Dedicated to my loving family
and my dearest friends….

Summary

The overall objective of the work done in this dissertation was to investigate thirty one isolates of entomopathogenic fungi, including 17 *B. bassiana* and 14 *M. anisopliae* isolates for their biochemical characteristics at the enzyme and molecular level and transformation, mass production and formulation aspects were studied as well.

The first aspect of the thesis describes the biochemical characterization of the thirty one isolates and selection of isolates based on virulence candidate enzymes. The extracellular enzyme activity of cuticle hydrolyzing enzymes such as protease, chitinase and lipase was investigated. The induction and repression mechanisms of these enzymes were studied using different medium constituents. Pr1 activity was induced by supplemented protein in the media whereas chitinase activity was repressed by glucose. In-Gel enzyme activity was studied and a predominant chitinase of 58 & 14.3 kDa was observed in activity gels for *B. bassiana* and *M. anisopliae* isolates respectively whereas a 97 and 66 kDa protease was frequent for both *B. bassiana* and *M. anisopliae*. Two isolates were finally screened out (*B. bassiana* (UB9) and *M. anisopliae* (UM10) isolates) and used for further studies. The protease and chitinase enzymes from these two isolates were partially purified and characterized. The purified fraction was characterized on the basis of temperature, pH and effect of inhibitors. Molecular weight of the enzymes was detected on SDS-PAGE. A conventional 33 kDa chitinase was purified from *B. bassiana* and 23 kDa chitinase was purified from *M. anisopliae*. 47 & 43 kDa protease was purified from *B. bassiana* and *M. anisopliae* respectively. The genes of virulence determinant enzymes viz. protease and chitinase were studied as well. *Chitinase* and *protease* specific primers (two primer pairs for each gene and each isolate) were designed based on the conserved sequences. PCR amplified products were sequenced (Xcelris Labs) and sequences were analyzed

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by BLAST (BAST N & BLAST X) and conserved domains in the amplified sequence were detected by CD finder. 351 & 312 bp and 434 & 438 bp amplified fragments were observed for *B.bassiana* and *M. anisopliae* chitinase gene respectively. A 504 & 517 bp and 535 & 551 bp amplified fragments were observed for *B. bassiana* and *M. anisopliae* protease gene respectively. Sequence alignments showed conserved sequences are present in the amplifications. Pathogenicity potential of *B. bassiana* (UB9) and *M. anisopliae* (UM10) isolates against cotton-bollworm *Helicoverpa armigera* was investigated. Bioassay was performed using Diet Surface Technique and 5 replicates were taken for each treatment. Fungus was reisolated from the mycosed cadaver of *H. armigera* and several virulent factors of indigenous conidia and insect passaged conidia were investigated. *M. anisopliae* (UM10) was more effective against 1st and 2nd instar larvae of *H. armigera* than *B. bassiana* (UB9).

The second aspect deals with the studies optimizing protoplast yield from *M. anisopliae* (UM10) and *B. bassiana* (UB9) and transformation of these strains to herbicide resistance. Several factors were investigated influencing protoplast release from mycelium (*B. bassiana* (UB9) and *M. anisopliae* (UM10)) including effective cell wall lysing enzyme, osmotic stabilizer, mycelium age and enzyme incubation time. 10 mg/ml Lysing enzyme (Sigma) was proved to be the best enzyme for protoplast release and 0.7 M KCl was best osmotic stabilizer. Mycelium age must be 40 hr to obtain the optimized yield and maximum Lysing enzyme incubation period should be 3 hrs. The plasmid pBARGEM7-2 containing bar gene from *S.hygroscopicus* was multiplied and extracted from *E.coli* DH5α and inserted into the fungal protoplast from two species, *M. anisopliae* and *B. bassiana* through a PEG fusion method. Confirmation of the transformation was checked by PCR amplification using a designed bar gene

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specific primer. Furthermore transformation was biochemically confirmed by SDS-PAGE using whole cell proteins of both transformed and native isolates. Transformed isolates showed an expected 22-23 kDa PAT Protein.

The third aspect of this thesis describes the cost-effective mass multiplication of fungal conidia by Solid State Fermentation and different formulations of conidia. Effective factors for mass multiplication of conidia were screened out using Plackett-Burman (PB) experiment. Optimization of the screened out factors were done by Response Surface Methodology. Furthermore the prediction efficacy of RSM was compared with the Machine Learning Technique (MLT). Moisture content and Inoculum age are most significant variables for mass production of conidia by SSF and Yeast extract seems to improve conidia yield in case of *B. bassiana*. Interestingly predictive capability of the Machine Learning Techniques (MLT) is more accurate than Response Surface Methodology (RSM). Formulation of mass produced conidia from *Metarhizium anisopliae* and *Beauveria bassiana* was investigated in dry matrices (Diatomaceous earth, Kaolinite and Fullers earth), oils (Coconut oil, Mustard oil and Soybean oil), oil and water emulsions and conidial entrapment by Na-alginate beads. Percentage of germination was checked during different storage time and temperature. Dry formulations especially kaolinite clay and emulsion of soybean oil and water comparably performed better and can be used for long term storage. Storage at low temperature facilitates increased viability while viability decreases with increasing storage time.

APPENDIX 1

List of Abbreviations

DDT	Dichloro diphenyl trichloroethane
BHC	Benzene hexachloride
EPF	Entomopathogenic Fungi
BCA	Biocontrol agents
IPM	Integrated Pest Management
CME	Critical milestone events
PEG	Poly ethylene glycol
PPT	Phosphinothricin
PAT	Phosphinothricin acetyl transferase
SSF	Solid State Fermentation
ARSEF	ARS Collection of Entomopathogenic Fungi
SDA	Sabouraud Dextrose Agar
TCA	Tri chloro acetic acid
DMAB	para-dimethyl-Amino benzaldehyde
BAPNA	Benzoyl-phenylalanine-valinearginine-*p*-nitroanilide
GAGE	Gelatin acrylamide gel electrophoresis
SEM	Scanning electron microscope
PNP-NADG	Para nitro phenyl N-acetylglucopyranoside
pNPP	*p*-nitrophenyl palmitate
GIcNAc	N-acetyl glucosamine
PMSF	phenylmethylsulfonyl fluoride
DEAE-cellulose	Diethyl amino ethyl cellulose
EDTA	Ethylene diamine tetraacetic acid
BLAST	Basic Local Alignment Search Tool
PP1	Protease primer 1
PP2	Protease primer 2
CP1	Chitinase primer 1
CP2	Chitinase primer 2
CD finder	Conserved Domain finder
RH	Relative humidity
GM medium	Glucose Minimal medium
YM medium	Yeast Extract Minimal medium
FDA	Fluorescein diacetate
RF	Regeneration frequency
FGSC	Fungal Genetics Stock Centre
LB	Luria-Bertani
TSS buffer	Transformation and Storage buffer
MIC	Minimum inhibitory concentration
RSM	Response Surface Methodology
CCD	Central Composite Design
MLT	Machine learning technique

DT	Decision Tables
BFW	Biscuit Factory Waste
PB design	Plackett-Burman design
OVAT	One Variable At a Time
RMSE	Root Mean Squared Error

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1.1 Introduction

The most essential strategy to increase crop productivity is effective pest management system. Almost 45 % of annual food production is lost to pest infestation especially in tropical countries and the damage is severe due to high temperature and humidity facilitating rapid multiplication of pests. The modernization of agriculture enhanced the use of pesticides leading to 50 folds increase in the pesticide use since 1950. At present, India is the largest producer of pesticides in Asia and ranks twelfth in the world for the use of pesticides with an annual production of 90,000 tons (The Energy and Resources Institute, India). Vast majorities of the population in India (56.7 percent) is engaged in agriculture and are therefore exposed to the pesticides used in agriculture (Gupta et al., 2004; Government of India. Tenth five-year plan: 2002-2007). However, the sporadic use causes significant environmental and human health problems. The recent Endosulfan fiasco in Kasargod (Kerala, India) addresses the issue of deleterious effects of this agrochemical on populace. Endosulfan, a ramification of ‘Green Revolution’ in India, was extensively used in Kerala Plantations from 1976 though banned in 2003 the biomagnified affect left almost 8000 victims with neurological disorders, skin and respiratory ailments. The impact is severe on children as most of them are bedridden since birth and carry congenital defects. The use of synthetic pesticides started in 1948–49 with the use of DDT (**d**ichloro **d**iphenyl **t**richloroethane) for malaria control and BHC (**b**enzene **h**exa**c**hloride) for locust control. The maladies of DDT led to the banning of this agrochemical in 1960-70. It crossed placental barrier and babies were born with DDT contamination. Even the penguins of South Pole are not spared, as traces of DDT were noticed in them. In India DDT residues were observed in 82 % of bovine milk samples collected from 12 states (Toteja et al., 1993). High concentrations of DDT and BHC residues were observed in human serum samples of the agriculture related people who had direct exposure to these pesticides (Subramaniam and Solomon, 2006).

The strong impetus to find an effective alternative to chemical pesticides focused on a more environment friendly sustainable approach that exploits natural predators of insect pest; the entomopathogenic fungi (EPF). Fungal biological control is an exciting and rapidly developing research area to control invertebrate pests, and as such biocontrol agents (BCAs) could be used where chemical pesticides have been banned (eg. organochlorines) or being phased out (e.g. methyl bromide) or where pests have developed resistance to conventional pesticides. Almost 700 species of entomopathogenic fungi have been reported however only ten of these are in commercial use. Incessant reports of these fungi from both temperate and tropical regions confirm the pivotal role of EPFs in pest management. The largest programme employing EPF for biocontrol was undertaken by Peoples Republic of China to treat 1,000,000 ha of pine forest with the *B. bassiana* conidia for 3 years to control pine moth (Xu, 1988). In Latin American countries especially in Brazil, *M. anisopliae* conidia is produced by small companies to treat 1, 00,000 ha of sugar cane annually for spittlebug control (Roberts and Hajek, 1992). Factors involving EPFs that should be studied includes the nature of the fungal strain, quantity and quality of the inoculum, physiological state of the host population and density of the host population exposed to the inoculum (Hurpin, 1973). The EPFs are often applied for inundative augmentation of the fungi in the area where indigenous pest populations are limited to control pests effectively hence fungi are applied in large amounts for a short term control (Weiser et al., 1976). The application process is relatively similar to chemical insecticides consequently the approach has been termed as mycoinsecticide (Shah and Pell, 2003). Pathogens suitable for inundative applications are readily culturable in natural or artificial media to produce infective units and can be genetically altered with ease which makes them more amenable to analysis than fastidious pathogens (St. Leger and Screen, 2001).

Perceived advantages could be environmentally compatible, broad or narrow targets depending on organism, less prone to resistance whereas on the contrary subject to environmental influences, too specific or slow acting and may lack persistence to give long-term control. Due to its contact mode of action, EPF could be very useful against different developmental stages of insect where ingestion of food is impaired or absent. EPF have comparative advantage over other biocontrol agents such as *Bacillus thuringiensis*, which has single site of action with a higher risk of resistance development rather a multiple step mechanism of action where the risk is minimal. Despite the delayed onset of mortality due to harsh environmental conditions in the field, the crop consumption is significantly reduced between the period of infection and death. Thus the perspective on BCAs to be advantageous or not is just a matter of opinion.

Entomopathogenic Hyphomycetes are grouped under the phylogenetically diverse Deuteromycota phyla and characterized by mycelial forms that bear asexual spores. Members of Hyphomycetes class under Deuteromycotina phylum contain over 40 entomopathogenic genera distributed worldwide (Khachatourians et al., 2002). In terms of ecological niche, EPFs are found both in aquatic and terrestrial habitats. The natural soil borne entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* can be used efficiently for the suppression of insect pests and can be used as a potential biocontrol agent and indeed can be implemented as a sustainable approach in Integrated Pest Management (IPM) system.

The host pathogen interaction could be attributed to the knowledge of associative events in the early phases of interaction and subsequent events of cuticle penetration with the help of an infectious structure ‘appresoria’. Once through the cuticle it evades host defense mechanism and proliferates as hyphal bodies i.e. yeast like structures which multiply by budding. The fungus spreads through the haemocoel and produces a number of allelochemicals which acts as toxin and responsible for death of the insect by triggering initially tetanic and then flaccid paralysis. Host death results from the cumulative effect of mechanical damage and enzymatic digestion of insect tissues, production of mycotoxins and finally utilizing the host as a source of carbon and nitrogen. After the nutrient exhaustion by the fungal parasite, fungi emerge from the host cadaver as spores to infect other hosts (Fig 1.1). The rate limiting step for chemical pest control agents is adsorption whereas EPFs have a multistep and complex process of physical interaction, germination and cuticle penetration and each process have physiological, physical and chemical properties (Jeffs et al., 1999). Variable LT_{50} values, ranging from 2 to 12 days, were observed for entomopathogenic *B. bassiana* isolated from different regions worldwide. The concept of “critical milestone events” (CME) is supposed to determine the minimum time required for the biocontrol process i.e. there is a threshold time which is required to reach the point where the insect host demise is irreversible and death is unavoidable (Hegedus and Khachatourians, 1996).

Fungal invasion initiates with the formation of infectious peg appresoria which secretes an array of extracellular cuticle hydrolyzing enzymes viz protease and chitinase.

Cuticle degrading enzymes from entomopathogenic fungi can be attributed for the comparison of isolates differing in pathogenicity. Virulent isolates should be selected and implemented in IPM.

The immense scope of genetically manipulating EPF is being explored in order to improve its performance. Strain improvements have been attempted with parasexual crossing or protoplast fusion but genetic engineering by directed addition of one or more genes is more flexible approach. Genes conferring resistance to herbicides makes it a lucrative option to use EPF along with the herbicides to protect the crop with dual mode of action.

The inundative strategy for biocontrol typically involves the mass production and application of a formulated product. Applied research and development of mycoinsecticides relies on the advancements in appropriate production and formulation techniques. Development of industrial scale production of EPF with economical downstream processing leads to the mass production by Solid State Fermentation. Formulation of the mass produced conidia addresses several major issues of viability of fungi, dispersibility, suspendibility and necessarily compatibility with other agricultural products required for crop protection.

This present study illustrates these above mentioned three aspects of EPF. Biochemical characterization of *B. bassiana* and *M. anisopliae* isolates were performed to study different aspects of the virulence determinant enzymes chitinase and protease. Improved selection of isolates based on the enzyme activity was also an objective in this study. Protoplast yield optimization and transformation were also studied for the improvement of the fungal strain. The cost-effective mass multiplication of EPF conidia was investigated and formulations of conidia to increase the viability after long term storage were also studied. Finally compatibility with a chemical pesticide was tested for dual application and effective implementation in the Integrated Pest Management.

1.2 Aspect 1: Biochemical Characterization of *B .bassiana* and *M .anisopliae* isolates

The proteins produced in the culture media have an important role in physiological processes of the fungi in the environment. However, the evaluation of these metabolites with respect to different parameters in screening programs and genetic breeding of isolates depends on the understanding of the basic aspects related to their development in the culture media.

The infection process occurs through the external cuticle of the insect host. The conidia of mitosporic fungi adhere to the host cuticle and germinate to produce an infectious propagule, appresoria (Inglis et al., 2001). In entomopathogenic fungi these infectious structures secretes extracellular enzymes in an orderly manner starting with proteolytic enzymes, then chitinases and finally lipase (St. Leger at al., 1986a). Proteins may account for up to 70 % of the cuticle hence proteases are the most important and earliest enzymes involved in the invasion followed by chitinase after the eventual exposure of chitin in the host cuticle after the proteolytic degradation of cuticular proteins (Smith et al., 1981; St. Leger at al., 1986b). Cuticle degrading enzymes from entomopathogenic fungi can be attributed for the comparison of isolates differing in pathogenicity. Highly pathogenic strains showed detectable amounts of extracellular chitinase, lipase, and protease activities (Samuels et al., 1989). These enzymes are utilized in cuticle penetration. Fungal proteases are considered critical in facilitating cuticle breach (Charnley and St. Leger et al. 1991). The studies of protease enzymes from

B. bassiana and *M. anisopliae* focused on the production of majorly serine type proteases which includes chymoelastases (Pr1), metalloprotease, and trypsin like enzyme (Pr2) and one minor cysteine type protease (Pr4). Several studies have been associated with protease production and role of proteases in virulence (Goettel et al., 1989; Kucera 1980; St. Leger et al., 1987). The existence of wide genetic variability in the production of these enzymes was also observed in EPFs (Braga et al., 1994; Oliveira and Messias, 1996). Natural variability was observed in the production of cuticle degrading chymoelastase type protease Pr1 among isolates of *M. anisopliae* (de Silva pinto et al., 2002). Pr1 is principal enzyme produced during the infection though Pr2 appears first in this process. It was reported that antiserum Pr1 protease interfered with the host cuticle penetration and ultimately resulted in reduced infection suggesting that the level of Pr1 directly related to the capacity of fungus to cause the disease (St. Leger et al., 1988). The role of trypsin type protease (Pr2) in invasion is not yet comprehended though it is considered to complement Pr1 protease in splitting the peptide bonds in the insect cuticle. Chitinase activity is often associated with the fungal virulence as studies utilizing *Beauveria bassiana* and *Nomuraea rileyi* have suggested that virulence against lepidopteron larvae may be correlated with chitinase activity (EL-Sayed et al., 1989; Samsinakova et al., 1971). Quantitative biochemical characterization of *B .bassiana* isolates was conducted by Daque and Arango (1998) with the goal to select the best isolate for the biocontrol of coffee berry borer, *Hypothenemus hampei* and this study was based on three criteria, 1) enzymatic activity, 2) factors which determine the process of virulence, 3) survival in natural conditions. Enzymatic response was variable for chitinase and lipase productions by isolates. The regulation of the genes encoding these enzymes is complex and may involve a combination of carbon-nitrogen induction/repression (Screen et al., 1998). Several human pathogenic fungi such as *Candida albicans* have shown that genes required for virulence are regulated in response to environmental signals native to host niche (Mekalanos, 1992), the feature particularly prominent in opportunistic saprophyte *Aspergillus nidulans* (Caddick et al., 1986). Regulation of gene expression by pH was observed in entomopathogenic *M. anisopliae* which includes cuticle degrading subtilisin type proteases and other putative virulent factors (St. Leger et al., 1998) though likelihood of altering the surrounding environment by entomopathogenic fungi as a mechanism of adaption and survival has not been extensively studied. Chitinolytic enzymes in EPF have been reported to be induced and repressed by end products of chitinase isozymes present within a chitinolytic system. *B. bassiana* also has a well developed chitinolytic system which can be induced by a number of chitooligosachharides (Smith and Grula, 1983). St. Leger et al. (1991) investigated the purified fractions of crude chitinase from *M. anisopliae* ME1 and observed different isozymes showing endo and exo chitinase activity. Barreto et al. (2004) has reported the effect of GlcNAc on chitinase production by *M. anisopliae*. Modern tools of biochemistry and molecular genetics could be useful for the study of pathogenicity, species dissemination and most importantly isolation of virulence determinant genes. Isolation and analysis of these genes could be useful in studying their regulation, structure-function relationship, evolution and synergistic relationship with other enzymes. A novel chitinase gene was isolated from *M. anisopliae* grown in a medium containing chitin as the sole carbon source, which has only 24-36 % sequence similarity with other fungal chitinase genes and molecular mass determination of the fused gene product showed it to be a 70 kDa protein (Kang et al., 1998). Chitinases from *T. harzianum* have been characterized (De la Cruz et al., 1992; Haran et al., 1995). Their coding genes have been cloned and their expression was monitored when parasitizing other fungi, thus establishing their role in infection (Chet et al., 1997; Zeilinger et al., 1999). Bogo et al. (1998) reported a complete sequence of *M. anisopliae* chitinase gene *chit1* with three short typical fungal introns and a 1521 bp ORF. Increased insect virulence was observed in *B. bassiana* strains overexperessing an engineered chitinase (Fan et al., 2007). A cDNA clone of protease gene was isolated from *B. bassiana*. The cDNA sequence revealed that Pr1 is synthesized as a large precursor containing signal peptide and a propeptide (Joshi et al., 1995). Insight to the mechanism of pathogenicity can provide the knowledge for the development of potent mycoinsecticide by identifying the virulence factors or else identifying the genes responsible for virulence and genetically modify the fungi to enhance virulence (St. Leger et al., 1996). The multigenic nature of pathogenicity could be investigated through isolation of several catabolic genes through homologous gene sequence probing of genomic DNA. Such studies in conjunction with cloning and sequencing of genes encoding cuticle degrading enzymes could develop improved isolates using rDNA technologies (Khachatourians et al., 2002).

1.3 Aspect 2: Protoplast studies and transformation of the selected isolate to herbicide resistance

Due to its position in phylum Deuteromycotina, Hyphomycetous EPFs have been difficult subjects for genetics studies. It requires a collection of biochemical and developmental information to build an isogenic background. The most preferred method to genetically manipulate the fungi exploits the isolation of the protoplasts.

Isolation of fungal protoplast is a constructive approach to understand the physiology, biology of fungi and most importantly the fungal genetics. Protoplast is referred to a cellular structure without the cell wall. The formation of protoplast is dependent on factors like culture condition, age of mycelium, pre incubation in reducing agents, choice of osmotic stabilizers and most importantly choice of enzymes and its concentrations. Fungal cell wall constitutes mainly different kinds of polysaccharides, generally glucosamines, in their acetylated form, for instance chitin or chitosan, polymers of N-acetyl glucosamine which is mostly present on the cell wall of the fungi imperfecti. The cell wall of the model filamentous fungi *Neurospora crassa* composed of four major types of complex, one peptide-polysaccharide complex, two glucose polymers and chitin (Mahadevan and Tatum, 1965). The mostly preferred technique for the production of fungal protoplasts is cell wall digestion by lytic enzymes, so the choices of the lytic enzymes in turn implicitly dependent upon the cell wall composition of different fungal taxa. The complete cell wall lysis of *Rhizopus delemar* was achieved by the combined action of purified lytic enzyme, protease and chitosanase from *Bacillus* R-4 and chitinase II from *Streptomyces orientalis* (Tominaga and Tsuzisaka, 1980). An enzyme preparation from *Trichoderma harzianum,* grown on *Schizophylhtm commune* hyphal walls, can release spheroplasts from 33 species of basidiomycetis which is much more effective than commercial enzyme preparation, Helicase (Vries and Wessels, 1973). Among the commercial lysing enzyme preparations Novozyme-234 (Novo Industry A/S, Denmark) was assumed to be the most effective one. It is a multienzyme preparation containing primarily, α-1, 3-glucan glucanohydrolase and small quantities of cellulose, protease, α-1, 3-glucan glucanohydrolase activity (Varma and Kwon-Chung, 1991). A combination of

Novozyme 234 and Cellulase released a high yield of protoplast from *Pleurotus* mycelia (Lau et al., 1985). Various species of *Aspergillus* released high amount of protoplast when *Aspergillus* mycelium is treated with Novozyme 234 and Cellulase CP (Hamlyn et al., 1981). Entomopathogenic fungus *Metarhizium flavoviridae* mycelium treated with 5 mg/ ml Novozyme 234 and Cellulase CP each, for 2 h at 30 ˚C, produced almost 10^8 protoplasts per ml (Valadares-Inglis and Inglis, 1997). A valuable alternative could be Lysing enzyme preparation from *Trichoderma harzianum* (Sigma Chemical, St. Louis, USA). Dhitaphichit and Pornsuriya (2005) reported that Lysing enzyme preparation (9 mg/ ml) can produce protoplasts up to 10^6 when incubated with *Pleurotus ostreatus* mycelia. Lysing enzyme incubation period is crucial for the protoplast release from the mycelia. Robinson and Deacon (2001) reported that, increase in the protoplast yield was achieved during the first 2.5 h, with the highest protoplast release at this time and then declining quickly. Typically 2-3 h lytic enzyme digestion of the fungal mycelia is enough to produce considerable amount of protoplast (Hamlyn et al., 1981). It has been observed that in case of *Phanerochaete chrysosporium*, protoplast release was linear through the 3 h lytic enzyme digestion, after that number of protoplast decreased considerably (Gold et al., 1983). Yield of protoplast is high using young mycelia. Protoplast isolated from filamentous fungi *Collybia veltipes* and *Pleurotus ostreatus* remarkably affected by the mycelium age. Almost 10^7 protoplasts were produced for each species with 2-3 days old culture; older mycelium 7-8 days old cultures conferred low yields (Yamada et al., 1983). Employing proper osmotic stabilizer is a vital issue in the preparation of protoplasts. A protoplast without its cell wall is quite vulnerable due to osmotic pressure of the environment therefore to maintain the stability of the protoplast a proper osmotic stabilizer is required. The preference of osmotic stabilizer could differ depending on fungal species. Both organic and inorganic stabilizers can be used though in most of the cases KCl, $MgSO_4$, sucrose or mannitol are frequently used in the range of 0.4-0.8 M (Bachman et al., 1959; Gull et al., 1972; Hamlyn et al., 1981). The viability of the isolated protoplasts is ensured by reviving the protoplasts into mycelial form. Yet again the osmotic stabilizer plays a very important role. The regeneration frequency is dependent on the choice of osmoticum. For *Rhizoctonia solani*, mannitol at a concentration of 1 M showed 87 % regeneration frequency whereas NaCl and sucrose showed very poor results (Robinson and Deacon, 2001). Uptake of DNA by endocytosis is a primary mechanism which occurs in mammalian cells and *S. cerevisiae* though in filamentous fungi the exact molecular mechanism is yet not elucidated (Khalil et al., 2006; Neukamm et al., 2002). The most common method for fungal transformation utilizes PEG fusion method although higher transformation frequency can be achieved through biolistic method (Barreto et al., 1997). Enhanced frequency of *B. bassiana* blastospore transformation was observed by restriction enzyme mediated integration and electroporation compared to other methods though PEG/ Ca^{2+} is favored for its simplicity and reproducibility (Jiang et al., 2007; Olmedo-MonWl et al., 2004). Couteaudier et al. (1996) reported that highly virulent hybrid strains of entomopathogenic fungi *B. bassiana* can be obtained by protoplast fusion using PEG/ Ca^{2+} method. A different approach uses *Agrobacterium* mediated genetic transformation (Dos Reis et al., 2004). The first established stable transformants in filamentous fungi were obtained utilizing PEG/ Ca^{2+} method for *Neurospora crassa* (Hinnen et al., 1978; Case et al., 1979). The concentration of Ca^{2+} used in the transformation of filamentous fungi is typically in the range of 10-50 mM which is actually the concentration of external Ca^{2+} that causes transient increase of cytosolic Ca^{2+} in filamentous fungi (Nelson et al., 2004). PEG is known to cause fusion of protoplasts in filamentous fungi (Anne and Peberdy 1975) hence fusion could be the probable way of extracellular DNA uptake in filamentous fungi (De Vries et al., 2003). However, according to Kuwano et al. (2008); the role of PEG in transformation was assumed to be intracellular rather than functioning outside of the cell.

