0.96±0.03	13.26±0.96
M45317	2.82±0.05	3.02±0.04	0.24±0.04	0.34±0.04	0.23±0.04
M51118	1.16±0.07	0.72±0.03	0.33±0.15	0.61±0.03	1.45±0.14
M51219	0.58±0.02	0.42±0.07	0.08±0.01	0.33±0.03	4.29±0.22
M79120	1.23±0.04	0.60±0.02	0.33±0.12	0.21±0.04	4.62±0.16
M79221	0.46±0.04	0.30±0.02	0.08±0.01	0.21±0.01	6.13±0.12
M79322	1.52±0.06	0.78±0.03	0.33±0.04	0.36±0.05	3.82±0.23
M81123	3.76±0.25	3.32±0.22	0.74±0.04	0.96±0.02	35.80±1.64
M91124	3.90±0.20	1.35±0.10	0.24±0.04	0.42±0.01	34.90±1.90
M91225	2.59±0.16	0.93±0.03	0.12±0.04	0.45±0.01	7.44±0.12
M91326	1.98±0.02	1.81±0.04	0.16±0.04	0.66±0.04	13.26±1.10
M91427	3.90±0.25	2.45±0.23	0.49±0.04	0.17±0.01	25.16±1.16
M91528	2.32±0.19	2.79±0.03	0.24±0.04	0.97±0.01	2.32±0.05

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M91629	5.10±0.16	3.18±0.23	0.58±0.04	ND	26.23±1.01
M91730	0.75±0.06	0.45±0.07	0.20±0.04	0.35±0.03	3.70±0.16
M91831	0.42±0.07	0.31±0.01	0.16±0.04	ND	3.26±0.14
M91932	0.55±0.08	0.84±0.01	0.24±0.04	0.33±0.01	4.28±0.17
M101133	3.30±0.29	2.91±0.28	0.29±0.04	0.68±0.05	12.50±1.41
M101234	2.91±0.04	1.19±0.04	0.27±0.02	ND	11.52±0.18
M101335	3.74±0.24	3.05±0.22	0.29±0.04	0.59±0.05	33.93±1.39
M101436	2.74±0.11	2.01±0.02	0.16±0.04	ND	5.26±0.06
M101537	3.40±0.30	3.14±0.21	0.29±0.04	0.17±0.01	12.26±1.06
M101638	2.66±0.06	1.80±0.13	0.24±0.04	ND	6.28±0.01
M101739	2.43±0.14	1.41±0.08	0.16±0.04	0.26±0.03	12.80±0.30
M101840	0.93±0.08	1.77±0.06	0.11±0.02	ND	8.74±0.38
M101941	1.89±0.11	1.80±0.13	0.12±0.04	ND	22.60±1.32
M102042	2.42±0.06	2.00±0.14	0.16±0.04	ND	6.80±0.09
M102143	2.94±0.23	1.68±0.03	0.16±0.04	0.81±0.03	12.40±1.06
M102244	4.74±0.05	1.72±0.02	0.24±0.04	0.73±0.05	6.33±0.51
M111145	2.92±0.30	1.74±0.04	0.20±0.04	0.25±0.03	25.04±0.94
M121146	4.65±0.07	1.23±0.03	0.20±0.04	1.81±0.03	5.02±0.03
M121247	2.67±0.09	1.26±0.04	0.16±0.04	0.28±0.01	20.65±0.94
M121348	1.67±0.21	0.66±0.05	0.24±0.04	0.29±0.03	1.96±0.04
M121449	2.63±0.13	1.85±0.03	0.08±0.04	ND	4.01±0.22
M131150	0.43±0.04	0.56±0.05	0.16±0.04	0.06±0.01	1.25±0.05
M141151	1.00±0.04	1.96±0.02	0.24±0.04	1.16±0.04	7.81±0.12
M141252	2.07±0.16	2.33±0.17	0.33±0.04	0.03±0.02	5.28±0.17
M151153	1.70±0.27	1.80±0.02	0.12±0.04	ND	12.36±0.14
M16154	2.41±0.10	1.71±0.04	0.16±0.04	1.55±0.03	11.20±0.27
M16255	2.77±0.05	2.22±0.02	0.20±0.04	0.27±0.02	5.70±0.44
M16356	2.63±0.26	2.03±0.07	0.20±0.04	ND	5.32±0.69
M16457	2.89±0.13	1.52±0.03	0.12±0.04	0.35±0.01	2.31±0.04
M16558	4.99±0.03	0.93±0.04	0.20±0.04	0.39±0.01	0.76±0.03

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M16659	2.43±0.04	1.98±0.03	0.08±0.04	0.16±0.02	6.40±0.50
M16760	3.59±0.19	2.72±0.22	0.29±0.04	0.46±0.01	27.60±1.03
M16861	2.91±0.04	1.00±0.09	0.24±0.04	0.69±0.04	10.76±0.80
M16962	2.95±0.03	1.00±0.11	0.20±0.04	1.89±0.06	0.42±0.03
M161063	1.01±0.16	1.07±0.13	0.29±0.04	1.24±0.12	13.22±1.45
M171264	5.70±0.16	2.10±0.12	0.41±0.04	0.21±0.05	25.64±0.66
M183365	6.90±0.12	2.38±0.08	0.33±0.04	0.48±0.07	23.24±0.70
M193166	3.80±0.25	2.30±0.18	0.20±0.04	0.19±0.04	24.78±0.25
M202367	4.93±0.30	2.40±0.20	0.66±0.11	0.37±0.05	22.22±0.77
M214268	5.40±0.22	2.30±0.11	0.58±0.04	0.42±0.03	21.65±0.37

*CDA activity determined by MBTH method as described in section 2.10.4.1

ND: Not detected

Table 3.4 Cuticle degrading enzymes produced by *Metarhizium* isolates in YPG medium

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M1311	0.21±0.006	0.77±0.06	0.09±0.02	0.38±0.05	5.77±0.06
M1322	1.86±0.006	1.54±0.03	0.15±0.02	3.50±0.26	1.51±0.05
M1333	2.39±0.010	1.47±0.05	0.22±0.05	3.39±0.28	2.87±0.05
M2104	0.37±0.010	1.03±0.06	0.13±0.05	2.37±0.21	3.08±0.05
M2305	0.43±0.010	0.74±0.05	0.11±0.05	2.54±0.26	6.63±0.06
M2416	1.75±0.026	1.34±0.06	0.19±0.05	2.69±0.29	3.62±0.05
M2427	1.56±0.026	0.87±0.06	0.13±0.02	1.16±0.03	6.57±0.06
M2508	0.27±0.012	0.35±0.04	0.19±0.05	2.86±0.02	6.34±0.06
M3419	0.61±0.038	1.40±0.04	0.12±0.02	2.45±0.03	6.71±0.07
M34210	1.28±0.015	0.93±0.04	0.11±0.05	6.46±0.12	5.60±0.05
M34311	2.66±0.031	1.45±0.04	0.27±0.02	3.21±0.23	9.30±0.06
M34412	0.51±0.010	0.96±0.02	0.27±0.03	1.21±0.05	5.01±0.06
M34513	1.15±0.026	0.98±0.04	0.12±0.01	0.52±0.02	4.12±0.09

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M42014	1.01±0.059	0.44±0.04	0.23±0.02	6.48±0.01	2.69±0.05
M45115	0.40±0.021	0.34±0.04	0.19±0.02	2.70±0.02	1.98±0.07
M45216	0.97±0.015	0.36±0.04	0.09±0.02	2.51±0.04	7.48±0.06
M45317	1.24±0.026	1.61±0.06	0.15±0.02	3.61±0.05	0.12±0.06
M51118	1.04±0.021	0.69±0.03	0.22±0.02	2.46±0.04	1.09±0.07
M51219	0.43±0.021	0.35±0.03	0.06±0.02	0.53±0.03	3.20±0.08
M79120	1.02±0.021	0.36±0.06	0.15±0.02	3.02±0.05	2.43±0.07
M79221	0.34±0.021	0.23±0.05	0.19±0.05	0.36±0.04	0.93±0.07
M79322	1.12±0.021	0.58±0.05	0.31±0.02	5.06±0.18	2.60±0.08
M81123	1.25±0.021	1.06±0.04	0.09±0.02	2.48±0.21	3.94±0.06
M91124	1.00±0.044	0.45±0.03	0.18±0.02	1.63±0.03	3.56±0.07
M91225	0.72±0.035	0.08±0.01	0.09±0.02	2.67±0.05	3.17±0.02
M91326	0.58±0.012	1.45±0.04	0.12±0.05	2.47±0.04	7.75±0.04
M91427	1.50±0.017	0.02±0.01	0.18±0.02	2.50±0.21	2.35±0.05
M91528	2.08±0.025	2.01±0.03	0.22±0.02	2.71±0.33	1.68±0.06
M91629	1.50±0.026	1.12±0.03	0.06±0.02	2.36±0.22	5.05±0.08
M91730	0.57±0.010	0.39±0.04	0.19±0.02	0.56±0.06	3.20±0.08
M91831	0.23±0.020	0.16±0.02	0.15±0.02	3.21±0.05	2.89±0.03
M91932	0.19±0.021	0.14±0.03	0.23±0.02	0.81±0.03	3.20±0.08
M101133	0.80±0.012	0.99±0.05	0.22±0.05	2.56±0.20	1.31±0.06
M101234	1.23±0.021	0.47±0.04	0.11±0.02	2.50±0.03	6.21±0.06
M101335	0.37±0.017	0.09±0.02	0.15±0.02	2.46±0.22	5.92±0.06
M101436	1.90±0.040	0.35±0.04	0.15±0.02	3.88±0.03	3.91±0.05
M101537	1.40±0.021	0.92±0.04	0.15±0.02	2.35±0.21	11.50±0.04
M101638	0.62±0.015	0.41±0.04	0.12±0.04	2.50±0.22	4.28±0.01
M101739	0.96±0.021	0.36±0.04	0.12±0.02	0.46±0.02	5.79±0.07
M101840	ND	1.75±0.06	0.08±0.01	0.23±0.02	5.32±0.05
M101941	1.03±0.026	0.03±0.02	0.09±0.01	0.33±0.04	2.30±0.06
M102042	0.93±0.021	1.88±0.04	0.15±0.02	0.20±0.01	1.19±0.05
M102143	0.28±0.021	0.27±0.04	0.06±0.01	1.86±0.02	3.96±0.05

Isolate No.	Chitinase (U/ml)	Protease (U/ml)	Lipase (U/ml)	CDA* (U/ml)	Chitosanase (U/ml)
M102244	0.67±0.015	1.31±0.05	0.09±0.02	1.19±0.04	3.60±0.06
M111145	1.73±0.029	1.41±0.04	0.12±0.02	0.63±0.03	6.53±0.06
M121146	0.64±0.015	0.11±0.02	0.08±0.02	2.39±0.05	3.19±0.07
M121247	1.53±0.031	1.16±0.03	0.13±0.02	0.73±0.02	1.83±0.08
M121348	0.24±0.031	0.48±0.05	0.13±0.02	1.18±0.03	1.27±0.05
M121449	1.90±0.038	1.50±0.03	0.06±0.02	2.22±0.03	2.34±0.07
M131150	0.23±0.031	0.15±0.03	0.12±0.02	1.34±0.03	0.84±0.03
M141151	0.38±0.025	0.13±0.02	0.15±0.02	2.94±0.04	2.43±0.05
M141252	1.23±0.030	0.03±0.02	0.27±0.02	2.50±0.13	2.36±0.07
M151153	0.23±0.021	0.68±0.02	0.09±0.04	0.69±0.03	10.25±0.07
M16154	1.08±0.026	1.65±0.06	0.12±0.02	4.02±0.04	8.00±0.04
M16255	0.75±0.044	0.94±0.03	0.19±0.02	1.39±0.03	3.54±0.04
M16356	1.04±0.045	0.77±0.04	0.13±0.02	0.99±0.04	2.39±0.06
M16457	0.17±0.020	0.04±0.01	0.11±0.02	2.13±0.04	1.19±0.04
M16558	2.25±0.038	0.01±0.001	0.09±0.02	2.73±0.02	0.35±0.07
M16659	0.81±0.045	0.36±0.02	0.16±0.02	1.24±0.03	2.80±0.10
M16760	0.94±0.026	1.87±0.04	0.22±0.02	2.49±0.20	5.88±0.06
M16861	0.82±0.050	0.41±0.04	0.12±0.01	3.67±0.04	6.91±0.09
M16962	0.84±0.038	0.93±0.03	0.18±0.02	4.32±0.05	0.24±0.02
M161063	0.38±0.09	0.72±0.03	0.11±0.04	2.26±0.25	2.52±0.05
M171264	2.32±0.21	0.96±0.08	0.12±0.02	0.45±0.18	4.66±0.07
M183365	1.40±0.18	1.08±0.04	0.12±0.04	1.43±0.09	3.86±0.12
M193166	0.96±0.03	0.88±0.10	0.09±0.02	0.24±0.20	3.24±0.11
M202367	1.05±0.07	0.94±0.17	0.22±0.02	0.63±0.12	2.60±0.11
M214268	0.98±0.10	1.12±0.10	0.19±0.02	0.85±0.08	4.52±0.09

*CDA activity determined by MBTH method as described in section 2.10.4.1

ND: Not detected

The pathogenic potential of sixty eight *Metarhizium* isolates against 3rd instar larvae of *H. armigera* was also evaluated. Though all the sixty eight *Metarhizium* isolates were pathogenic to *H. armigera*, the mortality varied between 20-97% within 14 d (Table 3.5).

Table 3.5 Mortality of *Metarhizium* isolates against 3rd instar larvae of *Helicoverpa armigera*

Isolate No.	**Mortality (%)**
M2104, M34311, M34412, M81123, M91427, M91629, M101133, M101335, M101537, M16760, M171264, M183365	>90
M1311, M1322, M1333, M2305, M2416, M2427, M34210, M34513, M45317, M91124, M91326, M101234, M101436, M101638, M102143, M102244, M111145, M121146, M121449, M141151, M141252, M16154, M16255, M16356, M16558, M16962, M161063, M202367, M214268	70-90
M2508, M3419, M42014, M45115, M45216, M51118, M79120, M79322, M91225, M91528, M101739, M101840, M101941, M102042, M121247, M151153, M16457, M16659, M16861, M193166	50-70
M51219, M91730, M91831, M91932, M121348, M131150, M79221	<50

The potential of *Metarhizium* isolates for the control of *H. armigera* has been previously reported in many studies. The four isolates of *M. anisopliae* obtained from insects and soil from different parts of Nepal showed mortality against 3rd instar larvae of *H. armigera* in the range of 84-92% within 14 d (Rijal et al., 2008b) whereas the treatment of 3rd and 4th instar larvae of *H. armigera* with *M. anisopliae* showed 93.33% mortality within 4 d (Sahayaraj and Borgio, 2010). Further, the pathogenicity of six *Metarhizium* isolates against *H. armigera* was assessed and it was observed that the mortality rates varied from 7.3-56.3%, 5.5-45.3%, 3.6-30.7% and 3.6-23.5% for 3rd, 4th, 5th and 6th instar larvae respectively indicating more sensitivity of the younger larvae compared to the older larvae (Kpindou et al., 2012).

The relationship between the extracellular CDE activities and pathogenic potential of all sixty eight *Metarhizium* isolates was evaluated by multivariate regression analysis (Table 3.6). After regression analysis, a model with high R value (0.79) was generated that was significant (p<0.0001) indicating the effect of CDEs on mortality. However, as the regression coefficient for lipase (0.79) and chitosanase (0.21) were not significant, the masking effect of chitinase, protease and CDA was explored. Elimination of chitinase and protease, singly and in combination, showed increase in the significance of lipase from 0.79 to 0.37, 0.16 and 0.01 and chitosanase from 0.21 to 0.10, 0.02 and 0.001, respectively. Similarly, elimination of CDA showed increase in the significance of lipase from 0.79 to 0.13. However, significance of chitosanase was decreased on elimination of CDA from 0.21 to 0.99. This observation suggested that chitosanase is dependent on CDA for its activity. There was no change in the significance of CDA after elimination of chitosanase suggesting CDA is not dependent on chitosanase for its activity. Thus, regression analysis of sixty eight isolates showed that alternate chitin metabolism pathway of chitin deacetylation involving CDA is also significant in the cuticle degradation.

As all five CDEs were important for initiating pathogenicity, they were considered together with mortality data for cluster analysis. The sixty eight *Metarhizium* isolates were clustered in two major groups on the basis of variability in mortality (Figure 3.1). *Metarhizium* isolates from the larger group were further grouped into eight clusters based on the different levels of CDEs and mortality displayed by the isolates. The chitinase (3.23–6.90 U/ml), protease (2.10–3.38 U/ml), lipase (0.29–0.99 U/ml), CDA (0.45–3.21 U/ml) and chitosanase (23.24–49.60 U/ml) activities were in the higher range for isolates showing >90% mortality (cluster 8) whereas the enzyme activities for the isolates exhibiting <40% mortality of *H. armigera* (cluster 9) were in the lower range viz. chitinase (0.43–0.75 U/ml), protease (0.30-0.84 U/ml), lipase (0.08–0.24 U/ml), CDA (0.36–1.34 U/ml) and chitosanase (1.25-6.13 U/ml).

Earlier, it has been reported that chitinases and proteases were virulence factors for entomopathogenicity (St. Leger et al., 1986c; St. Leger et al., 1996a). In the present investigations also, the CDEs like chitinase, protease and CDA showed high correlation with mortality. Kaur and Padmaja (2009) observed 50% relationship between enzyme production and virulence of *B. bassiana* isolates as the isolates

Table 3.6 Regression analysis of *Metarhizium* isolates based on percent corrected mortality of *Helicoverpa armigera* and *in vitro* cuticle degrading enzyme activities

Model Variables	Regression coefficient										Model summary				Significance				
	Chi		CDA		Chito		Pro		Lip										
	t	Beta	t	Beta	T	Beta	t	Beta	T	Beta	R	r^2	F	P	Chi	CDA	Chito	Pro	Lip
All CDEs	5.71	0.42	4.49	0.28	1.25	0.09	5.79	0.46	0.26	0.02	0.88	0.79	46.85	0.0001	0.00	0.000	0.21	0.00	0.79
(–) Chi	-	-	3.63	0.27	1.64	0.15	7.22	0.65	0.88	0.08	0.82	0.68	33.53	0.0001	-	0.001	0.10	0.00	0.37
(–) Pro	7.14	0.59	3.39	0.26	2.39	0.21	-	-	1.40	0.12	0.82	0.67	33.07	0.0001	0.00	0.001	0.02	-	0.16
(–) Chi and Pro	-	-	2.38	0.24	3.37	0.39	-	-	2.60	0.30	0.64	0.41	15.18	0.0001	-	0.020	0.001	-	0.01
(-) CDA	4.98	0.42	-	-	-0.01	-0.001	4.86	0.44	1.49	0.12	0.85	0.72	41.01	0.0001	0.00	-	0.99	0.00	0.13
(–) CDA and Pro	6.53	0.58	-	-	1.30	0.12	-	-	2.35	0.22	0.78	0.61	34.56	0.0001	0.00	-	0.19	-	0.02
(-) Chito	5.90	0.43	4.29	0.25	-	-	6.34	0.49	0.74	0.05	0.88	0.78	57.64	0.0001	0.00	0.00	-	0.00	0.46
(-) CDA and Chito	5.06	0.42	-	-	-	-	5.13	0.44	1.57	0.12	0.85	0.72	55.55	0.0001	0.00	-	-	0.00	0.12

Chi: chitinase; CDA: chitin deacetylase; Chito: chitosanase; Pro: protease; Lip: lipase

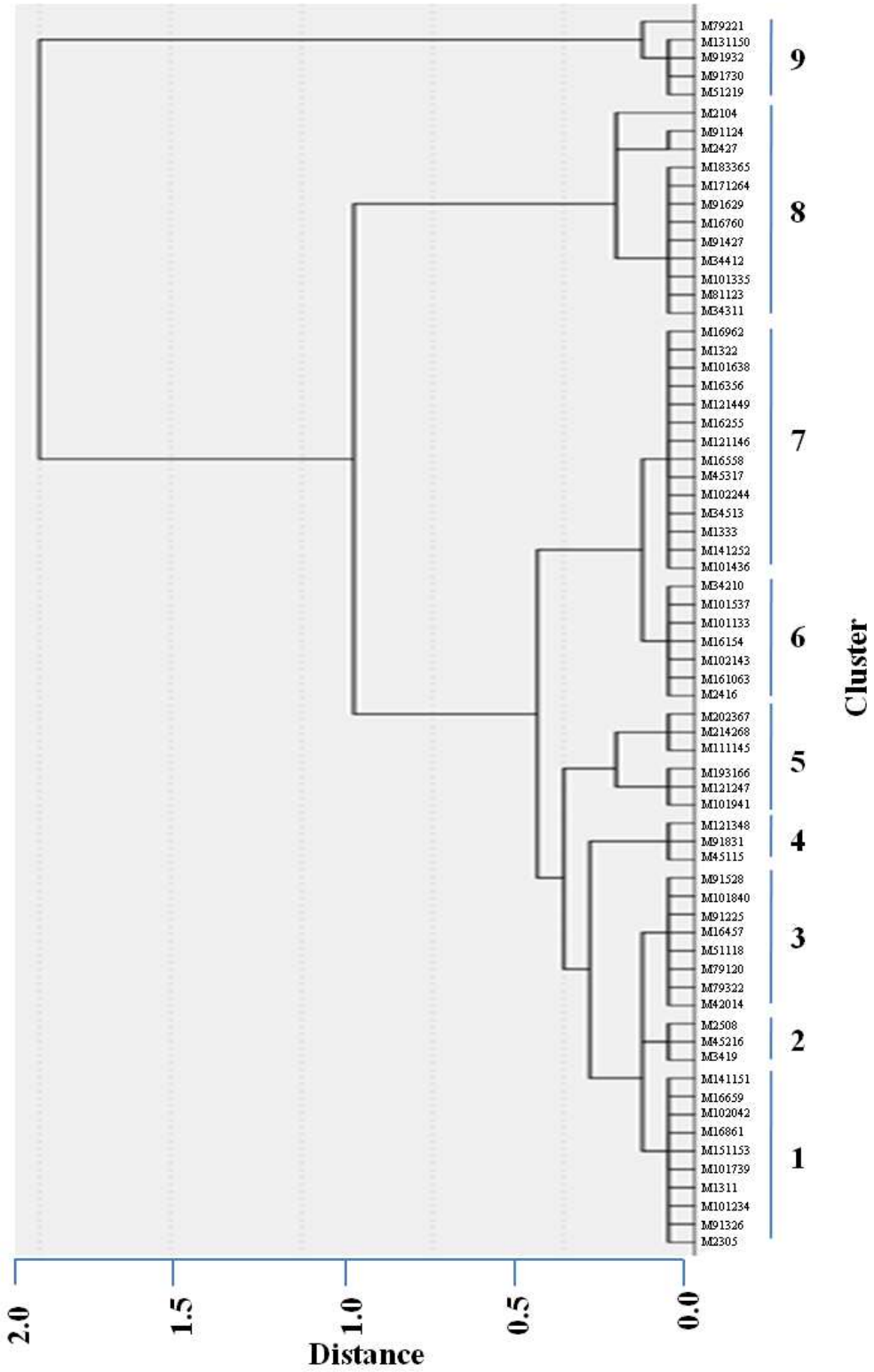


Figure 3.1 Average linkage cluster analysis of *Metarhizium* isolates based on percent corrected mortality of *Helicoverpa armigera* and *in vitro* cuticle degrading enzyme activities

categorized as highly or moderately virulent towards the 2nd instar larvae of *H. armigera* were found to be good enzyme secretors. In another study, it has been

demonstrated that the overproduction of endo-chitinase from *B. bassiana* increased the virulence towards the aphid, *M. persicae* (Fang et al., 2005). Further, the significance of any enzyme(s) was suggested to be dependent upon the cuticle characteristics and physiological state of the insect as well as the mechanism of invasion by the fungus. For instance, the lipase activity contributed to the initial phase of infection when the germinating conidium had to break the epicuticle layer to gain entry into the insect (Hegedus and Khachatourians, 1995).

Out of sixty eight *Metarhizium* isolates, the twelve isolates possessing >90% mortality were distributed in cluster 8 and cluster 6. Out of twelve isolates from cluster 8, ten isolates with >90% mortality were selected. The two isolates (M2427 and M91124) from cluster 8 that produced relatively lower chitinase, protease and CDA activities and percent mortality of *H. armigera* as compared to other isolates from the same group were not selected. On the other hand, out of seven isolates from cluster 6, only two isolates (M101133 and M101537) that showed >90% mortality and produced high levels of chitinase, protease, CDA and lipase activities were selected. These two isolates were grouped in the sixth cluster due to low levels of chitosanase. The remaining five isolates from cluster 6 with <90% mortality were not selected. The twelve selected isolates from cluster 8 and cluster 6 were further used for the determination of LT_{50}.