Various approaches have been investigated to select the transformants. The former version of selecting the transformant colony utilizes auxotrophic parental strains. However, this method is time consuming and labor intensive to screen the mutant parental strains. An efficient alternative is the use of dominant selectable resistance markers. Furthermore, variation in the electrophoretic patterns of particular enzyme was the basis for distinguishing isolates but such techniques are not suited to the efficient monitoring of strains under field conditions; introducing selectable heterologous genes by transformation could be an effective alternative (Moreira et al., 1996; Inglis and Inglis, 1997). The bar gene, which codes for the enzyme Phosphinothricin (PPT) acetyl transferase (PAT), is an established selectable marker of genetically modified crops and confers tolerance against glufosinate ammonium, an active ingredient of herbicide Basta. Glufosinate ammonium is a broad-spectrum contact herbicide which is used to control a wide range of weeds. Glufosinate ammonium inhibits the activity of an enzyme, glutamine synthetase, which is necessary for the production of glutamine and for ammonia detoxification produced during photorespiration and inorganic nitrogen assimilation (Wallsgrove et al., 1983). The application of glufosinate leads to reduced glutamine and increased ammonia levels in the plant tissues which is also found to inhibit appresoria formation and conidial germination in fungi (Ahn et al., 2008). Treatment of glufosinate ammonium on rice produced resistance against plant pathogenic *Magnaporthe grisea* and *Rhizoctonia solani* (Uchimiya et al., 1993; Tada et al., 1996). Glufosinate ammonium reduces fungal diseases such as brown patch and dollar spot caused by *R. solani* and *Sclerotinia homoeocarpa*, in transgenic glufosinate-resistant bentgrasses (Higgins et al., 2003). The effectiveness in the dual exploitation of glufosinate ammonium along with the fungi makes it a lucrative option in transformation as it can be used as a dominant selectable marker as well as the recombinant fungus can be used to control pests in combination with this herbicide. The resistance against glufosinate ammonium was conferred in entomopathogenic *Paecilomyces fumosoroseus* utilizing bar gene from *Streptomyces hygroscopicus* under the control of the *Aspergillus nidulans trp*C promoter and terminator sequences (Cantone and Vandenberg, 1999). PEG mediated co-transformation of hygromycin B resistance in conjunction with green fluorescent protein genes in *Verticillium fungicola* yielded transformation frequency of 102 transformants per µg of DNA (Amey et al., 2002). The goals of genetic modification of fungi intended to expand host range, increase the speed of kill by incorporating insect toxic genes and extend persistence. Genetically altered organisms are unique in nature and possess potential hazards which could only be evaluated once the pathogen has been released into the environment hence it would be desired to engineer fungi in such a way as to minimize their ability to persist in nature.

1.4 Aspect 3: Cost-effective mass multiplication of fungal conidia by Solid State Fermentation and formulation

Most entomopathogenic fungi produce large quantities of hydrophobic conidia under natural conditions. Emphasis has been given to the production of conidiospores as this infective propagule has ready to use characteristics. However under field conditions the infectivity of individual conidia is not significant therefore high doses of conidia, 10^{13}-10^{14} spores ha^{-1}, is required to control pests in the field (Bartlett and Jeronski, 1988). Solid media production of conidia is preferred as conidia produced in this method are typical aerial conidia i.e. conidia produced on solid media are considered to be similar to those produced on insect cadavers (Ye et al., 2006; Roberts and St. Leger, 2004). The major issue regarding the compatibility of the mass produced material with the formulation and application techniques should be considered. The lipophilic conidia and aerial hyphae produced during this process are more endurable to environmental stresses than the hyphae from submerged fermentation due to the presence of monomeric form of hydrophobin on the surface of mycelia rather than polymerizing as it happens in case of aerial hyphae (Jenkins and Prior, 1993; Wessels, 1997). But it has limitations in terms of volumetric productivity, on the contrary yields of conidia in submerged fermentation is low. Moreover extraction of conidia from the liquid media is costly and labor intensive process. The limitations of solid media productions could be managed by using relatively large quantities of inexpensive and nutritious solid substrates. Mass multiplication of fungal conidia utilizing cost effective solid substrates is a new area to exploit. Solid State Fermentation (SSF) is preferred in order to produce many industrially important enzymes and antibiotics due to high productivity, lower cost and easier downstream processing

(Adinarayana et al., 2003; Martins et al., 2002). SSF can be defined as growth of a microorganism on solid substrate in the absence or near absence of free liquid, but the substrate must possess enough moisture to support fungal growth (Moo-Young et al., 1983). The solid material can serve as a carbon and nutrient source promoting fungal growth or it can act as an inert matrix which is supplemented with carbon and nitrogen sources (Pandey, 2003). Conidia production of entomopathogenic fungi by SSF on whole grains is being practiced in Latin America for a long time (Grimm, 2001). This production process involves a biphasic system where a liquid culture is obtained at first by mycelium harvesting and then using the liquid culture as an inoculum for the solid substrate (Mendonca, 1992). Rice is the most popular solid substrate for conidia production of entomopathogenic fungi due to its complex nutritional source (Posada-Flórez, 2008; Ye et al., 2006). A comparative study shows production of 10^{13} conidia, costs the same as chemical insecticides used per hectare. This high production efficiency leads to the development of consciousness to mass produce *B. bassiana* conidia in a cost effective way (Wright et al., 2001). Lower production rate of conidia and aeration problem due to highly viscous culture restricts submerged fermentation from effective commercialization, giving an immense scope to the SSF (Wright et al., 2001). Advancements in the SSF lead to the optimization of culture conditions to maximize the conidia production. Conventionally one variable at a time approach was used in optimization study though simple; this method is laborious and time consuming. Though optimization of factors influencing product formation is an extensively studied area, very few reports are available on detailed study of fermentation variables using statistical methodologies or other useful mathematical modeling tools (Nair and Panda, 1997;

Pandey et al., 1994; Prakash et al., 2007). Optimization studies help in understanding interactions among the nutrients at varying concentrations. Response Surface Methods are empirical statistical tool that are being used for modeling and analyzing problems where the response is influenced by several independent variables and the aim is to maximize the process variables to get an optimum response (Montgomery, 2001). Filamentous fungi are the extensively used microorganisms in SSF due to their ability to grow on nutritionally complex solid substrates and produce a wide range of products (Lee et al., 2003; Castilho et al., 2000). Optimization of different variables could enhance the conidial yield. Physical factors in addition to media components could be studied in an effort to optimize the conidia yield. Proper nutritional and pH requirements have been documented in entomopathogenic fungi (Hallsworth and Magan, 1996). Yeast extract is observed to be essential for the vegetative growth of four entomopathogenic fungi (Im et al., 1988). Moisture content of the substrate is another key factor influencing conidia yield. Nampoothiri et al. (2004) reported an initial moisture content of 65.7 % as the best condition for chitinase production from *T. harzianum* while significantly affecting the enzyme production with increasing or decreasing moisture content level. Moisture requirement could be different for individual substrates. Deshpande (2001) reported higher water requirement for the production of *N. rileyi* on wheat compared to other substrates such as rice sorghum and barley. All the strategies involved in mass production of conidia, the quantity and quality of the mass produced material should be reliable.

The development of fermentation and formulation process must be a coordinated effort in order to reduce costs and maximize product efficacy. The widespread acceptance and use of mycoinsecticides will depend on improvements in the development of formulations that will enhance virulence, extend the shelf life of the pathogen, improve efficiency of application, and prolong field persistence (Wright et al., 2001). Eleven different technical grade active ingredients or formulation types have been identified till date, with technical concentrates (fungus-colonized substrates) (26.3 %), wettable powders (20.5 %) and oil dispersions (15.2 %) being most common (de Faria and Wright, 2007). Approximately 43 % of all products were developed by South American companies and institutions. Unfortunately, little progress has been made in the improvement of existing formulations. Oil-based formulations show promise for the control of some pests (Prior et al., 1988). It is assumed that oils reduce the dependency on saturated conditions normally required for successful infections (Bateman, 1997). Exactly how the oils may facilitate this is unclear. It is feasible that oils release nutrients or dilute fungistatic compounds in the epicuticle which may stimulate or inhibit germination and infection. Different vegetable oils and sometimes mineral oils are preferred for preparing conidial emulsion. Almost 70 % of *M. anisopliae* conidia retained stability after 10 days of invert oil emulsion prepared in various oils from plant origin (Batta, 2003). Dry formulations in solid matrices occasionally proved to give better stability compared to oil. Matrices like Kaolin, diatomaceous earth and talc can be used efficiently as conidial formulation using these materials could increase the shelf life of the product; almost 85 % of conidial viability was observed after 12 months of storage at 4 °C (Shi et al., 2008). The nutritional environment present in the production medium could influence the fitness of the conidia and their endogenous reserve (Wright et al., 2001). Shelf life of the unformulated conidia produced on barley was high compared to other cereals (Prakash et al., 2007). The combined use of different pathogens or use of EPFs along with agrochemicals with different biological attributes in terms of biotic and abiotic factors to maximize the control of target pests as well as a number of pests on same crops. Numerous instances of improved efficacy have been reported in biocontrol of pests combining insecticides with EPFs (Quintela and McCoy, 1998; Purwar and Sachan, 2006). Some commercially developed wettable *M.anisopliae* formulations are 'Bioblasts' to control termites, and 'Green-Muscles' to control locusts and grasshoppers.

1.5 Summary and Conclusion

In summary, the selection of virulent isolates based on extracellular enzyme production could be considered as an effective strategy to improve efficacy to biocontrol pest population. The knowledge of enzyme regulation and genes encoding those enzymes could facilitate this objective. Genetically altering the fungi for better field persistence could be another way to increase the effectiveness. The ability of entomopathogenic Hyphomycetes to produce infective propagules on solid substrate exploits various agro and agro-industrial residues in an effort to mass produce conidia. Preserving the product quality upon storage leads to the development of formulation techniques that increase the product's shelf-life and conidial viability. The influence of various agrochemicals on entomopathogenic fungi could develop better understanding of interactions of EPFs with agrochemicals. It may be possible to select isolates which are naturally less susceptible therefore implementation of EPFs with agrochemicals to target a wide array of pests in IPM is promising. In conclusion, the assessment of previous studies described in this chapter addresses the major issues regarding entomopathogenic fungi and led to the investigations presented in this thesis.

OBJECTIVES

1. Studies on general characteristics (Growth, Sporulation, Germination) of *B.bassiana* and *M. anisopliae*.
2. Screening of *B. bassiana* and *M. anisopliae* isolates for the production of enzyme, Chitinase and Protease.
3. Purification and characterization of the enzymes, Chitinase and Protease.
4. Studies on virulence candidate genes in relation to enzyme activity and virulence.
5. Protoplast studies and transformation of the selected isolate to glufosinate ammonium resistance.
6. Mass production of conidia using cost-effective solid substrates and statistical optimization of different factors.
7. Formulation of mass produced conidia (oil and wettable powders).

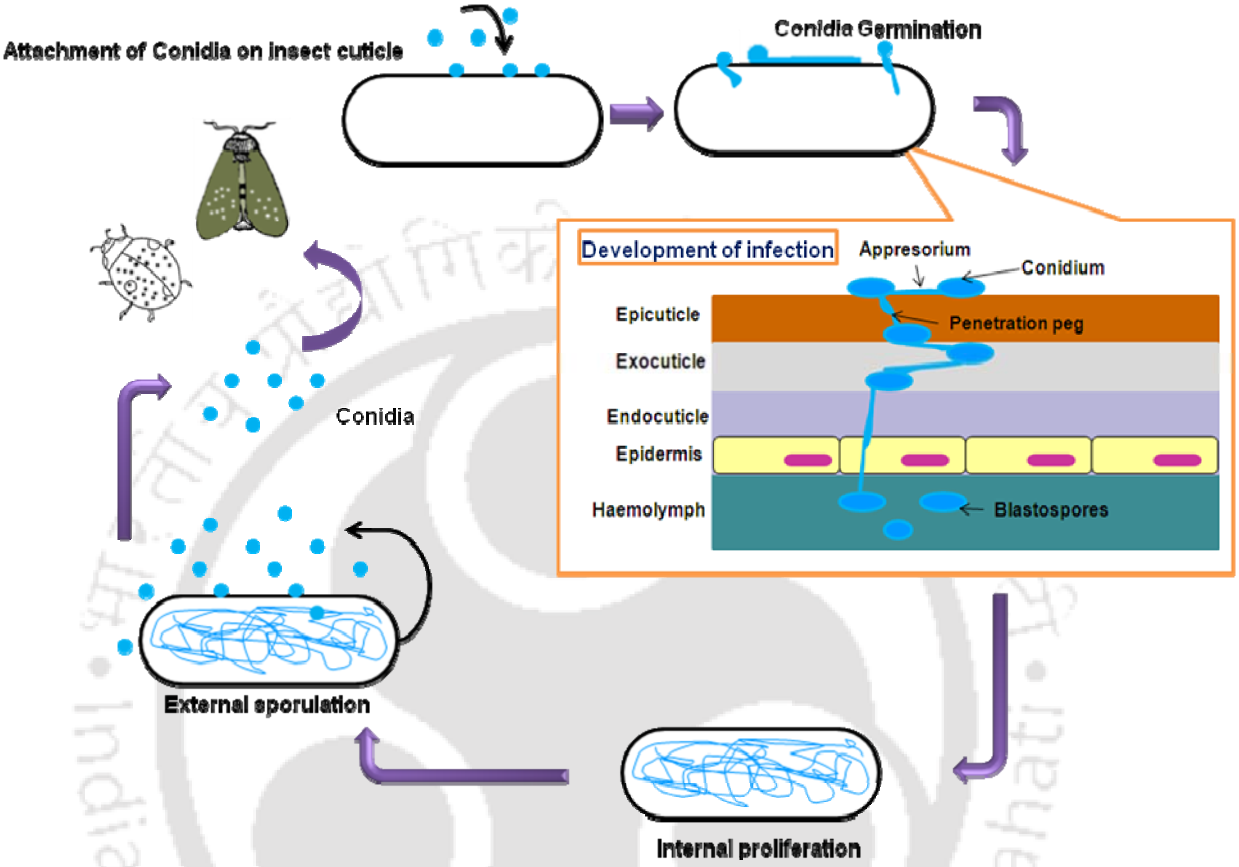
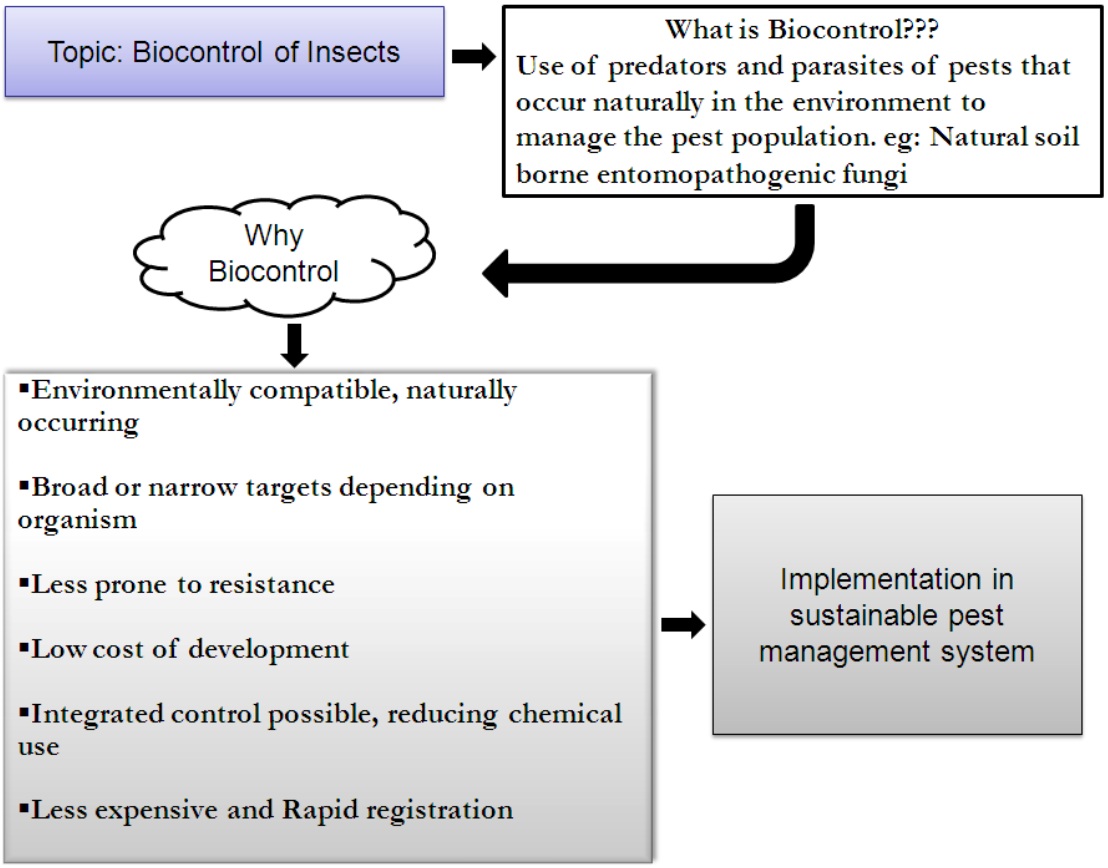


Fig 1.1. Generalized diagram of EPF infection and life cycle



What is Biocontrol???
Use of predators and parasites of pests that occur naturally in the environment to manage the pest population. eg: Natural soil borne entomopathogenic fungi

- Environmentally compatible, naturally occurring
- Broad or narrow targets depending on organism
- Less prone to resistance
- Low cost of development
- Integrated control possible, reducing chemical use
- Less expensive and Rapid registration

Flowchart 1.1. A synopsis of Biocontrol

Characterization of *B. bassiana* and *M. anisopliae* isolates for Growth, Sporulation and Germination

Introduction

The application of natural predators of insect pest for pest management is a preferred choice at present. The implementation of microbial pesticide for crop protection includes diverse genera of bacteria and fungi. The concern for the development of hyphomycete fungi as suitable biocontrol agent of insect pests leads to the isolation of various insect pathogenic fungi and amongst them the most studied are *Beauveria bassiana* and *Metarhizium anisopliae*. The conidia of mitosporic fungi adhere to the host cuticle and germinate to produce an infectious propagule, appresoria that produce a sequential release of extracellular enzymes to breach the insect cuticle (Inglis et al., 2001). Isolates of entomopathogenic fungi differs in their genetic ability and mortality. Penetration, colonization and sporulation capacity was observed to be high for *M. anisopliae* isolates compared to *B. bassiana* that ultimately results in earlier death of the infected insect (Moino et al., 2002). According to Heale et al. (1988) pathogenicity is the interaction of different virulence traits determining germination and invasion of host cuticle followed by successful growth inside the insect host ultimately causing the mortality. Highly virulent isolates of *B. bassiana* germinates quickly and can orientate on the cuticle (Charnley & St. Leger, 1991). Entomopathogenic fungi are mostly facultative with both parasitic and saprophytic stages of growth and easy to culture in synthetic media compared to obligate fungi which can only be cultured in-vivo (Ghanbary et al., 2009). The nutrient deprivation could be the cause for the shift of the mode of growth; from saprophytic to pathogenic. Acquaintance with the fungal growth, sporulation and germination rate could provide a primary basis for strain selection. Liu et al. (2003) characterized *B. bassiana* and *M. anisopliae* isolates for the management of tarnished plant bug depending on physiological characteristics viz, conidial viability, capacity of sporulation, speed of germination and growth rate. Present study illustrates the three primary aspects of entomopathogenic fungi i.e. growth, sporulation and germination in detail.

Materials & Methods

Microorganism and culture condition

Fungal cultures of *B.bassiana* and *M.anisopliae* were obtained from ARSEF (USDA-ARS Plant Protection Unit, Ithaca NY). The *B.bassiana* and *M.anisopliae* isolates were designated as UB1-UB16 and UM1-UM13 respectively and two isolates AB1 and AR1 were from India. The isolates with their accession number, host insect and geographical origin are detailed in Table1 and 2. The isolates were routinely subcultured on SDA (Sabourauds Dextrose Agar) slants at 28 °C.

Fungal growth and sporulation

Conidial suspension was made in 0.02 % Tween-80 solution using seven to ten day old cultures on SDA plates. A 200 µl of this conidial suspension (1×10^7 spores/ ml) were spread plated on SDA medium. After 3 days of incubation at 28 ˚C, 5mm agar disc were taken with the help of cork borer and placed in the middle of fresh SDA medium (5 replicates were maintained for each isolate) and incubated at 28 °C. Radial growth was measured from 3rd day onwards till 8th day. Sporulation was measured by taking a 5 mm agar disc at the end of 8th day and placed into 0.02 % Tween-80 solution and vortexed to suspend the spores. Spore count was determined by using a haemocytometer.

Germination

Agar slide technique was used for studying the rate of germination. Glass micro slides were spread with 1 ml SDA media and put in a Petri plate. Each plate contained two slides and was lined with blotting paper disc. Conidial suspension at 1×10^6 was made in 0.02 % Tween-80 solution. Approximately 100 µl of this solution were spread onto the SDA coated slides and plates containing the slides were incubated at 28 °C (2 replicates/ isolate). The slides were observed under compound microscope (40 x) for germination, every 2 h. A conidium was considered to be germinated when a distinct germ tube projected from it and was at least twice the diameter of the conidium (Milner et al., 1991). Percentages of germination were checked till 100 % germination. Almost 100 spores were scored per slide.

Results

Growth and sporulation

Assessment of growth, sporulation, and germination is important features in the basic characterization of entomopathogenic fungi. *B. bassiana* produced white colonies whereas characteristic green colonies were observed for *M. anisopliae* (Fig 1). Among the seventeen isolates tested UB12 demonstrated good results, 1.69 cm of radial growth after eight days of incubation period, followed by UB15, UB10 and UB9 with 1.59 cm, 1.44 cm, 1.38 cm respectively (Fig 2a). But in case of UB12 and UB15; less conidial yield was observed than UB9 and UB10 (Fig 3a). Highest sporulating isolate was found to be UB11 giving 8.5×10^8 spores. The isolates showing good growth do not necessarily have high levels of sporulation. Isolates UB10 showed moderate growth and sporulation. Growth was higher in case of *M. anisopliae* than *B. bassiana* isolates (Fig 2b). Isolate UM13 showed most excellent growth, 2.25 cm among all the isolates although sporulation capacity was less, 3.6×10^7 conidia/ ml compared to highest sporulating *B. bassiana* isolate UB11.

Germination

The time taken for 100 % conidial germination varied largely among isolates (Fig 4). Conidia from almost all the isolates started to germinate from the ten to twelve hour post inoculation. The *B. bassiana* isolate UB12 and AB1 showed relatively early germination when compared to other isolates as 100 % germination was recorded on 16th and 18th h respectively (Fig 4a). A higher time period was required for 100 % germination for isolate UB6, UB13, UB14, UB15, showing total conidial germination at 20th h. For highly sporulating isolates like UB10 and UB9, 100 % germination occurred almost on 28th and 30th h respectively. 100 % germination was recorded for all the seventeen isolates within 30 h of culture. Germination is one of the most important and the initial steps for cuticle penetration. In general *Metarhizium* isolates were found to be fast germinating ones but *B. bassiana* isolate UB12 shown an exception to reach 100 % germination level within sixteen hours and was the fastest germinating isolate among all the 31 isolates investigated followed by AB1, UM3,UM9, UM10 and UM13 reaching 100 % germination level within 20 h (Fig 4b).

Discussion

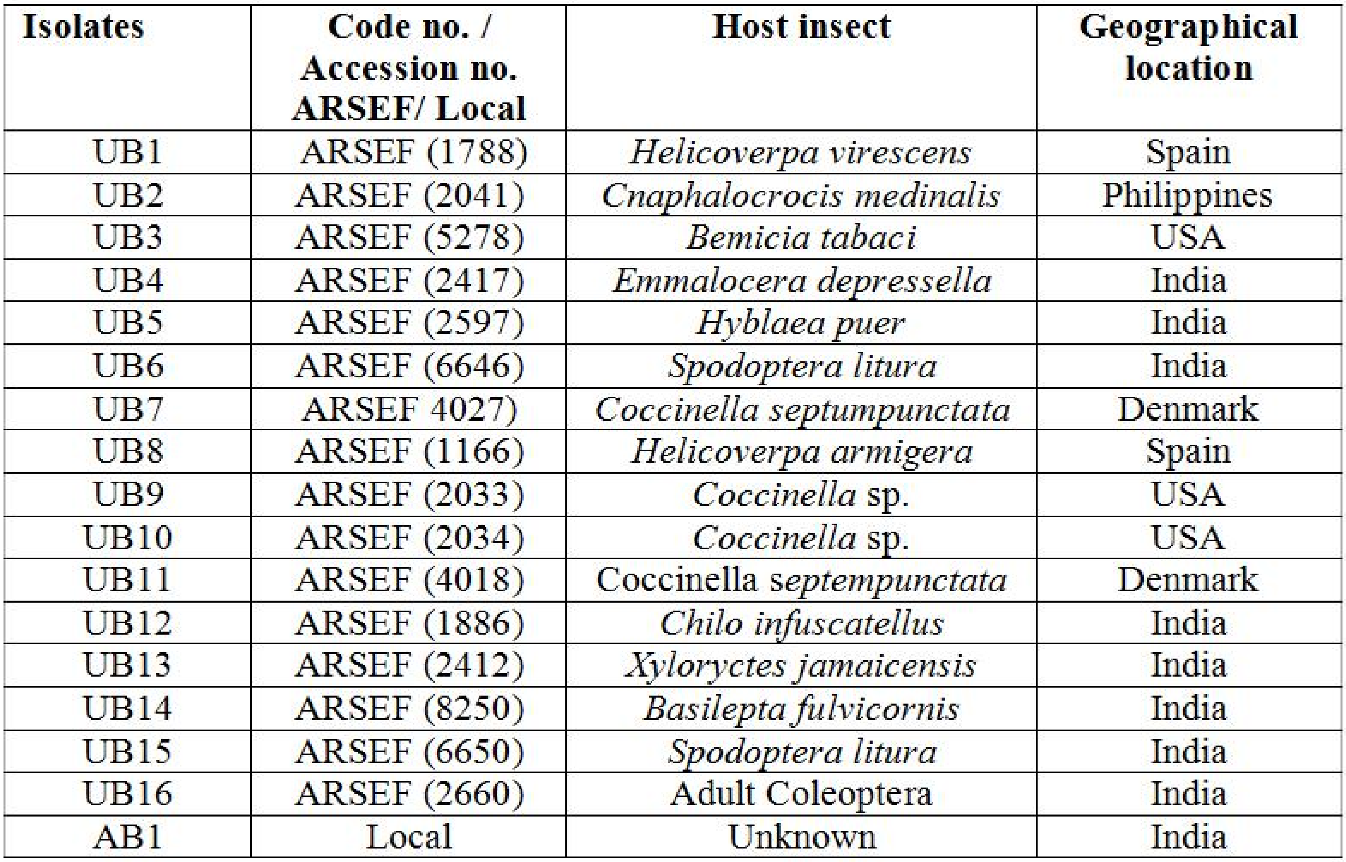
Imperfect fungi typically have profuse growth and sporulation in the media containing yeast extract (Clarkson and Charnley, 1986). In this study all the isolates showed appreciable growth on SDA. According to Sharma et al. (2002), the high level of growth could be due to the presence of peptone as a source of nitrogen. However the nutritional requirements for optimum growth and maximum conidiogenesis could be highly specific and different for isolates.

Sporulation was high in *M. anisopliae* isolates compared to *B. bassiana*. Sporulation occurs depending on the water and carbon-nitrogen source availability. *T. flavus* yielded highest sporulation in medium containing oligosaccharides whereas maximum biomass was formed in medium containing polysaccharides (Engelkes et al., 1997).Virulent isolates tend to germinate more quickly than the isolates with low virulence against the insect host (Magalhaes et al., 1990). The rate of formation of appresoria, the infection structure, is directly related to the degree of pathogenicity for entomopathogenic fungi (Prior et al., 1992). Almost 80 % germination was achieved within 25 h for highly virulent strain of *Metarhizium flavoviride* on 1 % yeast extract agar at 28 °C (Xavier-Santos et al., 1999). The correlation of pathogenicity with germination rate for fungus *Paecilomyces fumosoroseus* to Diamondback Moth, *Plutella xylostella* was investigated (Altre et al., 1999). Dillon and Charnley (1985) reported that presoaking the conidia in distilled water reduces the time period for conidial germination. A 20 h soaking period was sufficient for the germination of almost 90 % conidia within 7 h which indicates the necessity of high moisture level at the time of germination. Effect of temperature on the germination and pathogenicity towards the host insect could be significant.

Inference

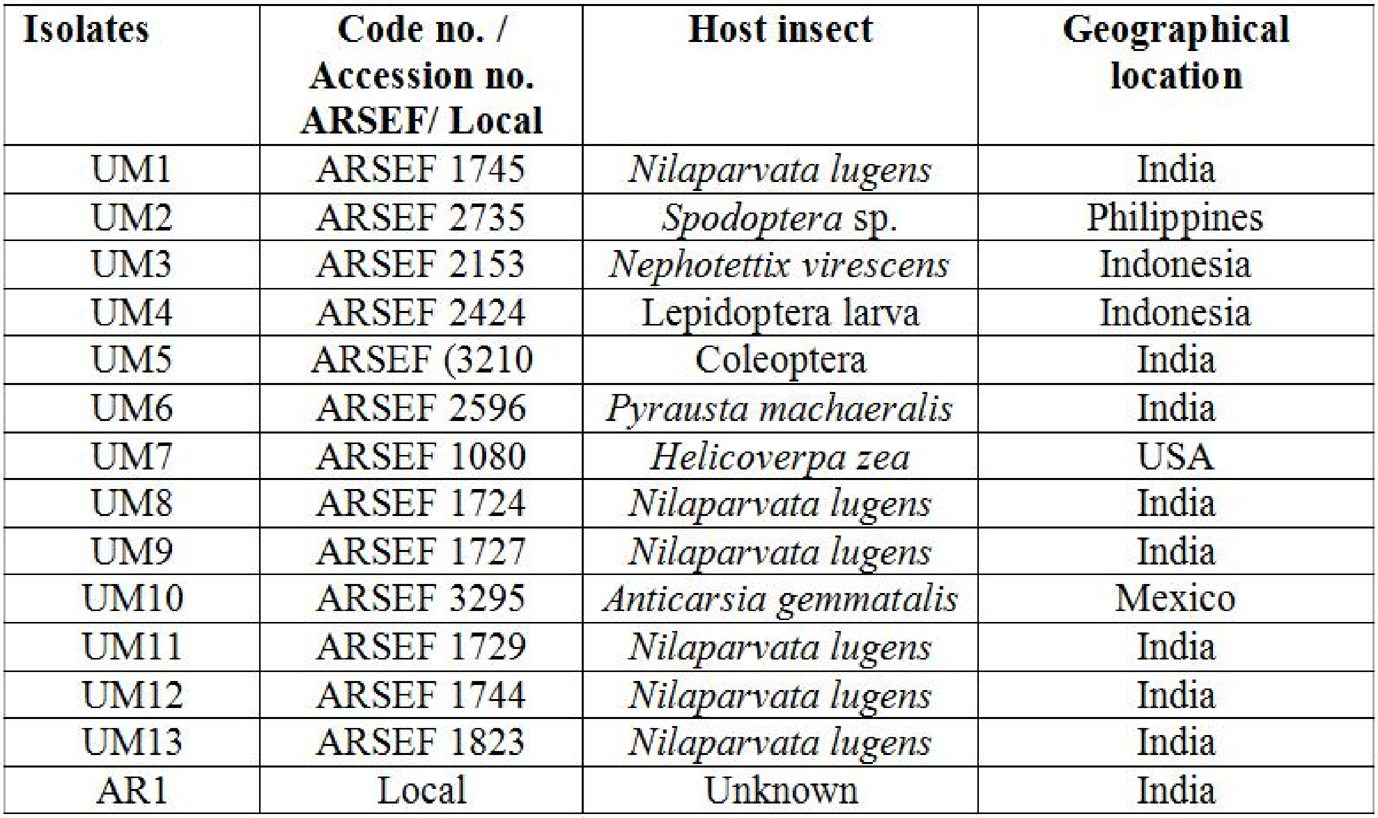
This study determines the basic features of the isolates that could provide a basis for further studies. Isolates with high growth, sporulation and germination potential could be considered for further study however the isolates should be characterized biochemically before concluding decision on robust isolates.

Table 1. Source of *B. bassiana* isolates



Isolates	Code no. / Accession no. ARSEF/ Local	Host insect	Geographical location
UB1	ARSEF (1788)	*Helicoverpa virescens*	Spain
UB2	ARSEF (2041)	*Cnaphalocrocis medinalis*	Philippines
UB3	ARSEF (5278)	*Bemicia tabaci*	USA
UB4	ARSEF (2417)	*Emmalocera depressella*	India
UB5	ARSEF (2597)	*Hyblaea puer*	India
UB6	ARSEF (6646)	*Spodoptera litura*	India
UB7	ARSEF 4027)	*Coccinella septumpunctata*	Denmark
UB8	ARSEF (1166)	*Helicoverpa armigera*	Spain
UB9	ARSEF (2033)	*Coccinella* sp.	USA
UB10	ARSEF (2034)	*Coccinella* sp.	USA
UB11	ARSEF (4018)	Coccinella *septempunctata*	Denmark
UB12	ARSEF (1886)	*Chilo infuscatellus*	India
UB13	ARSEF (2412)	*Xyloryctes jamaicensis*	India
UB14	ARSEF (8250)	*Basilepta fulvicornis*	India
UB15	ARSEF (6650)	*Spodoptera litura*	India
UB16	ARSEF (2660)	Adult Coleoptera	India
AB1	Local	Unknown	India

Table 2. Source of *M. anisopliae* isolates



Isolates	Code no. / Accession no. ARSEF/ Local	Host insect	Geographical location
UM1	ARSEF 1745	*Nilaparvata lugens*	India
UM2	ARSEF 2735	*Spodoptera* sp.	Philippines
UM3	ARSEF 2153	*Nephotettix virescens*	Indonesia
UM4	ARSEF 2424	Lepidoptera larva	Indonesia
UM5	ARSEF (3210	Coleoptera	India
UM6	ARSEF 2596	*Pyrausta machaeralis*	India
UM7	ARSEF 1080	*Helicoverpa zea*	USA
UM8	ARSEF 1724	*Nilaparvata lugens*	India
UM9	ARSEF 1727	*Nilaparvata lugens*	India
UM10	ARSEF 3295	*Anticarsia gemmatalis*	Mexico
UM11	ARSEF 1729	*Nilaparvata lugens*	India
UM12	ARSEF 1744	*Nilaparvata lugens*	India
UM13	ARSEF 1823	*Nilaparvata lugens*	India
AR1	Local	Unknown	India

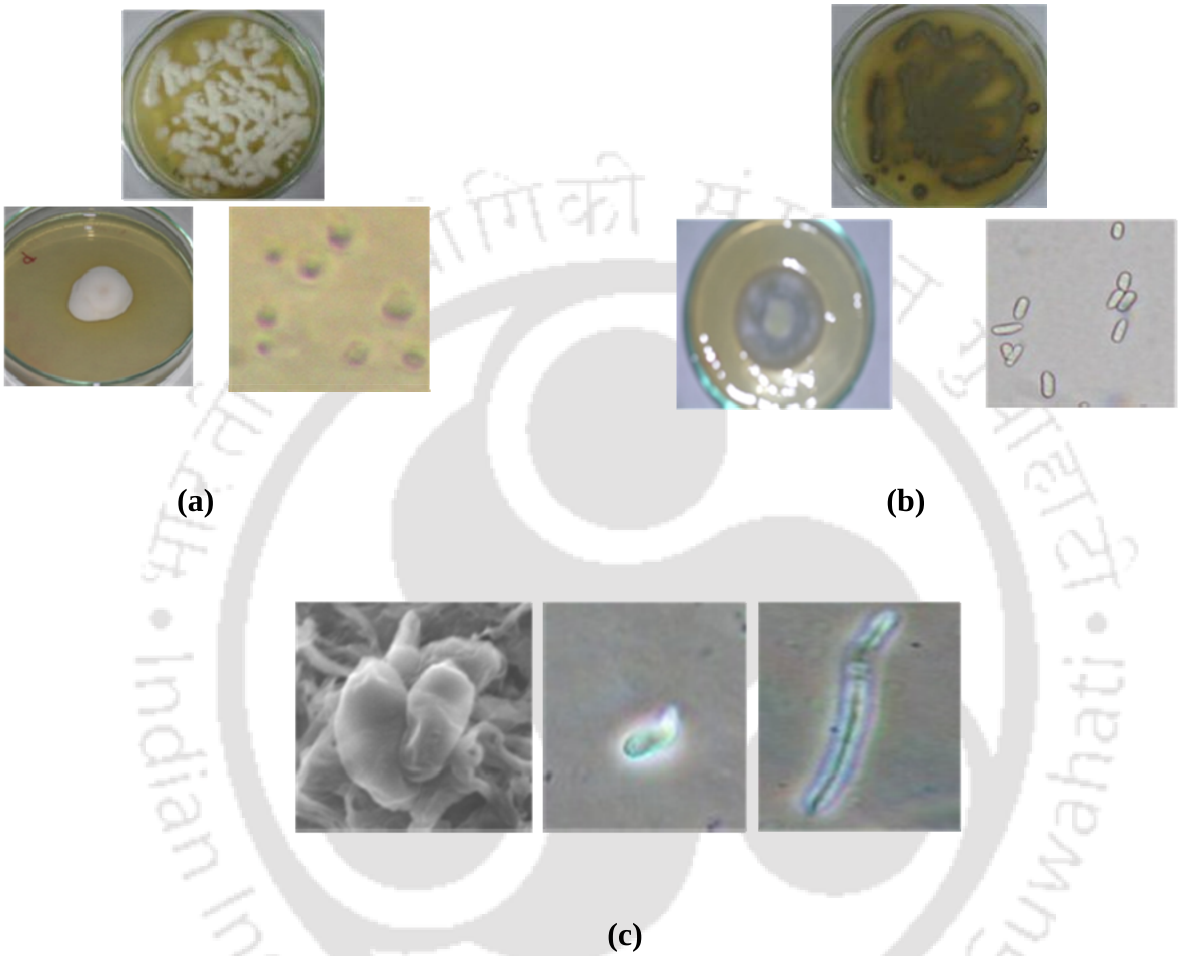
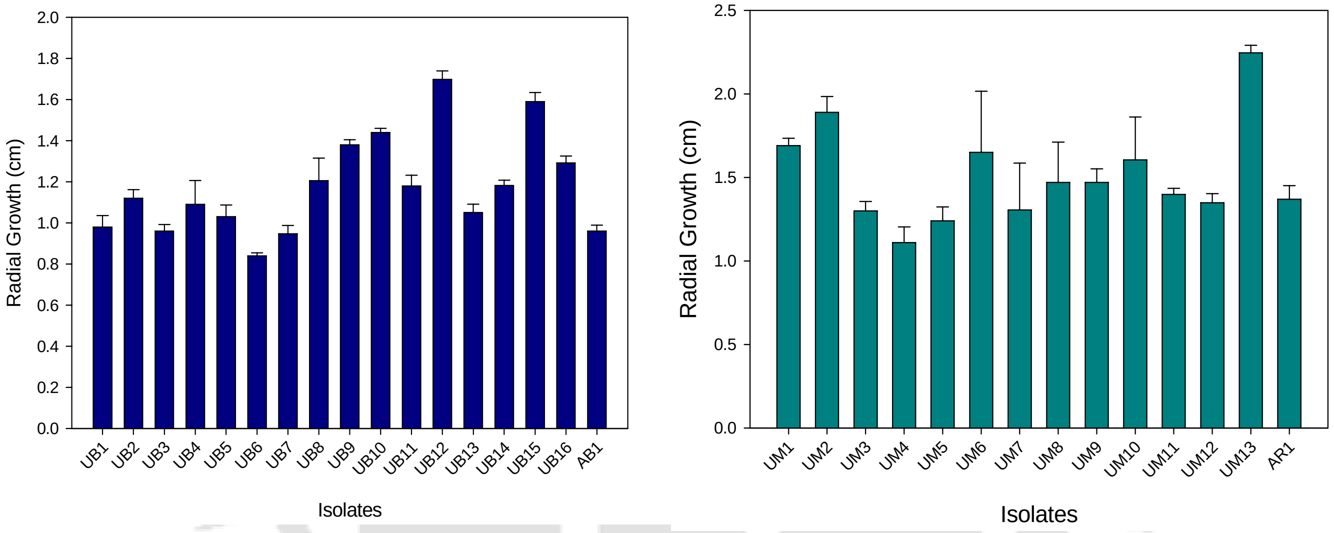
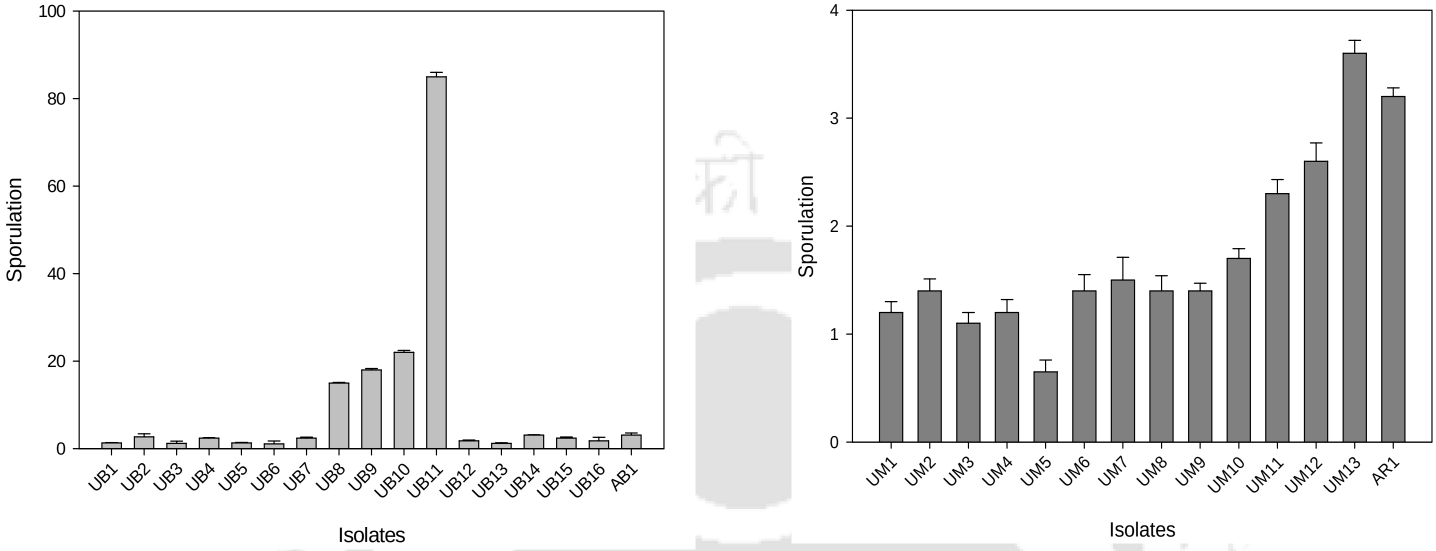


Fig 1. (a) *B. bassiana* white colony and round spores **,(b)** *M. anisopliae* green colony and oval spores, **(c)** (Left to right) SEM images of spores of *M. anisopliae* and light micrograph of germinating spores of *M. anisopliae*



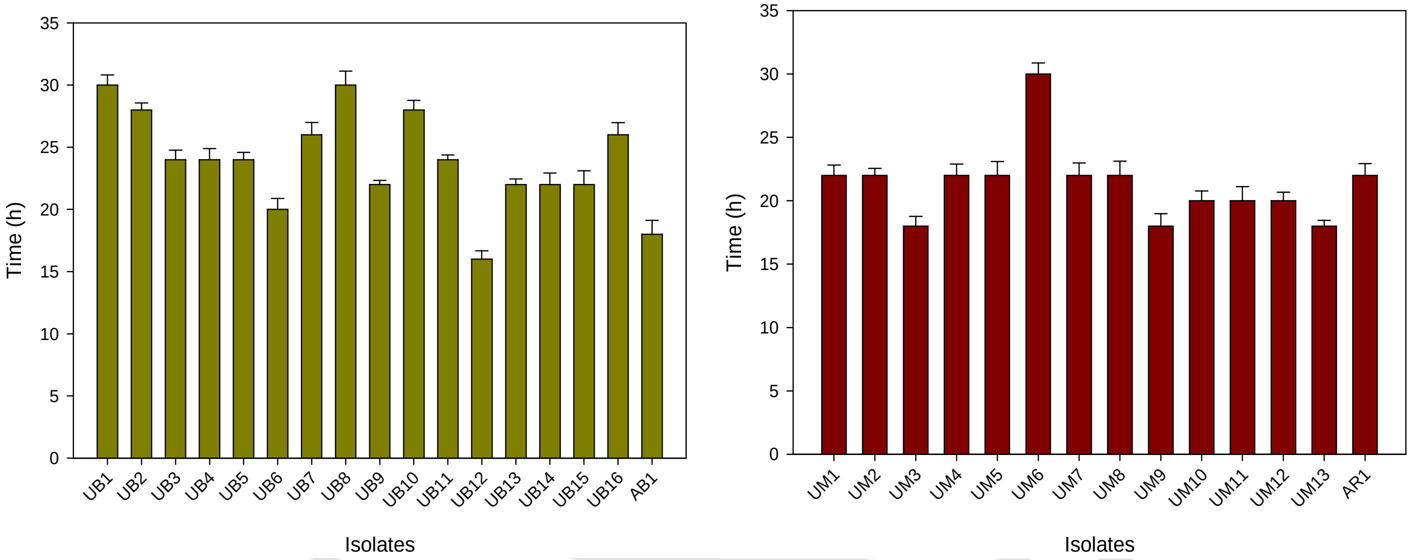
(a) **(b)**

Fig 2. Radial growth on 8th day of (a) seventeen *B. bassiana* and (b) fourteen *M. anisopliae* isolates



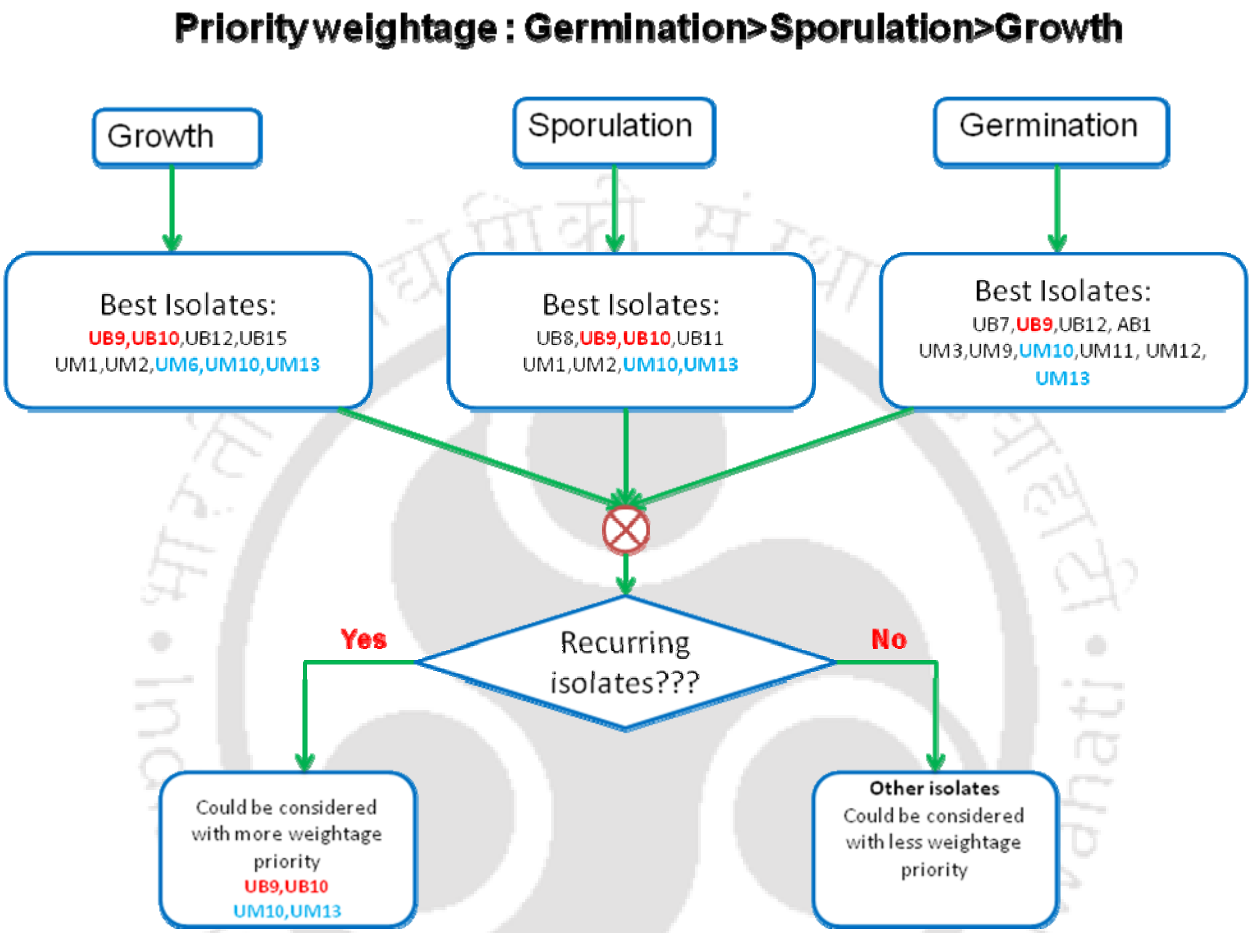
(a) **(b)**

Fig 3. Sporulation at the end of 8th day (y×10^7)(spores/ ml): (a) seventeen *B. bassiana* and (b) fourteen *M. anisopliae* isolates



(a) **(b)**

Fig 4. Time taken for 100 % conidial germination of (a) seventeen *B. bassiana* and (b) fourteen *M. anisopliae* isolates



Flowchart 2. Proposed scheme for selecting virulent isolates based on growth, sporulation and germination on priority basis

Table 2.1. Media and their constituents (Chitinase activity)

Media	**Media constituents**
Medium: I	Colloidal chitin (2%) + Basal salt medium
Medium : II	Colloidal chitin (2%) + Dextrose (2%)
Medium : III	Colloidal chitin (2%) + Yeast Extract (1%)
Medium : IV	Colloidal chitin (2%) + Dextrose (2%) + Yeast Extract (1%)

Table 2.2. Protease activity of *B. bassiana* isolates

Isolates	Enzyme and specific activity as on									
	Day 2		Day 4		Day 6		Day 8		Day 10	
	Enzyme activity (U/ ml)	Sp. Activity (mU/ mg)	Enzyme activity (U/ ml)	Sp. Activity (mU/ mg)	Enzyme activity (U/ ml)	Sp. Activity (mU/ mg)	Enzyme activity (U/ ml)	Sp. Activity (mU/ mg)	Enzyme activity (mU/ ml)	Sp. Activity (mU/ mg)
UB1	0.4247^{c}	1.26^{f}	0.5748^{d}	1.39^{g}	0.5718^{c}	2.37^{f}	0.5710^{d}	5.19^{c}	0.5705^{b}	0.87^{f}
UB2	0.4422^{c}	1.61^{f}	0.4367^{e}	2.46^{f}	0.3967^{de}	5.54^{c}	0.4010^{e}	7.65^{a}	0.4137^{d}	1.39^{e}
UB3	0.3538^{d}	2.01^{e}	**0.7633^{b}**	**3.45^{e}**	0.7466^{a}	3.37^{e}	0.6985^{c}	7.61^{a}	0.6088^{b}	10.41^{a}
UB4	0.0156^{h}	7.08^{a}	0.1109^{h}	4.32^{d}	0.1243^{g}	4.79^{d}	0.1279^{h}	4.38^{d}	0.1313^{f}	1.13^{f}
UB5	0.1757^{f}	1.57^{f}	0.3497^{f}	9.43^{a}	0.3467^{e}	1.59^{g}	0.3421^{f}	2.49^{f}	0.3401^{e}	3.71^{d}
UB6	0.1141^{g}	0.518^{g}	0.2081^{g}	7.43^{b}	0.2548^{f}	9.78^{a}	0.2842^{g}	1.65^{g}	0.3022^{e}	3.27^{d}
UB7	0.1423^{g}	1.17^{f}	0.1476^{h}	1.15^{g}	0.2355^{f}	1.75^{g}	0.3211^{f}	3.23^{e}	0.3371^{e}	3.39^{d}
UB8	0.5889^{a}	3.63^{c}	**0.5679^{d}**	**2.87^{f}**	0.7347^{a}	6.19^{b}	0.7582^{b}	5.30^{c}	0.6084^{b}	6.01^{b}
UB9	0.4336^{c}	3.25^{c}	**0.9021^{a}**	**3.09^{e}**	0.6216^{b}	3.89^{e}	0.5864^{d}	6.69^{b}	0.5211^{c}	14.58^{a}
UB10	0.6059^{a}	4.71^{b}	**0.6536^{c}**	**6.74^{c}**	0.4105^{d}	5.60^{c}	0.4526^{e}	6.24^{b}	0.4269^{d}	6.10^{b}
UB11	0.4204^{c}	3.43^{c}	0.4925^{e}	3.02^{e}	0.7583^{a}	5.03^{c}	0.8946^{a}	5.07^{c}	0.8277^{a}	5.05^{c}
UB12	0.1575^{g}	1.01^{f}	0.3723^{f}	1.42^{g}	0.4224^{d}	2.01^{f}	0.4014^{e}	4.31^{d}	0.3515^{e}	3.99^{d}
UB13	0.0524^{h}	0.546^{g}	**0.7747^{b}**	**2.49^{f}**	0.6706^{b}	2.96^{f}	0.6660^{c}	4.05^{d}	0.6454^{b}	9.22^{a}
UB14	0.5216^{b}	3.35^{c}	**0.5531^{d}**	**3.53^{e}**	0.5179^{c}	4.51^{d}	0.4745^{e}	4.26^{d}	0.4429^{d}	4.57^{c}
UB15	0.1755^{f}	1.92^{e}	0.2845^{g}	3.71^{e}	0.2059^{f}	2.50^{f}	0.1831^{h}	2.12^{f}	0.1819^{f}	2.06^{e}
UB16	0.2672^{e}	2.86^{d}	**0.5222^{d}**	**3.23^{e}**	0.5982^{c}	4.47^{d}	0.8405^{a}	5.43^{c}	0.8309^{a}	4.95^{c}
AB1	0.0360^{h}	0.070^{h}	0.0817^{i}	0.15^{h}	0.0804^{h}	0.16^{h}	0.1024^{h}	0.46^{h}	0.2031^{f}	1.71^{e}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey’s test