3.3 Determination of median lethal time (LT_{50}) against *Helicoverpa armigera*

The day-wise mortality of the twelve selected *Metarhizium* isolates was recorded to determine the fastest kill time. The *Metarhizium* isolate M34412, an isolate from custard apple, recorded lowest LT_{50} (3.3 d) whereas LT_{50} values for M81123, M34311, M91629 and M91427 were 3.3, 3.5, 3.6 and 4.1 d respectively. The LT_{50} values for the remaining seven isolates ranged between 4.6-6.8 d. The isolate from an insect host, M16760 that exhibited 90% mortality of *H. armigera* possessed highest LT_{50} value of 6.8 d which may be due to host specificity of that particular *Metarhizium* isolate (Table 3.7). All Chi-square values were not significant (α=0.05) indicating good fit of regression lines. Some mycoinsecticide products are commercially available which were prepared using the entomopathogenic fungi isolated from insect hosts. For instance, the product ‘Green Muscle or Green Guard’

Table 3.7 Median lethal time (LT_{50}) of twelve selected *Metarhizium* isolates against 3rd instar larvae of *Helicoverpa armigera*

Isolate No.	Chi-square value	LT_{50} (d)	Fiducial limit (d)
Cluster 6			
M101133	2.03	6.7	6.3 - 7.0
M101537	10.16	6.4	6.1 - 6.7
Cluster 8			
M2104	10.79	4.8	4.5 - 5.1
M34311	0.67	3.5	3.2 - 3.7
M34412	2.74	3.3	3.0 - 3.6
M81123	1.96	3.3	3.1 - 3.6
M91427	2.28	4.1	3.7 - 4.4
M91629	4.05	3.6	3.3 - 3.9
M101335	0.69	4.6	4.2 - 5.0
M16760	2.50	6.8	6.4 - 7.1
M171264	0.79	6.2	5.2 – 10.7
M183365	2.03	4.9	4.1 – 8.1

was prepared using *M. anisopliae* var. *acridium* isolated from mycosed acridoid insects (Copping, 1998). Earlier, out of ten *Metarhizium* isolates studied, the isolate with maximum mortality (92.5%) recorded least LT_{50} value (2.3 d) whereas the isolate with least mortality (50%) recorded maximum LT_{50} value (8.0 d) against 2nd instar larvae of *H. armigera* (Kumar and Chowdhry, 2004). Similarly, the LT_{50} values of the four *M. anisopliae* isolates studied by Rijal et al. (2008b) ranged between 5.3-6.8 d. The five isolates (M34311, M34412, M81123, M91427 and M91629) showing low LT_{50} values were selected for evaluating median lethal concentration (LC_{50}).

3.4 Determination of median lethal concentration (LC_{50}) against *Helicoverpa armigera*

The five selected *Metarhizium* isolates were studied at four different concentrations (1×10^3, 1×10^5, 1×10^7 and 1×10^9 conidia/ml) to determine the number of conidia required to kill 50% 3rd instar larvae of *H. armigera* i.e. LC_{50}. The *Metarhizium* isolate M34412 possessing low LC_{50} value (1.4×10^3 conidia/ml) was more virulent

than other isolates. The *Metarhizium* isolates M34311 and M81123 displayed the LC_{50} values 2.04×10^3 conidia/ml and 5.7×10^3 conidia/ml, respectively whereas M91427 and M91629 showed high LC_{50} values ($>16\times10^3$ conidia/ml) (Table 3.8). All Chi-square values were not significant ($\alpha=0.05$) indicating good fit of regression lines. As reported previously, the LC_{50} value of *M. anisopliae* against 3rd instar larvae of *H. armigera* was 6.0×10^5 conidia/ml (Nguyen et al., 2007) whereas in one of the similar studies, the LC_{50} value of *M. anisopliae* against 2nd instar larvae of *H. armigera* was reported to be 1.23×10^3 conidia/ml (Kumar and Chowdhry, 2004). Similarly, (Sahayaraj and Borgio, 2010) observed LC_{50} values of 1.25×10^6, 1.75×10^6 and 2.40×10^5 conidia/ml for *M. anisopliae* against 2nd, 3rd and 4th instar larvae of *H. armigera,* respectively. The LC_{50} values of the five studied *Metarhizium* isolates were found to be less compared to the above mentioned reports. The three *Metarhizium* isolates (M34311, M34412 and M81123) possessing low LC_{50} values were further studied for conidia production on a solid substrate, viability and settling time of conidia.

Table 3.8 Median lethal concentration (LC_{50}) of 5 selected *Metarhizium* isolates against 3rd instar larvae of *Helicoverpa armigera*

Isolate No.	Chi-square value	Slope	SE of slope	LC_{50} ($\times10^3$ conidia/ml)	Fiducial limit ($\times10^3$ conidia/ml)
M34311	1.47	0.299	0.0772	2.04	0.4–10.3
M34412	2.50	0.246	0.0762	1.40	0.1–1.9
M81123	2.95	0.281	0.0727	5.70	1.2–26.7
M91427	0.096	0.299	0.0726	35.8	9.1–140.7
M91629	3.31	0.266	0.0666	16.8	4.5–62.3

3.5 Production, viability and settling time for conidia of *Metarhizium* isolates

For commercialization of a mycoinsecticide, large scale production of infective conidia is essential. The production of conidia using three selected isolates (M34311, M34412 and M81123) was carried out by solid state fermentation using rice as a substrate. The conidial yields for all the three isolates were not significantly different

from each other and were in the range of 60-75 g/kg substrate (Table 3.9). The yield of conidia/g rice was >2.0×10^9 and the number of conidia/g first quality spore powder were in the range of 4.04×10^{10}-4.4×10^{10}. It has been reported that an average yield of conidia of *Metarhizium flavoviride* collected with the mycoharvester was 1.5×10^9 per g of rice and the number of conidia/g pure conidial powder were 5.0×10^{10} conidia (Jenkins et al., 1998).

Table 3.9 Conidia production, percent germination and sedimentation time (ST_{50}) of 3 selected *Metarhizium* isolates

Isolate	Yield (g/kg rice)	% Germination	ST_{50} in Tween-80 (h)	Fiducial limit (h)
M34311	60 ± 2.64	92 ± 2.64	2.47	2.26 - 2.69
M34412	67 ± 3.46	97 ± 1.73	2.30	2.11 - 2.52
M81123	75 ± 3.60	93 ±1.73	2.65	2.43 - 2.90

In the present study, the percent conidial germination for the three *Metarhizium* isolates ranged between 92-97% after 24 h (Table 3.9). As observed by Petlamul and Prasertsan (2012), the conidial germination of six *M. anisopliae* isolates on Czapeck Dox agar ranged between 39.83-70.82% after 48 h. According to Vijayavani et al. (2010), as *M. anisopliae* isolates that showed fast germination possessed high infectivity against *H. armigera*; conidial germination could be used as an important criterion to identify virulent isolates.

The hydrophobicity of conidia is one of the important parameters during fungus-insect interaction as it helps in adhesion of the fungus to the insect cuticle and higher the hydrophobicity of conidia, faster is the sedimentation. In the present study, the ST_{50} values for *Metarhizium* isolates M34412, M34311 and M81123 were 2.1, 2.2 and 2.5 h, respectively when determined using 0.2 M $(NH_4)_2SO_4$. As 0.1% (w/v) Tween 80 was used for maintaining the homogenous conidial suspension during bioassays as well as for spraying in the field, the sedimentation of conidia in 0.1% (w/v) Tween 80 was also studied. The ST_{50} values for *Metarhizium* isolates M34412, M34311 and M81123 were 2.3, 2.4 and 2.6 h, respectively in the presence of 0.1% (w/v) Tween 80 which were higher as compared to the ST_{50} values in the presence of 0.2 M $(NH_4)_2SO_4$. Further, out of three isolates, conidia of *Metarhizium* isolate

M34412 settled faster indicating greater hydrophobicity than the other two isolates. According to Kapoor (2012), the roughness of conidial surface was responsible for deciding the hydrophobicity and settling time of conidia of *M. anisopliae*.

3.6 Identification of *Metarhizium* isolates

The ITS1-5.8S-ITS4 regions of the *Metarhizium* isolates M34311, M34412 and M81123 were obtained by amplification using ITS1 and ITS4 fungal primers (White et al., 1990). Agarose gel electrophoresis of PCR products showed the presence of 550 bp amplicons. The purified PCR products were sequenced with ABI 3730 DNA analyzer. The ITS1-5.8S-ITS4 sequences were used to perform BLAST search. The sequence identity with the closest sequence was used to identify the isolate. The ITS1-5.8S-ITS4 sequences were homologous (100% identical) to the *M. anisopliae* var *anisopliae* strain sequences deposited in the NCBI database (LK995311 and KJ872681). The ITS1-5.8S-ITS4 sequences of *Metarhizium* isolates M34311, M34412 and M81123 were submitted to genebank under accession numbers KM875561, KM875560, KM875562, respectively.

In conclusion, on the basis of highest extracellular CDE activities and percent mortality against *H. armigera*; lowest LT_{50} and LC_{50} values as well as higher conidial germination and faster sedimentation time (ST_{50}), the *M. anisopliae* isolate M34412 was found to be the most effective isolate among sixty eight *Metarhizium* isolates as it possessed traits necessary for development of successful mycoinsecticide.

CHAPTER 4
CUTICLE DEGRADING ENZYMES AS BIOCHEMICAL AND MOLECULAR MARKERS OF *Metarhizium* ISOLATES

A. Molecular characterization of *Metarhizium* isolates based on polymorphism in protease (Pr1A) gene

Though all the cuticle degrading enzymes are important in insect killing process, major role was assigned to proteases in fungus-insect interaction as the proteolytic complex appeared first during *in vitro* cultivation which was in accordance with insect cuticular structure (St. Leger et al., 1986c). The proteases were considered as crucial enzymes in facilitating invasive mycosis of insects (Charnley and St. Leger, 1991). In case of *M. anisopliae*, chymoelastase (Pr1) was reported to play a major role in entomopathogenicity (St. Leger et al., 1987a). Further, *M. anisopliae* was also reported to produce a metalloprotease (St. Leger et al., 1994), a trypsin-like enzyme (Pr2) belonging to the serine protease group and a cysteine protease (Pr4) (Cole et al., 1993).

Among proteases, subtilisins Pr1 are important and most extensively studied proteases of *M. anisopliae* due to their intricate relation to strains abilities to penetrate, colonize and macerate insect host tissues (St. Leger et al., 1996b). The role of Pr1 in solubilizing cuticular proteins and making them available as nutrients was demonstrated by St. Leger et al. (1988), suggesting it as a virulence factor essential for rapid invasion through the host cuticle. The mutant strains of *M. anisopliae* lacking Pr1 gene exhibited decreased virulence towards *T. molitor* (Wang et al., 2002). Furthermore, the Pr1 activity of *M. flavoviride* isolates was significantly affected when insect cuticle was added in the mineral medium (without nitrogen source), while there was no appreciable variation in Pr2 activity (Pinto et al., 2002). The entomopathogens such as *B. bassiana* and *N. rileyi* also extracellularly produced proteases which contributed in the fungus-insect interaction (Dhar and Kaur, 2010) (Nunes et al., 2010). In case of *N. rileyi*, a positive correlation between the Pr1 and virulence against *Anticarsia gemmatalis* was seen (Nunes et al., 2010).

There are multiple isoforms of Pr1 viz. Pr1A, Pr1B, Pr1D, Pr1J and Pr1K in *M. anisopliae* genome which are related to the pathogenicity of the fungus. As large quantities of subtilisins are required during penetration of host cuticles, multiple isoforms of Pr1 are present. The multiple Pr1's may have diverged to perform different functions like role in pathogenesis, increased adaptability and host range as well as function in survival in various ecological habitats. Among them, Pr1A was predominantly expressed during penetration (Bagga et al., 2004).

The entomopathogenic fungi show variation in virulence because of the genetic variability and this can be observed by way of molecular markers. The molecular markers can detect differences in DNA which will help in understanding the genetic diversity and the structure of fungal populations. Molecular markers based on the polymerase chain reaction (PCR) technique such as amplified fragment length polymorphism (AFLP), internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP), inter-simple sequence repeats (ISSR) and intron splice site primer are used in characterizing inter- and intraspecific variations of *Metarhizium* and *Beauveria*. The genetic variability among eighty three isolates of *M. anisopliae* from Canadian soil was studied using allozymes, random amplified polymorphic DNA (RAPD) and RFLP of a subtilisin-like protease encoding gene and was correlated to the origin of isolation (Bidochka et al., 2001). A molecular technique was developed for the identification of *B. bassiana* isolates on the basis of presence of Pr1 gene which overcomed the difficulties with morphological identification (Safavi, 2010). The molecular techniques like ITS-RFLP, ISSR and intron splice site primer were used for studying the polymorphism among thirty seven *M. anisopliae* var. *anisopliae* isolates. The use of these techniques as molecular markers was evaluated so as to get information about the DNA fingerprinting of these isolates which can be further used in detection of these isolates in laboratory and field work (Tiago et al., 2011).

The difference in the virulence of sixty eight *Metarhizium* isolates obtained from different locations, insect hosts, and crop plants probably may be due to the genetic variation in Pr1A. In the present study, the incidence of Pr1A gene polymorphism in sixty eight *Metarhizium* isolates and its correlation with *in vitro* protease activities and mortality of *H. armigera* was studied.

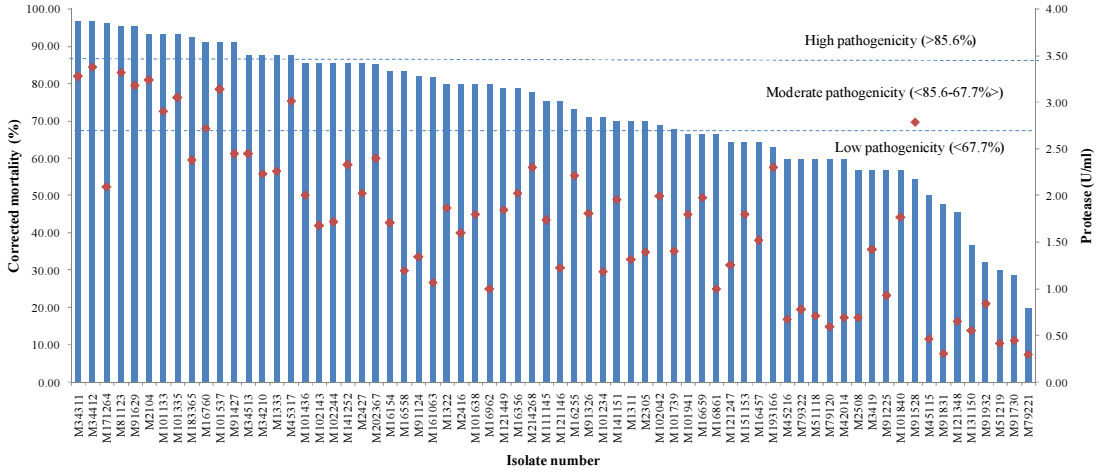
Results and discussion

4.1 Protease activity of *Metarhizium* isolates

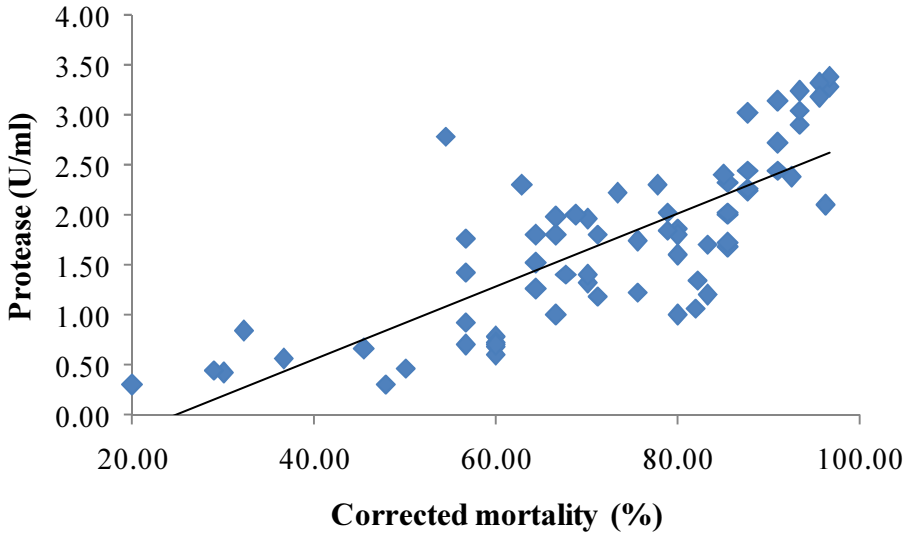
Proteins are major and outer cuticular components which mask micro-fibrillar chitin. The action of Prl on these proteins releases smaller peptides which are then further degraded and rendered amenable for catabolism by the amino-peptidases produced during penetration. A high level of *in vivo* protease activity was found to be associated with appressoria formation. Apparently, *Metarhizium* isolates showing

higher protease production will be better equipped for penetration of the host cuticle and subsequently exhibit more virulence than the isolates with low protease activity (St. Leger et al., 1987a).

In the present study, the sixty eight *Metarhizium* isolates showed variation in the *in vitro* production of extracellular cuticle degrading protease when grown in chitin containing medium. The *in vitro* protease activities of the sixty eight *Metarhizium* isolates in chitin containing medium ranged from 0.30–3.38 U/ml (Figure 4.1a).



(a)



(b)

Figure 4.1 Correlation of *in vitro* protease activity and percent corrected mortality of *Helicoverpa armigera* for *Metarhizium* isolates

Natural variability in the production of extracellular enzymes like chitinase and proteinase of *M. anisopliae* isolates was reported by Mustafa and Kaur (2009). Similarly, *M. flavoviride* isolates also showed a natural variability in the production of cuticle-degrading proteases. The expression of both Pr1 and Pr2 proteases was influenced by the substrates used for growth and highest protease activities (more Pr1 production than Pr2) were produced by all the *M. flavoviride* isolates in the medium containing cuticle from *Rhammatocerus schistocercoides* (Pinto et al., 2002).

4.2 Bioassay against *Helicoverpa armigera*

The corrected mortality of the 3rd instar larvae of *H. armigera* in bioassays with the sixty eight *Metarhizium* isolates ranged from 20-97% (Figure 4.1a). After normalization of corrected mortality by arcsine square root transformation and subsequent regression analysis, the isolates were divided into three groups according to the mean percentages (x) and standard deviation (SD). Isolates with mortality 0.5 SD above the mean were classified as highly pathogenic, those with mortality in the range of 0.5 SD above to 0.5 SD below the mean were classified as moderately pathogenic, and those with the mortality 0.5 SD below the mean were considered as possessing low pathogenicity. The *Metarhizium* isolates classified as highly pathogenic possessed mortality rates >85.6% whereas those classified as moderately pathogenic showed mortality rates between 67.7-85.6% and isolates possessing mortality rates <67.7% were considered as less pathogenic (Figure 4.1a).

Out of sixty eight *Metarhizium* isolates, sixteen were highly pathogenic, twenty seven were moderately pathogenic and remaining twenty five exhibited low pathogenicity. The *in vitro* protease activity for isolates from highly pathogenic group was in the range of 2.1-3.38 U/ml whereas for moderately pathogenic isolates the protease activity ranged between 1-2.4 U/ml and for most of the isolates possessing low pathogenicity, the protease activity was below 1 U/ml (Figure 4.1a). Thus, a positive correlation was observed between the protease activity and mortality of *H. armigera* by the *Metarhizium* isolates (Figure 4.1b, $p>0.001$, $r=0.783$).

The variability in Pr1 production of the entomopathogenic fungi may be directly related to variability in virulence, as the regulation of Pr1 gene expression determines the capacity of the fungus to cause insect disease (St. Leger et al., 1987a). The *M. anisopliae* proteases were reported to play important role in the control of mosquito larvae. The up-regulation of proteinase genes Pr1, Pr2 increased the percent

mortality whereas decreased larval mortality was seen in presence of protease inhibitors (Butt et al., 2013).

4.3 Amplification of Pr1A gene

The amplification of Pr1A gene was carried out for all the sixty eight *Metarhizium* isolates. Out of sixty eight isolates, sixty six *Metarhizium* isolates gave a single product of approximately 1.2 kb after amplification of genomic DNA using METPR2 and METPR5 primer pair (Figure 4.2) and was corresponding to the previously reported Pr1A sequences in the genebank database. The two *Metarhizium* isolates, viz. M102143 and M102244 from brinjal field possessing protease activity comparable to other isolates (1.68 and 1.72 U/ml, respectively) and 85% mortality against *H. armigera* did not show the presence of 1.2 kb PCR product. This may be due to the contribution of other proteases in the total activity than Pr1. The reason for the absence of PCR products for Pr1 in some strains of *Verticillium chlamydosporium* and *V. lecanii* was suggested to be difference in one or few critical bases within the primer binding sites on the homologous gene (Leal et al., 1997).

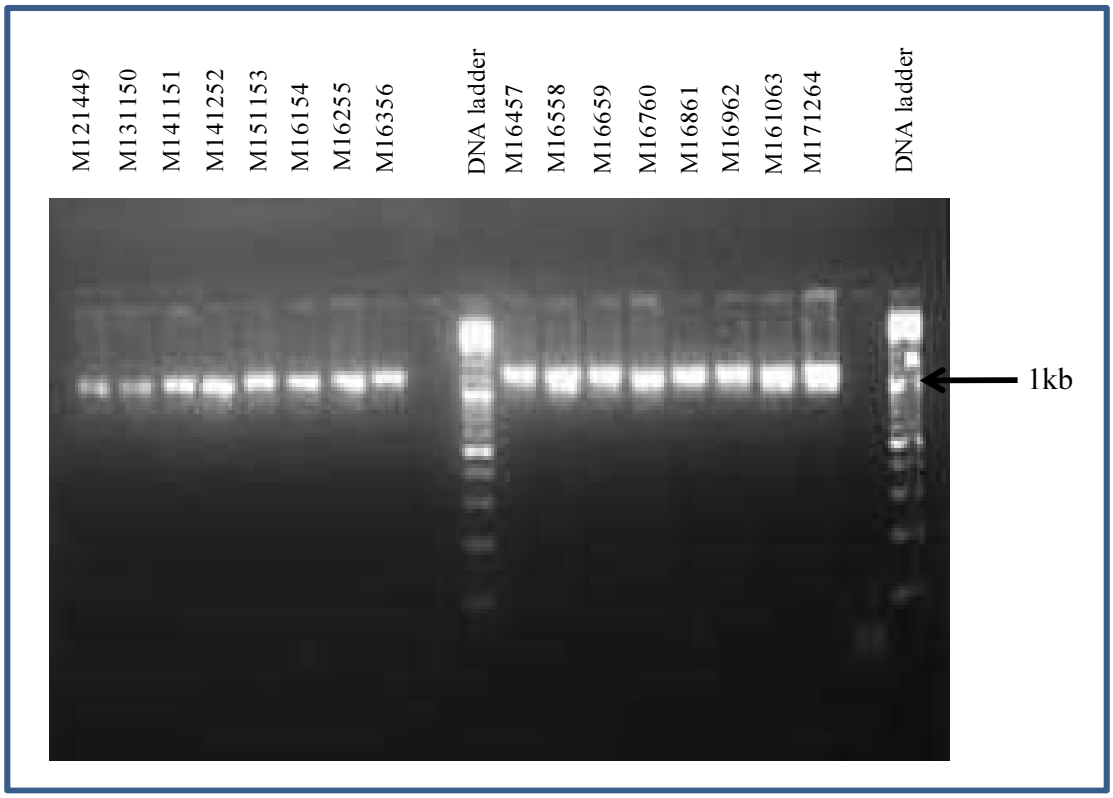


Figure 4.2 Representative electrophoretic profile of Pr1A amplicon

4.4 Restriction digestion of Pr1A PCR products

The Pr1A amplicons of the sixty six *Metarhizium* isolates were digested using restriction endonucleases *viz*. RsaI, MspI and DdeI to study the polymorphism in Pr1A gene. The digestion of Pr1A amplicons showed multiple polymorphisms with 7, 2 and 5 restriction patterns for RsaI, MspI and DdeI which were designated as A-G, H-I and J-N, respectively (Figure 4.3). In one of the earlier studies, the polymorphism in three subtilisin-like protease isoforms (Pr1A, Pr1B and Pr1C) from *Metarhizium* strains was studied and the greatest variation in RFLP patterns between all *Metarhizium* isolates for Pr1A and fewest differences for Pr1B and Pr1C were observed (Bidochka and Melzer, 2000).