Table 2.3. Protease activity of *M. anisopliae* isolates

Isolates	**Enzyme and specific activity as on**									
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
	Enzyme activity (U/ ml)	**Sp. Activity (mU/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (mU/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (mU/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (mU/ mg)**	**Enzyme activity (mU/ ml)**	**Sp. Activity (mU/ mg)**
UM1	0.4054^{c}	4.14^{a}	0.4360^{e}	3.74^{d}	0.4457^{e}	5.79^{d}	0.5118^{e}	3.20^{c}	0.4011^{e}	4.77^{b}
UM2	0.5398^{b}	4.12^{a}	**0.5750^{d}**	**4.76^{c}**	0.8122^{c}	9.81^{a}	0.4274^{f}	5.28^{a}	0.3037^{f}	2.92^{d}
UM3	0.3312^{d}	3.21^{b}	0.4380^{e}	4.33^{c}	0.4245^{e}	7.26^{b}	0.4382^{f}	4.77^{b}	0.3296^{f}	3.73^{c}
UM4	0.2701^{e}	1.87^{d}	**0.8548^{b}**	**3.78^{d}**	0.9676^{b}	5.46^{d}	0.9617^{ab}	4.61^{b}	0.2987^{f}	1.14^{e}
UM5	0.5194^{b}	2.76^{c}	**0.7520^{c}**	**3.33^{d}**	0.7859^{c}	4.32^{e}	0.7838^{c}	2.53^{d}	0.8781^{a}	3.84^{c}
UM6	0.6266^{a}	2.97^{c}	**1.1905^{a}**	**3.26^{d}**	1.8241^{a}	6.07^{c}	1.0479^{a}	3.76^{c}	0.9522^{a}	3.61^{c}
UM7	0.5737^{b}	3.15^{b}	**0.8550^{b}**	**4.36^{c}**	0.8639^{bc}	6.12^{c}	0.8681^{b}	3.96^{c}	0.7068^{b}	4.38^{b}
UM8	0.2179^{f}	0.447^{e}	0.2811^{g}	3.44^{d}	0.3471^{f}	3.40^{f}	0.5825^{e}	5.71^{a}	0.6046^{c}	3.99^{c}
UM9	0.5378^{b}	3.89^{b}	**0.5467^{d}**	**1.05^{e}**	0.5423^{d}	5.02^{d}	0.6408^{d}	4.25^{b}	0.6534^{c}	4.44^{b}
UM10	0.4797^{c}	4.65^{a}	**0.5929^{d}**	**7.16^{a}**	0.5992^{d}	4.66^{e}	0.6632^{d}	4.73^{b}	0.6639^{c}	5.35^{a}
UM11	0.2156^{f}	0.442^{e}	0.2330^{g}	4.37^{c}	0.4048^{e}	3.13^{f}	0.6082^{d}	3.74^{c}	0.5837^{d}	3.47^{c}
UM12	0.1711^{g}	0.345^{e}	0.2053^{g}	4.54^{c}	0.2639^{g}	3.35^{f}	0.6401^{d}	3.81^{c}	0.5907^{d}	3.68^{c}
UM13	0.3227^{d}	2.43^{c}	0.3352^{f}	5.08^{b}	0.3943^{e}	5.89^{d}	0.5812^{e}	4.37^{b}	0.4211^{e}	3.05^{c}
AR1	0.2755^{e}	0.742^{e}	0.3925^{f}	4.06^{c}	0.3840^{ef}	1.42^{g}	0.5794^{e}	2.29^{d}	0.5295^{d}	2.44^{d}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.4. Chitinase specific activity of *B. bassiana* isolates

Media	Isolates															
	UB1	**UB2**	**UB3**	**UB4**	**UB5**	**UB6**	**UB7**	**UB8**	**UB9**	**UB10**	**UB11**	**UB12**	**UB13**	**UB14**	**UB15**	**UB16**	**AB1**
Specific activity (U/ mg) on Day 2																	
MI	0.52aIH	0.29bJ	0.51bIH	0.22bJ	0.36bIJ	0.12bK	0.6bH	1.62bF	2.91aD	3.14aC	0.89bG	2.02aE	2.19aE	1.5bF	4.39aA	4.08aB	0.15Bk
MII	0.52aG	0.59aF	1.66aA	1.21aB	0.69aE	1.04aC	1.24aD	0.59cF	0.51bG	0.09cJ	1.06aC	0.78bD	0.44bH	0.48cH	1.66bA	0.29dI	0.78aD
MIII	0.06bH	0.08cGH	0.35bF	0.14bG	0.20bG	0.06bH	1.66aC	2.18aB	0.17cG	1.43b	1.31aD	0.06cH	0.31bF	2.7aA	1.56bD	0.76cE	0.06Bh
MIV	0.08bE	0.22bB	0.1cDE	0.09cE	0.22bB	0.10bDE	1.88aA	1.89bA	0.13cCD	1.88bA	1.89aA	0.08cE	0.16bC	1.89bA	1.88bA	1.88bA	0.16bC
Specific activity (U/ mg) on Day 4																	
MI	0.39aG	0.08cH	0.09cH	0.05bH	0.45aH	0.09bH	0.82cE	0.58cF	1.29aD	1.85aB	1.29aD	3.33aA	1.76aB	1.85bB	1.61aC	1.76aB	0.03bH
MII	0.34aDE	0.62aC	0.23aFG	0.16aG	0.11bI	0.25aF	4.19aA	0.54cC	0.39bD	0.48bC	0.27dF	0.3bF	0.34bDE	0.74cBC	0.79bB	0.39cD	0.15aG
MIII	0.12bGH	0.07cG	0.13bGH	0.08bH	0.03cI	0.08bH	2.46bB	1.65aD	0.17cG	1.88aC	0.72bF	0.05bH	0.21bG	2.7aA	1.02abE	0.69bF	0.05bH
MIV	0.11bEF	0.29bD	0.03dF	0.08aE	0.06cE	0.08bF	0.50dC	0.78bA	0.06dF	0.61bB	0.58cC	0.10bEF	0.17bE	0.51cC	0.81bA	0.75bA	0.24aD
Specific activity (U/ mg) on Day 6																	
MI	0.56aH	0.13aJ	0.34aI	0.06aJ	0.45aH	0.21aIJ	3.17aC	0.83aG	1.98aD	1.54aE	1.56aE	5.78aA	1.3aF	5.75aA	1.85aD	5.2aB	0.08aJ
MII	0.19bG	0.15aGH	0.08bI	0.09aI	0.11bI	0.29aF	1.26bB	0.29bF	1.37aB	0.69bD	0.37bDE	0.32bDEF	0.36bDE	1.17bC	1.89aA	0.67bD	0.13aHI
MIII	0.17bH	0.04bI	0.07bI	0.07aI	0.03cI	0.09bI	1.84bA	1.49aB	0.08bI	1.05aC	0.73bF	0.07cI	0.45bG	1.58bB	0.86bE	0.97bD	0.04aI
MIV	0.11bED	0.17aD	0.09bE	0.08aE	0.06cE	0.05bE	0.45cA	0.39bB	0.08bE	0.53bA	0.32bBC	0.05cE	0.05cCE	0.25cC	0.24cC	0.42bA	0.14aD
Specific activity (U/ mg) on Day 8																	
MI	0.07bH	0.12bH	0.82aD	0.08bH	1.09aC	0.37aG	0.64bF	0.49bG	1.57aB	1.01aCD	0.63aF	2.45aA	0.84aD	1.03aC	0.69aDEF	0.7bDEF	0.09bH
MII	0.28aEH	0.37aCD	0.08cG	0.24aE	0.27bEH	0.18bF	0.41bC	0.06cG	0.59bB	0.18dF	0.05cG	0.34bDH	0.44bC	0.12bF	0.15bF	0.91aA	0.16aF
MIII	0.03bG	0.04cG	0.47bE	0.07bG	0.03cG	0.23abF	1.72aA	1.12aB	0.25bF	0.77bC	0.65aD	0.03cG	0.02cG	1.71aA	0.66aD	1.02aB	0.06bG
MIV	0.08bE	0.12bCD	0.06cE	0.08bE	0.05cE	0.05cE	0.34bA	0.31bAB	0.08cE	0.32cAB	0.16bC	0.03cE	0.05cE	0.1cDE	0.07cE	0.27cB	0.08bE
Specific activity (U/ mg) on Day 10																	
MI	0.16bG	0.13bG	0.3bF	0.5bF	0.96aD	0.12aG	0.15bG	0.37aF	1.49aB	0.59bE	0.75aDE	2.14aA	0.31aF	1.22aC	0.39bF	0.31bF	0.22aG
MII	0.56aB	0.36aCD	0.1aG	0.75aA	0.46bC	0.18aF	0.16bF	0.07cG	0.46bC	0.29cD	0.06cG	0.66bAB	0.10bG	0.04bG	0.03cG	0.19cEF	0.23aE
MIII	0.04cG	0.05cG	0.18aF	0.08cG	0.03cG	0.04bG	0.94aB	0.28aE	0.09dFG	0.97aB	1.18aA	0.02cG	0.02bG	1.26aA	0.55aD	0.69aC	0.09bFG
MIV	0.09cC	0.07cC	0.05bC	0.07cC	0.04cC	0.03bC	0.09cC	0.15bB	0.15cB	0.28cA	0.14bB	0.03cC	0.04bC	0.05bC	0.03cC	0.13cB	0.09bC

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey’s test.

Table 2.5. Chitinase activity on 4th day in four media: *Beauveria bassiana*

Isolates	Enzyme activity (U/ ml) as on 4th day			
	Medium I	Medium II	Medium III	Medium IV
	Enzyme activity (U/ ml)	Enzyme activity (U/ ml)	Enzyme activity (U/ ml)	Enzyme activity (U/ ml)
UB1	**33.72^{b}**	1.73^{gh}	16.95^{d}	16.29^{d}
UB2	**52.99^{a}**	2.34^{fg}	10.98^{f}	17.27^{c}
UB3	**28.52^{d}**	2.89^{f}	10.09^{f}	7.41^{j}
UB4	6.84^{l}	1.45^{h}	2.43^{k}	5.59^{k}
UB5	**33.03^{b}**	2.01^{gh}	5.08^{j}	8.60^{i}
UB6	**29.88^{d}**	1.67^{gh}	4.77^{j}	18.14^{a}
UB7	7.60^{l}	9.99^{a}	10.65^{f}	9.65^{g}
UB8	8.15^{k}	7.17^{b}	9.53^{g}	10.91^{f}
UB9	**31.32^{c}**	4.46^{d}	9.64^{g}	9.14^{h}
UB10	14.38^{j}	4.94^{cd}	17.29^{c}	7.65^{j}
UB11	16.02^{i}	7.55^{b}	15.88^{e}	9.77^{g}
UB12	27.76^{e}	5.49^{c}	6.69^{i}	17.87^{b}
UB13	20.75^{g}	3.46^{ef}	36.98^{a}	17.60^{b}
UB14	16.55^{i}	4.89^{cd}	18.74^{b}	9.01^{h}
UB15	19.00^{h}	3.64^{e}	15.66^{e}	8.58^{i}
UB16	22.27^{f}	5.70^{c}	9.07^{h}	11.51^{e}
AB1	16.03^{i}	2.64^{f}	5.05^{j}	16.4^{d}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.6. Chitinase specific activity of *M. anisopliae* isolates

Media	Isolates													
	UM 1	**UM2**	**UM3**	**UM4**	**UM5**	**UM6**	**UM7**	**UM8**	**UM9**	**UM10**	**UM11**	**UM12**	**UM13**	**AR1**
Specific activity (U/ mg) on Day 2														
MI	0.34aB	0.11aC	0.09aD	0.12bC	0.36aB	0.67aA	0.10dC	0.07cD	0.04dD	0.11cC	0.10cC	0.20cB	0.18bB	0.11aC
MII	0.13bH	0.02bI	0.06aI	1.05aC	0.39aG	0.42aG	0.93bD	0.59aF	0.73bE	0.32bG	5.90aA	1.67aB	1.53aB	0.13aH
MIII	0.07cG	0.11aF	0.05aG	0.18bE	0.10bF	0.03cG	1.58aC	0.13bE	1.81aA	1.29aD	1.71bB	1.88aA	1.59aC	0.15aE
MIV	0.18bDE	0.10aF	0.06aF	0.13bF	0.13bF	0.17bF	0.45cB	0.43aB	0.23cC	0.15cE	0.07cF	0.65bA	0.18bDE	0.09aF
Specific activity (U/ mg) on Day 4														
MI	0.33aA	0.10aC	0.07aDE	0.11bC	0.15aB	0.07aDE	0.09cD	0.07dD	0.04cE	0.13cC	0.09dD	0.12cC	0.12dC	0.08bDE
MII	0.09cG	0.02bG	0.06aG	0.43aF	0.07bG	0.08aG	0.69bE	0.85aD	2.39aB	0.75bED	4.56aA	1.19aC	1.53aC	0.09bG
MIII	0.13bFG	0.14aFG	0.08aH	0.14bFG	0.08bH	0.02aI	1.62aB	0.25cF	1.81bA	1.48aC	1.75bA	1.16aD	0.54bE	0.10bAH
MIV	0.19bD	0.12aEF	0.05aF	0.10bEF	0.07bEF	0.05aF	1.15aA	0.43bC	1.25bA	0.59bB	0.39cC	0.71bB	0.31cC	0.19bD
Specific activity (U/ mg) on Day 6														
MI	0.22aA	0.10aB	0.05bCD	0.11bB	0.09bB	0.04bD	0.07cCD	0.06cCD	0.03cD	0.13cB	0.11dB	0.06cCD	0.12cB	0.06cD
MII	0.29aH	0.29aH	1.34aE	1.17aF	1.06aF	0.90aD	2.58aC	1.38aE	3.71aB	1.32aE	0.84bG	1.93aD	23.93aA	0.29aH
MIII	0.14aF	0.13aF	0.09bG	0.09bG	0.04bG	0.03bG	1.06bC	0.54bE	1.36bB	1.03aC	1.77aA	0.84bD	1.01bC	0.15bF
MIV	0.25aG	0.11aH	0.06bI	0.11bH	0.05bI	0.04bI	1.66bA	0.59bE	1.08bB	0.45bF	0.47cF	0.95bC	0.79bD	0.06cI
Specific activity (U/ mg) on Day 8														
MI	0.21aA	0.07bB	0.03aC	0.05aBC	0.03aC	0.01aC	0.08dB	0.04cC	0.02dC	0.20dA	0.04dC	0.05cBC	0.07cB	0.03bC
MII	0.01bD	0.09bD	0.03aD	0.07aD	0.09aD	0.04aD	1.11bBC	1.68aAB	1.06aBC	1.73aAB	2.66aA	1.02aC	0.57bCD	0.01bD
MIII	0.09bFG	0.15aF	0.08aG	0.05aG	0.03aG	0.02aG	0.64cB	0.22bE	0.33cD	0.72bA	0.32cD	0.38bD	0.46bC	0.14aF
MIV	0.24aE	0.15aF	0.08aG	0.09aG	0.04aH	0.03aH	2.28aA	0.38bD	0.69bC	0.46cD	0.65bC	1.14aB	1.09aB	0.08bG
Specific activity (U/ mg) on Day 10														
MI	0.16aB	0.20aB	0.09bC	4.70aA	0.09aC	0.04aE	0.07cC	0.04cE	0.01cE	0.19cB	0.04cE	0.08cC	0.07cC	0.09bC
MII	0.06bF	0.02bF	0.02bF	0.02bF	0.03aF	0.01aF	0.77bC	1.95aA	0.41bE	0.35bE	1.33aB	0.73aC	0.59aD	0.06bF
MIII	0.09bDE	0.15aD	0.14aD	0.04bE	0.02aE	0.08aDE	0.77bA	0.26bC	0.44bB	0.65aA	0.28bC	0.49bB	0.25bC	0.19aC
MIV	0.18aE	0.12aF	0.08bF	0.05bF	0.02aF	0.01aF	1.62aA	0.24bE	0.61aB	0.40bD	0.46bD	0.57bBC	0.49aC	0.06bF

Values followed by same lower case alphabets in the column and values followed by upper case alphabets in a row are statistically equivalent according to Tukey’s test.

Table 2.7. Chitinase activity on 6th day in four media: *Metarhizium anisopliae*

Isolates	**Enzyme activity (U/ ml) as on 6th day**			
	Medium I	**Medium II**	**Medium III**	**Medium IV**
	Enzyme activity (U/ ml)	**Enzyme activity (U/ ml)**	**Enzyme activity (U/ ml)**	**Enzyme activity (U/ ml)**
UM1	3.26^{c}	1.09^{f}	4.89^{c}	10.31^{a}
UM2	5.43^{b}	1.09^{f}	5.97^{c}	8.69^{b}
UM3	2.71^{c}	2.70^{be}	3.80^{d}	3.80^{d}
UM4	5.97^{b}	4.34^{d}	5.43^{c}	4.80^{d}
UM5	3.80^{c}	3.80^{d}	3.26^{d}	2.70^{d}
UM6	2.44^{c}	6.50^{c}	2.17^{d}	2.17^{d}
UM7	**7.59**^{a}	10.26^{a}	14.86^{a}	8.31^{b}
UM8	5.55^{b}	5.37^{c}	6.75^{b}	6.47^{c}
UM9	5.59^{b}	8.47^{b}	10.97^{a}	12.19^{a}
UM10	**7.87**^{a}	5.10^{c}	13.06^{a}	8.98^{b}
UM11	**7.76**^{a}	7.00^{b}	12.22^{a}	8.34^{b}
UM12	**7.76**^{a}	8.88^{b}	7.53^{b}	5.95^{c}
UM13	**7.48**^{a}	10.29^{a}	7.89^{b}	7.45^{c}
AR1	4.89^{b}	5.90^{c}	7.06^{b}	4.34^{d}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey’s test

Table 2.8. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *B. bassiana* isolates in minimal medium

Isolates	pH	Ammonia conc. (µg/ ml)	Oxalic acid conc. (µg/ ml)	Pr1		Pr2	
				Enzyme activity (U/ ml)	Sp. Activity (U/ mg)	Enzyme activity (U/ ml)	Sp. Activity (U/ mg)
UB1	6.99	0.022	33.5	0.76^{c}	0.17^{e}	**2.03^{a}**	0.45^{b}
UB2	7.01	0.065	43.0	1.49^{b}	0.38^{d}	0.15^{b}	0.04^{c}
UB3	7.05	0.068	60.5	0.88^{c}	1.26^{b}	0.16^{b}	0.05^{c}
UB5	7.02	0.054	85.5	0.35^{d}	3.89^{a}	0.16^{b}	1.78^{a}
UB9	7.01	0.059	75.5	**4.24^{a}**	0.67^{c}	0.17^{b}	0.09^{c}
UB10	7.03	0.055	94.5	0.28^{d}	0.12^{e}	0.17^{b}	0.07^{c}
UB13	7.02	0.052	26.0	**4.44^{a}**	0.33^{d}	0.16^{b}	0.01^{c}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.9. pH, ammonia, oxalic acid concentration, Pr1 and Pr2activity of *B. bassiana* isolates in MM supplemented with casein (1%)

Isolates	**pH**	**Ammonia conc. (µg/ ml)**	**Oxalic acid conc. (µg/ ml)**	**Pr1**		**Pr2**	
				Enzyme activity (U/ ml)	**Sp. Activity (U/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (U/ mg)**
UB1	7.17	0.015	-	**4.77^{a}**	0.05^{a}	0.14^{b}	0.001^{a}
UB2	7.08	0.027	-	**4.77^{a}**	0.05^{a}	0.09^{b}	0.009^{a}
UB3	7.06	0.037	-	2.56^{c}	0.04^{a}	0.13^{b}	0.002^{a}
UB5	7.05	0.033	-	3.77^{b}	0.05^{a}	0.18^{b}	0.009^{a}
UB9	7.06	0.026	-	**4.94^{a}**	0.06^{a}	**0.75^{a}**	0.003^{a}
UB10	7.02	0.029	-	1.87^{d}	0.03^{a}	0.11^{b}	0.002^{a}
UB13	7.11	0.035	-	0.07^{e}	0.001^{b}	0.14^{b}	0.009^{a}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey’s test

Table 2.10. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *B. bassiana* isolates in MM supplemented with colloidal chitin (2%)

Isolates	**pH**	**Ammonia conc. (µg/ ml)**	**Oxalic acid conc. (µg/ ml)**	**Pr1**		**Pr2**	
				Enzyme activity (U/ ml)	**Sp. Activity (U/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (U/ mg)**
UB1	7.17	0.015	-	3.53^{c}	0.24^{b}	0.11^{bc}	0.01^{a}
UB2	7.08	0.027	-	3.59^{c}	0.34^{a}	0.09^{bc}	0.01^{a}
UB3	7.06	0.037	-	3.02^{d}	0.26^{b}	0.18^{a}	0.02^{a}
UB5	7.05	0.033	-	3.79^{b}	0.39^{a}	0.09^{bc}	0.01^{a}
UB9	7.06	0.026	-	**4.36^{a}**	0.21^{b}	0.10^{bc}	0.01^{a}
UB10	7.02	0.029	-	1.53^{f}	0.15^{c}	0.05^{c}	0.01^{a}
UB13	7.11	0.035	-	2.66^{e}	0.26^{b}	0.09b^{c}	0.01^{a}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.11. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *M. anisopliae* isolates in minimal medium (MM)

Isolates	**pH**	**Ammonia conc. (µg/ ml)**	**Oxalic acid conc. (µg/ ml)**	**Pr1**		**Pr2**	
				Enzyme activity (U/ ml)	**Sp. Activity (U/ mg)**	**Enzyme activity (U/ ml)**	**Sp. Activity (U/ mg)**
UM4	7.04	-	3.5	0.32^{c}	0.16^{b}	0.13^{ab}	0.06^{bc}
UM6	7.05	-	1.5	0.39^{c}	0.19^{b}	0.17^{ab}	0.09^{bc}
UM7	7.06	-	3.5	0.37^{c}	0.17^{b}	0.14^{ab}	0.07^{bc}
UM10	7.06	-	4.5	1.16^{a}	0.33^{a}	0.11^{b}	0.03^{c}
UM11	7.04	-	21.5	0.21^{d}	0.09^{c}	0.13^{ab}	0.05^{c}
UM12	7.05	-	42.5	0.53^{b}	0.34^{a}	0.18^{a}	0.12^{a}
UM13	7.06	-	39	0.41^{c}	0.36^{a}	0.16^{ab}	0.14^{a}

Table 2.12. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *M. anisopliae* isolates in MM supplemented with casein (1%)

Isolates	pH	Ammonia Conc. (µg/ ml)	Oxalic acid conc. (µg/ ml)	Pr1		Pr2	
				Enzyme activity (U/ ml)	Sp. Activity (U/ mg)	Enzyme activity (U/ ml)	Sp. Activity (U/ mg)
UM4	7.68	0.0277	-	0.17^{c}	0.52^{c}	0.35^{c}	1.07^{d}
UM6	7.32	0.0354	-	0.18^{c}	0.56^{c}	0.19^{d}	0.59^{e}
UM7	7.72	0.0323	-	0.38^{b}	1.18^{b}	0.32^{c}	0.99^{d}
UM10	**8.21**	**0.2238**	-	**4.44^{a}**	7.43^{a}	**2.48^{a}**	7.55^{a}
UM11	7.61	0.0538	-	0.42^{b}	1.26^{b}	0.43^{b}	1.29^{c}
UM12	7.59	0.0531	-	0.46^{b}	1.40^{b}	0.41^{b}	1.25^{c}
UM13	7.69	0.0261	-	0.35^{b}	1.07^{b}	0.49^{b}	1.49^{b}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.13. pH, ammonia, oxalic acid concentration, Pr1 and Pr2 activity of *M. anisopliae* isolates in MM supplemented with colloidal chitin (2%)

Isolates	pH	Ammonia Conc. (µg/ ml)	Oxalic acid conc. (µg/ ml)	Pr1		Pr2	
				Enzyme activity (U/ ml)	Sp. Activity (U/ mg)	Enzyme activity (U/ ml)	Sp. Activity (U/ mg)
UM4	7.01	0.0054	-	4.29^{b}	0.76^{a}	0.99^{c}	0.18^{a}
UM6	7.03	0.0069	-	4.16^{c}	0.39^{c}	0.10^{e}	0.01^{d}
UM7	7.17	0.0169	-	4.27^{b}	0.16^{e}	0.98^{c}	0.07^{bc}
UM10	7.09	0.0223	-	**4.58^{a}**	0.49^{b}	**1.87^{a}**	0.11^{b}
UM11	7.08	0.0300	-	**4.52^{a}**	0.33^{cd}	0.13^{de}	0.01^{d}
UM12	7.10	0.0300	-	4.21^{b}	0.29^{d}	0.19^{d}	0.01^{d}
UM13	7.22	0.0292	-	4.16^{c}	0.15^{e}	1.22^{b}	0.04^{c}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