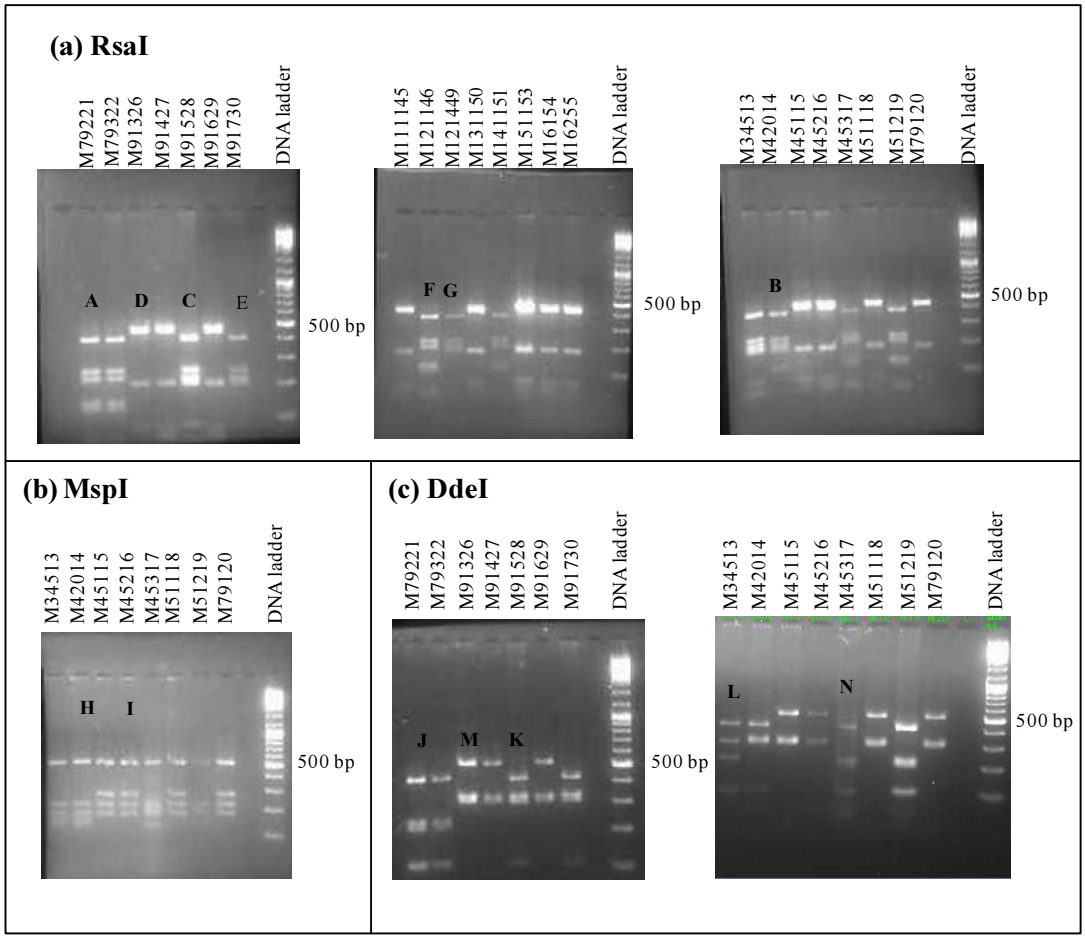


Figure 4.3 Representative electrophoretic profile of restriction digestion fragments of Pr1A amplicon digested with (a) RsaI (b) MspI and (c) DdeI

After combination of the restriction digestion patterns of Pr1A amplicons of sixty six *Metarhizium* isolates obtained using three restriction enzymes, 15 cumulative profile types were produced (Table 4.1). Similarly, as reported by Leal et al. (1997), the restriction patterns generated by digestion of Pr1 PCR products in fourty

Table 4.1 Distribution of *Metarhizium* isolates based on restriction digestion patterns and pathogencity category

Cumulative profile type	**Endonuclease profile type**			**Pathogenicity group**	**Isolate No.**
	RsaI	**MspI**	**DdeI**		
I	D	I	M	High pathogenicity (>85.6%)	M34311, M34412, M81123, M91427, M91629, M101133, M101335, M101537, M16760, M171264, M183365
				Moderate pathogenicity (67.7 – 85.6%)	M91124, M91326, M101234, M101436, M111145, M141252, M16154, M16255, M16962, M161063, M202367, M214268
				Low pathogenicity (<67.7%)	M45115, M45216, M51118, M79120, M91225, M91831, M101840, M131150, M151153, M16861
II	D	I	K	High pathogenicity	-----
				Moderate pathogenicity	M101739
				Low pathogenicity	-----
III	G	H	K	High pathogenicity	-----
				Moderate pathogenicity	M101638, M102042
				Low pathogenicity	-----
IV	B	H	K	High pathogenicity	M1333
				Moderate pathogenicity	M2305
				Low pathogeinicity	M42014

Cumulative profile type	Endonuclease profile type			Pathogenicity group	Isolate No.
	RsaI	MspI	DdeI		
V	C	H	K	High pathogenicity	-----
				Moderate pathogenicity	-----
				Low pathogenicity	M91528, M91932, M193166
VI	E	H	K	High pathogenicity	-----
				Moderate pathogenicity	-----
				Low pathogenicity	M91730
VII	C	H	L	High pathogenicity	M34513
				Moderate pathogenicity	M2416
				Low pathogenicity	M121247
VIII	G	H	L	High pathogenicity	-----
				Moderate pathogenicity	M121449
				Low pathogenicity	M101941
IX	E	H	L	High pathogenicity	M2104
				Moderate pathogenicity	M2427
				Low pathogenicity	M2508
X	B	I	L	High pathogenicity	-----
				Moderate pathogenicity	-----
				Low pathogenicity	M3419
XI	E	I	L	High pathogenicity	M34210
				Moderate pathogenicity	-----
				Low pathogenicity	-----
XII	A	H	J	High pathogenicity	-----
				Moderate pathogenicity	M1322
				Low pathogenicity	M79322, M121348, M79221

Cumulative profile type	Endonuclease profile type			Pathogenicity group	Isolate No.
	RsaI	MspI	DdeI		
XIII	F	H	J	High pathogenicity	-----
				Moderate pathogenicity	M16558, M121146, M16356, M141151
				Low pathogenicity	M16659, M16457
XIV	F	H	N	High pathogenicity	M45317
				Moderate pathogenicity	-----
				Low pathogenicity	M51219
XV	B	H	N	High pathogenicity	-----
				Moderate pathogenicity	M1311
				Low pathogenicity	-----

Metarhizium strains by three endonucleases namely DdeI, HpaII and RsaI produced 15 different profile types.

In the present study, cumulative profile type I (D-I-M pattern) was most prevalent as thirty three (50%) out of the sixty six isolates exhibited this profile. Out of the sixteen isolates from highly virulent group, eleven isolates showed D-I-M pattern, whereas remaining five exhibited five different cumulative profile types (IV, VII, IX, XI, XIV). Though ten isolates from low pathogenicity group also showed D-I-M profile, the *in vitro* protease activities were quite low (<1.0 U/ml for eight isolates). Also for two isolates M91528 and M193166 with high protease activity (>2.0 U/ml) but low virulence, the cumulative profile type was different i.e. V (C-H-K pattern). Next dominant cumulative profile type was XIII (F-H-J pattern) with four and two isolates from moderate and low pathogenicity groups, respectively.

4.5 Cluster analysis

In the present investigation, the cluster analysis using all the three variables i.e. protease activity, mortality against *H. armigera* and the data for polymorphism of Pr1A for sixty six *Metarhizium* isolates showed two major groups on the basis of variation in mortality (Figure 4.4). The *Metarhizium* isolates were further grouped

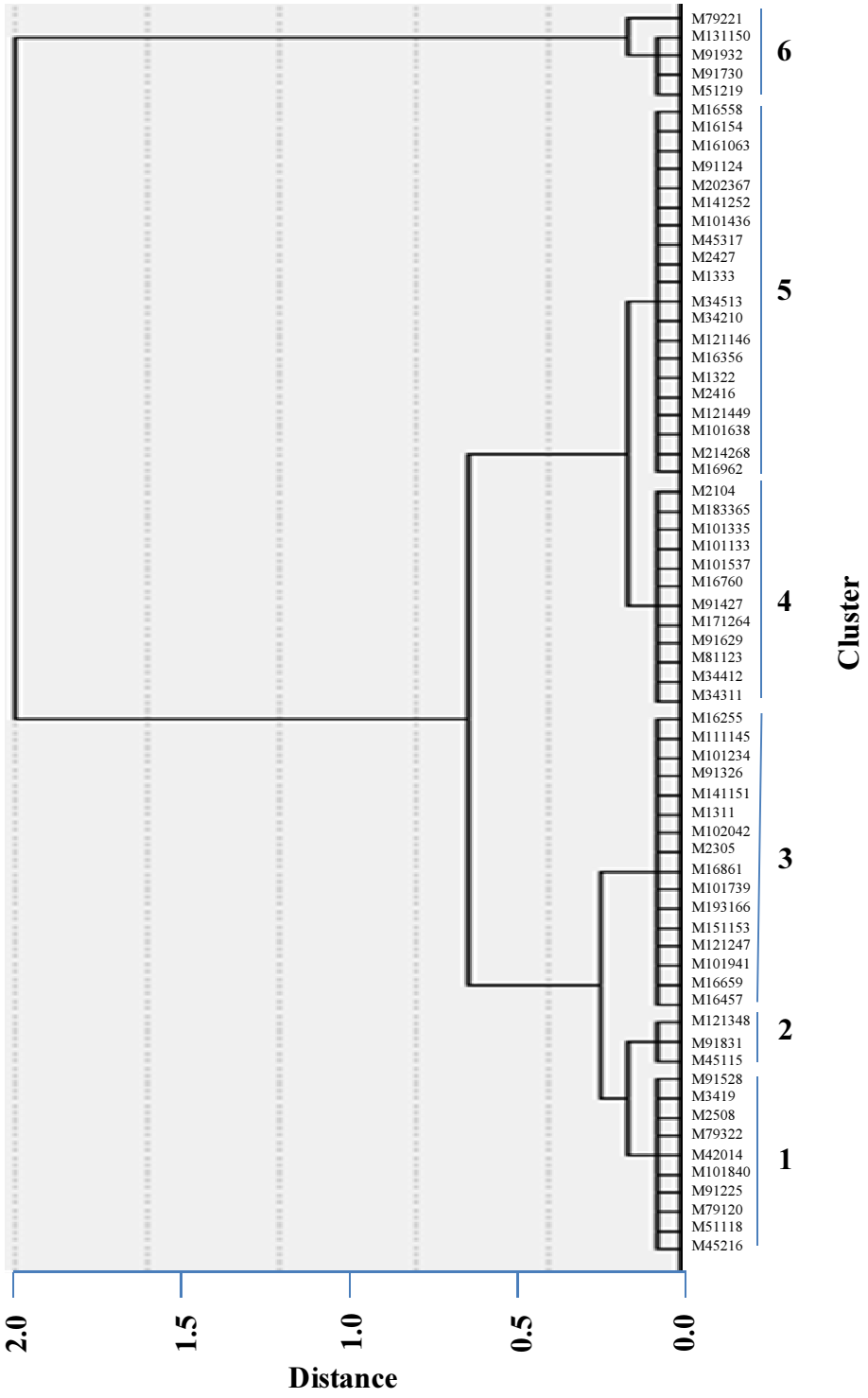


Figure 4.4 Average linkage cluster analysis of *Metarhizium* isolates based on percent corrected mortality of *Helicoverpa armigera,* restriction digestion patterns of Pr1A amplicon and *in vitro* protease activity

into six clusters on the basis of protease activity and restriction digestion patterns. It was observed that, the sixteen isolates from the highly pathogenic group were divided

in two clusters (cluster 4 and cluster 5); the twelve isolates possessing high protease activity (>2.0 U/ml) and >90% mortality were grouped together in cluster 4 whereas the remaining four isolates were grouped in cluster 5 as they possessed 87.78% mortality. Out of the twelve isolates from cluster 4, eleven isolates showed cumulative profile type I (D-I-M pattern) whereas only one isolate showed cumulative profile type IX (E-H-L pattern). Based on the cluster analysis, the correlation between protease activity, virulence against *H. armigera* and the cumulative profile type I (D-I-M pattern) of sixty six *Metarhizium* isolates could be established. Further, this analysis also supported the selection of *M. anisopliae* M34412 (possessing cumulative profile type I) as the most effective isolate among sixty eight *Metarhizium* isolates.

Further, the correlation between genetic polymorphism of entomopathogenic fungi and geographic origin or insect host has been reported. Leal et al. (1997) observed correlation between the profile of restriction fragments of Pr1 gene and geographic origin for certain groups of *Metarhizium* strains. On the contrary, Velasquez et al. (2007) showed that the intra-specific differentiation of Chilean isolates of *M. anisopliae* revealed by RAPD, SSR and ITS markers was not associated with the geographical origin of isolates. Similarly, the RAPD pattern of five *N. rileyi* strains showed high degree of homology irrespective of their geographic origin and the insect host from which they were isolated (Vargas et al., 2003). It would be interesting to categorize all sixty six *Metarhizium* isolates for geographical, crop and insect origin.

Butt et al. (2006) reported that Pr1 and destruxins could be used as markers to monitor the virulence of entomopathogenic fungi during mass production. Recently, it has been reported that out of five *M. anisopliae* isolates studied, the three most virulent isolates to cattle tick *Rhipicephalus microplus* possessed highest Pr1 activities. Therefore, it was suggested that Pr1 activity can be used as a biochemical tool for screening of the virulent isolates of *M. anisopliae* (Perinotto et al., 2014). On the basis of present investigation, it can be proposed that the protease activity along with the polymorphism of Pr1A gene can be used as biochemical and molecular tool to identify the most effective *Metarhizium* isolates against *H. armigera* and as a quality control parameter of a bio-control organism.

B. Effect of repeated *in vitro* sub-culturing and *in vivo* passage on cuticle degrading enzyme production and virulence of *Metarhizium anisopliae* against *Helicoverpa armigera*

The commercial success of any mycoinsecticide depends significantly on the virulent nature of the infective propagules viz. conidia sprayed in the field and; for the large scale production of mycoinsecticide, stability of the fungal strain during repeated *in vitro* conidial sub-culturing on artificial media is desirable. The entomopathogenic fungi degenerate as a result of loss of virulence and change in morphology, when they are successively sub-cultured on artificial media and the loss in virulence of the entomopathogenic fungi can be restored by passage through the insect host (Adames et al., 2011; Butt et al., 2006; Fargues and Robert, 1983; Schaerffenberg, 1964).

In many studies, the effect of repeated *in vitro* sub-culturing and *in vivo* passage on the virulence of entomopathogenic fungi has been reported. For instance, in case of *N. rileyi*, after 6 conidial transfers on Sabouraud maltose and yeast extract agar, a decrease in the ability to produce yeast-like hyphal bodies was observed whereas after 10 conidial transfers, avirulent progeny towards velvetbean caterpillar, *A. gemmatalis* was found to be developed (Morrow et al., 1989). Further, a strain of *B. bassiana* showed decreased virulence against *Leptinotarsa decemlineata* after 16 conidial transfers on an artificial medium (Schaerffenberg, 1964) whereas *A. aleyrodis* lost its virulence towards greenhouse whitefly, *Trialeurodes vaporariorum* after 19 sub-cultures on artificial medium (Fransen et al., 1987). On the contrary, morphological and physiological changes in *V. lecanii* were observed after repeated sub-culturing on different solid media without significant attenuation towards an aphid host, *Macrosiphoniella sanborni* whereas passage through an aphid host did not enhance the virulence (Hall, 1980). In case of *B. bassiana*, no decrease in the virulence towards *Bemisia argentifolii* was observed following 15 repeated *in vitro* transfers (Brownbridge et al., 2001). Further, *Paecilomyces farinosus* also apparently retained its virulence towards the English grain aphid, *Sitobion avenae* after 15 repeated conidial transfers (Hayden et al., 1992). Similarly, no change in the virulence of *P. fumosoroseus* was observed towards *Diuraphis noxia* or *Plutella xylostella* even after 30 *in vitro* transfers. It was also observed that different host passages had varying effects on virulence. For instance, the virulence of *P. fumosoroseus* towards *D. noxia* was decreased after 15 passages in *P. xylostella* and was not regained even

after five passages in *D. noxia*. Also, there was no change in the virulence of *P. fumosoroseus* towards the insect hosts, *D. noxia* and *P. xylostella* after 15 passages in *D. noxia* (Vandenberg and Cantone, 2004). The contradictions in all these reports were attributed to considerable inter- and intra-species variation and the genetic stability of the entomopathogenic fungi.

Considerable variation in the extent of the effect of repeated *in vitro* sub-culturing on morphological, biochemical, molecular characteristics, and most importantly, on the virulence and host specificity of the entomopathogenic fungal strains has been observed. Though the attenuation of virulence has been observed in nearly all the entomopathogenic fungi, different factors were reported to be responsible for the attenuation. For instance, in case of *N. rileyi*, the attenuation process was associated with the sporulation and multiple vegetative transfers did not decrease its virulence towards *A. gemmatalis* (Morrow et al., 1989). The attenuation of virulence was also reported to be due to the effect of *in vitro* cultivation conditions on different virulence factors involved in the killing process. The virulent conidia of *M. anisopliae* possessing low endogenous C: N ratios and high germination rates when transferred on a medium with high C: N ratio, their virulence towards 4^{th} and 5^{th} instar larvae of *Galleria mellonella* and *T. molitor* was lost (Shah et al., 2005). The use of different methodologies viz. single-spore or multi-spore transfers on different organic media, method of bioassay also contributed significantly to the attenuation of virulence. However, the morphological and physiological changes seen during single- and multi-spore transfers could not be correlated with virulence in all cases. For instance, no correlation between sporulation capacity and virulence of fourty four isolates of *Aschersonia* against whiteflies *B. argentifolii* and *T. vaporariorum* was found (Meekes et al., 2002).

Considering all these reports, it was thought worthwhile to monitor the effect of repeated *in vitro* sub-culturing and *in vivo* passage on different aspects those were proposed quality control parameters of virulence. Therefore, the effect of repeated *in vitro* sub-culturing and *in vivo* passage on growth, conidial germination, appressorium formation and cuticle degrading enzyme production of the most effective isolate *M. anisopliae* M34412 identified in the present study was determined. The virulence of *M. anisopliae* M34412 against *H. armigera* and the genotypic changes as a result of repeated *in vitro* sub-culturing and *in vivo* passage was also assessed. The part of this work has been published in Nahar et al. (2008).

Results and discussion

The *in vitro* conidial sub-culturing of *M. anisopliae* M34412 on PDA up to 40 transfers and the *in vivo* passage of the 40th sub-culture in 3rd instar larvae of *H. armigera* for 5 times was carried out as described in section 2.6.

4.6 Growth, conidial germination and appressorium formation

The repeated *in vitro* sub-culturing did not show significant effect on the morphological and cultural characteristics viz. size of conidia and vegetative growth on PDA plates of 1st and 40th sub-cultures of *M. anisopliae* M34412. The average size of the 1st and 40th sub-culture conidia was 7.11±0.02×3.05±0.03 µm. The vegetative growth of the 1st and 40th sub-culture on PDA was observed at 28ºC for 8 d and these sub-cultures grew profusely on PDA plates with dark green sporulation without any sectoring. As reported earlier by Shah and Butt (2005), the formation of sectors on artificial media was strain specific and influenced by nutritional conditions. During studies using two strains of *M. anisopliae* and one strain of *Metarhizium brunneum*, Ansari and Butt (2011) observed significant differences in sector frequency. According to them, the sector formation was influenced by the number of sub-cultures; no sectors were produced by the 1st sub-culture and more sectors were produced by the 12th sub-culture. Further, in the present investigation, the germination of conidia on YPG agar was also not affected by repeated *in vitro* sub-culturing and *in vivo* passage of *M. anisopliae* M34412. All the sub-cultures (1st, 20th and 40th) and passaged cultures (40th 1st *in vivo* passage and 40th 5th *in vivo* passage) of *M. anisopliae* M34412 showed 90% germination on YPG agar within 24 h. Ansari and Butt (2011) obtained similar results for the freshly produced conidia on rice by three strains of *Metarhizium*. The germination rates of 1st and 12th sub-cultures of all the studied *Metarhizium* species/strains were not significantly different.

In the present study, the appressorium formation in the 1st sub-culture of *M. anisopliae* M34412 was 96.9±0.46% (Table 4.2). It was found to be decreased to 86.6 ±5.12% and 76.7±3.36% for the 20th and 40th sub-cultures, respectively due to repeated *in vitro* sub-culturing on PDA. The appressorium formation in 40th sub-culture increased to 82.2±7.76% and 89.8±5.47% after 1st and 5th *in vivo* passage in *H. armigera*, respectively (Table 4.2). A nutrient gradient associated with contact of the

Table 4.2 Effect of repeated *in vitro* conidial sub-culturing and *in vivo* passage on appressorium formation by *Metarhizium anisopliae*

***M. anisopliae* sub-culture**	**Appressorium formation (%)**
1st	96.9 ± 0.46
20th	86.6 ± 5.12
40th	76.7 ± 3.36
40th *	82.2 ± 7.76
40th **	89.8 ± 5.47

40th* and 40th** - 40th 1st and 5th *in vivo* passaged cultures respectively as described in section 2.6

germ tube to rigid insect surface was suggested to be the main stimulus triggering appressorium formation (St. Leger et al., 1989). In *M. flavoviride*, the appressorium formation was affected by the concentration of yeast extract used for the initial conidial germination and further appressorium development on the hydrophobic surface (Xavier-Santos et al., 1999).

4.7 Production of cuticle-degrading enzymes

In the present investigation, when conidial sub-cultures and passaged cultures were used as an inoculum, significant variation in the constitutive production of CDEs in YPG medium was observed (Table 4.3). The chitinase, protease, CDA and chitosanase activities of the 1st sub-culture decreased by 27.45%, 21.87%, 25.61% and 22.55% respectively after *in vitro* sub-culturing for 40 times. The *in vivo* passages of the 40th sub-culture in 3rd instar larvae of *H. armigera* for 5 times resulted in a gradual increase in the CDEs; 27.02%, 24%, 27.77% and 22.16% for chitinase, protease, CDA and chitosanase respectively (Table 4.3).

A similar trend for the production of CDEs in chitin containing medium was observed when conidial sub-cultures and passaged cultures were used as an inoculum (Table 4.4). There was 20.45%, 14.79%, 26.82% and 19.38% decrease in the chitinase, protease, CDA and chitosanase activities of the 40th sub-culture in chitin containing medium as compared to the activities of the 1st sub-culture. The enzyme activities increased gradually by 18.73%, 16.31%, 30% and 14.89%; for chitinase, protease, CDA and chitosanase respectively in 40th 5th passaged culture than the 40th sub-culture (Table 4.4).

Table 4.3 Effect of repeated *in vitro* conidial sub-culturing and *in vivo* passage on the extracellular production of cuticle-degrading enzymes in YPG medium by *Metarhizium anisopliae*

***M. anisopliae* sub-culture**	**Chitinase (U/ml)**	**Protease (U/ml)**	**Lipase (U/ml)**	**CDA*** (U/ml)**	**Chitosanase (U/ml)**
1st	0.51 ± 0.01	0.96 ± 0.02	0.27 ± 0.03	1.21 ± 0.05	5.01±0.06
20th	0.45 ± 0.01	0.81 ± 0.03	0.26 ± 0.02	1.06 ± 0.04	4.36±0.08
40th	0.37 ± 0.02	0.75 ± 0.01	0.23 ± 0.02	0.90 ± 0.01	3.88±0.07
40th *	0.43 ± 0.03	0.84 ± 0.02	0.23 ± 0.02	0.97 ± 0.01	4.27±0.09
40th **	0.47 ± 0.02	0.93 ± 0.01	0.26 ± 0.02	1.15 ± 0.03	4.74±0.05

40th* and 40th** - 40th 1st and 5th *in vivo* passaged cultures respectively as described in section 2.6; ***CDA activity determined by MBTH method as described in section 2.10.4.1

Table 4.4 Effect of repeated *in vitro* conidial sub-culturing and *in vivo* passage on the extracellular production of cuticle-degrading enzymes in chitin containing medium by *Metarhizium anisopliae*

***M. anisopliae* sub-culture**	**Chitinase (U/ml)**	**Protease (U/ml)**	**Lipase (U/ml)**	**CDA*** (U/ml)**	**Chitosanase (U/ml)**
1st	3.96 ± 0.12	3.38 ± 0.16	0.99 ± 0.04	0.41 ± 0.01	32.40±1.13
20th	3.69 ± 0.03	3.20 ± 0.03	0.91 ± 0.08	0.33 ± 0.02	28.81±1.12
40th	3.15 ± 0.02	2.88 ± 0.03	0.91 ± 0.08	0.30 ± 0.02	26.12±1.34
40th *	3.39 ± 0.03	3.17 ± 0.02	0.91 ± 0.08	0.31 ± 0.03	28.36±1.03
40th **	3.74 ± 0.04	3.35 ± 0.03	0.95 ± 0.04	0.39 ± 0.04	30.01±0.76

40th* and 40th** - 40th 1st and 5th *in vivo* passaged cultures respectively as described in section 2.6; ***CDA activity determined by MBTH method as described in section 2.10.4.1

However, the lipase production in both YPG and chitin containing medium was not significantly affected (<15%) when conidial sub-cultures and passaged cultures were used as an inoculum.

The results of the present study were in accordance with some of the previous reports where the levels of enzyme activities in virulent strains were found to be more than avirulent strains. As reported earlier, the virulent isolates of *N. rileyi* showed high levels of both endo- and exo-chitinase activity than avirulent isolates (El-Sayed et al., 1989). In some of the recent studies, the influence of repeated sub-culturing on Pr1 activity was determined (Ansari and Butt, 2011; Safavi, 2012; Shah et al., 2007). The Pr1 activity of the conidia harvested from Sabouraud dextrose agar (SDA) and mycosed cadavers of *T. molitor* decreased from 1st to 12th subculture but the conidia produced on rice showed increase in Pr1 activity from 1st to 12th sub-culture in all three studied strains of *Metarhizium* (Ansari and Butt, 2011). Similarly, Shah et al. (2005) reported that the virulent conidia of *M. anisopliae* from insects contained higher levels of Pr1A and other pathogenicity-related gene transcripts than inoculum from artificial media. Further, the release of proteolytic, chitinolytic and lipolytic enzymes by *N. rileyi* in presence of a cellulose substrate as well as larval cuticle was studied and it was found that the time and quantity of the enzymes expressed were influenced by the nature of the substrate (El-Sayed et al., 1993). Also, the induction of chitinolytic enzymes in the biocontrol agent *T. harzianum* was investigated during parasitism on *Sclerotium rolfsii* and only live mycelium of *S. rolfsii* was found to induce enzyme production suggesting its dependence on vital elements from the host (Inbar and Chet, 1995).

The effect of repeated vegetative transfers of *M. anisopliae* M34412 on the production of CDEs was also determined in the present investigation. When mycelium inoculum from the 10th serial vegetative transfer was used, the chitinase, protease, lipase and chitosanase activities decreased by 8.47%, 4.54%, 2.85% and 9.23%, respectively in YPG medium and; by 9.02%, 1.17%, 1.05% and 6.46%, respectively in chitin containing medium as compared to the 1st vegetatively transferred mycelium inoculum. However, when mycelium inoculum of 10th vegetative transfer was used, the CDA activity decreased significantly in both YPG (19.8%) and chitin containing (23.2%) medium (Table 4.5). This suggested the possible role of CDA in the penetration of insect cuticle. According to Morrow et al. (1989), the repeated vegetative transfer of *N. rileyi* did not affect the virulence against *A. gemmatalis*.

Table 4.5 Effect of repeated *in vitro* vegetative transfers on the extracellular production of cuticle-degrading enzymes by *Metarhizium anisopliae*

***M. anisopliae* sub-culture**	**Chitinase (U/ml)**	**Protease (U/ml)**	**Lipase (U/ml)**	**CDA* (U/ml)**	**Chitosanase (U/ml)**
YPG medium					
1st	0.59±0.02	1.10±0.01	0.35±0.01	1.240±0.03	5.20±0.05
10th	0.54±0.01	1.05±0.02	0.34±0.01	0.994±0.04	4.72±0.06
Chitin containing medium					
1st	4.10±0.09	3.40±0.02	0.95±0.01	0.460±0.04	33.70±1.1
10th	3.73±0.06	3.36±0.02	0.94±0.01	0.353±0.05	31.52±0.09

*CDA activity determined by MBTH method as described in section 2.10.4.1

The conidia of entomopathogenic fungi get pre-adapted for the pathogenic life style depending on the environmental conditions (St. Leger et al., 1991a). It was reported that the secondary metabolites, destruxins, harmful to the host's immune system were not produced by attenuated cultures of *M. anisopliae* (Wang et al., 2003). Further, it was suggested that the attenuated conidia germinate and infect their hosts marginally slower than non-attenuated conidia partly due to the lack of the right set of enzymes to facilitate host penetration in attenuated conidia. The attenuated cultures also did not undergo differentiation in the hemocoel and did not produce the metabolites that normally suppress the host defense (Butt et al., 2006).

The present results suggested that the constitutive activities of the 40th sub-culture may be mainly responsible for the growth as *M. anisopliae* can also grow saprophytically. Further, the decrease in the levels of enzyme activities during *in vitro* repeated sub-culturing from 1st to 40th sub-culture can be attributed to the decrease in the activities involved in functions other than growth, viz. virulence. This could be one of the reasons for the increase in the activities after insect passages. As explained by Shah et al. (2005), the conidia from mycosed insects were more virulent as they possessed 30–50% low carbon content and fewer endogenous reserves like glycogen and lipid than conidia from artificial media.

4.8 Insect bioassay

The virulence of *in vitro* sub-cultures and *in vivo* passaged cultures of *M. anisopliae* M34412 against 3rd instar larvae of *H. armigera* was assessed at the concentration of 1×10^7 conidia/ml. The mortality decreased from 97.68% for the 1st sub-culture to 86.58% for the 40th sub-culture due to repeated *in vitro* subculturing whereas the insect passages of conidia of the 40th sub-culture increased the mortality to 95.46% for the 40th 5th *in vivo* passaged culture (Table 4.6). The virulence of *M. anisopliae* against 3rd instar nymphs of the grasshopper, *Uvarovistia zebra* reduced by 17% in 4th sub-culture whereas there was a slight increase in the virulence after two passages through *U. zebra* (Mohammadbeigi, 2013).

The LT_{50} of the *in vitro* sub-cultures and the *in vivo* passaged cultures was determined to study the influence of repeated *in vitro* sub-culturing and *in vivo* passage on the speed of kill of *H. armigera* by *M. anisopliae*. The LT_{50} value of the 1st sub-culture was 3.4 d and it increased to 4.5 and 5.6 d for the 20th and 40th sub-cultures, respectively suggesting 1st sub-culture conidia were more virulent as compared to 20th and 40th sub-culture. The LT_{50} values decreased after passage of the 40th sub-culture on *H. armigera* to 4.4 and 3.7 d for the 40th 1st *in vivo* and 40th 5th *in vivo* passaged cultures, respectively (Table 4.6). All Chi-square values were not significant ($\alpha=0.05$) indicating good fit of regression lines. Similar studies were carried out by Shah et al. (2007) using two strains of *M. anisopliae*. It was observed that, after successive sub-culturing of both the strains up to 9th sub-culture, the virulence was declined and the LT_{50} values increased from 3.74 d to 4.46 d for one strain and from 4.79 d to 5.57 d for the other strain.

The effectiveness of the biocontrol preparation depends on the number of conidia required to kill individual larva. The LC_{50} value for the 1st sub-culture was 0.17×10^4 conidia/ml which increased to 0.7×10^4 and 3×10^4 conidia/ml for the 20th and 40th sub-cultures, respectively. The conidial transfers of the 40th sub-culture through

Table 4.6 Effect of repeated *in vitro* conidial sub-culturing and *in vivo* passage on percent corrected mortality, median lethal time (LT_{50}) and median lethal concentration (LC_{50}) of *Metarhizium anisopliae* sub-cultures against 3rd instar larvae of *Helicoverpa armigera*

***M. anisopliae* sub-culture**	**Mortality (%)**	**LT_{50} (d)**	**Fiducial limit (d)**	**Chi-square value**	**LC_{50} ($\times 10^4$ conidia/ml)**	**Fiducial limit ($\times 10^4$ conidia/ml)**	**Chi-square value**
1st	97.68	3.4	3.1–3.6	12.33	0.17	0.04 –0.72	3.57
20th	91.02	4.5	4.2–4.7	4.78	0.70	0.17–2.90	4.36
40th	86.58	5.6	5.2–5.9	4.32	3.0	0.82–11.0	5.22
40th *	91.02	4.4	4.1–4.8	7.84	0.74	0.18–3.0	5.01
40th **	95.46	3.7	3.5–4.0	12.23	0.23	0.05–1.0	4.22

40th* and 40th** - 40th 1st and 5th *in vivo* passaged cultures respectively as described in section 2.6

3rd instar larvae of *H. armigera* resulted in lowering the LC_{50} values from 3×10^4 to 0.74×10^4 and 0.23×10^4 conidia/ml for the 40th 1st *in vivo* and 40th 5th *in vivo* passaged cultures, respectively (Table 4.6). All Chi-square values were not significant ($\alpha=0.05$) indicating good fit of regression lines. The results suggested that increase in the number of *in vitro* sub-cultures decreased the virulence whereas increase in the number of *in vivo* passages in 3rd instar larvae of *H. armigera* increased the virulence. Similar trend was observed for one out of two *B. bassiana* isolates studied. The LC_{50} values of one of the isolates against *T. molitor* increased from 1.05×10^7 conidia/ml for the 1st sub-culture to 9.93×10^7 conidia/ml for the 15th sub-culture whereas the LC_{50} values were varying among different sub-cultures for the second isolate (Safavi, 2012). Further it was observed in the present study that when a lower concentration of conidia (1×10^3 conidia/ml) was used for the bioassay, the effect of *in vitro* sub-culturing and *in vivo* passage on virulence was more pronounced (Figure 4.5).

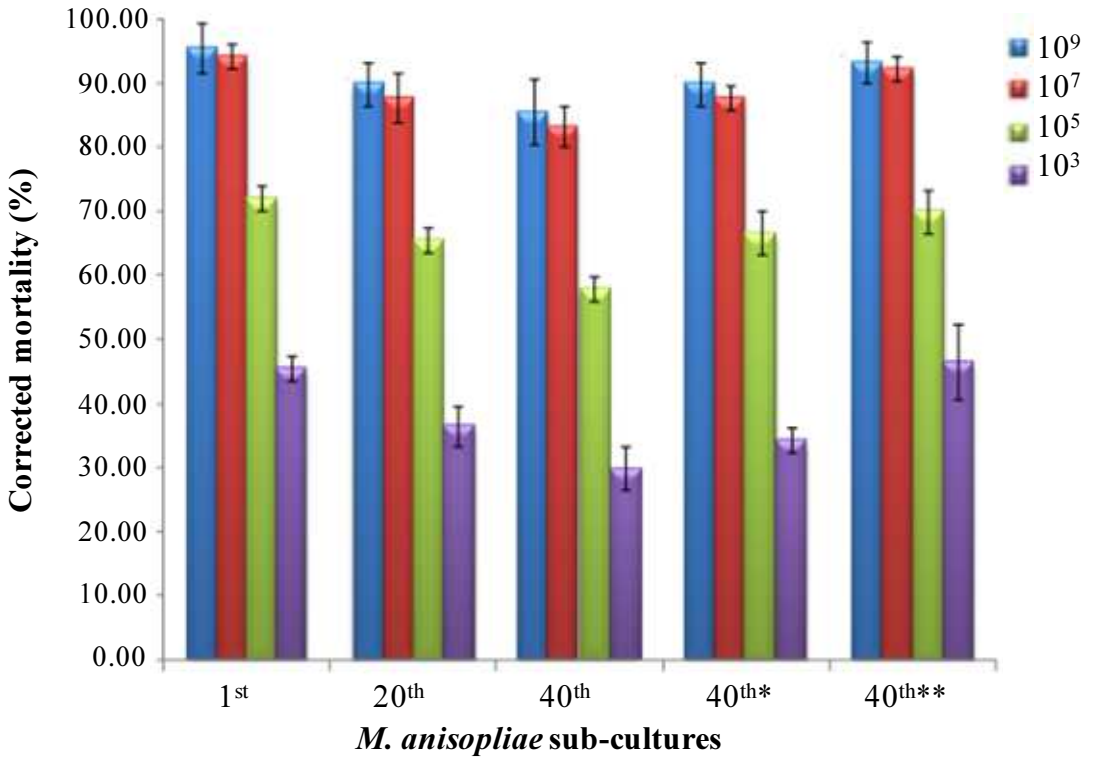


Figure 4.5 Effect of repeated *in vitro* sub-culturing and *in vivo* passage on median lethal concentration (LC_{50}) of *Metarhizium anisopliae* sub-cultures against 3rd instar larvae of *Helicoverpa armigera*

Several reasons were suggested for increase in the virulence after passage in the suitable host. As suggested by Adames et al. (2011), the adhesion of conidia and rate

of germination were increased due to repeated passages through a suitable host which ultimately caused a faster invasion to the host. On the contrary, in some of the earlier studies it was reported that passage in the insect host did not enhance the virulence as the strains were at their full insecticidal potential or as the strains changed irreversibly physiologically (Butt et al., 2006).

4.9 Genetic analysis

The similar isolates of entomopathogenic fungi can be better differentiated using RAPD patterns which is a sensitive and appropriate method (Milner et al., 2002). The RAPD analysis of the 1st, 10th, 20th, 30th, 40th *in vitro* sub-cultures and 40th 1st *in vivo* passaged culture was carried out using the five primers of ten nucleotides viz. 947G, OPA13, OPA07, OPA03 and 935G as described in section 2.16.4. The significant visual differences in the banding pattern with three primers OPA07, OPA03 and 935G were observed due to repeated *in vitro* sub-culturing and *in vivo* passage in the 3rd instar larvae of *H. armigera* (Figure 4.6). The number of bands with primer OPA07

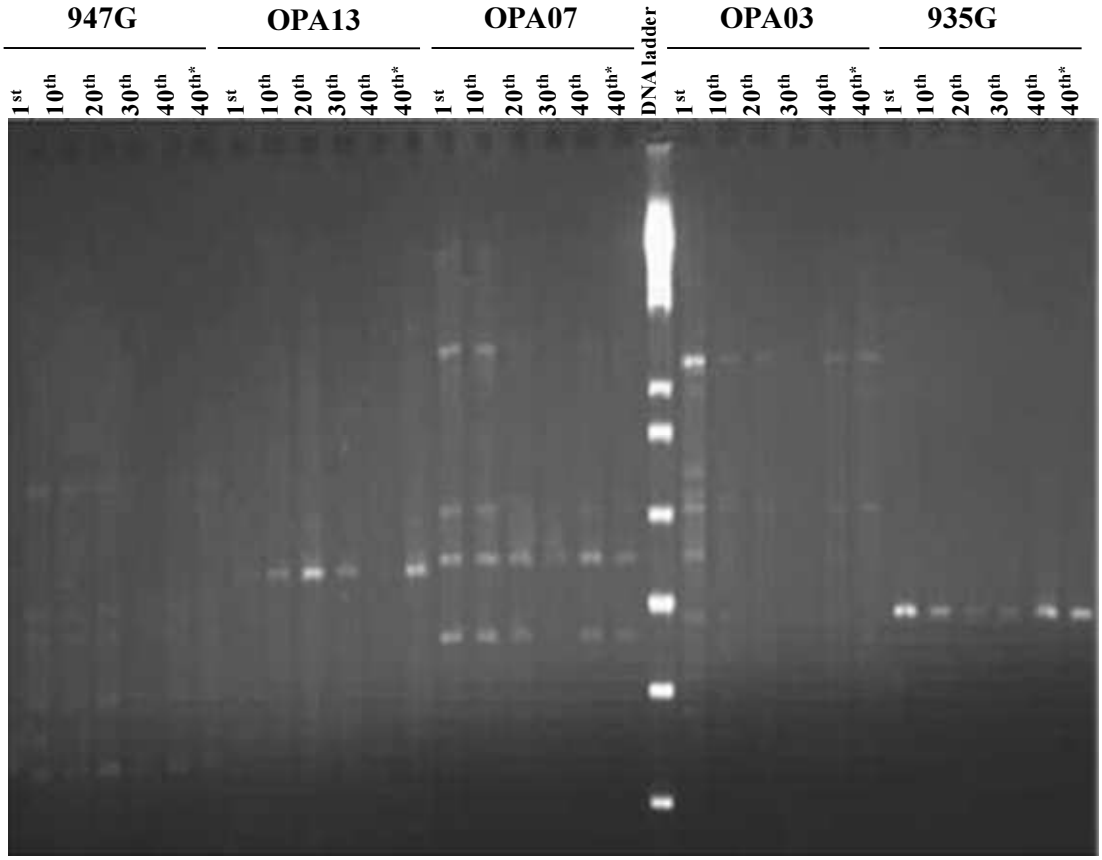


Figure 4.6 Representative agarose gel electrophoresis of RAPD-PCR products for *in vitro* conidial transfers and *in vivo* passage of 40th sub-culture of *Metarhizium anisopliae*

decreased from 1st to 40th sub-culture. The NCBI blast results of OPA07 showed significant alignment with serine peptidase from *Aspergillus fumigatus*. In case of the primer OPA03, the intensity of bands decreased from the 1st to 40th sub-culture and increased slightly in 40th 1st *in vivo* passaged culture. The OPA03 primer was reported to be used for the amplification of lysophospholipase, it was also used for the amplification of the calmodulin gene. The biochemical processes controlled by the Ca-calmodulin signalling system were involved in the induction of appressorium in *Colletotrichum trifolii* pathogenic to red pepper (Warwar and Dickman, 1996). Further, the intensity of bands decreased from 1st to 30th sub-culture and increased slightly in 40th *in vitro* sub-culture and 40th 1st *in vivo* passaged culture for the primer 935G. Thus, the genetic analysis of the *in vitro* sub-cultures and *in vivo* passaged cultures of *M. anisopliae* showed correlation with the biochemical data.

The present study demonstrated the effect of repeated *in vitro* sub-culturing and *in vivo* passage on appressorium formation, production of CDEs as well as RAPD pattern, thereby on virulence of *M. anisopliae* M34412 against *H. armigera*. Recently, Ansari and Butt (2011) observed decrease in the Pr1 activity of conidia sub-cultured on SDA. However, no correlation between decline in Pr1 activity and virulence was observed as the 12th sub-culture with low levels of Pr1 possessed the LT_{50} values comparable to the 1st sub-culture. Safavi (2012) observed that the Pr1 activity of *in vitro* transferred conidia of *B. bassiana* was low even in presence of mealworm cuticle indicating other determinants in insect body regulated Pr1 gene induction. In the present investigation, though all the CDE activities were influenced as a result of repeated *in vitro* sub-culturing and *in vivo* passage in 3rd instar larvae of *H. armigera*, the effect on CDA activity was more pronounced during both conidial and vegetative transfers of *M. anisopliae* and was correlated with the virulence against *H. armigera*. Therefore, the present investigation suggested that along with other CDEs, CDA could be used as marker to monitor the virulence of *M. anisopliae* during mass production. Further detailed investigations are necessary to clarify the importance of CDA in *M. anisopliae*.

CHAPTER 5

BIOCHEMICAL AND MOLECULAR STUDIES OF CHITIN DEACETYLASE

A. Evaluation of *Metarhizium* isolates based on chitin deacetylase activity

To improve understanding of the different stages of fungus-insect interaction, the mechanism by which the fungus invades into the host body must be identified. As CDEs are one of the important virulence factors, study of regulation of enzyme activities in response to infection-structure differentiation is of critical importance. Some of the earlier studies have reported the differentiation specific formation of CDA in plant pathogenic fungi. The spores of obligate biotrophic rust fungus *U. viciae-fabae* start germination after attachment to the plant cuticle. Once the germination is initiated, it undergoes differentiation to form appressorium above the stomatal opening and then enters the intercellular space of the mesophyll by means of a penetration hypha. It was reported that the differentiation structures of *U. viciae-fabae* formed in the intercellular space showed differential labelling pattern with wheat germ agglutinin (WGA) because of altered surface composition and the differential WGA binding pattern was attributed to conversion of chitin to chitosan by CDA (Deising et al., 1995). Similar studies were carried out in plant pathogenic fungus *M. grisea* for finding the genes important in the recognition of physical factors from solid surfaces that induced appressorium formation. It was observed that the null mutants of CBP1 (chitin binding protein) gene were not differentiated into appressorium on artificial surface. Therefore, the CBP1 gene showing homology to fungal CDAs was considered to be involved in the sensing of factors from solid surfaces for differentiation into appressorium (Kamakura et al., 2002).

The CDA has been described from different sources, most notably zygomycetous fungi that possess high chitosan content in their cell walls. Similarly, the deacetylation of chitin in the cell walls of ascomycetous and basidiomycetous fungi is also reported and these fungi represent a source for novel CDA with potentially differing and interesting enzymatic properties. As the industrial-scale production of chitosan from crustacean chitin has attracted great interest in use of enzymatic method instead of chemical hydrolysis, engineering a high CDA activity would be required.

In case of *M. anisopliae* M34412, there was a marked decrease in extracellular CDA activity as compared to other CDEs due to repeated *in vitro* conidial sub-culturing as well as mycelial transfers and further passage in 3rd instar larvae of *H. armigera* increased the CDA activity (Nahar et al., 2008). As CDA was observed to

be one of the important CDEs in the virulence of *Metarhizium* against *H. armigera*, it was interesting to study the differentiation specific formation of CDA so as to know the possible role of this enzyme in establishing stable relationship of the fungus with the insect. In the present study, one of the sixty eight *Metarhizium* isolates was selected and intracellular CDA activity in different morphological forms of this *Metarhizium* isolate was studied in order to understand the differentiation specific production of CDA.

Results and discussion

5.1 Selection of *Metarhizium* isolate for chitin deacetylase studies

In the present study, the sixty eight *Metarhizium* isolates showed variation in the *in vitro* production of the extracellular CDA in YPG as well as chitin containing medium. The *in vitro* CDA activities of *Metarhizium* isolates in YPG medium ranged from 0.20–6.48 U/ml in 3 d and in chitin containing medium ranged between 0.03-2.06 U/ml in 4 d as mentioned in section 3.2. This difference in the extracellular CDA activity in YPG medium and chitin containing medium may be due to media components. Earlier, it has been observed that in *Rhizopus oryzae*, the CDA activity was significantly affected by the fermentation technique as well as the chitin substrate used. The culture fluid of the *R. oryzae* grown in submerged fermentation exhibited a low activity of 0.008 U/ml, 0.013 U/ml and 0.036 U/ml with natural chitin, reprecipitaed chitin and partially deacetylated chitin (PDC), respectively as a substrate in 15 d whereas the CDA activity of the fermentation fluid grown on soy residue in solid state fermentation was higher; 0.18 U/ml, 0.91 U/ml and 0.61 U/ml with the above three substrates, respectively in 15 d (Aye et al., 2006). Further, in *S. brevicaulis*, the capacity of extracellular CDA production was increased due to addition of different inorganic salts (Cai et al., 2013).

For finding the suitable organism producing enzyme, screening of diverse candidate genes and organisms should be done and the traditional method of screening of pure organisms by cultivation is a standard approach (Zhao et al., 2011). In the current study, the screening of suitable organism was done based on *in vitro* extracellular CDA activity in YPG medium and corrected mortality against *H. armigera*. The cluster analysis using extracellular CDA activity in YPG medium and

mortality against *H. armigera* for sixty eight *Metarhizium* isolates showed two major groups based on variation in mortality (Figure 5.1).

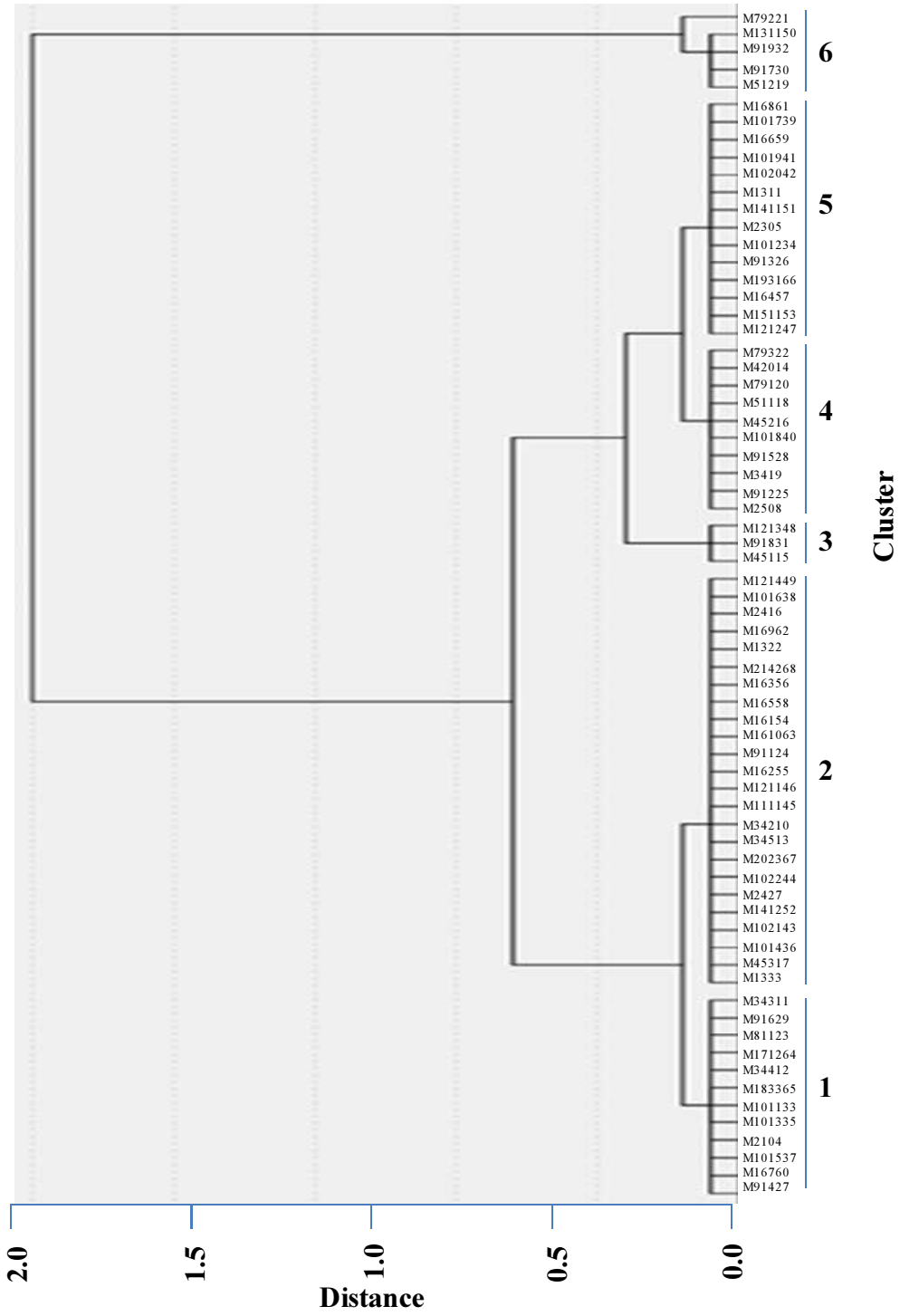


Figure 5.1 Average linkage cluster analysis of *Metarhizium* isolates based on percent corrected mortality of *Helicoverpa armigera* and *in vitro* constitutive chitin deacetylase activity

The *Metarhizium* isolates were further grouped into six clusters on the basis of extracellular CDA activity. Though only CDA activity and mortality data was used for cluster analysis, the isolates were compared for other CDE activities also. It was observed that, the twelve isolates possessing >90% mortality were grouped together in cluster 1. The chitinase activity in these twelve isolates ranged between 3.23–6.90 U/ml, protease activity between 2.10–3.38 U/ml and CDA activity between 0.45–3.21 U/ml (Table 5.1). Out of these twelve isolates, nine isolates possessed >2 U/ml CDA activity. However, the chitinase activity in these nine isolates was also high (>3.2 U/ml). Therefore, the high mortality of *H. armigera* with these isolates could be attributed to the combination of chitinase and CDA activity. On the contrary, the cluster 2 contained twenty four isolates possessing mortality in the range of 73–88% (Table 5.1). Out of twenty four isolates, eighteen isolates showed >80% mortality. The seventeen isolates out of eighteen possessed >2 U/ml chitinase activity and CDA activity in the range of 0.52-6.46 U/ml. The only isolate M161063 showing 82% mortality possessed 1.01 U/ml chitinase activity, 2.26 U/ml CDA activity and 13.22 U/ml chitosanase activity. Inspite of possessing low chitinase activity, the high mortality of *H. armigera* with *Metarhizium* isolate M161063 could be because of high CDA and chitosanase activity. This may be due to the possible reliance on CDA in the cuticular degradation process for insect pathogenesis. High amounts of CDA may be compensating for the chitinase levels (Nahar et al., 2004a). Further comparison of the isolate M161063 with other seventeen isolates showed that these isolates either possessed <82% mortality or >82% mortality. The >82% mortality could be due to chitinase alone or chitinase-CDA or chitinase-CDA-chitosanase combination. Therefore, the *Metarhizium* isolate M161063 supposed to be utilizing alternate mechanism of chitinase metabolism was selected for further studies on chitin deacetylase.