Table 2.14. Exochitinase activity by *B. bassiana* isolates

Isolates	**Enzyme Activity (U/ ml)**	**Sp. activity (U/ mg)**
UB1	7.72^{c}	0.77^{b}
UB2	8.63^{b}	0.88^{a}
UB3	**9.47^{a}**	0.37^{d}
UB5	7.14^{c}	0.49^{c}
UB9	7.31^{c}	0.74^{b}
UB10	4.56^{e}	0.24^{e}
UB13	5.50^{d}	0.26^{e}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey’s test

Table 2.15. Exochitinase activity by *M. anisopliae* isolates

Isolates	**Enzyme Activity (U/ ml)**	**Sp. activity (U/ mg)**
UM4	4.55^{c}	0.29^{c}
UM6	4.00^{c}	0.24^{c}
UM7	5.81^{b}	0.36^{b}
UM10	3.81^{c}	0.34^{b}
UM11	5.84^{b}	0.17^{d}
UM12	5.95^{b}	0.26^{c}
UM13	$\mathbf{7.19^{a}}$	0.98^{a}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test

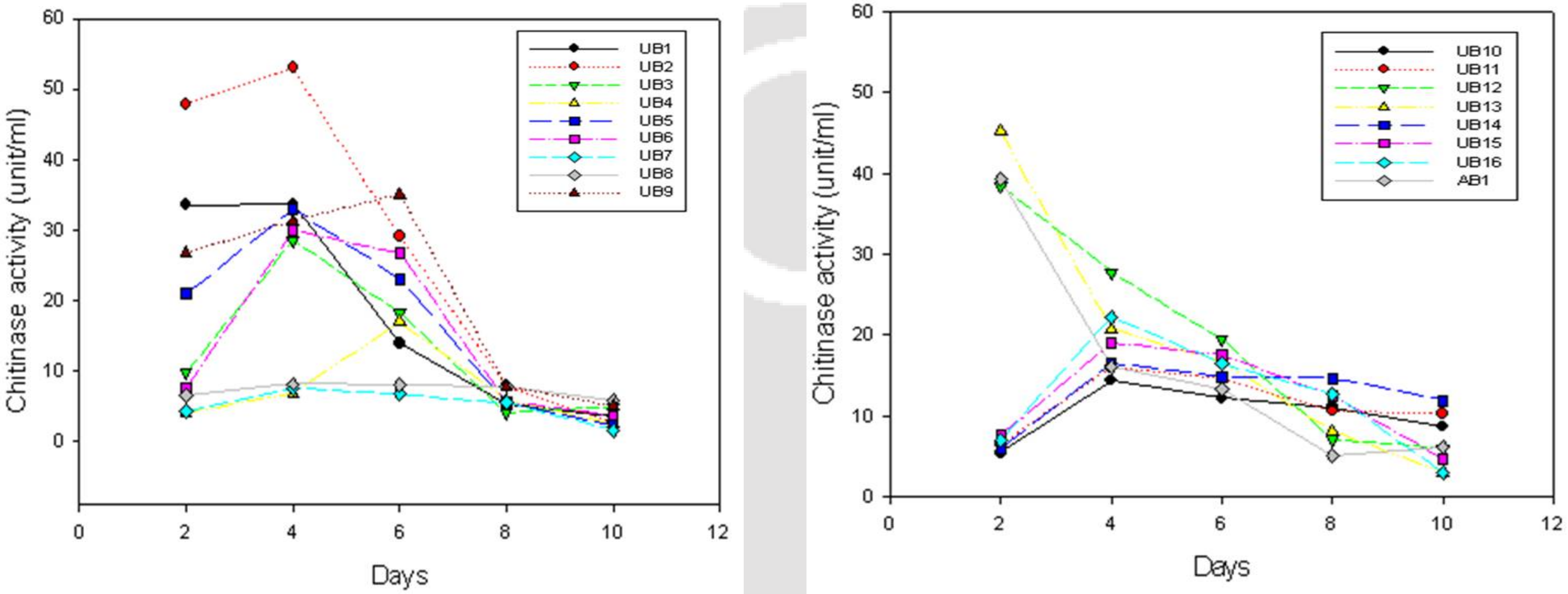


Fig 2.1. Chitinase activity on Basal salt medium containing Colloidal chitin (Medium I) as a sole source of carbon (*Beauveria bassiana*)

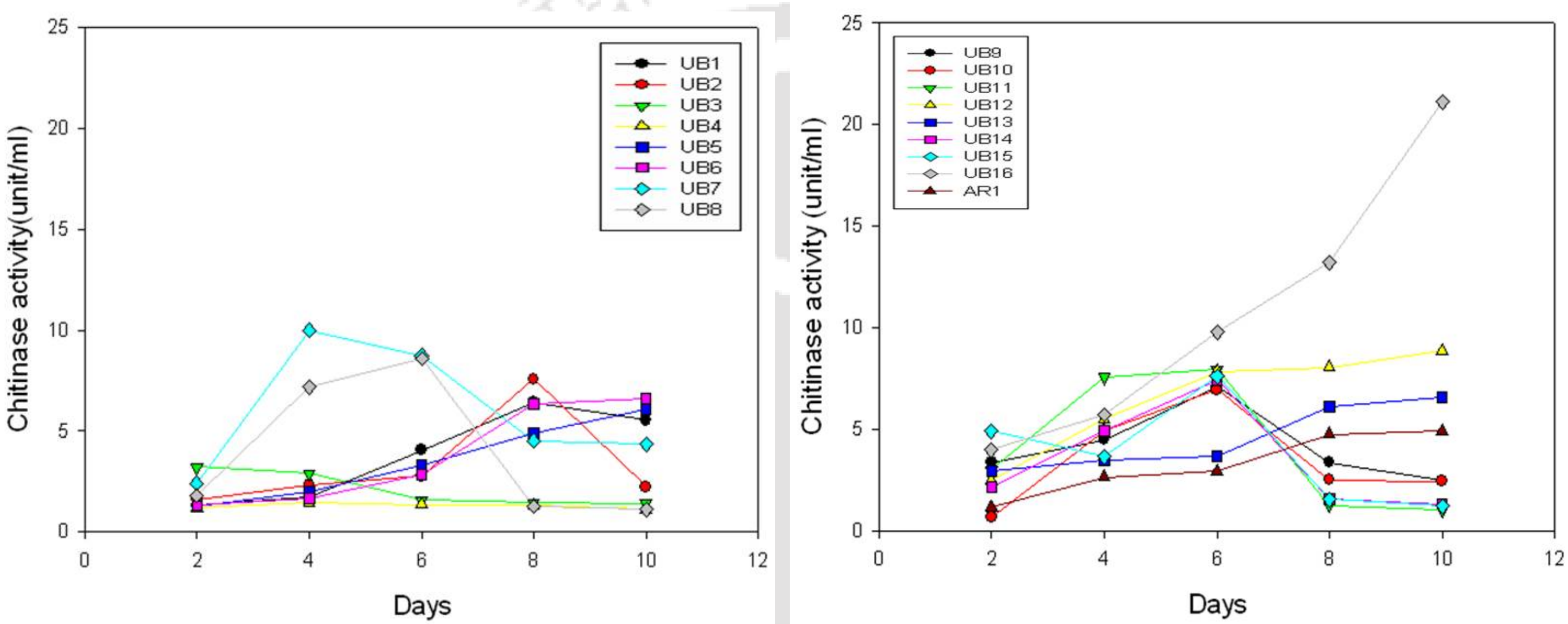


Fig 2.2. Chitinase activity on Medium containing Colloidal chitin and Dextrose (Medium II) as source of carbon (*Beauveria bassiana*)

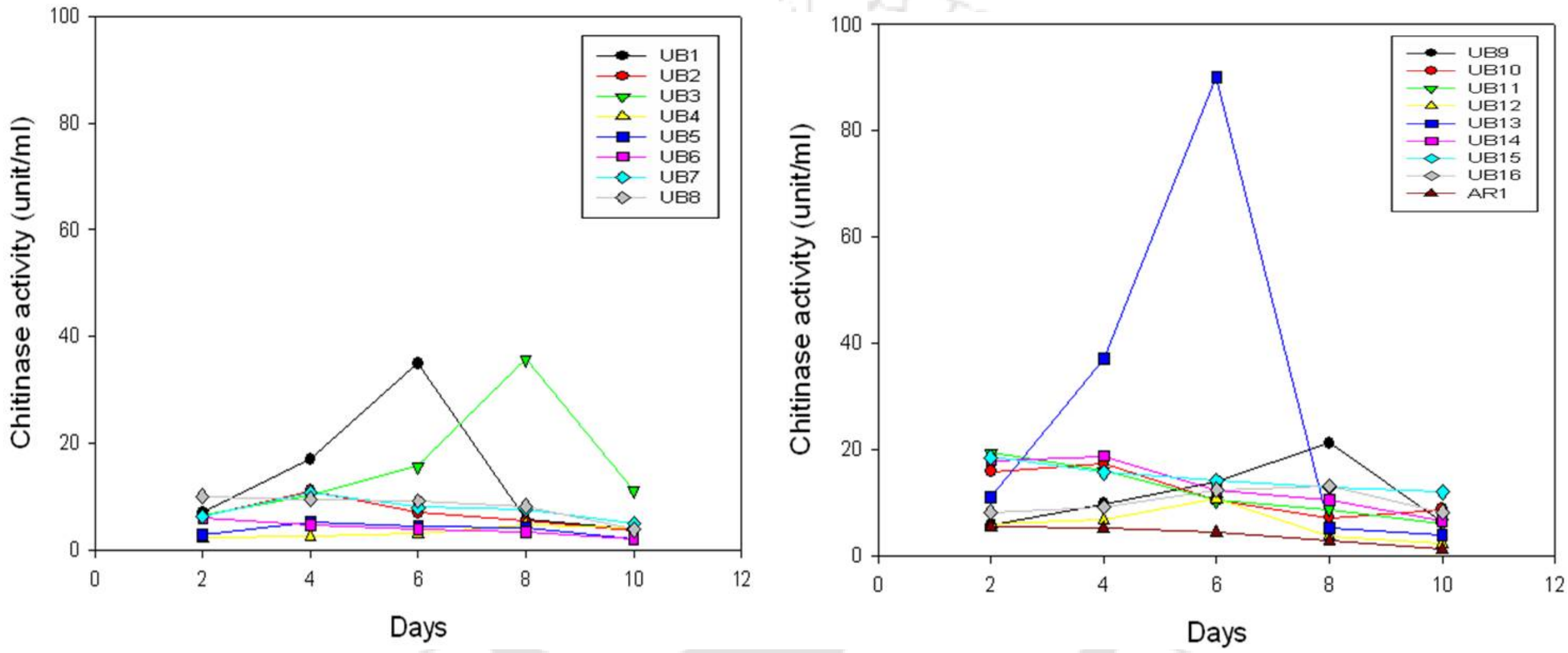


Fig 2.3. Chitinase activity on Medium containing Colloidal chitin as source of carbon and Yeast extract (Medium III) as nitrogen source (*Beauveria bassiana*)

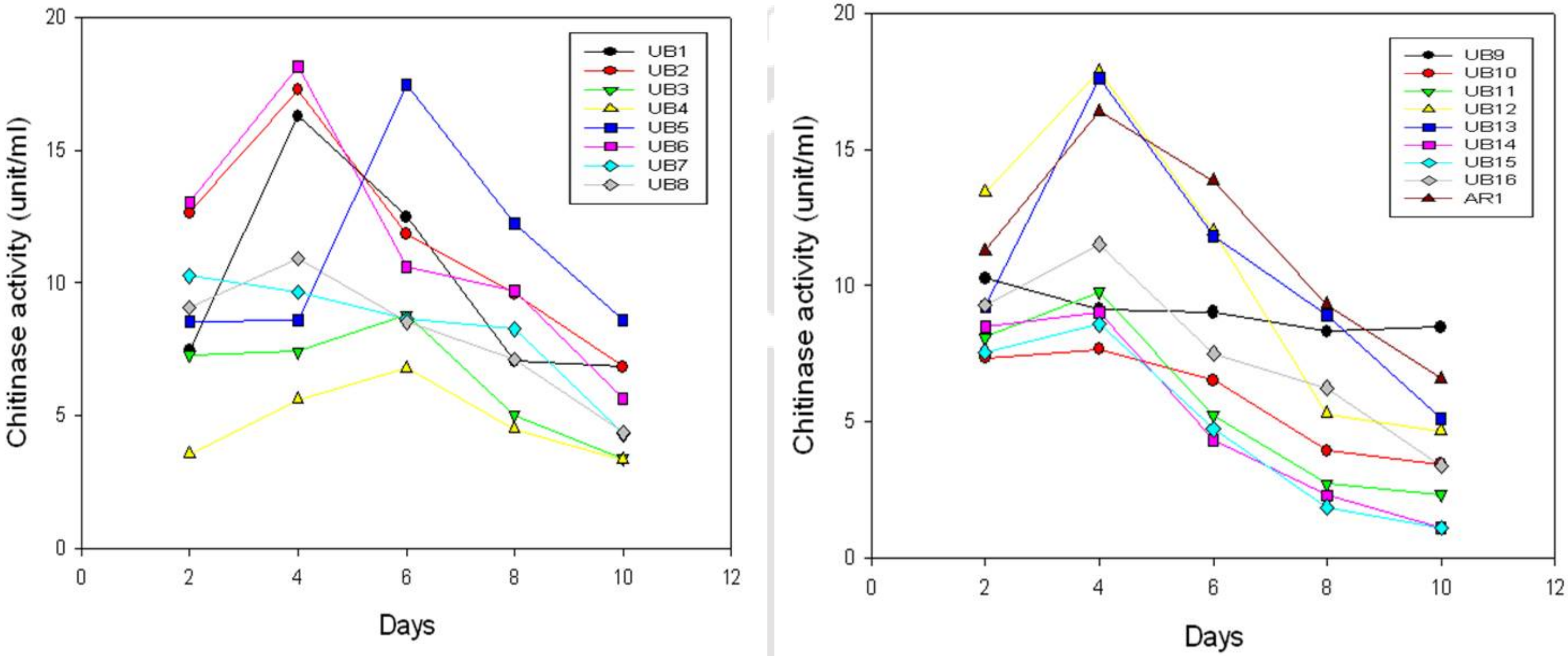


Fig 2.4. Chitinase activity on Medium containing Colloidal chitin and Dextrose as source of carbon and Yeast extract (Medium IV) as nitrogen source (*Beauveria bassiana*)

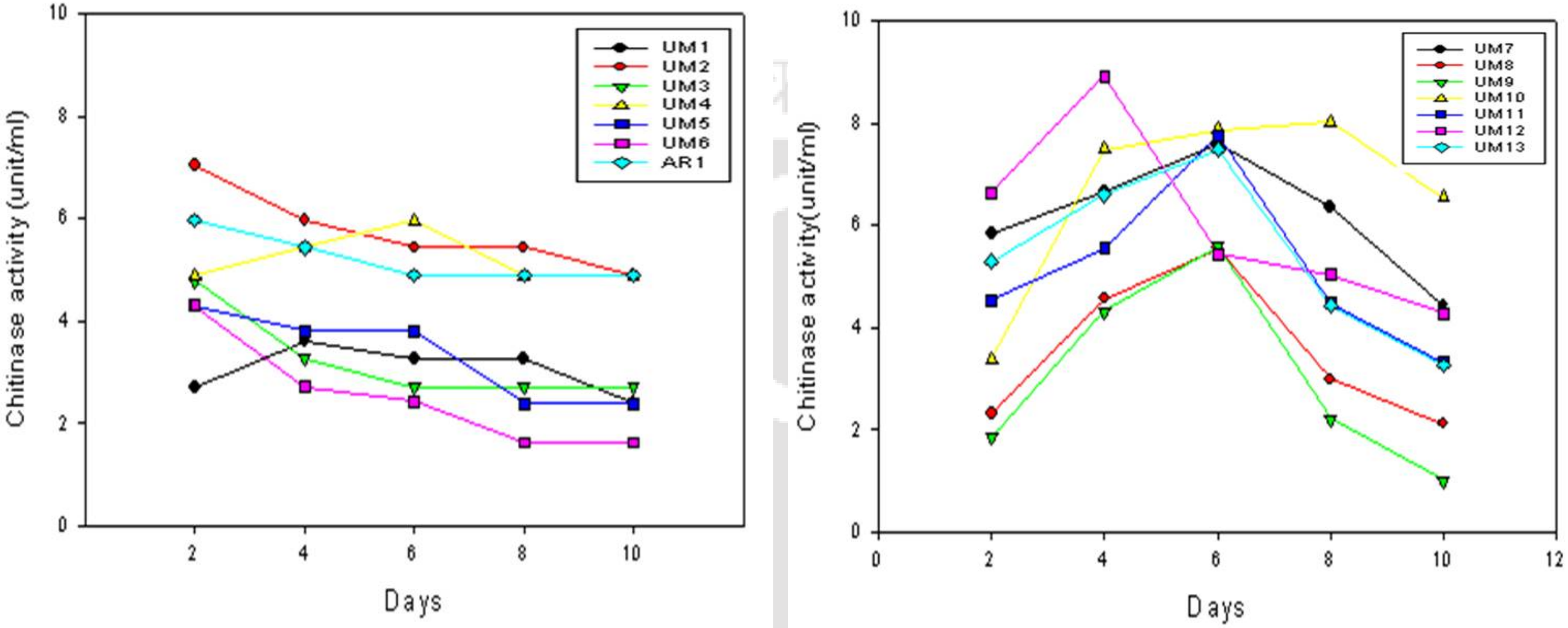


Fig 2.5. Chitinase activity on Basal salt medium containing Colloidal chitin (Medium I) as a sole source of carbon (*Metarhizium anisopliae*)

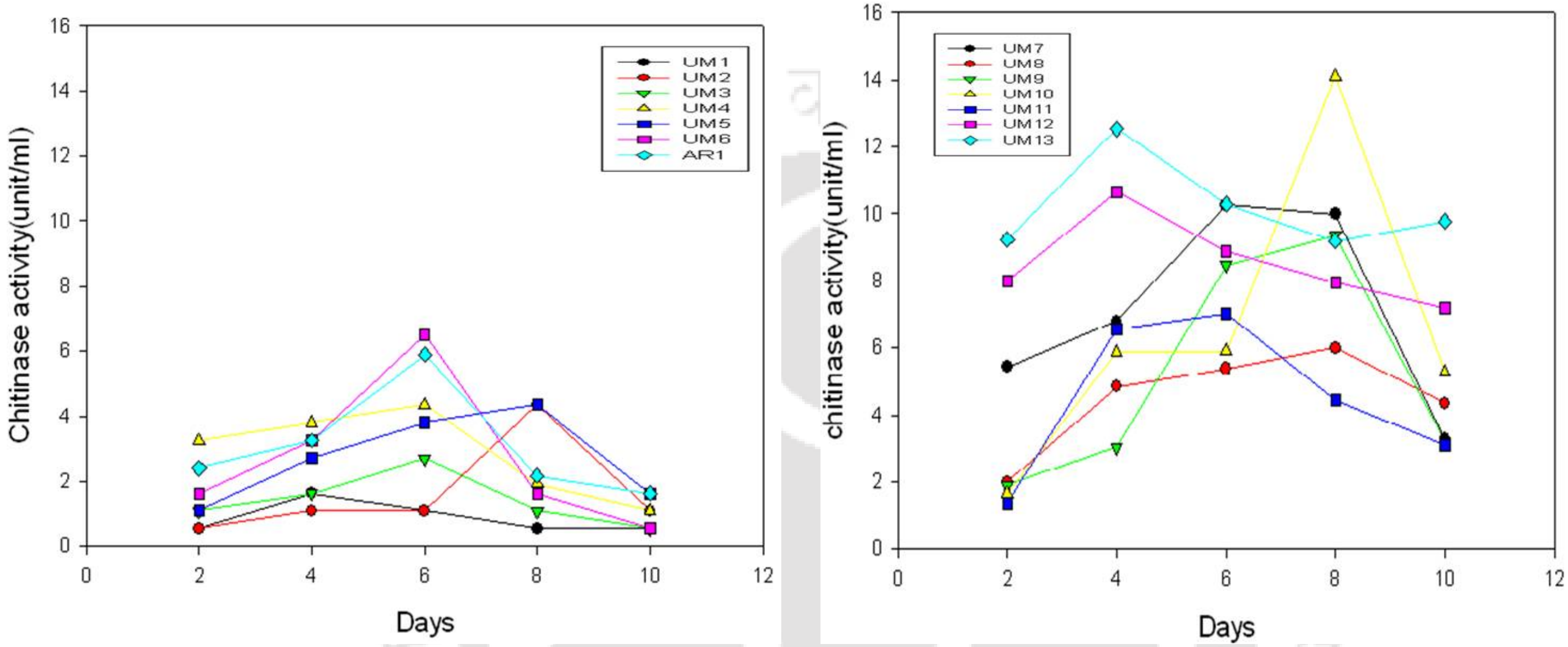


Fig 2.6. Chitinase activity on Medium containing Colloidal chitin and Dextrose (Medium II) as source of carbon (*Metarhizium anisopliae*)

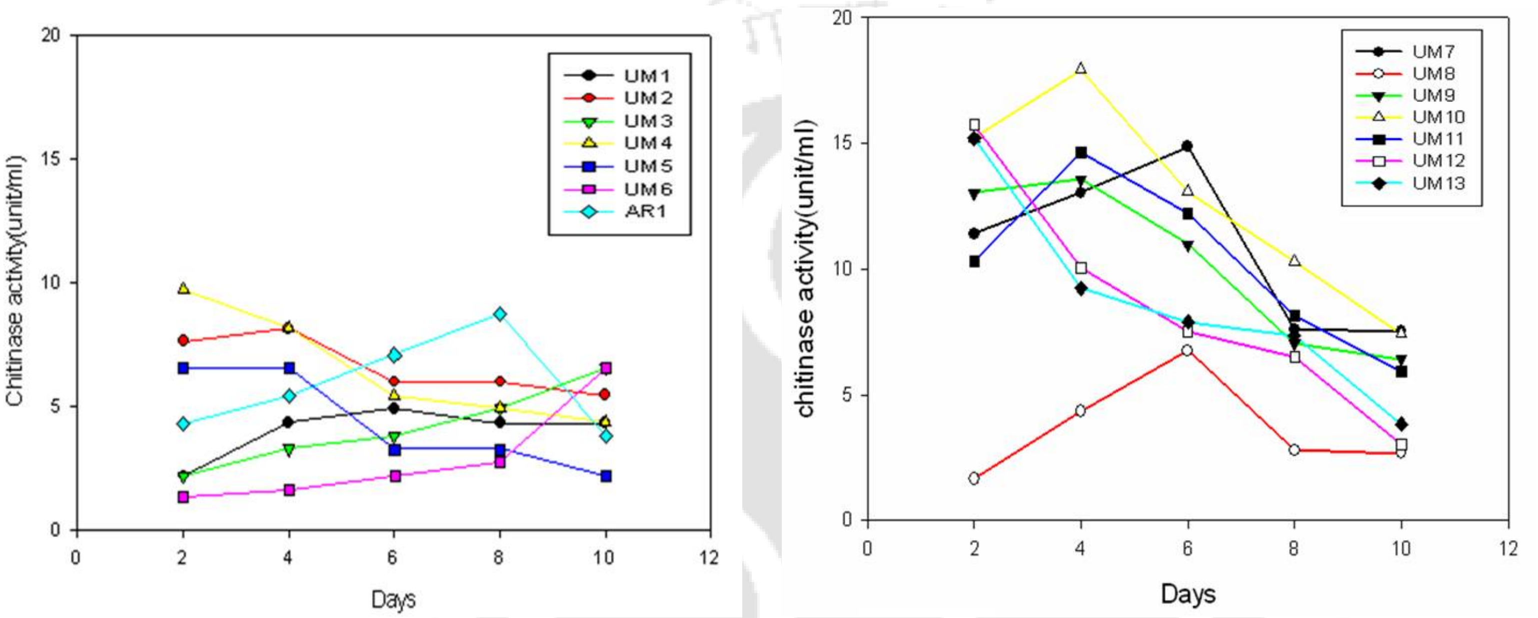


Fig 2.7. Chitinase activity on Medium containing Colloidal chitin as source of carbon and Yeast extract (Medium III) as nitrogen source
(*Metarhizium anisopliae*)