Some recent studies have used traditional methods for isolation of CDA producing fungi from environmental soils. More than hundread fungal isolates were screened for CDA production and a *Mortierella* species DY-52 was identified as high extracellular CDA producer possessing 0.60 U/ml CDA activity in 5 d (Kim et al., 2008). Similarly, the CDA producing fungi were isolated from soil and among more than hundread fungal isolates; *Absidia corymbifera* DY-9 was screened as high extracellular CDA producer (Zhao et al., 2010). Further, a strain of *Penicillium oxalicum* ITCC 6965 was isolated from residual materials of sea food processing

Table 5.1 Selection of *Metarhizium* isolate for chitin deacetylase studies

Isolate No.	**Chitinase (U/ml)**	**Protease (U/ml)**	**Lipase (U/ml)**	**CDA* (U/ml)**	**Chitosanase (U/ml)**	**Mortality *(%)***
Cluster 1						
M91427	3.90	2.45	0.498	2.50	25.16	91.11
M101537	3.40	3.14	0.290	2.35	12.26	91.11
M16760	3.59	2.72	0.290	2.49	27.60	91.11
M183365	6.90	2.38	0.332	1.43	23.24	92.59
M2104	3.23	3.24	0.498	2.37	49.60	93.33
M101133	3.30	2.91	0.290	2.56	12.50	93.33
M101335	3.74	3.05	0.290	2.46	33.93	93.33
M81123	3.76	3.32	0.747	2.48	35.80	95.56
M91629	5.10	3.18	0.581	2.36	26.23	95.56
M171264	5.70	2.10	0.415	0.45	25.64	96.29
M34311	3.53	3.28	0.747	3.21	35.70	96.67
M34412	3.96	3.38	0.996	1.30	32.40	96.67
Cluster 2						
M16255	2.77	2.22	0.207	1.39	5.70	73.33
M111145	2.92	1.74	0.207	0.63	25.04	75.56
M121146	4.65	1.23	0.207	2.39	5.02	75.56
M214268	5.40	2.30	0.581	0.85	21.65	77.78
M121449	2.63	1.85	0.083	2.22	4.01	78.89
M16356	2.63	2.03	0.207	0.99	5.32	78.89
M1322	2.39	1.87	0.415	3.50	3.11	80.00
M2416	2.73	1.60	0.249	2.69	14.28	80.00
M101638	2.66	1.80	0.249	2.50	6.28	80.00
M16962	2.95	1.00	0.207	4.32	0.42	80.00
M161063	**1.01**	**1.07**	**0.290**	**2.26**	**13.22**	**82.00**
M91124	3.90	1.35	0.249	1.63	34.90	82.22
M16154	2.41	1.71	0.166	4.02	11.20	83.33
M16558	4.99	0.93	0.207	2.73	0.76	83.33
M202367	4.93	2.40	0.664	0.63	22.22	85.18
M2427	2.82	2.03	0.415	1.16	42.60	85.55

M101436	2.74	2.01	0.166	3.88	5.26	85.56
M102143	2.94	1.68	0.166	1.86	12.40	85.56
M102244	4.74	1.72	0.249	1.19	6.33	85.56
M141252	2.07	2.33	0.332	2.50	5.28	85.56
M1333	2.65	2.27	0.498	3.39	4.04	87.78
M34210	3.50	1.24	0.664	6.46	10.20	87.78
M34513	2.90	2.45	0.498	0.52	6.20	87.78
M45317	2.82	3.02	0.249	3.61	0.23	87.78

*CDA activity determined by MBTH method as described in section 2.10.4.1

industries that produced a novel CDA. The mutant of *P. oxalicum* (SAEM-51) prepared using ethidium bromide and microwave irradiation showed improved levels of CDA (0.21 U/ml) as compared to the wild type strain (0.10 U/ml) (Pareek et al., 2011).

5.2 Identification of *Metarhizium* isolate M161063

The ITS1-5.8S-ITS4 region of the *Metarhizium* isolate M161063 was obtained by amplification using ITS1 and ITS4 fungal primers (White et al., 1990). Agarose gel electrophoresis of PCR product showed the presence of 550 bp amplicon. The purified PCR product was sequenced with ABI 3730 DNA analyzer. The ITS1-5.8S-ITS4 sequence was used to perform BLAST search. The sequence identity with the closest sequence was used to identify the isolate. The ITS1-5.8S-ITS4 sequence was homologous (100% identical) to the *M. anisopliae* var *anisopliae* strain sequences deposited in the NCBI database (LK995311 and KJ872681). The ITS1-5.8S-ITS4 sequence of *Metarhizium* isolate M161063 was submitted to genebank under accession number KM875563.

5.3 Intracellular chitin deacetylase activity in different morphological forms of *Metarhizium anisopliae* M161063

In the present investigation, the intracellular CDA activity was determined in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h) and blastospores (24 h) of *M. anisopliae* M161063 (Figure 5.2). The morphological forms were selected in such a way that they represented the differentiation structures of *Metarhizium* throughout the insect pathogenesis.

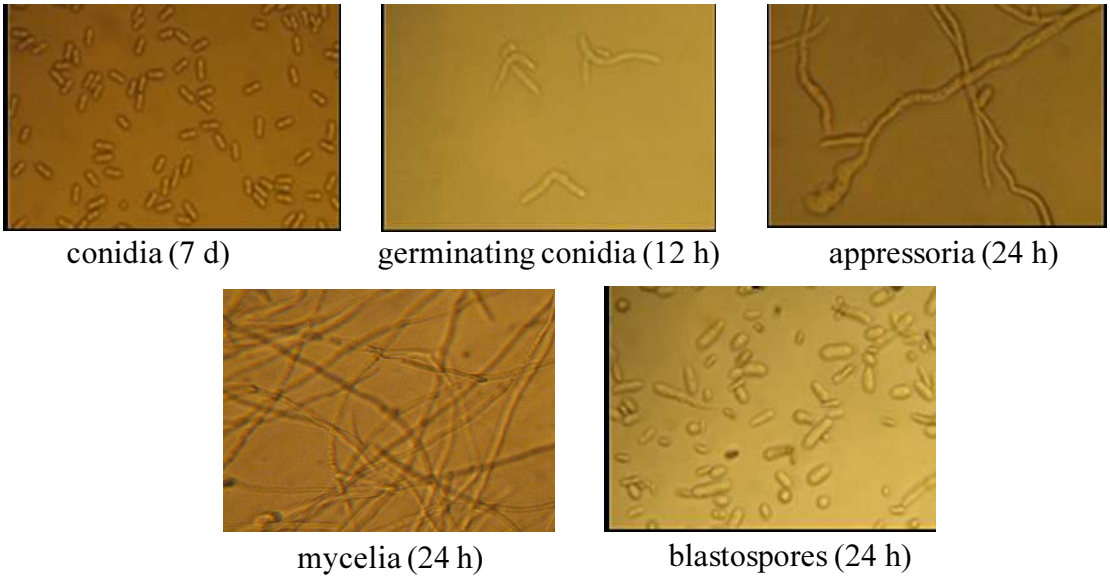


Figure 5.2 Morphological forms of *Metarhizium anisopliae* M161063

The intracellular CDA activity of *M. anisopliae* M161063 increased progressively in germinating conidia (12 h) (0.02 U/mg), appressoria (24 h) (0.04 U/mg) and mycelia (24 h) (0.08 U/mg) than conidia from PDA slants (7 d) (0.005 U/mg). Subsequently, there was decrease in the intracellular CDA activity in blastospores (24 h) (0.01 U/mg) (Figure 5.3). The increase in intracellular CDA activity in infection structures viz. in appressoria (24 h) and subsequently in mycelia (24 h) of *M. anisopliae* M161063 revealed the possible role of CDA in self defense of the fungus during its entry through the insect cuticle i.e. for the modification of the fungal cell wall chitin to chitosan so as to combat against insect chitinases. Further decrease in intracellular CDA activity in blastospores (24 h) suggested that once the fungus enters the haemolymph, some other mechanisms and not CDEs are responsible for further invasion of the fungus in the insect body.

The hyphae of plant pathogenic fungi were reported to produce specialized infection structures for invasion of plant tissue and a variety of cell wall-degrading enzymes were secreted by these infection structures in a highly regulated fashion in order to penetrate the plant cell wall (Mendgen et al., 1996). Other studies with CDA also documented that the production of CDA was stage specific. In *U. viciae-fabae*, CDA activity was significantly increased in extracellular material isolated from substomatal vesicles and older structures but not in 24 h old germ tubes grown on non-inductive glass plates indicating differentiation-specific regulation of CDA

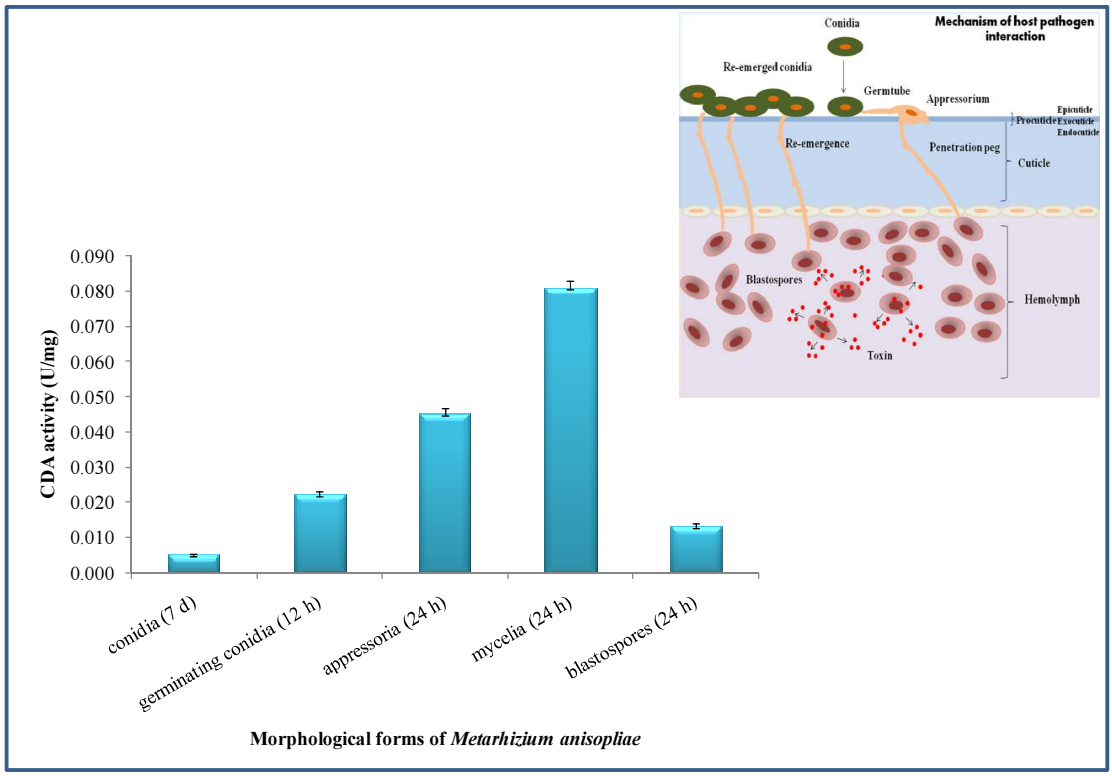


Figure 5.3 Chitin deacetylase activity in different morphological forms of *Metarhizium anisopliae* M161063 (CDA activity determined by Bergmeyer's method as described in section 2.10.4.2)

(Deising and Siegrist, 1995). Similarly, in *S. cerevisiae* when CDA activity was detected at different time intervals, it was found to be accumulated during the process of sporulation and no CDA activity was detected during vegetative growth (Christodoulidou et al., 1996). Thus, the previous reports supported the present investigation as the trend of the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 was observed to be stage specific. The results of the present study thus provided a basis for predicting the possible role of CDA in developing stable relationship between the fungus and insect.

B. Sequencing of chitin deacetylase gene from *Metarhizium anisopliae* M161063

For widespread use of the mycoinsecticides for the control of insect pests, the efficacy of the entomopathogenic fungi used should be high, the dose and time required to kill the insects should be low and it should be active against wide host range. The efficacy of the entomopathogenic fungi can be improved by manipulating the desired traits of these fungi using modern techniques in genetic engineering and biotechnology (Sandhu et al., 2012). However, for the development of a hypervirulent strain, the mechanism of entomopathogenesis should be known. A number of different genes were reported to be involved in the pathogenicity of entomopathogenic fungi when transcriptomic and genetic study of the fungal infection process was carried out (Khan et al., 2012). With the help of molecular biology techniques, the specific pathogenic genes can be isolated and their role as virulence determinants in entomopathogenesis can be investigated so as to produce strains with enhanced virulence.

The molecular characterization of CDA in different classes of fungi viz. zygomycetous and ascomycetous fungi has been carried out to either confirm the biological role of CDA or to further exploit its applications on commercial scale. In case of *M. rouxii*, as the CDA showed similarities to rhizobial nodB proteins and a polysaccharide deacetylase from *Bacillus stearothermophilus*, it was proposed that CDAs with improved deacetylation efficiency can be produced using the conserved catalytic domain (Kafetzopoulos et al., 1993b) whereas the CDA gene from *Gongronella butleri* was cloned and sequenced for its potential applications in chitosan production (Maw et al., 2002). Further, in *S. cerevisiae*, two CDA genes (CDA1 and CDA2) were reported to be located at 745662-746527 bp and 747939-748877 bp on chromosome XII separated by distance of 1.4 kb. Both these genes were sporulation specific and the products of both the genes accounted for total CDA activity as the double disrupted strains showed lack of CDA activity (Christodoulidou et al., 1996). Similarly, two putative CDA genes (EhCDA1 and EhCDA2) each containing a single intron were identified in the protist *Entamoeba histolytica* whereas three CDA genes (EiCDA1-EiCDA3) were identified in *Entamoeba invadens* one of which (EiCDA1) contained an intron and the CDA in *Entamoeba* were reported to be responsible for modification of chitin to chitosan in the cyst wall (Das et al., 2006). In the deuteromycetous fungus *C. lindemuthianum*, the CDA gene was sequenced for the first time and was overexpressed in *E. coli* (Tokuyasu et al., 1999b) whereas in *C.*

neoformans, the gene encoding a mannoprotein named MP98 that stimulated T cell responses when cloned and sequenced, showed the presence of a polysaccharide deacetylase domain. Thus, the role of CDA in immunogenicity was also demonstrated (Levitz et al., 2001). In some of the reports, studies related to sources of CDA, its characteristics, biological function, gene cloning and expression has been carried out so as acquire the active recombinant enzyme for its extensive use (Matsuo et al., 2005; Mishra et al., 1997; Shrestha et al., 2004).

The focus of the present study was to obtain the sequence of CDA gene from *M. anisopliae* M161063 which could be further used for biological function analysis of CDA in *M. anisopliae*. This section describes the amplification of CDA gene from *M. anisopliae* M161063, comparison of the deduced amino acid sequence of *M. anisopliae* CDA with already reported deacetylases by multiple sequence alignment and phylogenetic analysis.

Results and discussion

5.4 Cloning of the CDA gene from *Metarhizium anisopliae* M161063

For sequencing of CDA gene from *M. anisopliae* M161063, degenerate primers (CDAF1, CDAF2, CDAR1 and CDAR2) were designed from the polysaccharide deacetylase domain region of the available CDA sequences of ascomycetous fungi; *A. fumigatus*, *C. lindemuthianum* and putative polysaccharide deacetylase domains of *M. anisopliae* and *B. bassiana* (Figure 5.4). In previously reported other fungi viz. *C. lindemuthianum*, *G. butleri* and *M. racemosus*, the CDA genes were sequenced using primers designed towards the conserved central region of the CDA gene (Maw et al., 2002; Tokuyasu et al., 1999b; Xia-Yun et al., 2007).

The PCR amplification with *M. anisopliae* cDNA as a template was carried out using different primer combinations. The amplification using primer set CDAF1-CDAR2 gave a single amplicon of 600 bp (Figure 5.5) which was purified and cloned into pGEM-T easy vector and transformed to *E. coli* JM109 competent cells as described in section 2.16.9.4. The plasmid was isolated from the positive clones and sequenced.

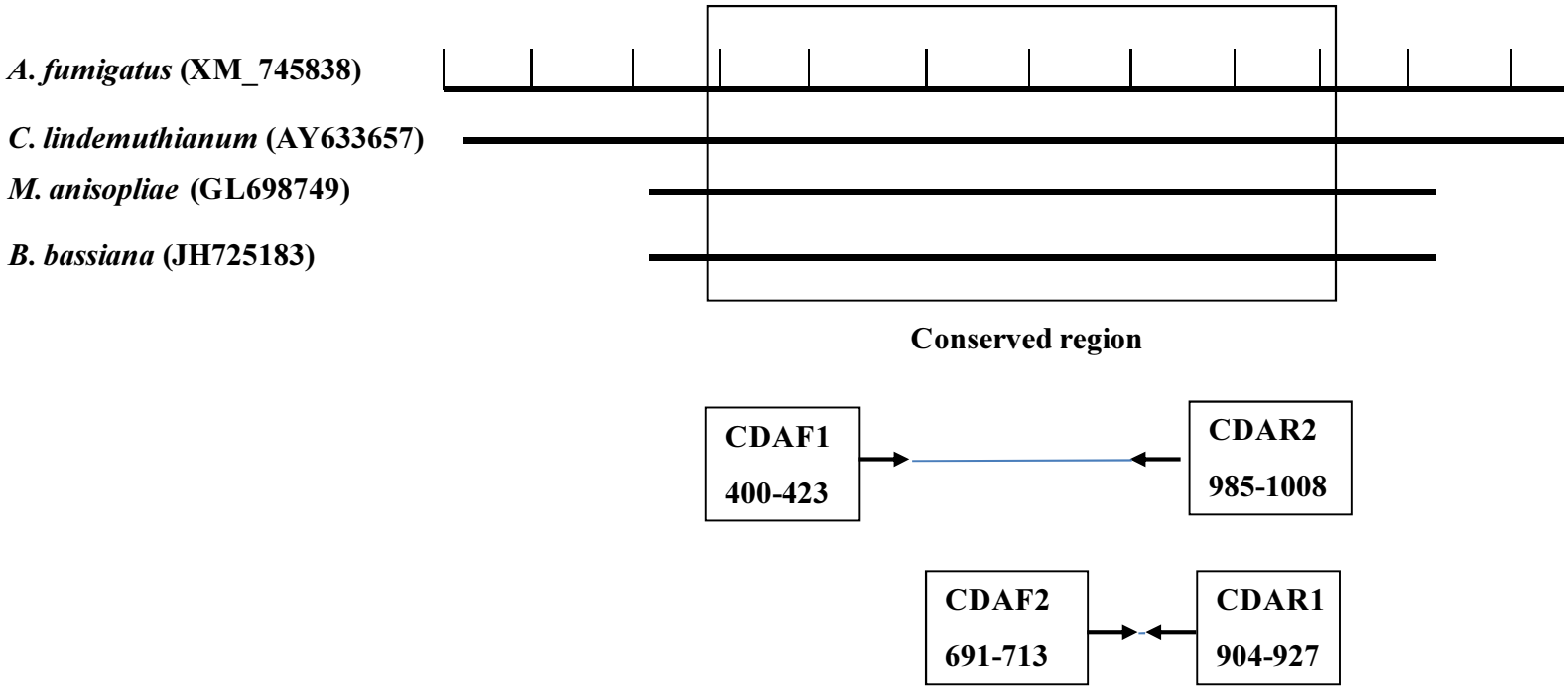


Figure 5.4 Schematic representation of the strategy used to determine the chitin deaectylase gene from *Metarhizium anisopliae* M161063. The box represents the polysaccharide deacetylase domain region of CDA sequences from ascomycetous fungi. The sequences of the primers (5'-3') used were: CDAF1–GCCBTVACBTATGABGACGGTCC; CDAF2–CCVAMBTABATGMGHSCRCCGTA; CDAR1–GACHGTCTGCTBGTGCACGTCGTG; CDAR2–ATCGYCVADRCAHTCGCCVACKGT where B–C, G or T; V–A, C or G; M–A or C; H–A, C or T; S–G or C; R–A or G; Y–C or T; D–A, G or T; K–G or T

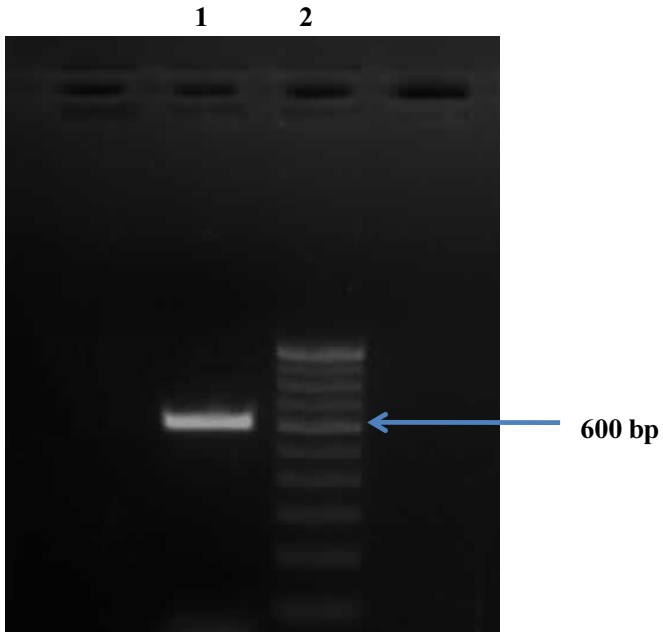


Figure 5.5 PCR amplification of *Metarhizium anisopliae* cDNA using CDAF1 and CDAR2 primers. Lane 1, PCR amplified product (600 bp) and Lane 2, DNA ladder

5.5 Sequence analysis

The plasmid from the positive clones was sequenced using the ABI prism 3730 DNA analyzer. The sequencing gave a 594 bp sequence. The BLAST search of this sequence showed higher identity scores with nucleotide sequences of other reported CDA genes in NCBI GenBank database. This 594 bp partial CDA sequence was submitted to NCBI GeneBank under accession number KM875564 and it encoded a sequence of 198 amino acid residues. The nucleotide sequence and deduced amino acid sequence for the cloned CDA gene obtained with CDAF1-CDAR2 primer set are shown in Figure 5.6. The deduced amino acid sequence of *M. anisopliae* CDA contained active site, zinc binding site and NodB motifs. As only partial CDA sequence of *M. anisopliae* M161063 was determined, two out of nine asparagine residues served as potential *N*-glycosylation sites (Asn^{71}, Asn^{173}- highlighted in grey) (Figure 5.6). In *G. butleri* and *R. nigricans*, the complete gene possessed an ORF of 1290 and 1341 nucleotides encoding a sequence of 430 and 447 amino acid residues possessing nine and eight potential *N*-glycosylation sites, respectively (Jeraj et al., 2006; Maw et al., 2002). Similarly, the 1344 bp ORF of *M. racemosus* encoded a sequence of 448 amino acid residues (Xia-Yun et al., 2007). In *C. lindemuthianum*, the CDA ORF consisted of two regions; one from the start codon encoding a deduced preprodomain of 27 amino acids, and the other encoding a mature CDA of 221 amino

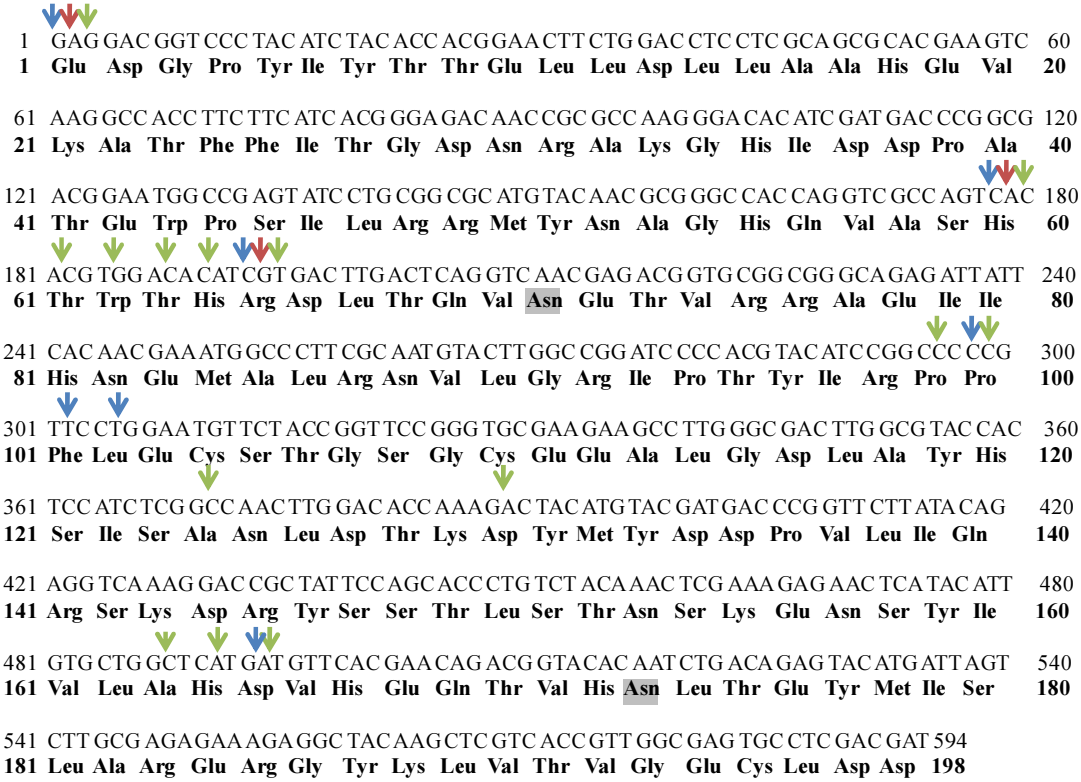


Figure 5.6 Partial nucleotide sequence and the deduced amino acid sequence of chitin deacetylase from *Metarhizium anisopliae* M161063. Blue arrows indicate active site; red arrows indicate zinc binding site and green arrows indicate the NodB motifs. Predicted *N*-glycosylation sites are highlighted in grey. The sequence was submitted to GeneBank under accession no. KM875564

acids (Tokuyasu et al., 1999b) whereas out of predicted 458 amino acids of *C. neoformans* mannoprotein (MP98), the *N*-terminal amino acid sequence started at amino acid 52 which suggested that the first 51 amino acids were not part of the mature protein (Levitz et al., 2001). Furthermore, the two putative CDAs in *E. histolytica* were made up of 275 and 262 amino acids, respectively whereas the three putative CDAs in *E. invadens* varied in length from 253 to 284 amino acids (Das et al., 2006).

The BLAST analysis of the deduced amino acid sequence of CDA from *M. anisopliae* M161063 showed similarities with other reported fungal deacetylases (Table 5.2). The amino acid sequence of CDA shared >50% identity with deacetylases of ascomycetous fungi from the database like CDA of *Colletotrichum gloeosporioides*, *Cordyceps militaris* and *A. niger*; bifunctional xylanase/deacetylase of *Fusarium oxysporum* and *Verticillium alfalfae* and polysaccharide deacetylase of

Table 5.2 BLAST analysis of amino acid sequence of CDA gene of *Metarhizium anisopliae* M161063

Organism	**Accession No.**	**Query cover (%)**	**Identity (aa) (%)**
Metarhizium anisopliae CDA	KFG84684	100	97
Metarhizium robertsii xylanase/ CDA and chitin binding domain protein	EXU96282	100	97
Colletotrichum gloeosporioides CDA	XP_007285779	100	65
Fusarium oxysporum bifunctional xylanase/ deacetylase	ENH69902	100	63
Verticillium alfalfae bifunctional xylanase/ deacetylase	XP_003002355	100	59
Cordyceps militaris CDA	XP006673297	100	59
Beauveria bassiana polysaccharide deacetylase	XP_008601714	100	58
Aspergillus niger CDA	XP_001394100	100	55

B. bassiana. It showed maximum identity (97%) with CDA of *M. anisopliae* (KFG84684) and xylanase/CDA of *Metarhizium robertsii* (EXU96282) (Table 5.2).

As previously reported, in zygomycetous fungus *G. butleri*, the maximum homology (48% identity) between the CDA sequences of *G. butleri* and *M. rouxii* was observed followed by *Phycomyces blakesleeanus*, *Blumeria graminis* and *C. lindemuthianum* that showed 30%, 14% and 12% identity, respectively (Maw et al., 2002). The predicted amino acid sequence of CDA from *R. nigricans* shared relatively high identity (40-55%) with CDAs of zygomycetous fungi viz. *P. blakesleeanus*, *G. butleri*, *R. oryzae* and *M. rouxii* than basidiomycetous (34%) and ascomycetous (29%) fungi (Jeraj et al., 2006). Similarly, the nucleotide sequence of *M. racemosus* CDA shared relatively higher identities (39-75%) with fungi from Mucoraceae whereas showed relatively lower homology (about 20%) with CDAs of *S. cerevisiae* (Xia-Yun et al., 2007). Further, cda1 gene from *S. pombe* that encoded a protein of 320 amino acids possessed a putative polysaccharide deacetylase domain showing 27% identity to the NodB proteins of the genus *Rhizobium* (Matsuo et al., 2005) whereas the ORFs of CDA1 (284 amino acids) and CDA2 (274 amino acids) genes of *S. cerevisiae*

exhibited 24.5% and 27.4% homology with *M. rouxii* CDA, respectively (Christodoulidou et al., 1996). In *C. neoformans*, out of 458 amino acids in the full-length protein, amino acids 160-290 of MP98 contained the polysaccharide deacetylase domain and it showed 40% identity to the *M. rouxii* Cda1 protein (Levitz et al., 2001).

CDA is composed of approximately 400 amino acids, and the region that displays homology to the other proteins (polysaccharide deacetylase domain) is located in the central part of the gene. The amino terminal and the carboxyl-terminal regions do not display any significant similarity to any other known protein sequence. The multiple sequence alignments (ClustalW) of the deduced amino acid sequence of *M. anisopliae* CDA with deacetylases of fungi as well as bacterial deacetylases including NodB like protein of *S. meliloti*, xylanase D from *Cellulomonas fimi*, peptidoglycan deacetylase from *S. pneumoniae* and acetylxylan esterase A from *S. lividans* demonstrated that the obtained CDA sequence was from the polysaccharide deacetylase domain as it shared conserved amino acids of the polysaccharide deacetylase domain located in the middle portion of other deacetylases (Figure 5.7).

Further conserved domain search analysis using the deduced amino acid sequence of *M. anisopliae* CDA displayed the presence of five conserved catalytic domains (Figure 5.8). It showed the presence of catalytic NodB homology domain of *C. lindemuthianum* CDA and similar proteins (cd10951), polysaccharide deacetylase domain which includes NodB protein from *Rhizobium* (pfam01522), polysaccharide deacetylase family sporulation protein PdaB of Gram-positive bacteria (TIGR02764), CDA1 domain for predicted xylanase/CDA (OG0726) and spore_pdaA domain from delta-lactam-biosynthetic de-*N*-acetylase (TIGR02884) (Figure 5.8). The earlier reports suggested that the deduced amino acid sequence of CDA from *M. rouxii* showed striking sequence similarities with the various rhizobial nodB proteins and a *B. stearothermophilus* protein encoded by an uncharacterized open reading frame (Kafetzopoulos et al., 1993b). The analysis of conserved polysaccharide deacetylase domain of *G. butleri* showed the presence of a 147 residue functional domain encoded in the middle part of the gene (143-290 amino acids). This functional domain shared high similarity with CDAs of fungi as well as bacterial deacetylases

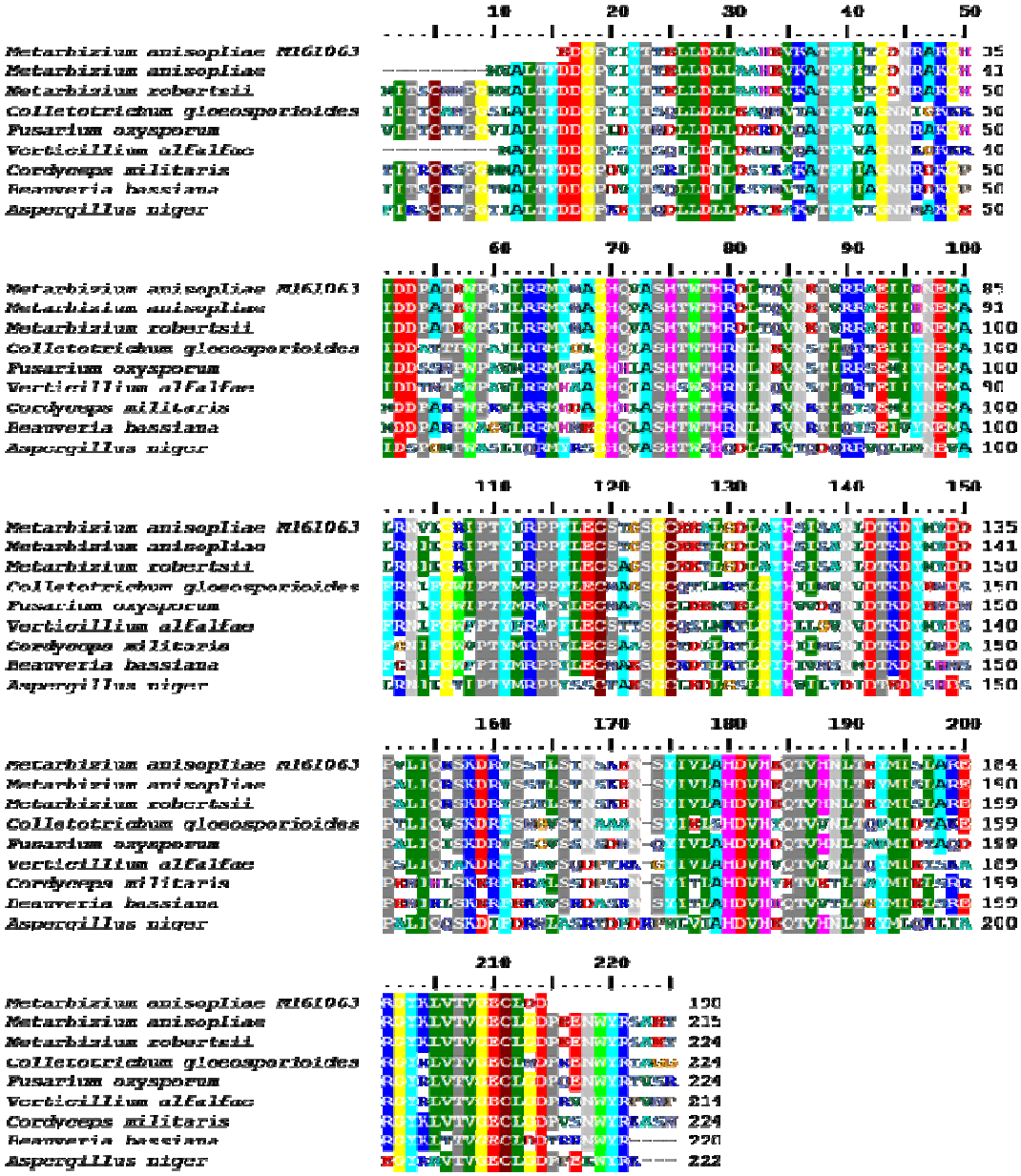


Fig. 5.7 Comparison of the deduced amino acid sequences of the conserved polysaccharide deacetylase domains in different deacetylases. Gene bank accession nos.: *Metarhizium anisopliae* M161063, KM875564; *Metarhizium anisopliae*, KFG84684; *Metarhizium robertsii*, EXU96282; *Colletotrichum gloeosporioides*, XP_007285779; *Fusarium oxysporum*, ENH69902; *Verticillium alfalfae*, XP_003002355; *Cordyceps militaris*, XP006673297; *Beauveria bassiana*, XP_008601714; *Aspergillus niger*, XP_001394100. The shaded regions indicate residues identical in atleast 6 of the compared sequences.

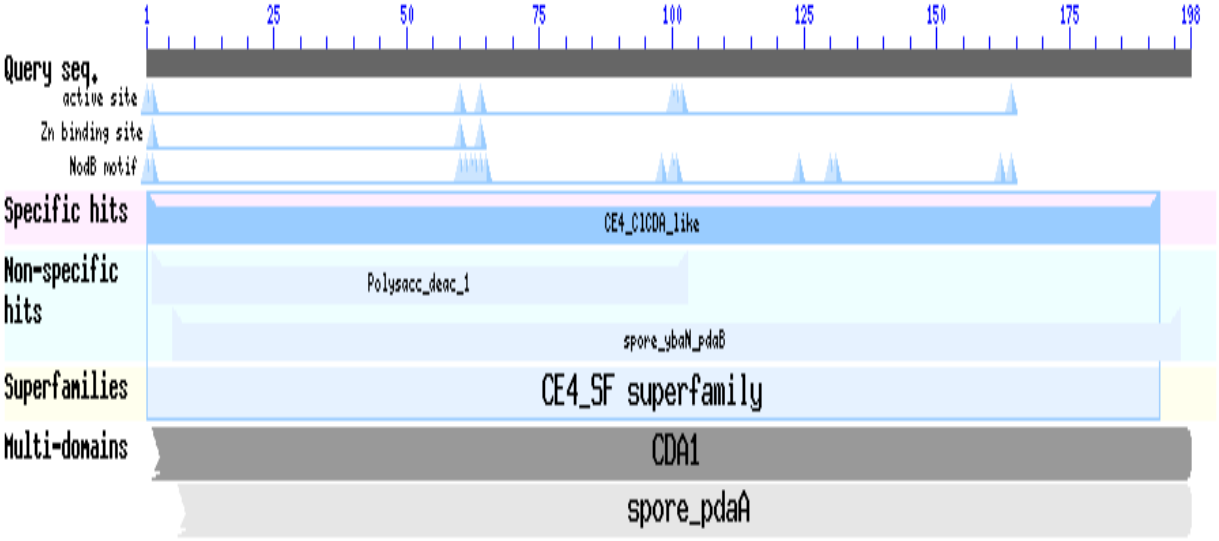


Figure 5.8 Conserved catalytic domains on chitin deacetylase sequence of *Metarhizium anisopliae* M161063

including the NodB-like protein from *S. meliloti* and *B. subtilis*, xylanase D from *C. fimi* and acetylxylan esterase A from *S. lividans* (Maw et al., 2002). In both *R. nigricans* and *M. racemosus*, the CDA sequences consisted of nucleotides encoding a conserved polysaccharide deacetylase domain located in the middle, covering 34% (115-304 amino acids) and 32% (170-315 amino acids) of the entire sequence, respectively (Jeraj et al., 2006; Maw et al., 2002; Xia-Yun et al., 2007).

5.6 Phylogenetic analysis

The evolutionary relationship among CDAs has been determined by many researchers by carrying out phylogenetic analysis. The phylogenetic tree constructed using the eukaryotic and representative bacterial chitin or polysaccharide deacetylases showed spotty distribution of deacetylases suggesting the possibility of lateral gene transfers (Das et al., 2006).

In the present investigation, the taxonomic and phylogenetic study of *M. anisopliae* CDA gene was carried out by comparing the deduced amino acid sequence of CDA with amino acid sequences of other fungal and bacterial deacetylases. For alignment of amino acid sequences and construction of phylogenetic tree, maximum parsimony and bootstrap method included in the ClustalW program was used as described in section 2.16.11. The phylogenetic tree was mainly divided into three clusters. The cluster one was made up of the fungi from ascomycota and

basidiomycota division. The *M. anisopliae* isolate M161063 joined *M. anisopliae* and *M. robertsii* to form one node which was further linked to a second node containing *C. militaris* and *B. bassiana*. The third node made up of *Paracoccidioides lutzii* and *A. niger* joined the previous two nodes. These three nodes were further linked to three separate clades containing *C. lindemuthianum*, *V. alfalfae* and *Magnaporthe oryzae*. All these fungi belonged to fungi from ascomycota division. The fungi from ascomycota division further joined the basidiomycota division containing *Coprinopsis cinerea* and *Flammulina velutipes*. The second cluster was made up of two nodes; one node made up of fungi from zygomycota division viz. *M. racemosus*, *G. butleri* and *Rhizopus stolonifer* and the other node containing the yeast *S. cerevisiae* belonging to ascomycota division. As *Streptococcus pneumoniae* was outgrouped, it formed distinct third cluster (Figure 5.9). Thus, the phylogenetic tree constructed coincided with the fungal taxonomic classification and it was in accordance with the observations of earlier researchers.

Similar studies with *G. butleri* suggested that the phylogenetic tree constructed using the nucleotide sequences of CDA from different fungi matched the taxonomic classification of fungi. The phylogenetic tree was divided into 3 clusters. *G. butleri*, *M. rouxii* and *P. blakesleeanus* belonging to zygomycota division were clustered together and *B. graminis*, *C. lindemuthianum* and *S. cerevisiae* belonging to ascomycota division formed another cluster whereas *Schizophyllum commune* alone formed a distinct third cluster (Maw et al., 2002). In case of *M. racemosus* also, the phylogenetic analysis according to the deduced amino acid sequences matched the classical fungal taxonomy (Xia-Yun et al., 2007).

In the current study, the partial sequence of CDA gene from *M. anisopliae* M161063 was obtained that showed homology with other fungal CDA genes especially with the amino acid sequences of CDAs from ascomycetous fungi. As the presence of multiple CDAs have been reported in fungi and insects, it would be interesting to know the possible existence of another CDA gene in *M. anisopliae*. The partial CDA gene sequence was further used for designing the specific primers for qRT-PCR studies for determining the differential expression of CDA in *M. anisopliae*.

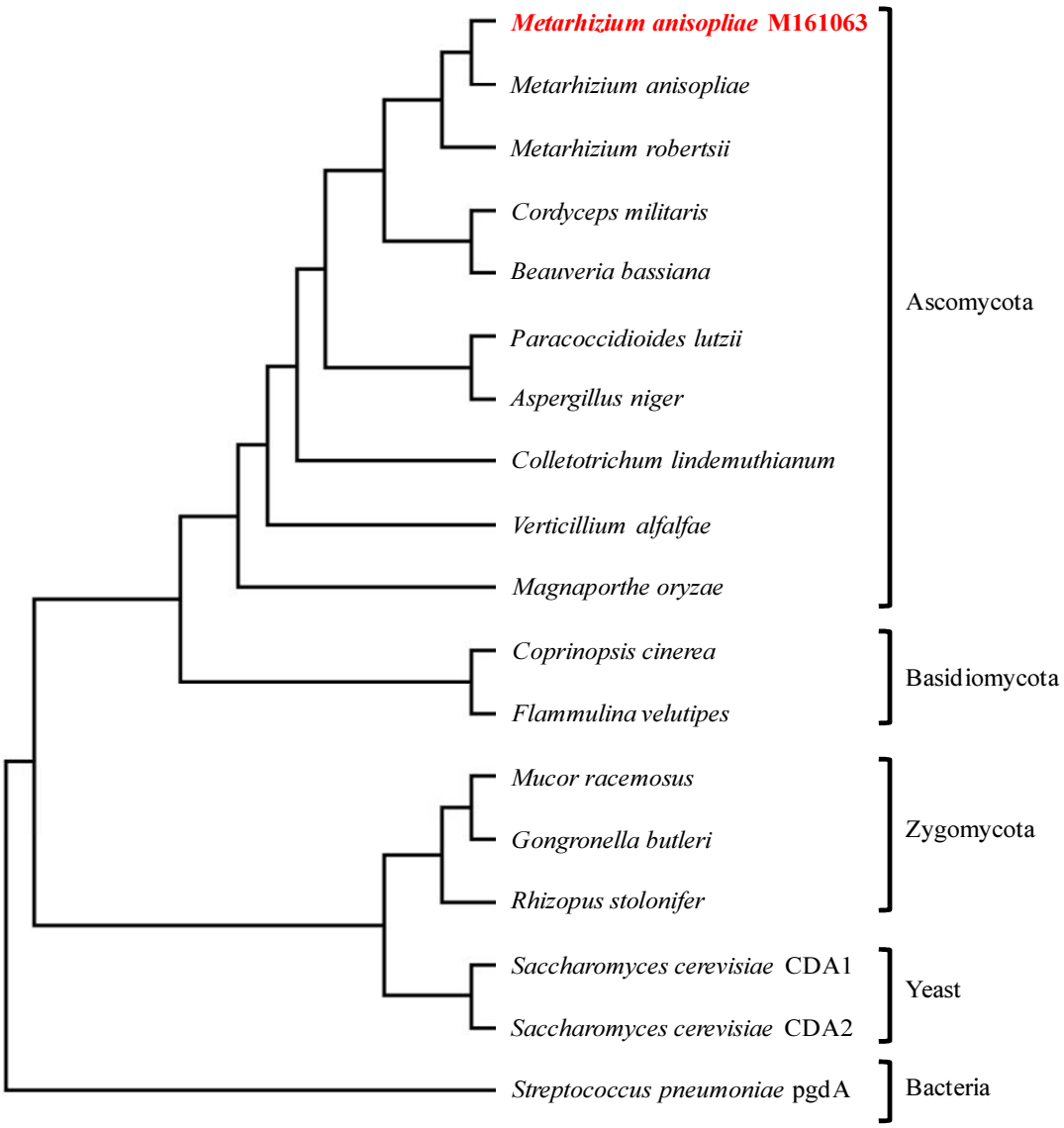


Figure 5.9 Phylogenetic tree derived from amino acid sequences of different deacetylases. Gene bank accession nos.: *Metarhizium anisopliae* M161063, KM875564; *Metarhizium anisopliae*, KFG84684; *Metarhizium robertsii*, EXU96282; *Cordyceps militaris*, XP006673297; *Beauveria bassiana*, XP_008601714; *Paracoccidioides lutzii*, XP_002793041; *Aspergillus niger*, XP_001394100; *Colletotrichum lindemuthianum*, AAT68493; *Verticillium alfalfae*, XP_003002355; *Magnaporthe oryzae*, XP_003712529; *Coprinopsis cinerea*, XP_001838434; *Flammulina velutipes*, ADX07295; *Mucor racemosus*, ABO38856; *Gongronella butleri*, AAN65362; *Rhizopus stolonifer*, AAX11701; *Saccharomyces cerevisiae* CDA1, NP_013410; *Saccharomyces cerevisiae* CDA2, NP_013411; *Streptococcus pneumoniae*, CAB96552.

C. Expression studies of chitin deacetylase from *Metarhizium anisopliae* M161063

Gene expression analysis is important for studying the biological role of known genes and the timing of gene expression is crucial in order to elucidate their function in pathogenesis or fungal development. The cellular events such as survival, growth and differentiation are reflected in altered patterns of gene expression. Therefore, it is necessary to evaluate the expression pattern of virulence genes of entomopathogenic fungi so as to understand molecular mechanism of fungus-insect interaction which can be further exploited to increase their efficacy.

The expression studies of enzymes from the polysaccharide deacetylase group have been carried out to understand their role in growth and development. The study of acetyl xylan esterase II gene from *Penicillium purpurogenum* correlated the expression with carbon source and pH (Chavez et al., 2004). The transcriptional profiling of the mycelium and yeast cells of human pathogenic fungus *P. brasiliensis* was carried out. The expression levels of different genes in both morphological phases were assessed by cDNA microarrays and northern blot analysis (Felipe et al., 2005). Furthermore, the expression pattern of insect CDAs has been reported to differ according to developmental stage and tissue. In the red flour beetle *T. castaneum*, the expression profiles of nine genes encoding CDA-like proteins in various tissues and developmental stages were studied by qRT-PCR (Arakane et al., 2009). Similarly, the stage and tissue specific expression of CDA genes (CfCDA2a and CfCDA2b) from the spruce budworm, *Choristoneura fumiferan* was studied by Quan et al. (2013). Further, the *H. armigera* genes whose expression could be advantageous for the increased insecticidal characteristics of the baculovirus were identified by checking the change in expression of host genes in response to baculovirus infection (Jakubowska et al., 2010).

The gene expression studies are usually carried out by quantification of mRNA of interest under different conditions. The quantification of mRNA is performed using techniques like Northern hybridization analysis, ribonuclease protection assay, cDNA microarray technology (Bustin, 2000; Suzuki et al., 2000). However, these techniques usually require microgram quantities of total RNA in order to detect specific mRNAs. It is also difficult to estimate less than fourfold difference in expression because of the low sensitivity of these assays. In addition, genes with

low levels of expression often cannot be detected at all by these assays. On the contrary, qRT-PCR is an *in vitro* method for enzymatically amplifying defined sequences of RNA. Using this method, the simultaneous measurement of gene expression in many different samples can be done and is especially suitable when only a small number of cells are available (Vandesompele et al., 2002). This method has advantages over other conventional methods, such as reduced hands-on time, technical ease, low reagent cost, and high throughput.

Irrespective of the method used, data normalization is required in order to correct the sample-to-sample variation caused due to variation in the amount of starting material, enzymatic efficiencies, differences in overall transcriptional activity of tissues or cells and differences in RNA quantity and quality. The data normalization can be done using the endogenous (already existing in the sample) and exogenous (characterized RNA and DNA spiked into the sample during its preparation) controls (Fang and Bidochka, 2006). The endogenous controls commonly include the use of a single housekeeping gene that is considered to be constitutively expressed. The housekeeping genes to be used for data normalization should be abundant, remain constant in proportion to total RNA and should not be affected by the experimental treatments. However, a single housekeeping gene can show considerable variation in the expression in different samples and can lead to erroneous results (Thellin et al., 1999). As the expression of housekeeping genes can vary depending on the developmental stage or environmental stimuli, it can lead to misinterpretation of the expression profile of a target gene. Therefore, the housekeeping genes showing constant expression regardless of life stages should be identified to obtain reliable and accurate expression levels of target genes (Suzuki et al., 2000). Usually, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Wall and Edwards, 2002), *β-actin* (Kreuzer et al., 1999), *tubulin* (Brunner et al., 2004), or *rRNA* (Bhatia et al., 1994) are considered for use as normalizers. Further, the use of several reference genes was suggested to get more accurate and reliable normalization of gene expression data and a procedure was developed to select internal controls based on the mean pairwise variation of a gene from all other tested control genes (Vandesompele et al., 2002). In one of the previous reports, six housekeeping genes in *M. anisopliae* were investigated for their expression stability during conidiogenesis, germination and insect pathogenesis and their further use as reference genes for studying the transcription profiles of virulence genes viz. a subtilisin-like protease (*pr1*), a

regulator of G protein signaling gene involved in conidiogenesis (*cag8*), the nitrogen response regulator gene (*nrr1*) and a hydrophobin gene (*ssga*) (Fang and Bidochka, 2006).