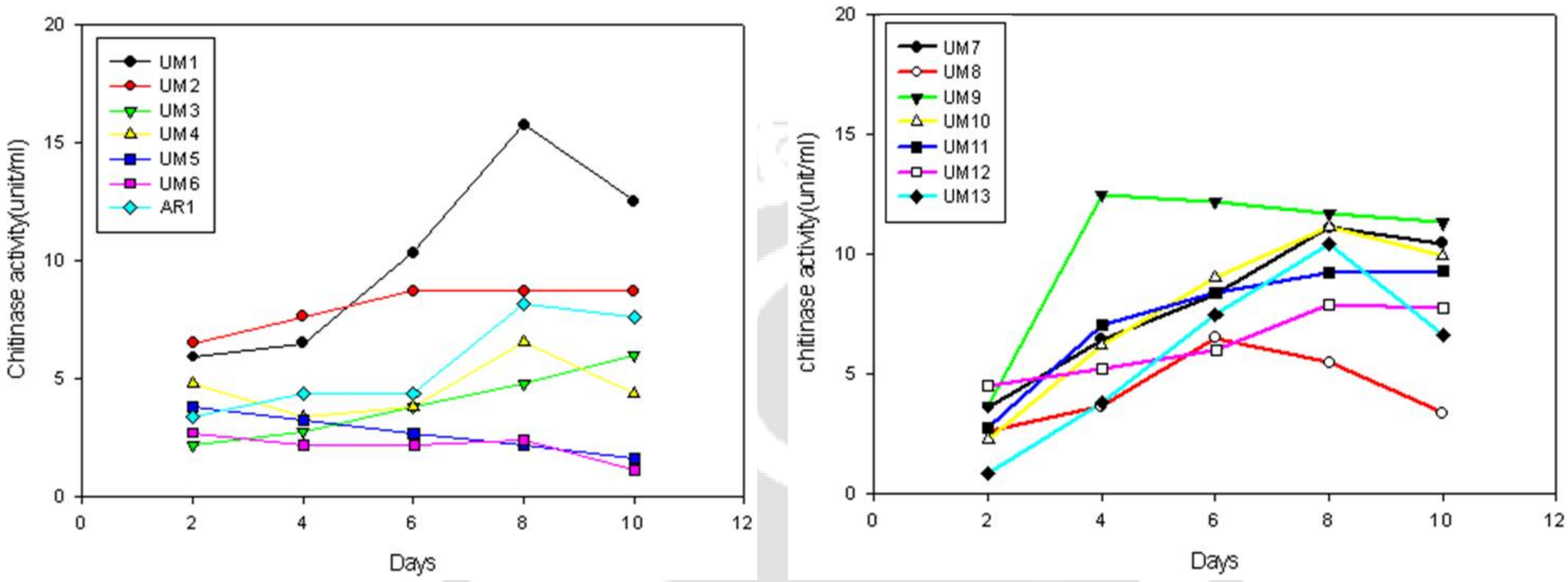
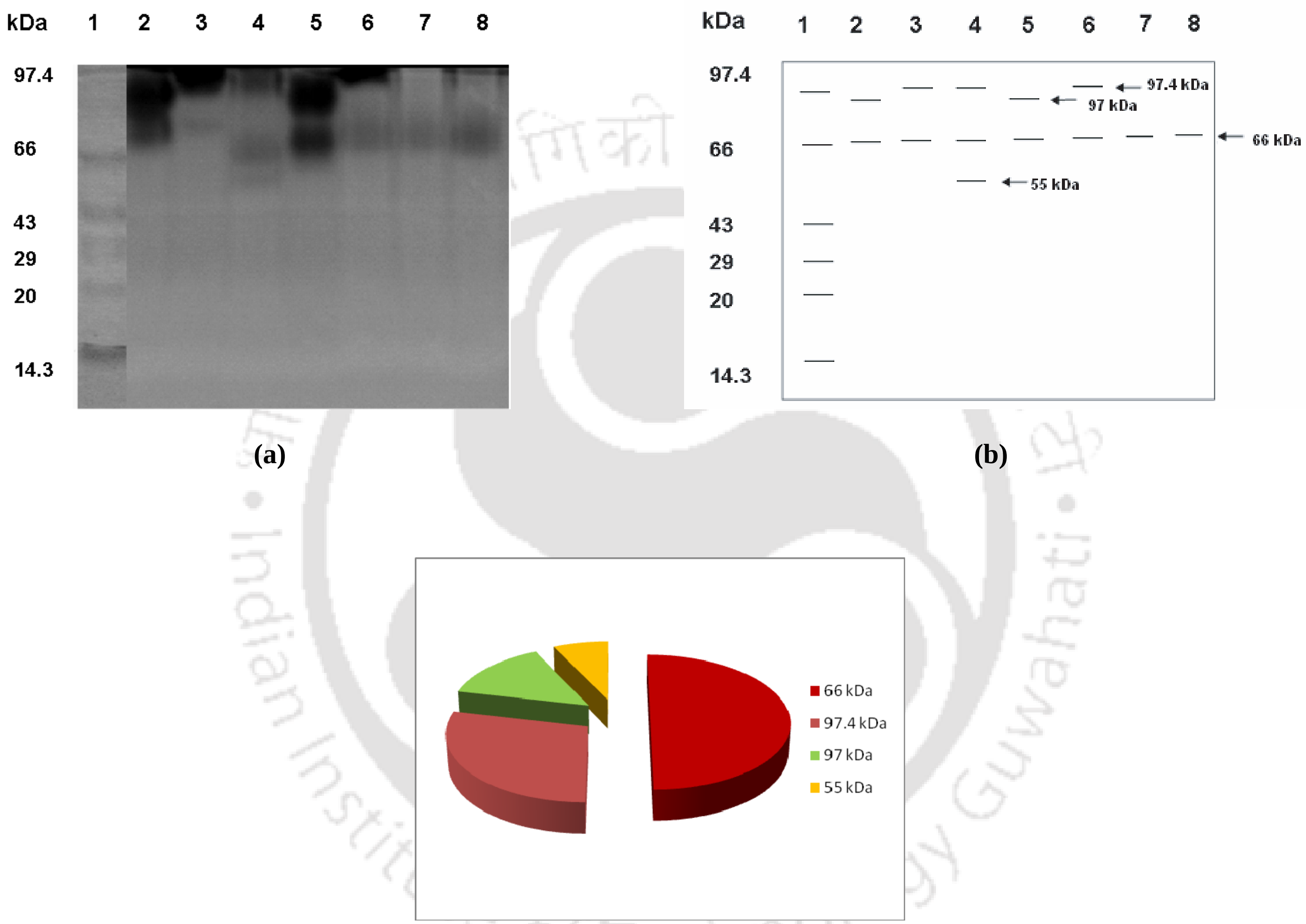


Fig 2.8. Chitinase activity on Medium containing Colloidal chitin and Dextrose as source of carbon and Yeast extract (Medium IV) as nitrogen source. (*Metarhizium anisopliae*)

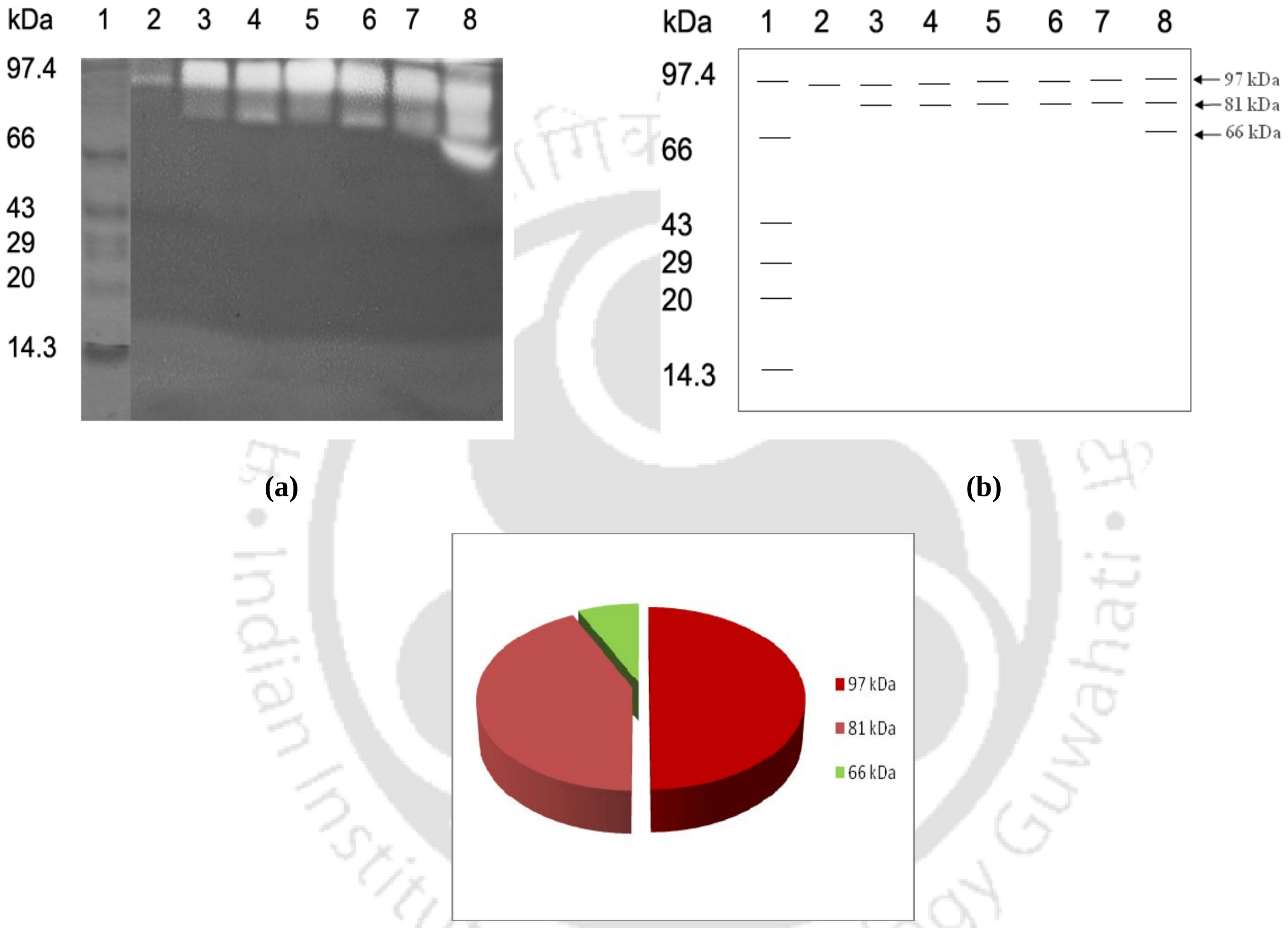


(a)

(b)

(c)

Fig 2.9. Protease Activity Gel: *B. bassiana.* (a) Lane 2,3,4,5,6,7,8 corresponds to strain UB1,UB2,UB3,UB5,UB9,UB10,UB13, (b) schematic representation of (a), (c) Pie chart showing the frequency of occurrence of a particular protease in seven isolates



(a)

(b)

(c)

Fig 2.10. Protease Activity Gel: *M. anisopliae*.(a) Lane: 2,3,4,5,6,7,8 corresponds to strain UM4, UM6, UM7, UM10, UM11, UM12, UM13, (b) schematic representation of (a), (c) Pie chart showing the frequency of occurrence of a particular protease in seven isolates

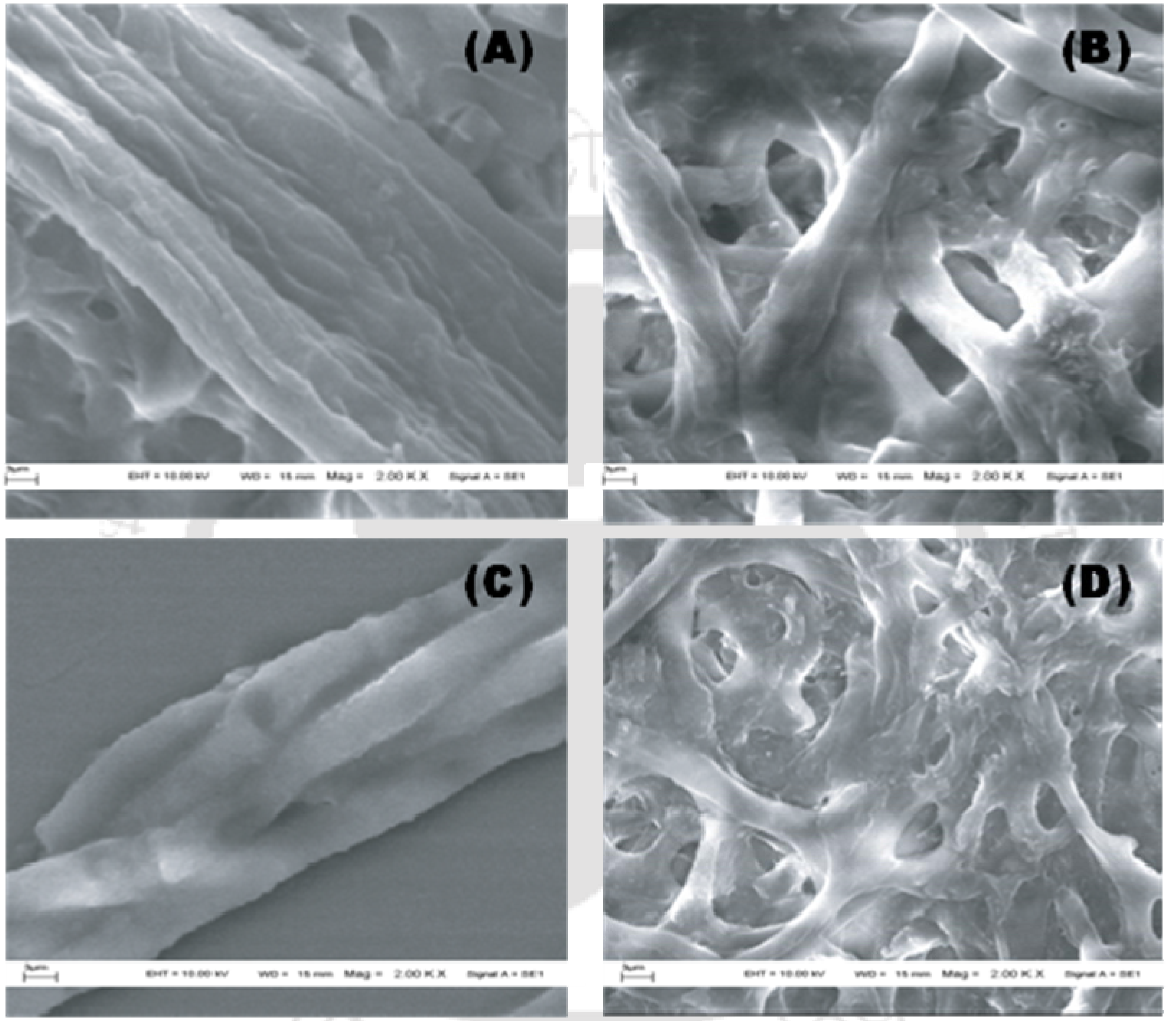
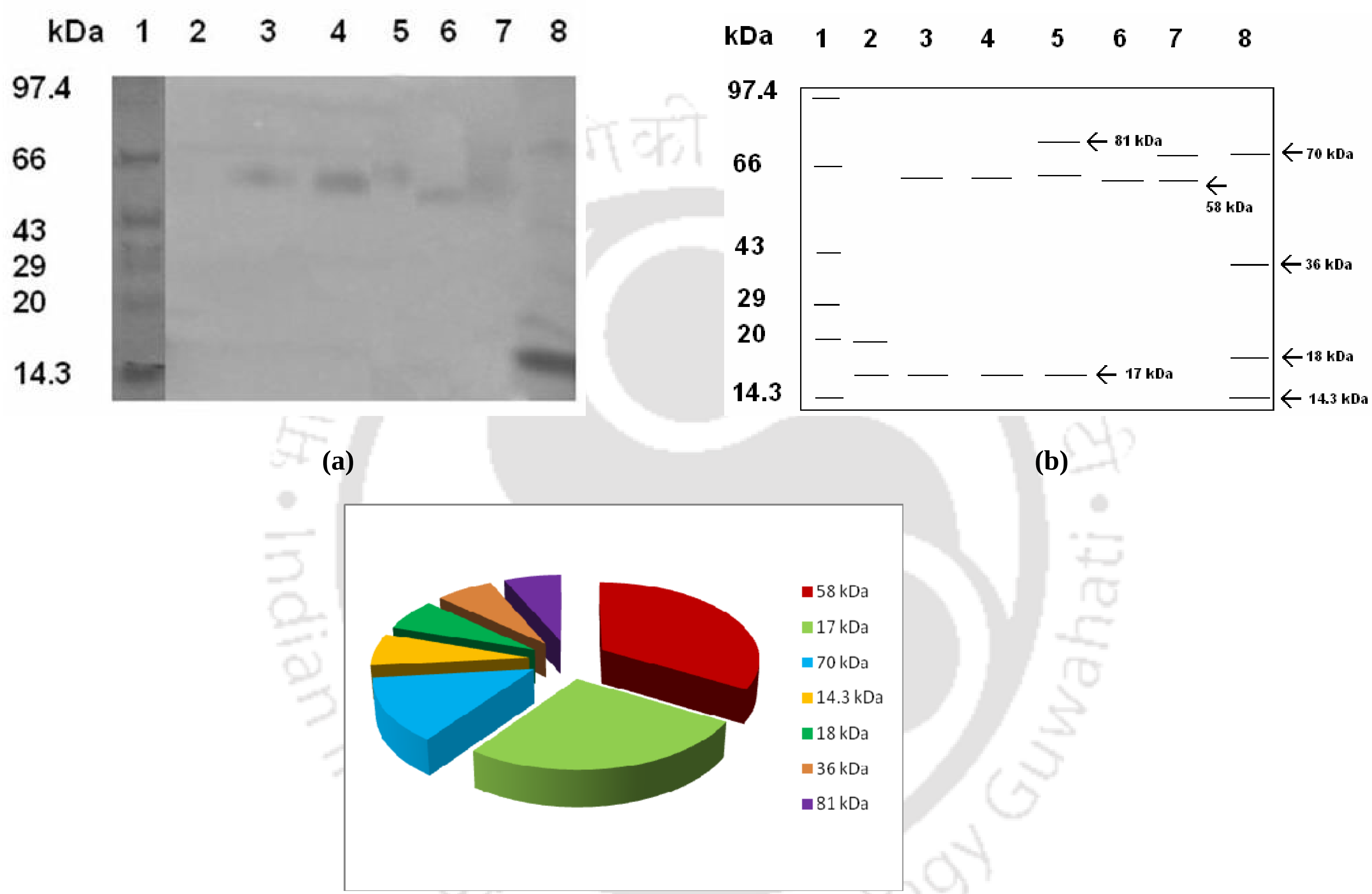


Fig 2.11. Difference in the surface topology of mycelium grown in different medium using SEM image of mycelium in four different media. (A) Minimal media (MM), (B) SD Broth, (C) MM + Casein, (D) MM + Colloidal chitin

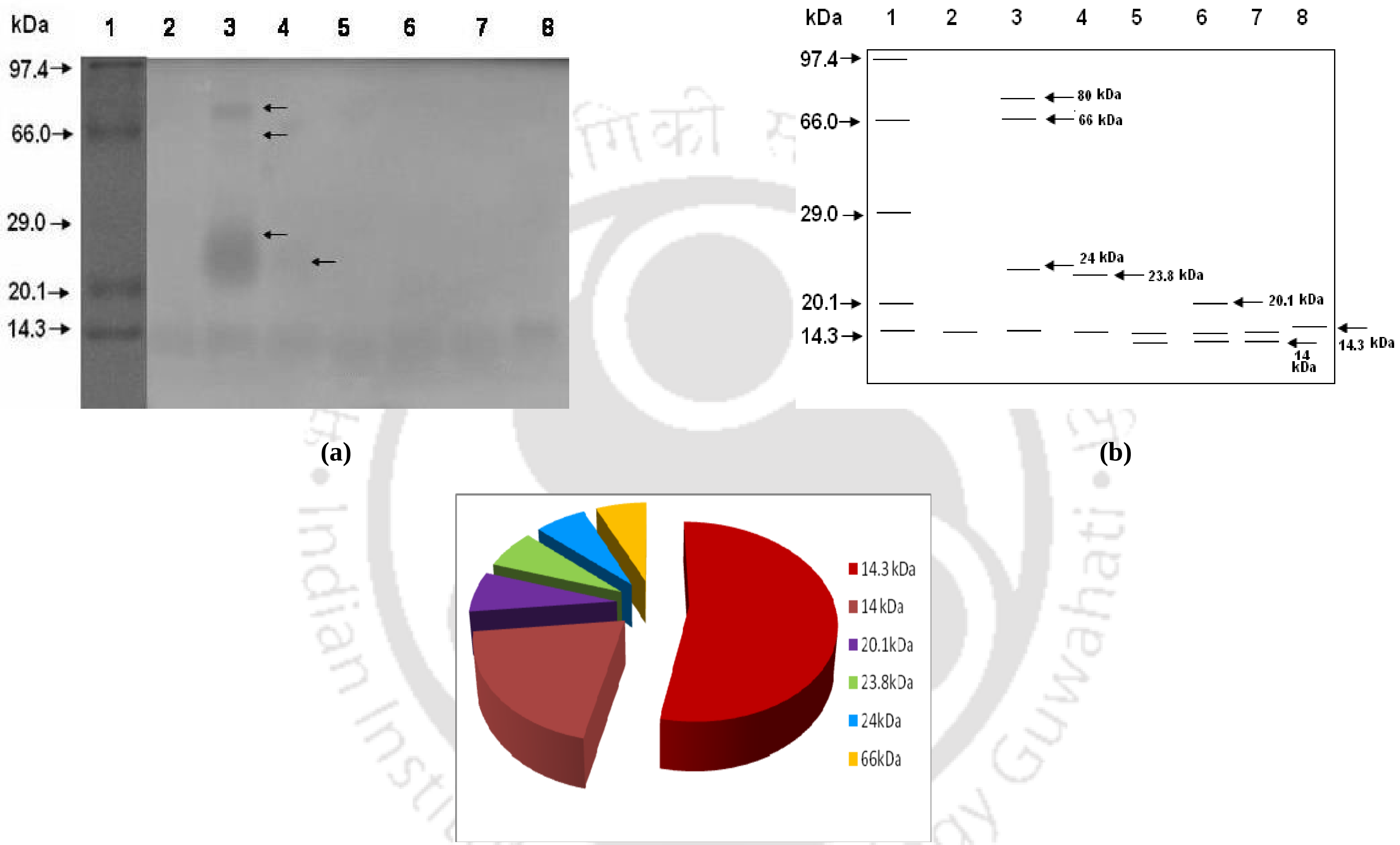


(a)

(b)

(c)

Fig 2.12. Chitinase activity Gel: *B. bassiana.* (a) Lane 2,3,4,5,6,7,8 corresponds to strain UB1,UB2,UB3,UB5,UB9,UB10,UB13. (b) schematic representation of (a), (c) Pie chart showing the frequency of occurrence of a particular chitinase in seven isolates

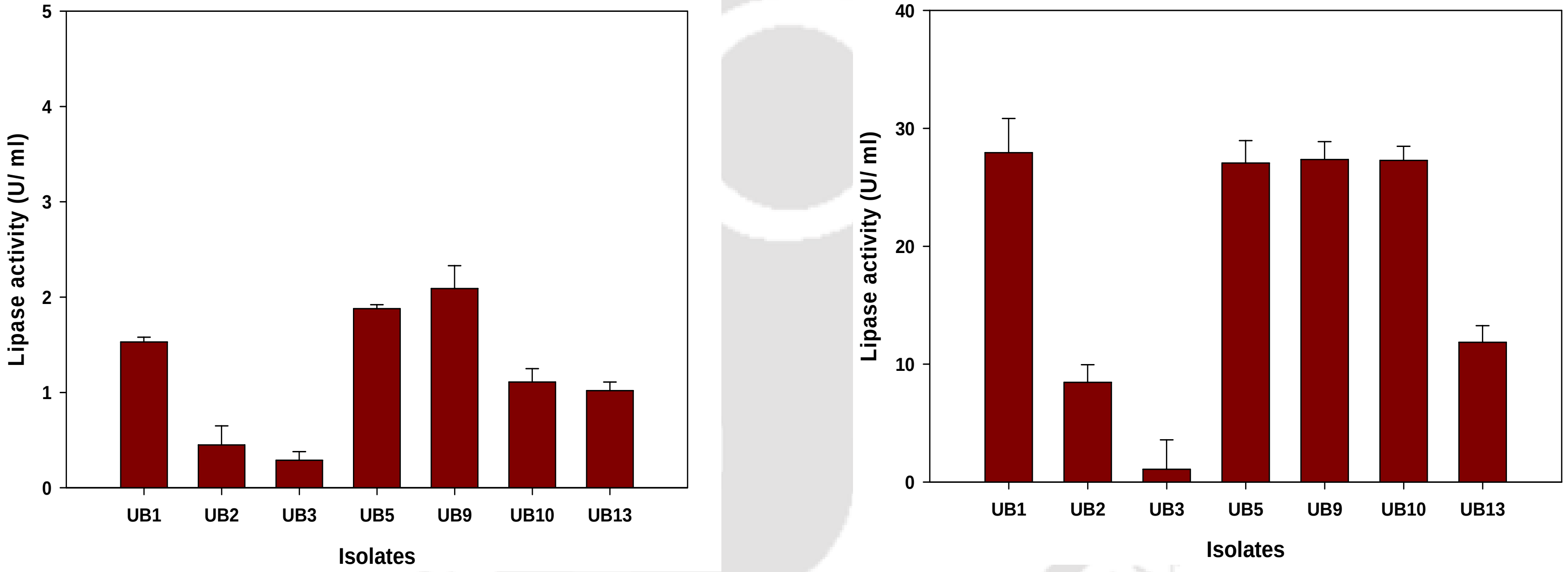


(a)

(b)

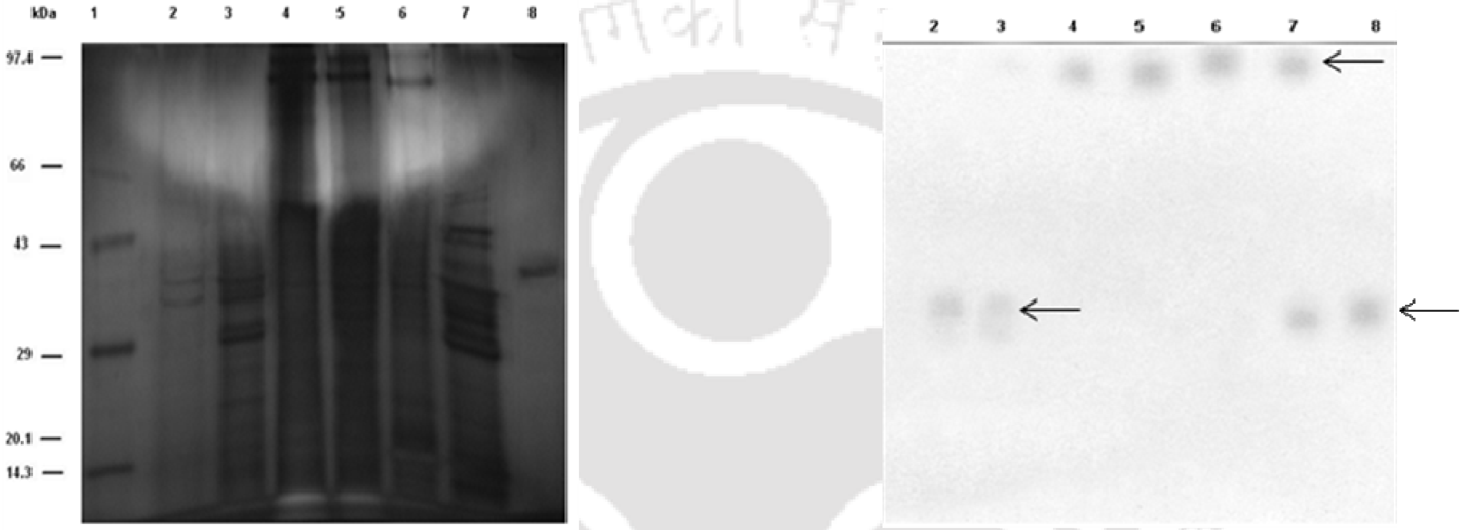
(c)

Fig 2.13. Chitinase activity Gel: *M. anisopliae*.(a) Lane: 2,3,4,5,6,7,8 corresponds to strain UM4, UM6, UM7, UM10, UM11, UM12, UM13, (b) schematic representation of (a), (c) Pie chart showing the frequency of occurrence of a particular chitinase in seven isolates