The intracellular CDA activity was found to be varying in different morphological forms of *M. anisopliae* M161063 (section 5.3) indicating the differentiation-specific regulation of CDA during fungus-insect interaction. To further understand the advantages of CDA expression during the entry of *M. anisopliae* through insect cuticle, we analyzed the change in expression of CDA gene in different morphological forms viz. conidia from PDA slants (7 d); germinating conidia (12 h); appressoria (24 h); mycelia (24 h, 48 h, 72 h) and blastospores (24 h, 48 h, 72 h) of *M. anisopliae* M161063. The present section describes the evaluation of five housekeeping genes on the basis of expression stability and further use of the most stable housekeeping gene as a reference gene to study the differential expression of CDA in different morphological forms of *M. anisopliae* M161063.

Results and discussion

5.7 Selection of housekeeping genes and specificity of the primer sets

The five commonly used housekeeping genes viz. *18S RNA*, *GAPDH*, *Ubc*, *Tub-a* and *Tub-b* involved in different cellular functions were evaluated for their use as reference gene in qRT-PCR studies. The primers for *18S RNA* and *GAPDH* were as described by Fang and Bidochka (2006) whereas the primers for *Ubc, Tub-a* and *Tub-b* were designed from the sequences of *Metarhizium* (section 2.16.7) retrieved from genebank database. The primers were designed to amplify ~200 bp of these genes by PCR (Table 5.3). The amplicons obtained were purified and cloned into pGEM-T easy vector and transformed to *E. coli* JM109 competent cells as described in section 2.16.9.4. The plasmid was isolated from the positive clones and sequenced using the ABI prism 3730 DNA analyzer. The partial sequences of housekeeping genes showed the corresponding sequences of respective housekeeping genes previously reported in the genebank database.

Table 5.3 Genes investigated in this study

Gene	Gene full name	Function	Primer sequence (5'-3')	Length of the PCR product (bp)	Tm (°C)
18S RNA	Ribosomal RNA	A component of 18S ribosome	F – GGCATCAGTATTCAGTTGTC R – GTTAAGACTACGACGGTATC	170	81.0
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	F – GACTGCCCCGCATTGAGAAG R – AGATGGAGGAGTTGGTGTTG	170	87.0
Ubc	Ubiquitin conjugating enzyme	Carrier of ubiquitin	F – GACTACCCTTTCAAGCCACCAAA R – GAGATGGTGAGAGCAGGGCTCCA	130	84.5
Tub-a	Tubulin-a	Globular protein forming microtubules	F – AACAACTATGCCCGTGGYCACTAC R – CCACCGAAGGAGTGGAAGA	120	84.5
Tub-b	Tubulin-b	Globular protein forming microtubules	F – CTTGATGTTGTCCGTCGCGAGGC R – ACAACGGTGTCGGAAACCTTGGG	190	87.0
CDA	Chitin deacetylase	Virulence determinant	F – ACGTGGACACATCGTGAC R – ACCGGTAGAACATTCCAGGAA	140	87.0

The specificity of all the primer sets was examined by qRT-PCR. The PCR efficiencies of all the primer sets were above 95% according to the standard curves. The standard curve for *GAPDH*, as a representative for the five housekeeping genes studied is shown in Figure 5.10. Fang and Bidochka (2006) reported >95% PCR efficiencies for the six studied housekeeping genes in *M. anisopliae*.

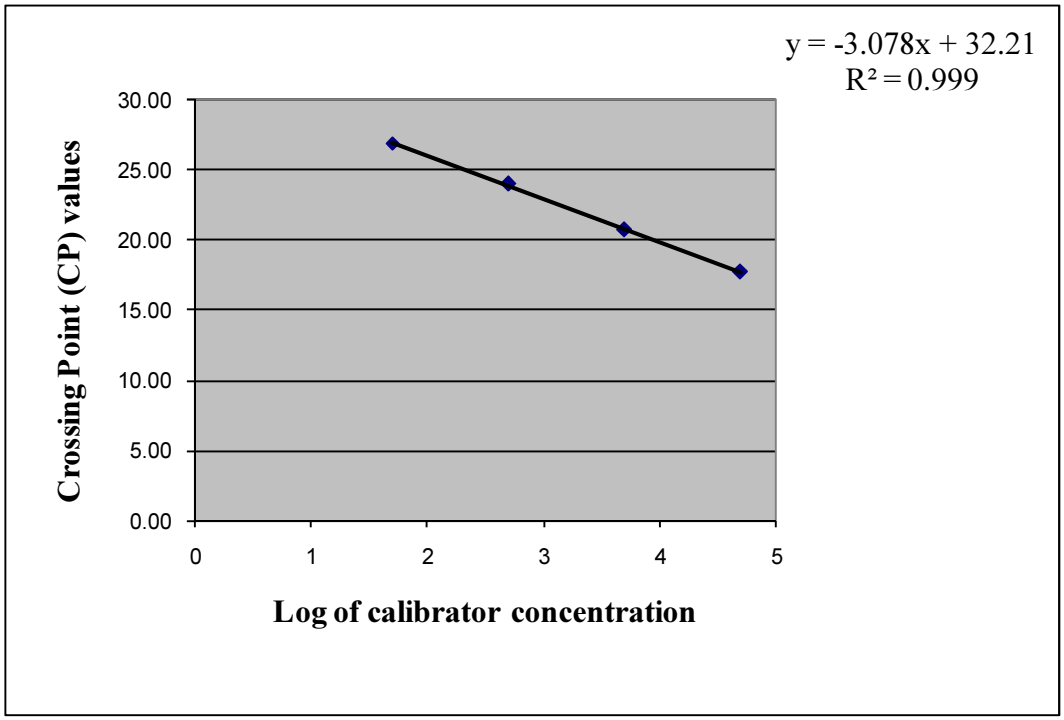


Figure 5.10 Standard curve for GAPDH obtained by correlation of the CP values and log of calibrator concentration

After completion of the amplification reaction, melting curve analysis was done. The appearance of a single peak in the melting curve analysis confirmed amplification of a specific transcript and the absence of primer dimers (Figure 5.11a). The amplified products were further analyzed by agarose gel electrophoresis. A single amplicon of expected size (Table 5.3) was detected for all primer sets (Figure 5.11b) indicating specificity of all the primer sets used in qRT-PCR. The amplification mixtures without cDNA template served as control and showed absence of amplicons. Earlier, in plant pathogenic fungus *Phytophthora parasitica*, the specificity of primer sets for eighteen housekeeping genes was determined by melting curve analysis in qRT-PCR and further by agarose gel electrophoresis (Yan and Liou, 2006).

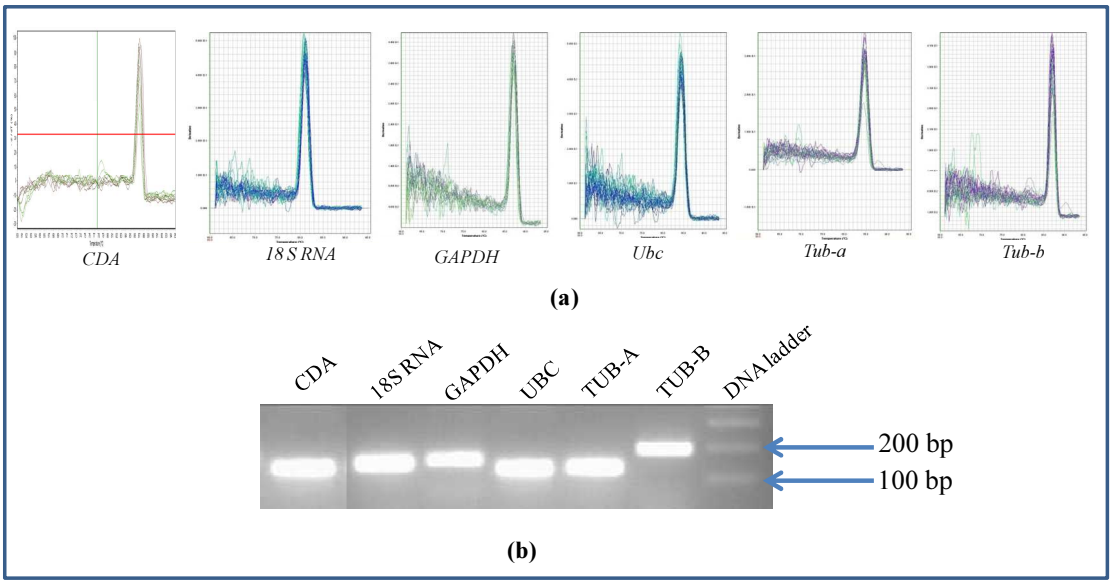


Figure 5.11 Confirmation of primer specificity and amplicon size of studied genes (a) Melting curves generated for genes investigated (b) Agarose gel electrophoresis showing specific qRT-PCR products of the expected size for each gene

5.8 Expression profile of the housekeeping genes

The transcription level of all the five housekeeping genes in different morphological forms of *M. anisopliae* isolate M161063 viz. conidia from PDA slants (7 d), 12 h germinating conidia, 24 h appressoria, blastospores (24 h, 48 h, 72 h) and mycelia (24 h, 48 h, 72 h) was determined by analyzing the cDNA from all the samples by qRT-PCR as mentioned in section 2.16.7. The transcription profiles of the five housekeeping genes for each cDNA sample are shown individually in Figure 5.12. The number of cycles needed for the amplification signal to reach a specific threshold level of detection is known as the crossing point (CP) value. It is inversely correlated with the amount of cDNA template present in the PCR amplification reaction (Rasmussen, 2001). As shown in Figure 5.12, the CP values for *Tub-a* and *Tub-b* obtained from different cDNA samples were higher than those of other three housekeeping genes which indicated a relatively low levels of transcription of these two genes in most of the morphological forms of *M. anisopliae*. On the contrary, genes encoding *18S RNA* displayed relatively low CP values, indicating higher expression of this gene in *M. anisopliae*. Such a candidate could be useful as the housekeeping gene for *M. anisopliae*. In *P. parasitica* also the expression profiles of the studied housekeeping genes were presented in the form of absolute CP values.

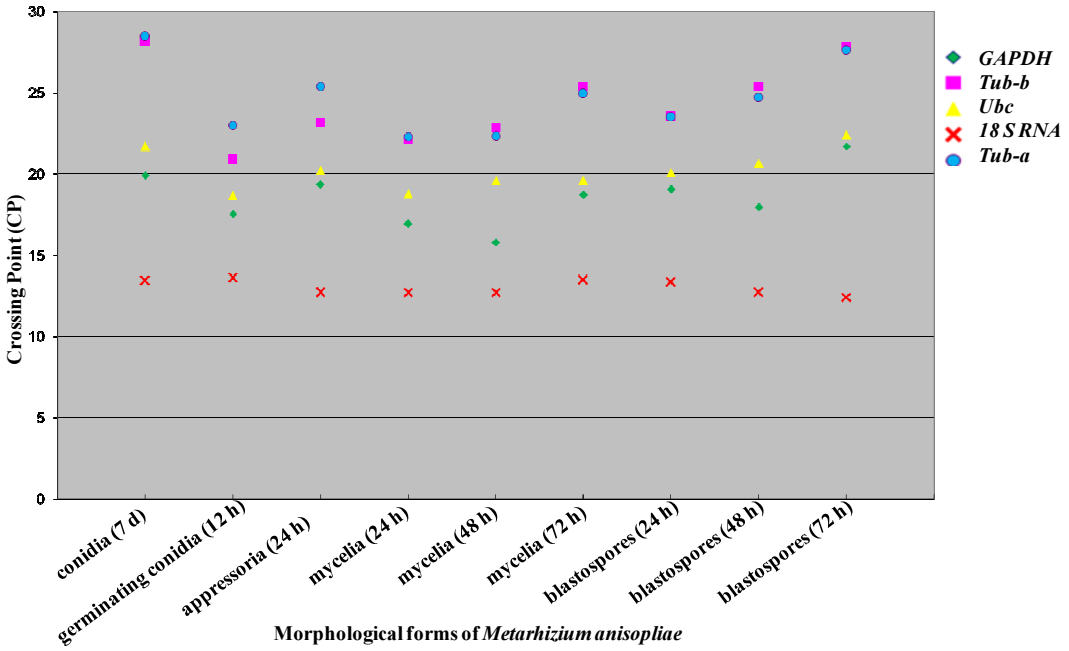


Figure 5.12 The transcription profile of each housekeeping gene in absolute CP values over all cDNA samples of *Metarhizium anisopliae*

Out of 18 housekeeping genes studied, *HGPRT* possessed relatively higher CP values due to low level of transcription in most life stages of this fungus whereas the genes encoding translation elongation factors displayed relatively low CP values indicating its high expression (Yan and Liou, 2006).

5.9 Expression stability measure and ranking of selected housekeeping genes

The constantly expressed genes in different morphological forms of *M. anisopliae* were identified by determining the gene expression stability of each housekeeping gene using an Excel based spreadsheet software application named *Bestkeeper*, developed by Pfaffl et al. (2004). The *Bestkeeper* software determines the stably expressed genes by calculating the standard deviation (SD) and the coefficient of variation (CV) based on the CP values for housekeeping genes. The expression of housekeeping genes exhibiting the lowest variation can be considered as most stable whereas those exhibiting the highest variation can be considered as the least stable. The housekeeping genes with SD>1 are inconsistent. Using this software, the *Bestkeeper Index* can be calculated from the genes considered as stably expressed (Pfaffl et al., 2004) and the most stably expressed genes can also be determined based on the coefficient of correlation to the *Bestkeeper Index* (Zhu et al., 2013). However,

the use of Pearson correlation coefficient is invalid for the groups of differently expressed genes showing heterogeneous variance (Pfaffl et al., 2004).

In the present investigation, the expression stability of five housekeeping genes in different morphological forms of *M. anisopliae* was estimated based on the inspection of calculated variations (SD and CV values) (Table 5.4). According to *Bestkeeper* analysis, the stabilities of the investigated housekeeping genes were *18S RNA>Ubc>GAPDH>Tub-a>Tub-b* in all morphological forms of *M. anisopliae* (Table 5.4). The *Bestkeeper Index* calculated for the five housekeeping genes showed an SD of CP = ±0.93 cycles. The SD (±CP) value was higher for *GAPDH* (1.33), *Tub-a* (1.71) and *Tub-b* (2.07). Therefore, these genes were excluded one by one from the *Bestkeeper Index* calculation, as they were not reliable reference genes. After the exclusion of *Tub-b* from the analysis, the variation in *Bestkeeper Index* decreased (SD = ±0.78 cycles) whereas after further exclusion of *Tub-a* and *GAPDH* subsequently, the variation in *Bestkeeper Index* was SD = ±0.62 and ±0.35 cycles, respectively (Table 5.4).

The genes expressed constantly at specific life stages of *M. anisopliae*, such as vegetative stage, mycelia, blastospores and conidia were identified by performing the analysis using *Bestkeeper* software with subsets of cDNA samples and the gene expression stability of each housekeeping gene was recalculated. The housekeeping genes were ranked on the basis of their expression stability (SD±CP) at specific life stages and were compared with ranking in all morphological forms of *M. anisopliae*, designated as 'all stages'. The comparison of stability ranking of the five housekeeping genes at different life stages of *M. anisopliae* to be in order of most stable to least stable is presented in Table 5.5. For the vegetative stage, which included cDNAs from mycelia, appressoria and blastospores, the expression of *18S RNA* and *Ubc* was the most constant as they exhibited SD (±CP) values of 0.30 and 0.78, respectively whereas *GAPDH*, *Tub-a* and *Tub-b* were least stable as they exhibited SD (±CP) values of 1.38, 1.46 and 1.61, respectively. Similarly, for mycelia at different time intervals viz. 24 h, 48 h, and 72 h; *18S RNA* (SD = ±0.33 cycles) and *Ubc* (SD = ±0.36 cycles) were most stable genes and *GAPDH* (SD = ±1.06 cycles), *Tub-a* (SD = ±1.18 cycles) and *Tub-b* (SD = ±1.27 cycles) were least stable. Further, for blastospores at different time intervals viz. 24 h, 48 h, and 72 h; *18S RNA* (SD = ±0.34 cycles) and *Ubc* (SD = ±0.89 cycles) were most stable genes.

Table 5.4 Expression stability of five housekeeping genes in different morphological forms of *Metarhizium anisopliae* evaluated by *Bestkeeper* software program

Factor	***GAPDH***	***Tub-b***	***Ubc***	***18S RNA***	***Tub-a***	***Bestkeeper* (n=5)**	***Bestkeeper* (n=4)**	***Bestkeeper* (n=3)**	***Bestkeeper* (n=2)**
n	9	9	9	9	9	9	9	9	9
GM [CP]	18.46	24.24	20.14	12.98	24.58	19.58	18.56	16.90	16.17
AM [CP]	18.53	24.36	20.18	12.99	24.66	19.61	18.59	16.92	16.18
Min [CP]	15.78	20.89	18.69	12.41	22.24	18.17	17.19	15.77	15.44
Max [CP]	21.68	28.16	22.37	13.57	28.45	21.53	20.18	18.19	17.05
SD [± CP]	1.33	2.07	**0.94**	**0.39**	1.71	0.93	0.78	0.62	0.35
CV [% CP]	7.18	8.49	4.65	3.03	6.93	4.72	4.21	3.69	2.16

Descriptive statistics of five housekeeping genes based on their crossing point (CP) values. Abbreviations: n: number of samples; GM [CP]: the geometric mean of CP; AM [CP]: the arithmetic mean of CP; Min [CP] and Max [CP]: the extreme values of CP; SD [± CP]: the standard deviation of the CP; CV [% CP]: the coefficient of variance expressed as a percentage on the CP level. Results from three independent experiments are shown. In the last four columns the *BestKeeper* index is computed together with the same descriptive parameters, for five genes (*GAPDH*, *Tub-b*, *Ubc*, *18S RNA* and *Tub-a*) or for four genes after removal of *Tub-b* (*GAPDH*, *Ubc*, *18S RNA* and *Tub-a*) or for three genes after removal of *Tub-a* (*GAPDH*, *Ubc* and *18S RNA*) or for two genes after removal of *GAPDH* (*Ubc* and *18S RNA*).

Table 5.5 Comparison of expression stability of five housekeeping genes at different life stages

Rank	**All stages (n=9)**		**Vegetative (n=7)**		**Mycelia (n=3)**		**Blastospores (n=3)**		**Conidia (n=2)**	
	Gene	**CV±SD**	**Gene**	**CV±SD**	**Gene**	**CV±SD**	**Gene**	**CV±SD**	**Gene**	**CV±SD**
1	***18sRNA***	**3.03±0.39**	***18sRNA***	**2.37±0.30**	***18sRNA***	**2.54±0.33**	***18sRNA***	**2.68±0.34**	***18sRNA***	**0.64±0.09**
2	***Ubc***	**4.65±0.94**	***Ubc***	**3.85±0.78**	***Ubc***	**1.88±0.36**	***Ubc***	**4.25±0.89**	*GAPDH*	6.29±1.18
3	*GAPDH*	7.18±1.33	*GAPDH*	7.47±1.38	*GAPDH*	6.17±1.06	*GAPDH*	7.26±1.42	*Ubc*	7.44±1.50
4	*Tub-a*	6.93±1.71	*Tub-a*	5.99±1.46	*Tub-a*	5.09±1.18	*Tub-b*	5.87±1.50	*Tub-a*	10.62±2.73
5	*Tub-b*	8.49±2.07	*Tub-b*	6.64±1.61	*Tub-b*	5.43±1.27	*Tub-a*	6.16±1.55	*Tub-b*	14.82±3.63

Abbreviations: n: number of samples

Whereas, *GAPDH* (SD = ±1.42 cycles), *Tub-b* (SD = ±1.50 cycles) and *Tub-a* (SD = ±1.55 cycles) were least stable. The ranking of genes in vegetative, mycelial and blastospore stage were similar to their ranking in 'all stages'. Finally, for conidial stage which included conidia from PDA slants (7 d) and germinating conidia (12 h), only *18S RNA* was the most stable gene exhibiting SD = ±0.09 cycles whereas *GAPDH* (SD = ±1.18 cycles), *Ubc* (SD = ±1.50 cycles), *Tub-a* (SD = ±2.73 cycles) and *Tub-b* (SD = ±3.63 cycles) were most unstable as they exhibited SD (±CP) values more than 1 (Table 5.5). On the basis of all above observations, *18S RNA* was found to be most stable gene followed by *Ubc* out of five housekeeping genes analyzed, in different morphological forms of *M. anisopliae* M161063.

In some of the earlier studies in *M. anisopliae*, the expression stability of six housekeeping genes (*act*, *gpd*, *18S RNA*, *tef*, *try* and *ubi*) was evaluated using the software *geNorm*. Out of six housekeeping genes investigated, three housekeeping genes (*tef*, *gpd* and *try*) were identified as most suitable reference genes for qRT-PCR analysis of genes expressed during conidiogenesis, germination and pathogenesis (Fang and Bidochka, 2006).

Many studies have demonstrated the use of *Bestkeeper* software for qRT-PCR data analysis (Li et al., 2012; Maroufi et al., 2010; McCulloch et al., 2012; Shi et al., 2013; Silva et al., 2014; Zhu et al., 2013). The expression stability of 16 reference genes was evaluated for their suitability for quantitative gene expression analysis by qRT-PCR in *Candida glabrata*, following fluconazole treatment. The reference genes *RDN5.8*, *UBC13* and *PGK1* were selected as most suitable and stably expressed reference genes based on the evaluation using four different software programs: hkgFinder, geNorm, BestKeeper and NormFinder. The evaluation using BestKeeper software was done by determining the correlation between each reference gene and BestKeeper index (Li et al., 2012). Further, Shi et al. (2013) ranked the housekeeping genes in a potato beetle, *Leptinotarsa decemlineata* on the basis of CV±SD values in BestKeeper software. The expression stability was also assessed using geNorm and Normfinder algorithms. Out of 9 housekeeping genes studied, the *ribosomal protein RP4* and *RP18* were found to be most stable reference genes and *ACT1* and *ACT2* were found to be the most unstable genes in *L. decemlineata*, irrespective of the software programs used.

5.10 Analysis of chitin deacetylase gene expression in different morphological forms of *Metarhizium anisopliae* M161063

The expression of CDA in different morphological forms of *M. anisopliae* M161063 was studied, using the most consistently expressed housekeeping gene i.e. *18S RNA* (Table 5.4 and Table 5.5) as a reference. The primers for studying the expression of CDA by qRT-PCR were designed from the partial CDA sequence of *M. anisopliae* M161063 as mentioned in section 5.5. The primer sequences of the CDA gene and the length of the amplified PCR product are listed in Table 5.3. Further, a single peak was obtained during melting curve analysis and CDA amplicon of expected size was detected during analysis of qRT-PCR products by agarose gel electrophoresis (Figure 5.11a and b). The data obtained after qRT-PCR analysis of CDA gene was normalized with *18S RNA*. The results of the current investigation (Figure 5.13) showed a 1.1-fold repression of CDA in conidia from PDA slants (7 d) whereas in germinating conidia

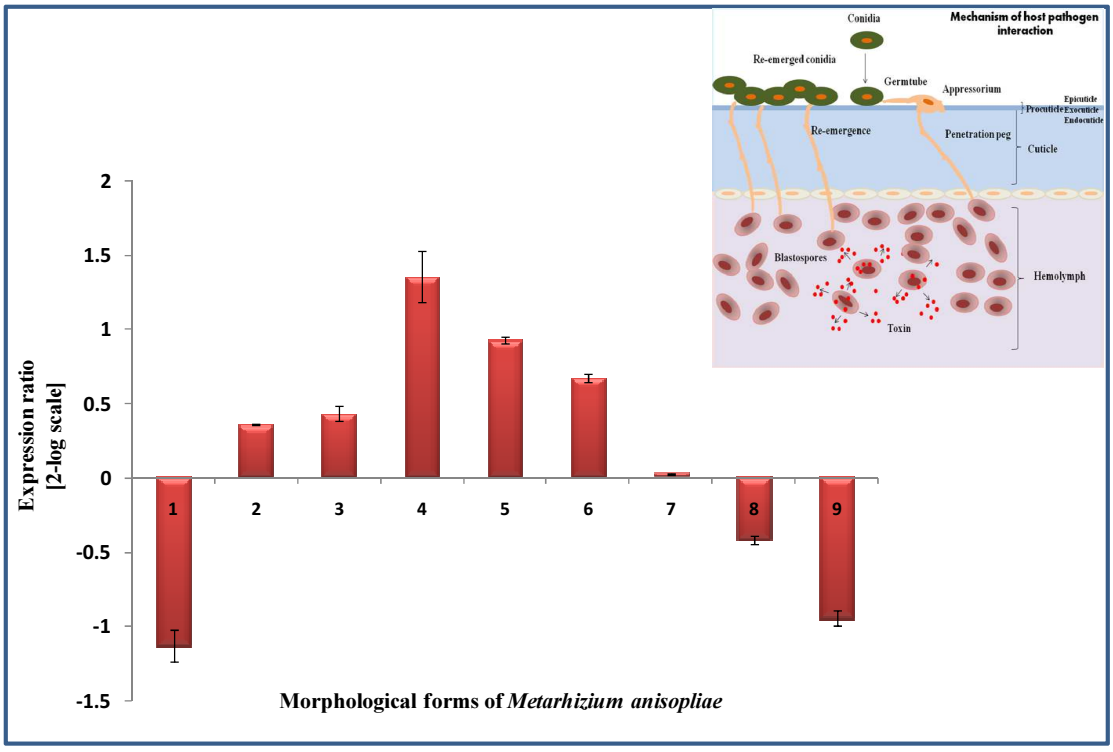


Figure 5.13 Quantification of the CDA transcript by qRT-PCR. The amount of CDA transcript present in different morphological forms of *Metarhizium anisopliae* M161063 was measured by qRT-PCR and normalized with the transcript of *18S RNA*. The morphological forms used for the analysis were: (1) conidia (7 d); (2) germinating conidia (12 h); (3) appressoria (24 h); (4) mycelia (24 h); (5) mycelia (48 h); (6) mycelia (72 h); (7) blastospores (24 h); (8) blastospores (48 h); (9) blastospores (72 h)

(12 h) and appressoria (24 h) it increased by 0.3-fold and 0.4-fold, respectively. Subsequently, the expression of CDA gene was enhanced to higher level i.e. 1.3-fold in mycelia (24 h). The mycelia (48 and 72 h) and blastospores (24 h) showed a lower expression level of CDA compared to mycelia (24 h) and the expression was 0.93 fold, 0.67 fold and 0.03 fold lower, respectively. Further, the CDA expression was again repressed in blastospores (48 h) (0.4-fold) and blastospores (72 h) (0.9-fold).

While studying the relative expression levels of four genes (*Pr1, nrr1, cag8 and ssga*) in germination, conidiogenesis and pathogenesis in *M. anisopliae*, the *Pr1* was found to be expressed only in emerging mycelia from insect cadavers and not in conidia or mycelia grown in YPD broth or YPD agar (Fang and Bidochka, 2006). In *H. armigera*, the qRT-PCR analysis showed the downregulation of *Hacda5a* in the initial hours after baculovirus infection. A decrease in expression of *Hacda5a* at 6 h (4.8-fold) and 24 h (2.6-fold) post infection was observed whereas *Hacda5a* expression returned to the regular level at 48 h post infection. The downregulation of *Hacda5a* was suggested to reduce the PM permeability further decreasing the entrance of baculovirus (Jakubowska et al., 2010). The multiple genes encoding CDA-like proteins were reported to be present in *T. castaneum* and the expression pattern of these genes differed according to developmental stages and tissues. The group I and group IV CDA gene transcripts were expressed at all stages of development. Similarly, the group III transcripts were observed in all stages except for the pupal stage. However, the group II and group V CDA gene transcripts showed highest level of transcript accumulation only in the pupal stage and larval stage, respectively (Arakane et al., 2009). In *P. brasiliensis*, the CDA was proposed to be involved in the synthesis of new components during cell growth and budding of yeast cells, as high expression of CDA was observed in yeast cells. Therefore, CDA could be used as a specific drug target for paracoccidioidomycosis as it is absent in humans (Felipe et al., 2005). In *S. pombe*, abnormal spore formation in *cda1* disruptant and the increased expression of *cda1* mRNA during sporulation process, 12 h after initiation of meiosis supported the role of chitosan in spore formation (Matsuo et al., 2005). The expression of acetyl xylan esterase II gene from *P. purpurogenum* was found to be repressed by glucose and induced by xylan whereas it was expressed at neutral pH, but not under alkaline or acidic conditions (Chavez et al., 2004).

In this study, the systematic analysis of five housekeeping genes by qRT-PCR provided *18S RNA* as a suitable reference gene for studying the expression of CDA in

different morphological forms of *M. anisopliae* M161063. The upregulation of CDA in germinating conidia (12 h), appressoria (24 h) and mycelia (24 h) promoted the hypothesis that CDA might be involved in self defense of *M. anisopliae* and by increase in its expression; the fungus might be protecting itself against insect chitinases during its entry through the insect cuticle. Further downregulation of CDA expression in blastospores (48 h and 72 h) indicated the possibility of involvement of other mechanisms for the survival of the fungus in the insect haemolymh. Thus, the results of the qRT-PCR studies supported the trend of the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 (section 5.3) suggesting the differentiation specific regulation of CDA in *Metarhizium*. The present investigation has extended the understanding of the role of CDAs during fungus-insect interaction which could be used in future for improvement of the insecticidal properties of *Metarhizium* species for the control of insect pests.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The review of literature discussed in the first chapter has highlighted the importance of CDEs as one of the virulence factors during the establishment of the entomopathogenic fungi in the insect body. The role of CDEs viz. chitinase, protease and lipase in pathogenicity of different entomopathogenic fungi has been widely studied. However, the studies emphasizing the significance of CDA in fungus-insect interaction have not been documented. Therefore, the present investigation was initiated to study the significance of CDEs especially CDA from *Metarhizium* species. The results of the present study are summarized as follows.

The entomopathogenic fungal isolate highly virulent for the target host and genetically and biologically stable should be selected for the development of an effective mycoinsecticide. In the present study, the sixty eight *Metarhizium* isolates obtained from the soil samples of different crop fields (fifty eight isolates) and from the insect hosts (ten isolates) were evaluated based on the extracellular *in vitro* production of CDEs in YPG and chitin containing medium and the pathogenic potential against 3rd instar larvae of *H. armigera*. The induced CDEs like chitinase, protease, lipase and chitosanase from chitin containing medium and the constitutive CDA activity from YPG medium being higher were used for the evaluation of *Metarhizium* isolates. The mortality of sixty eight *Metarhizium* isolates varied between 20-97% within 14 d. The sixty eight *Metarhizium* isolates were grouped into eight clusters based on the different levels of CDEs and mortality displayed by the isolates. The isolates possessing >90% mortality against *H. armigera* exhibited higher CDE activities whereas those possessing <40% mortality displayed lower CDE activities. Out of sixty eight *Metarhizium* isolates, the twelve isolates possessing >90% mortality were further used for the determination of LT_{50}. Out of twelve selected isolates with LT_{50} values in the range of 3.3-6.8 d, the five isolates M34311, M34412, M81123, M91427 and M91629 with lowest LT_{50} values (3.3-4.1 d) were selected for the evaluation of LC_{50}. Based on the LC_{50} values (1.4×10^3-5.7×10^3 conidia/ml), the three isolates (M34311, M34412, M81123) out of five were further studied for conidia production on a solid substrate, viability and settling time of conidia. Subsequently, the conidia production using the *Metarhizium* isolate M34412 was 67 g/kg rice and it also exhibited higher conidial germination (97%) and faster sedimentation time (ST_{50}-2.3 h) in 0.1% (w/v) Tween 80 as compared to other two isolates; M34311 and M81123. The three *Metarhizium* isolates were identified up to species level by ITS1-5.8S-ITS4 sequencing and were found to be strains of *M.*

anisopliae. Therefore, based on highest extracellular CDE activities and percent mortality against *H. armigera*; lowest LT_{50} and LC_{50} values as well as higher conidial germination and faster sedimentation time (ST_{50}), *M. anisopliae* M34412 was found to be the most effective isolate among sixty eight *Metarhizium* isolates as it possessed traits necessary for development of successful mycoinsecticide.

Some of the earlier studies have suggested the use of Pr1 activity as a biochemical tool for screening of the virulent isolates of *M. anisopliae*. In the present investigation, the polymorphism in Pr1A gene of sixty eight *Metarhizium* isolates was studied and its correlation with *in vitro* protease activity and mortality of *H. armigera* was determined. The *in vitro* protease activities of the sixty eight *Metarhizium* isolates in chitin containing medium ranged between 0.30–3.38 U/ml. On the basis of mortality of *H. armigera*, the sixty eight *Metarhizium* isolates were classified as: highly pathogenic (sixteen isolates), moderately pathogenic (twenty seven isolates) and less pathogenic (twenty five isolates) possessing the mortality rates >85.6%, 67.7-85.6% and <67.7%, respectively. A positive correlation was observed between the protease activities of sixty eight *Metarhizium* isolates and mortality of *H. armigera* as the protease activities for the highly pathogenic, moderately pathogenic and less pathogenic groups ranged between 2.1-3.38 U/ml, 1-2.4 U/ml and <1 U/ml, respectively. The amplification of the Pr1A gene using METPR2 and METPR5 primer pair gave a single amplicon of approximately 1.2 kb in sixty six *Metarhizium* isolates. After digestion of Pr1A amplicons using restriction endonucleases namely RsaI, MspI and DdeI, multiple polymorphisms with 7, 2 and 5 restriction patterns designated as A-G, H-I and J-N, respectively were obtained. When the restriction digestion patterns of Pr1A amplicons of sixty six *Metarhizium* isolates were combined, 15 cumulative profile types were produced. The cumulative profile type I (D-I-M pattern) was most prevalent as out of sixty six *Metarhizium* isolates thirty three (50%) isolates exhibited this profile and among these, eleven isolates were from highly pathogenic group. Further, the sixty six *Metarhizium* isolates were grouped into six clusters based on the three variables i.e. protease activity, mortality against *H. armigera* and the data for polymorphism of Pr1A gene. Out of sixteen isolates from highly pathogenic group, the twelve isolates possessing high protease activity (>2.0 U/ml) and >90% mortality were grouped together. Out of these twelve isolates, eleven isolates exhibited cumulative profile type I (D-I-M pattern) whereas only one isolate showed cumulative profile type IX (E-H-L pattern). Based on the cluster analysis, the

correlation between protease activity, virulence against *H. armigera* and the cumulative profile type I (D-I-M pattern) of sixty six *Metarhizium* isolates was established which also supported the selection of the *M. anisopliae* M34412 as the most effective isolate among sixty eight *Metarhizium* isolates. Therefore, the protease activity along with the polymorphism of Pr1A gene can be used as biochemical and molecular tool to identify the most effective *Metarhizium* isolates against *H. armigera* and as a quality control parameter of a bio-control organism.

For the large scale production of mycoinsecticide, the stability of the fungal strain during repeated *in vitro* conidial sub-culturing on artificial media is desirable. Therefore, the effect of repeated *in vitro* sub-culturing and *in vivo* passage of the most effective isolate *M. anisopliae* M34412 on different aspects considered as parameters of virulence was monitored. No significant effect on the morphological and cultural characteristics of *M. anisopliae* was observed. Further, germination efficiency of *M. anisopliae* conidia was also unaffected as conidia of all the sub-cultures (1^{st}, 20^{th} and 40^{th}) and passaged cultures (40^{th} 1^{st} *in vivo* passage and 40^{th} 5^{th} *in vivo* passage) showed 90% germination on YPG agar within 24 h. However, the appressorium formation was significantly affected (~20% reduction from 1^{st} to 40^{th} sub-culture) after repeated *in vitro* sub-culturing on PDA and was increased by 17% after 5 passages of 40^{th} sub-culture in *H. armigera*. The CDE activities such as chitinase, protease, CDA and chitosanase of 40^{th} sub-culture were decreased by 21-28% and 15-27% in YPG and chitin containing medium, respectively as compared to 1^{st} sub-culture. The *in vivo* passages of the 40^{th} sub-culture in 3^{rd} instar larvae of *H. armigera* for 5 times resulted in a gradual increase in these CDE activities. However, the lipase production in both YPG and chitin containing medium was not significantly affected (<15%) by repeated *in vitro* sub-culturing. Similarly, when mycelial inoculum from the 10^{th} serial vegetative transfer was used, no significant decrease (<10%) in the chitinase, protease, lipase and chitosanase activities in YPG and chitin containing medium was seen whereas the CDA activity was decreased significantly in both YPG (19.8%) and chitin containing (23.2%) medium as compared to the 1^{st} vegetatively transferred mycelium inoculum, suggesting the possible role of CDA in the penetration of insect cuticle. The conidia of 1^{st} sub-culture were found to be more virulent as mortality of *H. armigera* decreased by ~11% in 40^{th} sub-culture and subsequently, the mortality increased by ~10% after passage of the 40^{th} sub-culture for 5 times in *H. armigera*. The LT_{50} value of 1^{st} sub-culture was lower (3.4 d) as

compared to 40th sub-culture (5.6 d) and passage of the 40th sub-culture in *H. armigera* again decreased the LT_{50} value to 3.7 d. Similar trend for the effect of repeated *in vitro* sub-culturing on the LC_{50} values of 1st (0.17×10^4 conidia/ml) and 40th (3×10^4 conidia/ml) sub-culture and of *in vivo* passage on 40th 5th *in vivo* passaged culture (0.23×10^4 conidia/ml) was observed. During RAPD analysis, significant visual differences in the banding pattern with three primers OPA07, OPA03 and 935G were observed due to repeated *in vitro* sub-culturing and *in vivo* passage in the 3rd instar larvae of *H. armigera* which supported the biochemical data. The present study showed that the repeated *in vitro* sub-culturing has attenuated the virulence of *M. anisopliae* M34412 against *H. armigera* by altering the factors responsible for pathogenicity. Among all the virulence factors, the CDE activities were significantly influenced and the effect on CDA activity was more pronounced suggesting its possible use as marker to monitor the virulence of *M. anisopliae* during mass production.

The CDEs being one of the important virulence factors, study of regulation of enzyme activities in response to infection-structure differentiation can improve the understanding of the mechanism by which the fungus invades into the host body. As CDA was observed to be one of the important CDEs in the virulence of *M. anisopliae* against *H. armigera*, it was important to know the role of this enzyme in establishing stable relationship of the fungus with the insect. For finding the suitable organism for studies of CDA, the sixty eight *Metarhizium* isolates were screened based on cluster analysis using *in vitro* extracellular CDA activity in YPG medium and corrected mortality against 3rd instar larvae of *H. armigera*. The sixty eight *Metarhizium* isolates were grouped into six clusters based on the different levels of extracellular CDA activity and mortality displayed by the isolates. Out of sixty eight *Metarhizium* isolates, the twelve isolates possessing >90% mortality were grouped together (cluster 1) and the high mortality of *H. armigera* in this cluster was attributed to the combination of chitinase and CDA activity. Further, out of twenty four isolates from cluster 2 possessing mortality in the range of 73–88%, the *Metarhizium* isolate M161063 showing 82% mortality and low chitinase activity (1.01 U/ml) was selected for further studies, as high mortality of *H. armigera* with this *Metarhizium* isolate could be because of high CDA (2.26 U/ml) and chitosanase (13.22 U/ml) activity. The *Metarhizium* isolate M161063 was identified up to species level by ITS1-5.8S-ITS4 sequencing and was found to be a strain of *M. anisopliae*. The intracellular CDA

activity in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h) and blastospores (24 h) of *M. anisopliae* M161063 was determined in order to understand the differentiation specific production of CDA. The intracellular CDA activity of *M. anisopliae* M161063 increased progressively in germinating conidia (12 h) (0.02 U/mg), appressoria (24 h) (0.04 U/mg) and mycelia (24 h) (0.08 U/mg) than conidia from PDA slants (7 d) (0.005 U/mg) and subsequently decreased in blastospores (24 h) (0.01 U/mg). The increased intracellular CDA activity in appressoria (24 h) and subsequently in mycelia (24 h) of *M. anisopliae* M161063 revealed the importance of CDA in self defense of the fungus during entry through the insect cuticle. The trend of the intracellular CDA activity in *M. anisopliae* M161063 was observed to be differentiation specific and it thus provided a basis for predicting the role of CDA in developing stable relationship between the fungus and insect.

For widespread use of the mycoinsectides for the control of insect pests, the entomopathogenic fungi used should be highly efficient and the efficacy can be improved by manipulation of the desired traits of these fungi using modern techniques in genetic engineering and biotechnology. However, for the development of hypervirulent strains, the specific pathogenic genes involved in virulence should be isolated and investigated using the molecular biology techniques. Therefore, the molecular characterization of CDA gene from *M. anisopliae* M161063 was carried out for its further use in confirming the role of CDA in fungus-insect interaction. For amplification of CDA gene from *M. anisopliae* M161063, degenerate primers (CDAF1-CDAR2) designed towards the conserved polysaccharide deacetylase domain of previously reported fungl CDA genes from the NCBI database were used. The partial CDA nucleotide sequence of 594 bp encoding a sequence of 198 amino acid residues was obtained that shared maximum identity with deacetylases of ascomycetous fungi from the database. The two (Asn^{71}, Asn^{173}) out of nine asparagine residues of *M. anisopliae* CDA were glycosylated and the amino acid sequence also displayed the presence of five conserved catalytic domains. Further, the phylogenetic tree was constructed using the deduced amino acid sequence of *M. anisopliae* CDA with amino acid sequences of other fungal and bacterial deacetylases. The phylogenetic tree was mainly divided into three clusters; the fungi from ascomycota and basidiomycota division formed cluster one whereas the fungi from zygomycota division and the yeast *S. cerevisiae* belonging to ascomycota division formed second

cluster and; *Streptococcus pneumoniae* being outgrouped formed distinct third cluster. The *M. anisopliae* isolate M161063 joined *M. anisopliae* and *M. robertsii* forming one node of the first cluster. Thus, the results of the phylogenetic analysis coincided with the taxonomic classification of fungi. The present study determined the partial sequence of CDA gene that was further used for studying the differential expression of CDA from *M. anisopliae* M161063.

The biological role of fungal virulence genes during fungus-insect interaction can be better elucidated by carrying out gene expression analysis. As variation in the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 was observed, the present section aimed at further analyzing the change in expression of CDA gene in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h, 48 h, 72 h) and blastospores (24 h, 48 h, 72 h) of *M. anisopliae* M161063 using qRT-PCR. However, reliable and accurate expression levels of target genes can be obtained by normalization of data using the housekeeping genes showing constant expression. Therefore, in the current study, five housekeeping genes namely *18S RNA*, *GAPDH*, *Ubc*, *Tub-a* and *Tub-b* were evaluated by qRT-PCR for the expression stability. The transcription profiles of the five housekeeping for the cDNA sample of each morphological form were obtained in the form of crossing point (CP) values. Out of five housekeeping genes studied, genes encoding *18S RNA* displayed relatively low CP values, indicating high expression of this gene in *M. anisopliae* whereas the CP values for *Tub-a* and *Tub-b* were higher indicating relatively low levels of transcription of these two genes in most of the morphological forms of *M. anisopliae*. The gene expression stability of each housekeeping gene was determined using the software; *Bestkeeper* in which the standard deviation (SD) and the coefficient of variation (CV) based on the CP values was calculated. The housekeeping genes exhibiting lowest variation were considered as most stable whereas those exhibiting highest variation were considered as least stable. The stabilities of the investigated housekeeping genes were *18S RNA>Ubc>GAPDH>Tub-a>Tub-b* in all morphological forms of *M. anisopliae*. The *Bestkeeper Index* showed an SD of CP = ±0.93 cycles for the five housekeeping genes. This variation in *Bestkeeper Index* decreased to ±0.78, ±0.62 and ±0.35 cycles after the exclusion of three most unstable housekeeping genes i.e. *Tub-b*, *Tub-a* and *GAPDH*, respectively from the calculations. Furthermore, the expression stabilities of the housekeeping genes at specific life stages of *M.*

anisopliae viz. vegetative stage, mycelia, blastospores and conidia were also identified. The housekeeping gene *18S RNA* was the most stable gene followed by *Ubc* in vegetative, mycelial and blastospore stage as they exhibited SD (±CP) values <1 whereas *GAPDH*, *Tub-a* and *Tub-b* exhibiting SD (±CP) values >1 were most unstable genes. Finally, for conidial stage only *18S RNA* was the most stable gene whereas the remaining four housekeeping genes were most unstable. Subsequently, the expression of CDA in different morphological forms was studied by qRT-PCR using the primers designed based on the partial CDA sequence of *M. anisopliae* M161063 and the data obtained was normalized using *18S RNA* as a reference gene. The conidia from PDA slants (7 d) showed 1.1 fold repression of CDA whereas in germinating conidia (12 h), appressoria (24 h) and mycelia (24 h), it increased by 0.3-fold, 0.4-fold and 1.3 fold, respectively. Further, mycelia (48 and 72 h) and blastospores (24 h) showed a lower expression level of CDA compared to mycelia (24 h) and the CDA gene was again repressed in blastospores (48 h and 72 h). As expression of CDA in germinating conidia (12 h), appressoria (24 h) and mycelia (24 h) was upregulated, it was suggested to be involved in self defense of *M. anisopliae* for protection against insect chitinases. Thus, the results of the qRT-PCR studies supported the trend of the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 suggesting the differentiation specific regulation of CDA in *Metarhizium*. The present investigation has extended the understanding of the role of CDAs during fungus-insect interaction which could be used in future for improvement of the insecticidal properties of *Metarhizium* species for the control of insect pests.

Thus, the salient findings of the thesis can be summarized as follows:

- Screening of sixty eight *Metarhizium* isolates obtained from the soil samples of different crop fields and insect hosts was carried out. Based on the extracellular *in vitro* production of CDEs and mortality against *H. armigera*, LT_{50} and LC_{50} values as well as conidial germination rate and ST_{50} values, *M. anisopliae* M34412 was selected as the most effective isolate.
- The polymorphism in Pr1A gene of sixty eight *Metarhizium* isolates was studied and was observed to be correlated with *in vitro* protease activity and mortality of *H. armigera*. Out of the twelve isolates from highly pathogenic group possessing high protease activity (>2.0 U/ml) and >90% mortality of *H. armigera*, eleven isolates exhibited cumulative profile type I (D-I-M pattern). The protease activity

along with the polymorphism of Pr1A gene supported the selection of *M. anisopliae* M34412 as the most effective isolate.

- Due to repeated *in vitro* sub-culturing, the virulence of *M. anisopliae* M34412 against *H. armigera* was attenuated as the factors responsible for pathogenicity were affected and subsequent *in vivo* passage in *H. armigera* helped in regaining the virulence. The effect of repeated *in vitro* sub-culturing on CDA activity was more pronounced suggesting its possible use as marker to monitor the virulence of *M. anisopliae* during mass production.
- Out of sixty eight *Metarhizium* isolates, *M. anisopliae* M161063 possesing 82% mortality, high CDA (2.26 U/ml) and chitosanase (13.22 U/ml) activity and; low chitinase activity (1.01 U/ml) was selected for further biochemical and molecular studies of CDA. The intracellular CDA activity of *M. anisopliae* M161063 varied in different morphological forms and was increased in the differentiation structures viz. appressoria and mycelia predicting its role in self defense of the fungus during entry through insect cuticle.
- The partial nucleotide sequence (594 bp) of CDA gene from *M. anisopliae* M161063, encoding 198 amino acid residues was obtained and was further used for studying the differential expression of CDA in *M. anisopliae*.
- The five housekeeping genes namely *18S RNA*, *GAPDH*, *Ubc*, *Tub-a* and *Tub-b* in *M. anisopliae* M161063 were evaluated for the expression stability by qRT-PCR and *18S RNA* was selected as the most stable housekeeping gene. Using *18S RNA* as a reference gene, the expression pattern of CDA gene in different morphological forms of *M. anisopliae* M161063 was studied by qRT-PCR. The expression of CDA was upregulated in differentiation structures essential at the time of entry of the fungus through insect cuticle and thus extended the understanding of the role of CDAs during fungus-insect interaction.

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LIST OF PUBLICATIONS

Research papers:

1. Vidhate, R., Ghormade, V., **Kulkarni, S**., Mane, S., Chavan, P., Deshpande, M. V. 2013. Mission mode collections of fungi with special reference to entomopathogens and mycopathogens. *Kavaka* 41, 33-42.
2. Ghormade, V., **Kulkarni, S**., Doiphode, N., Rajamohanan, P. R., Deshpande, M. V. 2010. Chitin deacetylase: A comprehensive account on its role in nature and its biotechnological applications, In: Méndez-Vilas A. (Ed), *Current Research, Technology and Education Topics in Applied Microbiology, and Microbial Technology*. Formatex Research Centre, pp. 1054-1066.
3. **Kulkarni, S. A**., Ghormade, V., Kulkarni, G., Kapoor, M., Chavan, S. B., Rajendran, A., Patil, S. K., Shouche, Y., Deshpande, M. V. 2008. Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *Biocontrol Science and Technology* 18, 809-828.
4. Nahar, P. B., **Kulkarni, S. A**., Kulye, M. S., Chavan, S. B., Kulkarni, G., Rajendran, A., Yadav, P. D., Shouche, Y., Deshpande, M. V. 2008. Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Biocontrol Science and Technology* 18, 337-355.

Papers/posters presented in Symposia/Conferences/Meetings:

1. **Joshi (Kulkarni), S**., Ghormade, V., Deshpande, M. V. Perspectives of chitin deacetylase: Biological role and futuristic significance. Research Scholar Meet (RSM), National Chemical Laboratory, Pune, 2014.
2. **Joshi (Kulkarni), S.**, Neurgaonkar, P., Sali, S., Ghormade, V., Khire, J. M., Deshpande, M. V. Identification of virulence markers for the entomopathogenic fungus *Metarhizium anisopliae*. Indo-Mexico workshop, National Chemical Laboratory, Pune, 7^{th}-9^{th} October, 2013.
3. **Joshi (Kulkarni), S.**, Ghormade, V., Kapoor, M., Yadav, P. D., Deshpande, M. V. Biochemical and molecular characterization of proteases and their role in fungus-insect interaction. Research Scholar Meet (RSM), National Chemical Laboratory, Pune, 2012.
4. **Kulkarni, S.**, Ghormade, V., Kulkarni, G., Rajendran, A., Chavan, S., Shouche, Y., Deshpande, M. V. *Metarhizium anisopliae*: Identification of back-up strains

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5. Nahar, P., Yadav, P., Chavan, S., Kulkarni, G., Rajendran, A., **Kulkarni, S.**, Shouche, Y., Deshpande, M. V. *Metarhizium anisopliae*: Effect of *in vitro* sub-culturing on the fungus-insect interaction. International Conference on the Biology of Yeasts and Filamentous Fungi, NCL, Pune, 15th-17th February, 2007.
6. Kapoor, M., Kondawar, V., Chavan, S., **Kulkarni, S.**, Deshpande, M. V. Morphological transitions in entomopathogenic fungi *Metarhizium*, *Nomuraea* and *Beauveria*. International Conference on the Biology of Yeasts and Filamentous Fungi, NCL, 15th-17th February, 2007.
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