(a) **(b)**

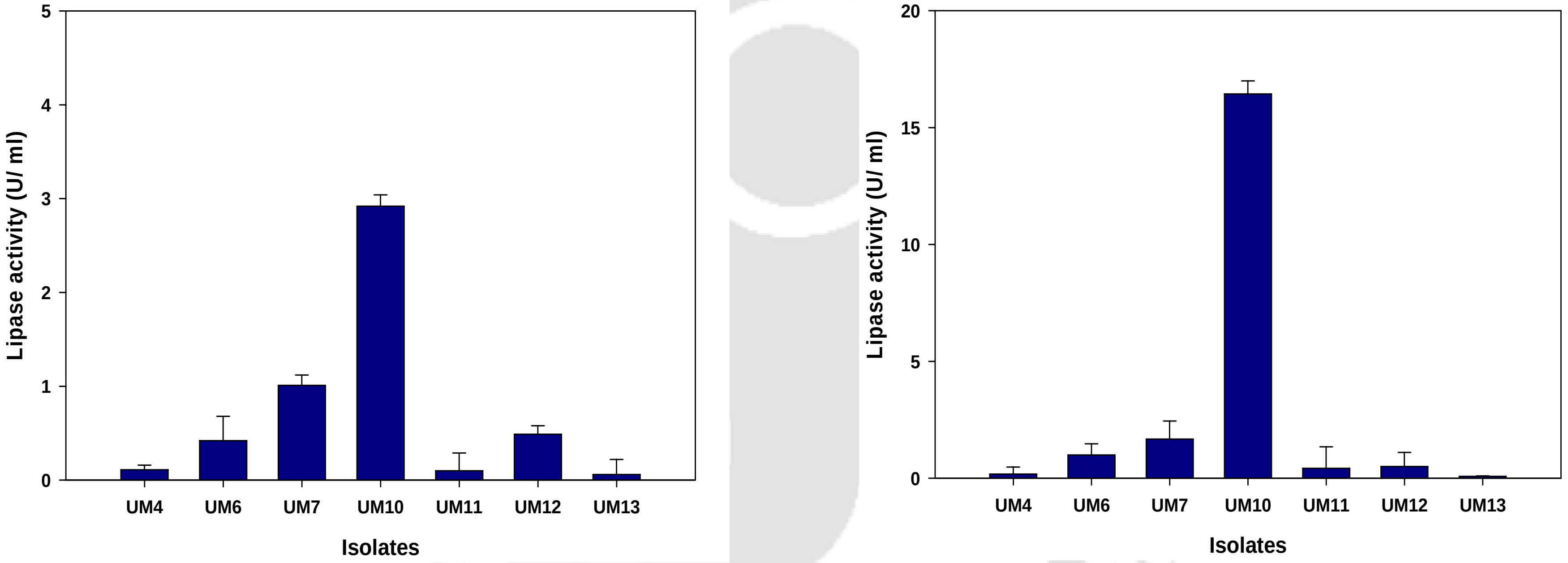
Fig 2.14. Lipase activity by *B. bassiana* isolates in (a) Basal Salt medium and (b) Basal Salt medium supplemented with 2 % Olive oil



(a)

(b)

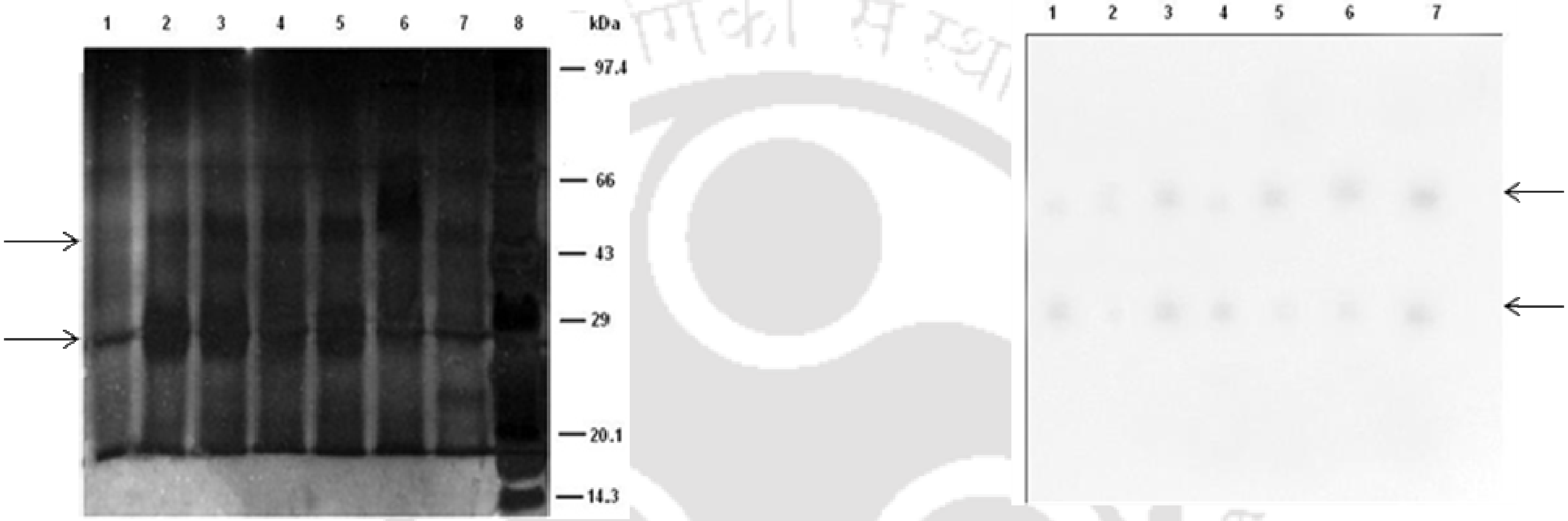
Fig 2.15. Lipase activity Gel: *B. bassiana.* (a) Silver stained Gel: Lane 2,3,4,5,6,7,8 corresponds to isolates UB1,UB2,UB3,UB5,UB9,UB10,UB13, (b) Activity spots observed after gel overlay: Lane2 & 3: 40 kDa, Lane 4& 5: 89 kDa, Lane 6 & 7: 95 kDa, lane 7 & 8: 42 kDa



(a)

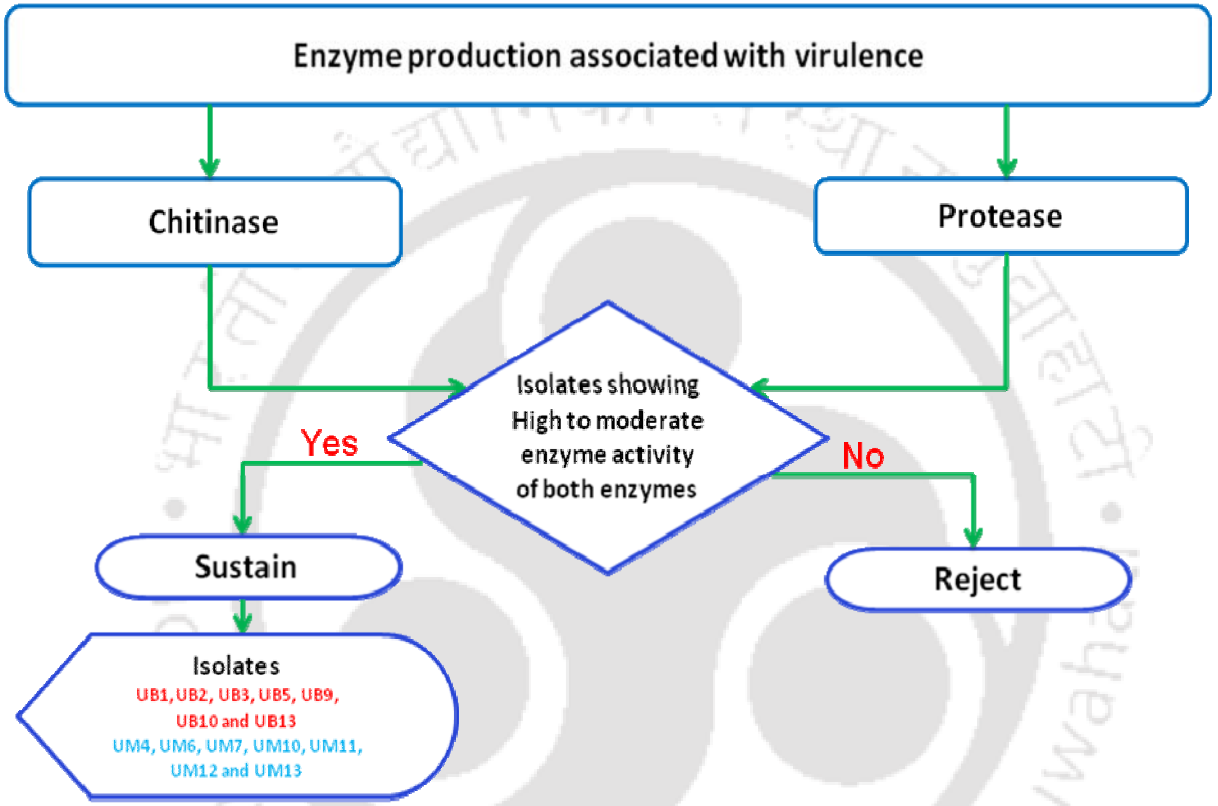
(b)

Fig 2.16. Lipase activity by *M. anisopliae* isolates in (a) Basal Salt medium and (b) Basal Salt medium supplemented with 2 % Olive oil



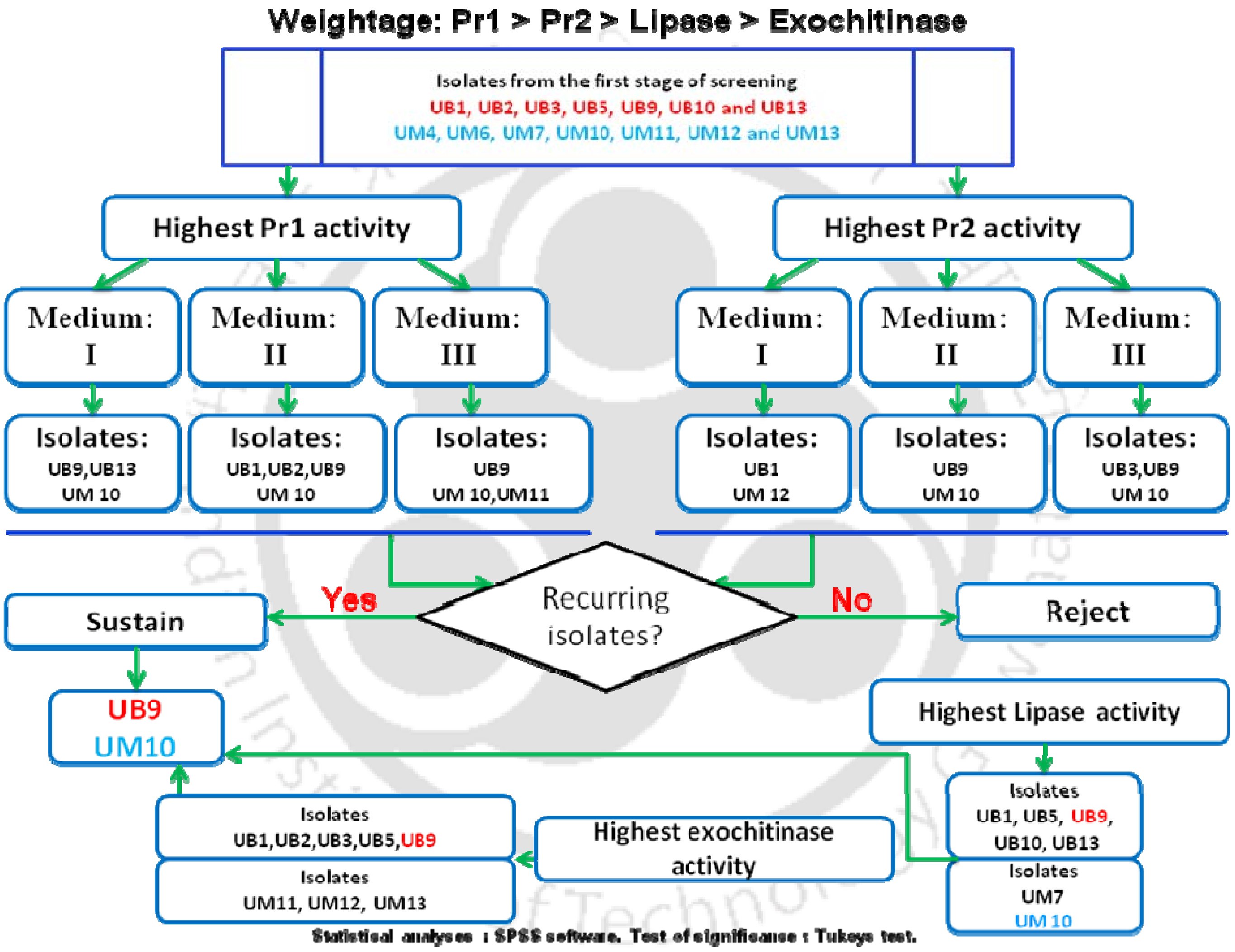
(a) (b)

Fig 2.17. Lipase activity Gel: *M. anisopliae.* (a) Silver stained Gel: Lane: 1,2,3,4,5,6,7 corresponds to isolates UM4,UM6,UM7,UM10,UM11,UM12,UM13, (b) Activity spots observed after gel overlay: 31 and 50 kDa

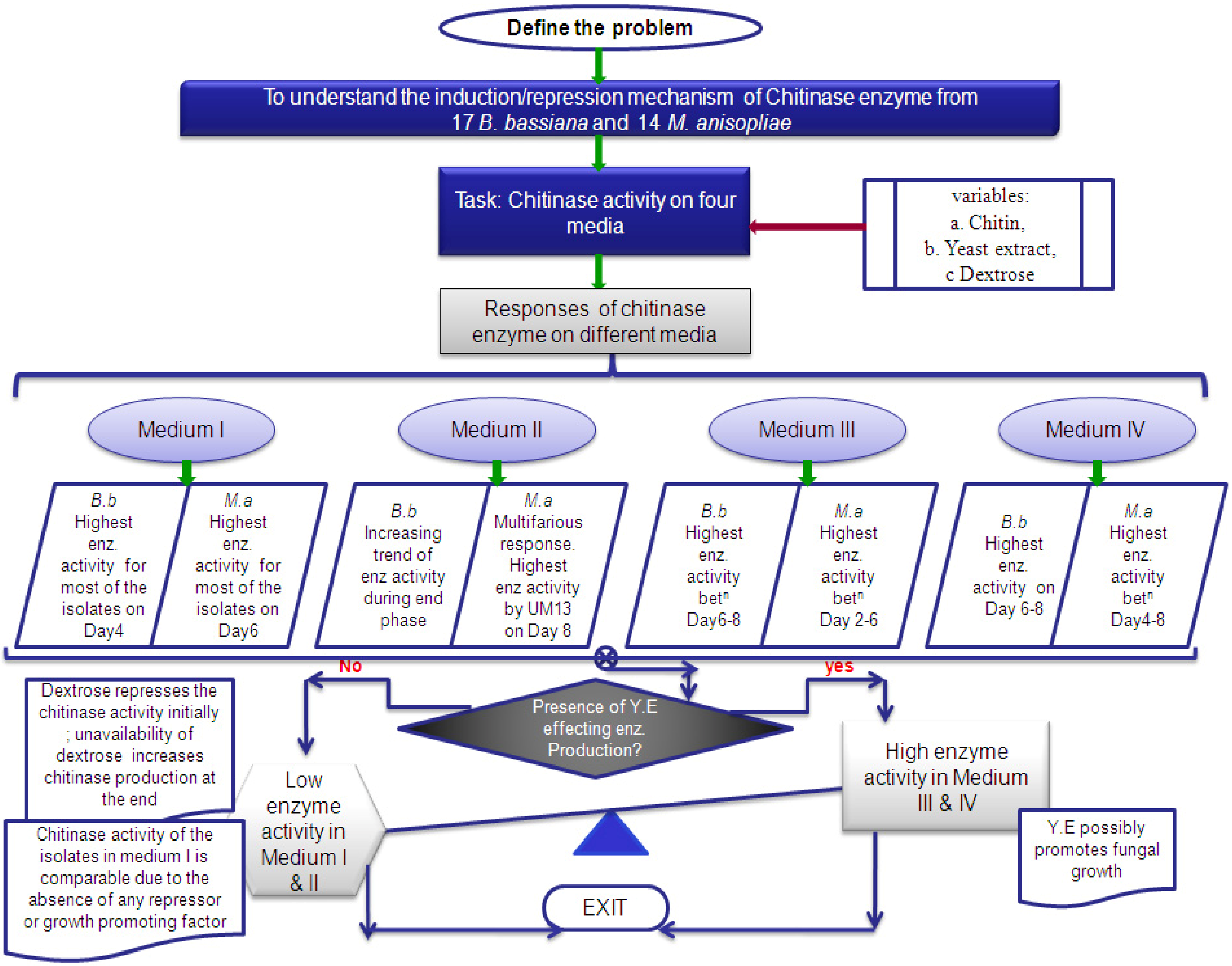


Statistical analyses : SPSS software. Test of significance : Tukeys test.

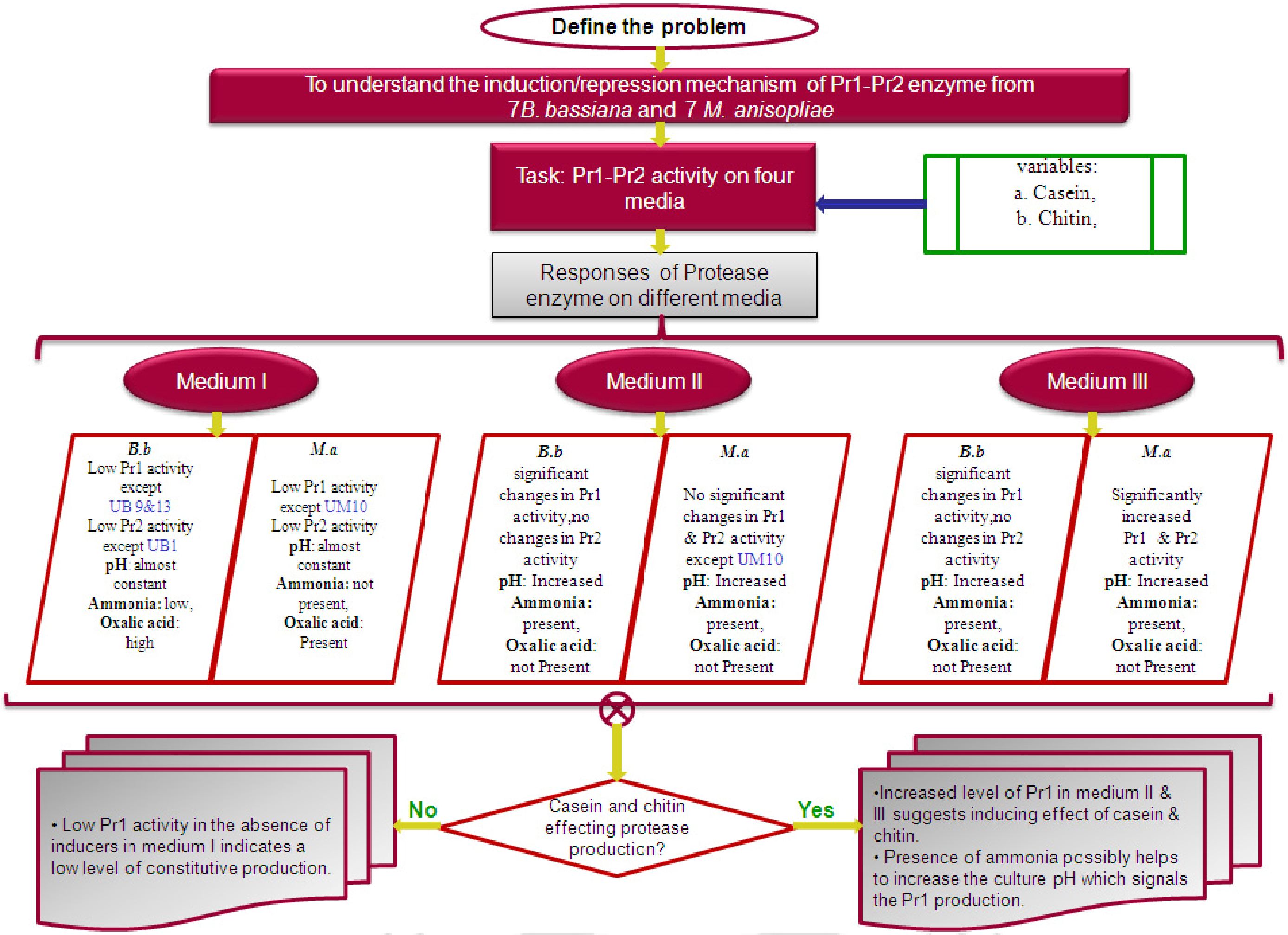
Flowchart 2.1. First stage of screening of isolates based on enzyme production associated with virulence, chitinase & protease



Flowchart 2.2. Second stage of screening based on Pr1 activity



Flowchart 2.3. Summary of Experiment 2 (Chitinase activity in seventeen *B. bassiana* and fourteen *M. anisopliae* isolates)



Flowchart 2.4. Summary of Experiment 3 (Pr1 and Pr2 activity of seven *B. bassiana* and seven *M. anisopliae* isolates)

3.1 Introduction

The mode of invasion of entomopathogenic fungi through insect cuticle is considered to occur by a combination of mechanical pressure and enzymatic degradation (St.Leger et al., 1986) and for this purpose a series of extracellular cuticle hydrolyzing enzymes are secreted by the fungi. The fungi can even diversify the production of enzymes and other virulent factors in response to the different insects (Moino et al., 1998). Proteases and chitinases are the most important enzymes among in invasion. The most essential enzymes released by infective conidia during multiplication on insect are proteases characterized as chymotrypsin-like serine proteinases (Chrzanowska et al., 2001). *M. anisopliae* produces various extracellular serine proteases mainly subtillisin type proteases, trypsin like proteases and metalloproteases and several families of exo-acting peptidases which are believed to be important in host cuticle breeching (St.Leger et al., 1995; St.Leger et al., 1996). Well developed chitinolytic systems has been observed in many entomopathogenic fungi as the production of chitinolytic enzymes are often associated with virulence and cuticle penetration (El sayed et al., 1989, Jackson et al., 1985). Chitin is the second most abundant polymer in nature after cellulose. It is a polymer of *N*-acetylglucosamine (β-1, 4 linked 2-acetamido-d-glucose), which constitutes almost 30 % of insect cuticle. Chitinases (EC 3.2.1.14) cleave the β-1, 4-glycosidic bonds of chitin which is the polymer of *N*-acetyl-β-dglucosamine (GlcNAc). Endochitinases, exochitinases (EC 3.2.1.14), β N-acetylglucosaminidases and chitobiases (EC 3.2.1.30) have been characterized in many EPFs (Courdon et al., 1984; St. Leger et al., 1991). These enzymes are capable of hydrolyzing chitin to its oligomers and monomer. Depending on their cleavage patterns they are divided into endo and exochitinase (Fig 3.1). Endochitinase degrade chitin at any point in the chain forming random sized oligomers. Usually β-N-acetylglucosaminidase is defined as an enzyme releasing non reducing N-acetylglucosamine residues from chitin but have highest affinity for dimer, chitobiose $(GlcNAc)_2$ and convert it into two monomers (Horsch et al., 1997). Typically N-acetylglucosaminidase along with chitobiase is cumulatively referred as exochitinase. Entomopathogenic *B. bassiana* and *M. anisopliae* produce a range of chitinolytic enzymes in medium containing chitin. St. Leger et al. (1993) observed the presence of multiple extracellular chitinase isozymes for *M. anisopliae.* Although the established fact of protease and chitinase in virulence; the enzymes have been poorly characterized. The objective of this chapter is to purify the extracellular chitinase and protease enzyme and characterization of the purified enzymes based on the effect of temperature, pH and influence of enzyme inhibitors.

3.2 Materials and Methods

Fungal isolates

The fungal isolates, *M. anisopliae* (ARSEF 3295) and *B. bassiana* (ARSEF 2033) were obtained from ARSEF (USDA-ARS Plant Protection Unit) and the culture was maintained periodically on Sabouraud dextrose agar slants.

Media and culture conditions for protease production

Conidial suspension of $1x10^6$ conidia/ ml was inoculated into 500 ml basal salts medium (0.1 % KH_2PO_4, 0.05 % MgSO4, 0.05 % NaCl at 20 % (v/v) containing casein (1 % w/v) (Sigma). The pH of the culture medium was adjusted to 8.0 and the cultures were incubated at 28 °C and 180 rpm for 72 h.

Media and culture conditions for chitinase production

Colloidal chitin was prepared as described in chapter 2. A 1×10^6 spores/ ml were inoculated in 500 ml of basal salt media (0.05 % $MgSO_4$, 0.5 % NaH_2PO_4) supplemented with 2 % (w/v) colloidal chitin and incubated for 72 h at 28 °C and 180 rpm.

Purification of protease

The fungal culture was centrifuged at 8000 rpm for 20 min and supernatants were filtered through Whatman filter paper No.1 and then through a 0.22 µm filter. 50-70 % saturation, showing maximum protease activity, was achieved by adding ammonium sulfate to the filtrate. The solution was allowed to stand overnight at 4 °C and the precipitate was collected by centrifugation at 13000 rpm for 15 min. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and subjected to gel-permeation chromatography on Sephadex G-150 column (25 × 3 cm) equilibrated with equilibration buffer, 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl at a flow rate of 50 ml/ h. Fractions showing proteolytic activity were pooled out and lyophilized.

Protease assay

Protease assays were done by the method described in chapter 2 (Materials and Methods: Experiment 1).

Effect of temperature, pH and inhibitors on protease activity

The effect of temperature on the protease activity of purified fractions was determined by incubating the reaction mixture at different temperatures ranging from 10 °C to 60 °C whereas effect of pH was measured by incubating the reaction mixture using Britton-Robinson universal buffer of pH 4.0 to pH 10.0. Purified protease was tested for inhibition by phenylmethylsulfonyl fluoride (PMSF) and EDTA. The final concentrations of these compounds in the assay mixture were 10 mM.

SDS-PAGE

SDS-PAGE of the purified fractions were carried out on a 12.5 % polyacrylamide gel and stained by silver staining method.

Purification of chitinase

Ion-exchange chromatography of extracellular protein extracts was performed. The liquid cultures were centrifuged at 8000 rpm for 20 min and the supernatants were filtered through Whatman filter paper No.1 and then through a 0.22 μm filter. Ninety percent saturation was achieved by adding ammonium sulfate to the filtrate. The solution was allowed to stand overnight at 4 °C and the precipitate was collected by centrifugation at 13000 rpm for 15 min. The precipitate was dissolved in 40 ml of 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The solution was dialyzed overnight against three changes of the same buffer at 4 °C. The dialyzed solution was centrifuged at 13000 rpm for 15 min and the precipitates were discarded. The supernatant was applied to DEAE-cellulose column (45 × 1.5 cm) which was previously equilibrated with the same Tris-HCl buffer. Three column volumes were used to wash and then eluted with 350 ml of linear salt gradient from 0 to 0.5 M NaCl in the same buffer at a flow rate of 60 ml/ h. The purified fractions were collected and purified by lyophilizing.

Chitinase assay

Chitinase assays were done by the method described in chapter 2 (Materials and Methods: Experiment 2 and Experiment 4).

Effect of temperature and pH on chitinase activity

The effects of temperatures on the chitinase activity were tested by incubation of the reaction mixture at various temperatures, 20 °C to 70 °C. Chitinase activity was measured in citrate-phosphate buffer at pH values 2.0 to 8.0.

SDS-PAGE

SDS-PAGE of the purified fractions were carried out on a 12.5 % polyacrylamide gel and stained by silver staining method.

3.3 Results

Protease purification

Protease productions in basal salt media supplemented with 1 % casein were analyzed for *B. bassiana* and *M. anisopliae* isolates. Protease activity of the crude was 1.33 and 1.28 U/ ml for *B. bassiana* and *M. anisopliae* isolate respectively and protease activity as well as specific activity increased in the subsequent purification steps. Protease was fractioned at 50-70 % saturation of ammonium sulfate from culture supernatant. Following gel filtration, two peaks with proteolytic activity were eluted with saline gradient for *B. bassiana* (Fig 3.2a) while three peaks were observed for *M. anisopliae* (Fig 3.2b) and in both the cases enzyme peaks corresponded to the protein peaks. The specific activity of the gel filtration fraction increased for both the isolates (Table 3.1 and 3.2). Fold purification of *B. bassiana* protease after gel filtration was 19.25 which was not much higher compared to the fold purification after ammonium sulfate precipitation, 11.25 (Table 3.1). Purification fold was almost 2.5 times more from ammonium sulfate precipitation to gel filtration in case of *M. anisopliae* though protease yield was low (3.54 %) (Table 3.2). The same trend in enzyme yield after purification was also observed for *B. bassiana.*

Effect of temperature, pH and inhibitors on protease activity

Stability of protease activity was tested over a wide range of temperature. The optimum temperature for protease activity was 30 °C for both *B. bassiana* and *M. anisopliae* isolates (Fig 3.3a & 3.4a). Protease in purified fractions from *M. anisopliae* was relatively stable as almost 36 % and 58 % activity remained at 20 °C and 40 °C respectively which was contrary to the trend observed in protease activity for *B. bassiana* isolate as only 19 and 18 % activity was observed at 20 °C and 40 °C respectively.

Protease activity was stable in the pH range of 7.0 to 9.0, though stability was high in purified fractions from *B. bassiana* compared to *M. anisopliae* though a sharp decrease at pH 10.0 was observed (Fig 3.3b & 3.4b).

Inhibition by PMSF was significant as only 4.27 % and 8.25 % activity remained for *B. bassiana* and *M. anisopliae* isolates respectively. Diminutive decrease in protease activity was observed using EDTA compared to control.

SDS-PAGE

Purified fractions were analyzed on SDS-PAGE. A 47 kDa protease along with 36 kDa protease were observed in the gel filtration fractions of *B. bassiana* (Fig 3.5a) whereas 43 kDa protease along with 36 & 26 kDa were observed in case of *M. anisopliae* purified fractions (Fig 3.5b).

Chitinase purification

Due to the unavailability of the media protein (basal salt media supplemented with 2 % (w/v) colloidal chitin), the culture supernatant was saturated to 90 % with ammonium sulfate. The DEAE-Cellulose chromatography produced two peaks, one major and one minor, of chitinase activity for *B. bassiana* (Fig 3.6 a). Almost 3.69 fold increase in specific activity of chitinase was observed for *B. bassiana* though percent yield was only 1.74 (Table 3. 3). Interestingly the minor peak showed high exochitinase activity with 28 fold purification and 8.04 % yield (Table 3.4). A single sharp peak was observed in case of *M. anisopliae* (Fig 3.6 b). Chitinase activity as well as specific activity of chitinase increased after final step with the purification fold of 2.41 and % yield of 1.76 (Table 3.5). Exochitinase activity of this purified fraction was 3.51 U/ ml though specific activity was low (Table 3.6).

Effects of temperature and pH

Purified chitinase was found to be stable at a range of 30-50 °C with a rapid decrease at 60 °C and only 20 % activity of purified chitinase from *B. bassiana* remained at 70 °C (Fig 3.7a). Optimum pH for this fraction was pH 4.0 though chitinase was reasonably stable at pH 5.0 while decreased at pH 6.0 (Fig 3.7b). The optimum temperature for *M. anisopliae* chitinase was 40 ºC. Chitinase activity was relatively stable at low temperatures as almost 80 % of the chitinase activity retained at 30 °C (Fig 3.8a). The activity was stable in the pH range of 4.0-5.0 with maximum chitinase activity at pH 5.0 and then a sharp decrease at pH 6.0 (Fig 3.8b).

SDS-PAGE

The major peak contained a 33 kDa chitinase whereas the minor one showed the presence of a 50 kDa chitinase for *B. bassiana* (Fig 3.9a). The single peak of *M. anisopliae* purified chitinase showed a relatively low molecular weight of 23 kDa (Fig 3.9b).

3.4 Discussion

Protease purification

Polymorphic enzyme production was observed for both *B. bassiana* and *M. anisopliae* in different culture conditions and has been described in previous chapter. In this study the purification and characterization of extracellular protease and chitinase was attempted. Purification of the enzymes could be correlated with the increase in the specific activity in the subsequent steps. Moreover increase in the enzyme activity after ammonium sulfate precipitation suggests dominance of protein - protein aggregation over protein salt or protein water interaction (Qader et al., 2009). Proteases from both the isolates are observed to be heat labile and subjected to inactivation at acidic pH as almost no activity was observed at pH 4.0 and only 20 % activity remained when using purified proteases from *M. anisopliae* and *B. bassiana* respectively. The optimum pH for protease activity was around pH 8.0 which implies that the enzymes are alkaline proteases. Proteases purified from *B. bassiana* and *M. anisopliae* culture media were strongly inhibited by PMSF which indicates the majority of purified proteases to be classified as serine proteases and not metalloproteases as EDTA inhibition was low. Interestingly a 36 kDa protease was observed for both *B. bassiana* and *M. anisopliae* isolates which is probably a serine protease. Serine proteases are abundant in fungi and mostly produced extracellularly with the molecular weights ranging from 18 kDa to 35 kDa and favors alkaline pH (North, 1982). Bidochka and Khachatourians (1987) purified a 35 kDa serine protease from *B. bassiana* culture media which has an optimum activity at pH 8.5 and 37 °C. The 26 kDa protease from *M. anisopliae* is possibly an elastase as one 25 kDa elastase with optimum pH of 8.0-9.0 was reported in *M. anisopliae* by St. Leger et al. (1986). Entomophthoralean fungus *Zoophthora radicans* produced a range of serine proteases ranging in size from 25 to 37 kDa with a large amount of 46 kDa metalloprotease which is in line with the present study (Xu et al., 2006). Relatively high molecular weight proteases could be classified as metalloproteases, though production in culture media may be low compared to serine proteases as suggested by the inhibition study.

Chitinase purification

Several chitinase isozymes are involved in the cuticle hydrolysis. The number of isozymes could be the result of post translational modifications, as glycosylation produces a wide range of molecular weights. Anion exchange chromatography of the *B. bassiana* culture media showed two distinct activities of chitinase responsible for chitin degradation whereas only one type of chitinase was purified from *M. anisopliae*. The 33 kDa chitinase purified from *B. bassiana* was also reported from several fungi and grouped in endochitinases. The optimum pH of the purified fraction was pH 4.0 for *B. bassiana* and pH 5.0 for *M. anisopliae* which agrees with the findings of St. Leger et al. (1991), both chitinase and exochitinase activities had optimum pH around 5.0. Interestingly most of the purified fungal chitinases have similar optimum pH and thermostability characteristics, active at pH 4.0 to pH 7.0 range and the optimum temperature ranging from 20 - 40 °C (Ulhoa and Perberdy, 1992; De La Cruz et al., 1993; Di Pietro et al., 1993; Li et al., 2005). These types of chitinases are grouped into family 18 of the glycohydrolase superfamily which predominantly groups bacterial chitinases. The 50 kDa chitinase may be an exochitinase as reported by Vishwanathan et al. (2006); a 50 kDa chitinase isoform was detected in mycoparasitic fungi *Trichoderma harzianum* degraded both chitobiose and chitotetrose. A novel low molecular weight chitinase, 23 kDa, was purified from *M. anisopliae* which has both endo and exochitinase activity and different from earlier reported chitinases from *M. anisopliae* but observed in mycoparasitic fungus *L. fungicola*, though optimum pH for the activity was different. Chitinase with both endo and exo chitinase activity was also detected in the culture supernatants of *M. anisopliae* grown in medium containing chitin as sole source of carbon (Kang et al., 1999). Chitin affinity study showed a wide range of chitin binding proteins, 15-89 kDa were observed for *M. anisopliae* whereas a single protein 45 kDa was detected in *B. bassiana* culture supernatants. The 45 kDa protein from *B. bassiana* may be a chitinase as chitinases of this molecular weight were also observed in *B. bassiana* as well as *M. anisopliae* during host cuticle penetration (Havukkala et al., 1992, St. Leger et al., 1996). Chitin binding protein of similar molecular weight, 43 kDa, secreted by *P. aeruginosa* showed chitin binding capability but no chitinolytic activity (Folders et al., 1999). Almost a 23 kDa protein was also observed in *M. anisopliae* culture supernatant which was similar to the chitinase recovered after ion exchange chromatography. Hence these proteins observed in chitin affinity study could be chitin binding proteins without chitinase activity or may be chitinase isozymes which were not detected in anion exchange chromatography.

3.5 Inference

The multiplicity of enzymes confers a core challenge to determine the role of that particular enzyme in adaptation to new environment or in pathogenicity. Purification and characterization of the extracellular proteins could develop better understanding of these enzymes which could be useful to decipher the physiological role of proteases and chitinases. Moreover studies of these virulent candidate genes at molecular level are necessary in order to determine the importance of these enzymes in pathogenicity, which is topic of the next chapter.

Table 3.1. Protease activity from *B. bassiana* (UB9) at different stages of purification

	Protease activity (U/ ml)	**Sp. Activity (U/ mg)**	**Fold purification**	**% yield**
Crude	1.33±0.07	0.08±0.04	1	-
Ammonium sulfate precipitation	4.59±0.19	0.9±0.06	11.25	6.90
Gel filtration fraction	6.78±0.13	1.54±0.10	19.25	4.18

Table 3.2. Protease activity from *M. anisopliae* (UM10) at different stages of purification

	Protease activity (U/ ml)	**Sp. Activity (U/ mg)**	**Fold purification**	**% yield**
Crude	1.28±0.10	0.14±0.05	1	-
Ammonium sulfate precipitation	2.56±0.15	0.75±0.10	5.36	4
Gel filtration fraction	5.67±0.41	1.95±0.11	13.93	3.54

Table 3.3. Chitinase activity from *B. bassiana* (UB9) at different stages of purification

	Chitinase activity (U/ ml)	**Sp. Activity (U/ mg)**	**Fold purification**	**% yield**
Crude	22.37±2.10	1.52±0.10	1	-
Ammonium sulfate precipitation	35.99±1.34	2.98±0.54	1.96	3.22
DEAE fraction	48.72±2.08	5.62±0.35	3.69	1.74

Table 3.4. Exochitinase activity from *B. bassiana* (UB9) at different stages of purification

	Exochitinase activity (U/ ml)	Sp. Activity (U/ mg)	Fold purification	% yield
Crude	0.43±0.04	0.04±0.01	1	-
Ammonium sulfate precipitation	2.79±0.58	0.75±0.06	18.75	12.98
DEAE fraction	4.32±0.76	1.12±0.09	28	8.04

Table 3.5. Chitinase activity from *M. anisopliae* (UM10) at different stages of purification

	Chitinase activity (U/ ml)	**Sp. Activity (U/ mg)**	**Fold purification**	**% yield**
Crude	12.45±1.26	0.85±0.05	1	-
Ammonium sulfate precipitation	16.77±1.82	1.69±0.09	1.98	2.69
DEAE fraction	27.39±1.08	2.05±0.10	2.41	1.76

Table 3.6. Exochitinase activity from *M. anisopliae* (UM10) at different stages of purification

	Exochitinase activity (U/ ml)	Sp. Activity (U/ mg)	Fold purification	% yield
Crude	2.67±0.20	0.09±0.01	1	-
Ammonium sulfate precipitation	2.98±0.07	0.24±0.03	2.67	2.23
DEAE fraction	3.51±0.15	0.39±0.01	4.33	1.05

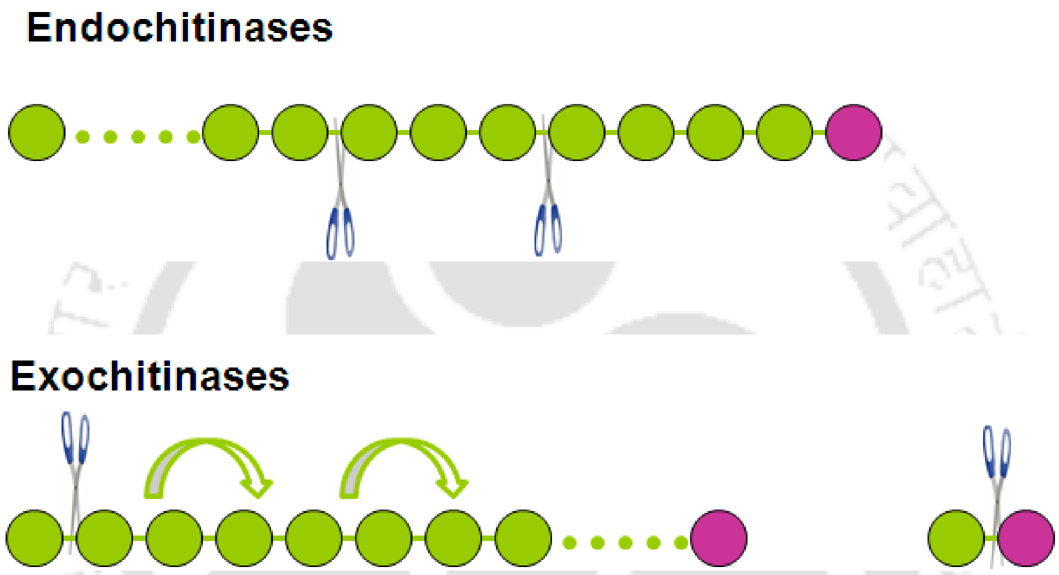
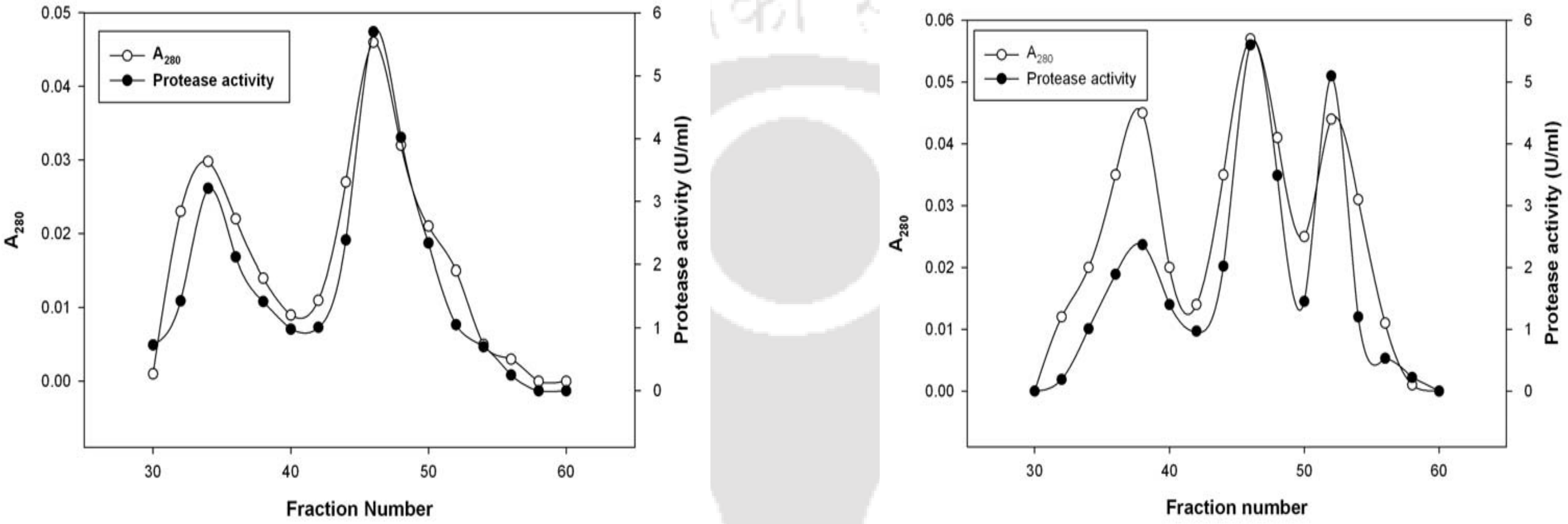


Fig 3.1. Schematic representation of cleavage patterns of chitinolytic enzymes. The N-acetyl glucosamine (GlcNAc) units are shown in green and the reducing end sugar is marked in pink. Dotted lines indicate polymer chains are longer than shown in the figure.



(a) **(b)**

Fig 3.2. Elution profile of protease activity (a) *B. bassiana*, (b) *M. anisopliae*

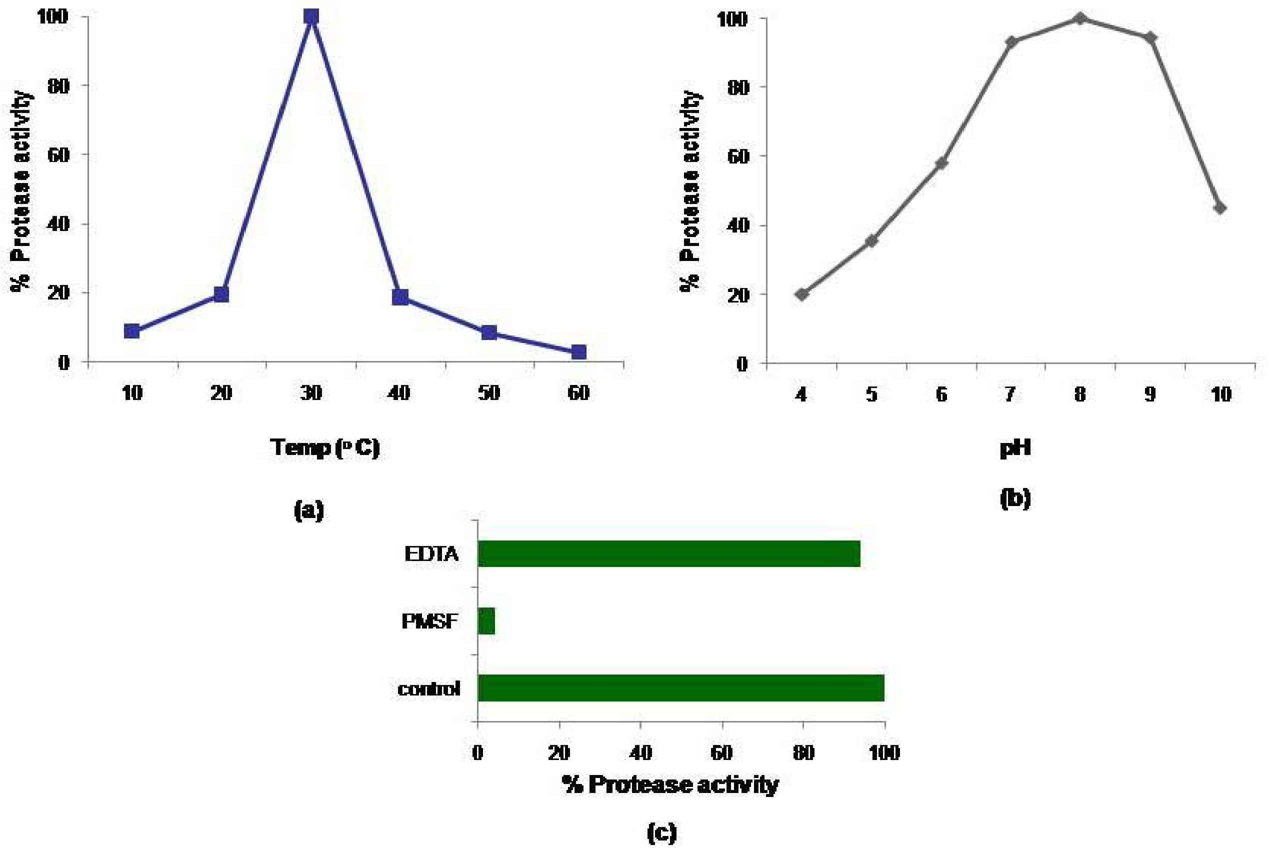


Fig 3.3. Effects of (a) Temperature, (b) pH and (c) inhibitors on purified protease from *B. bassiana* (UB9)

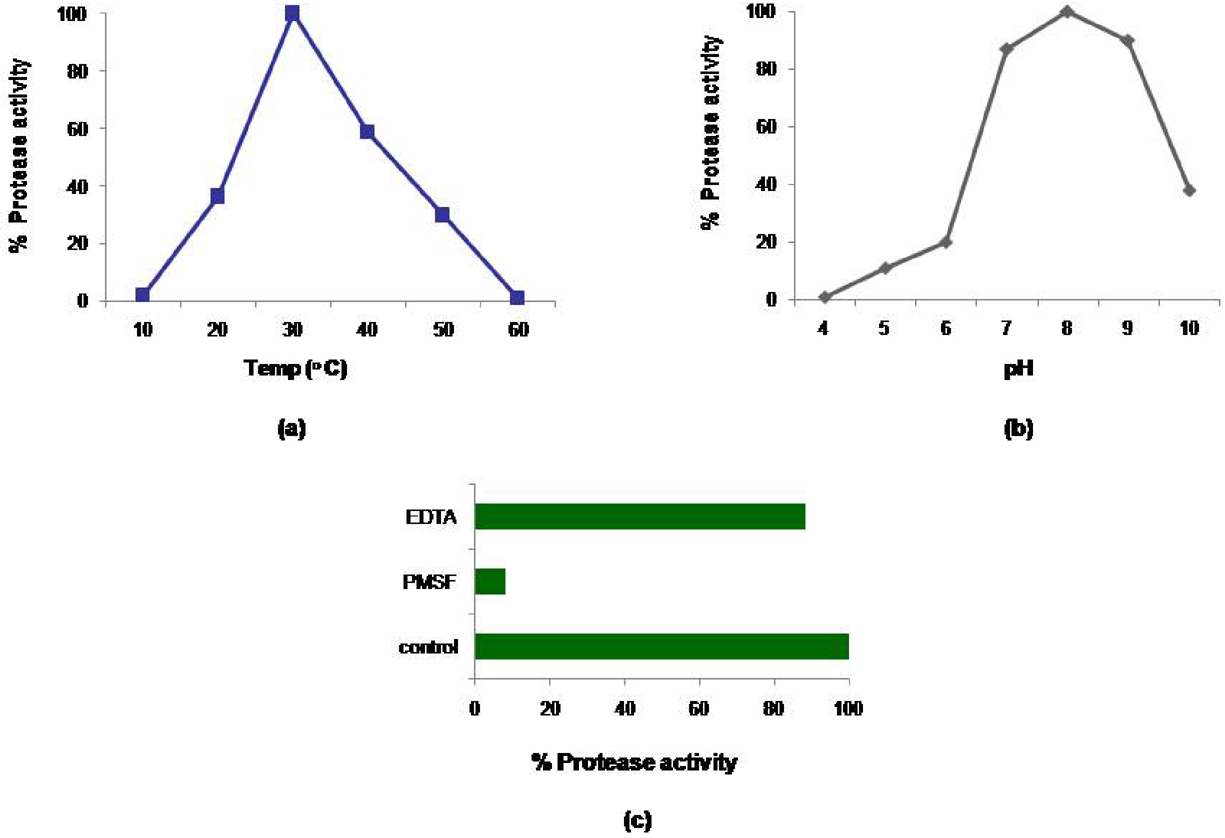
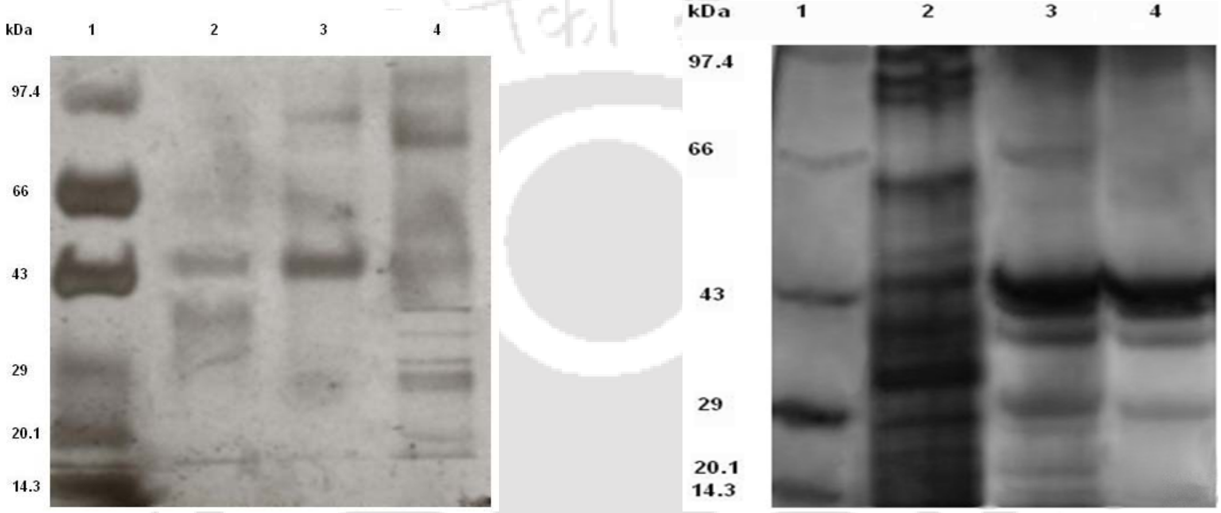


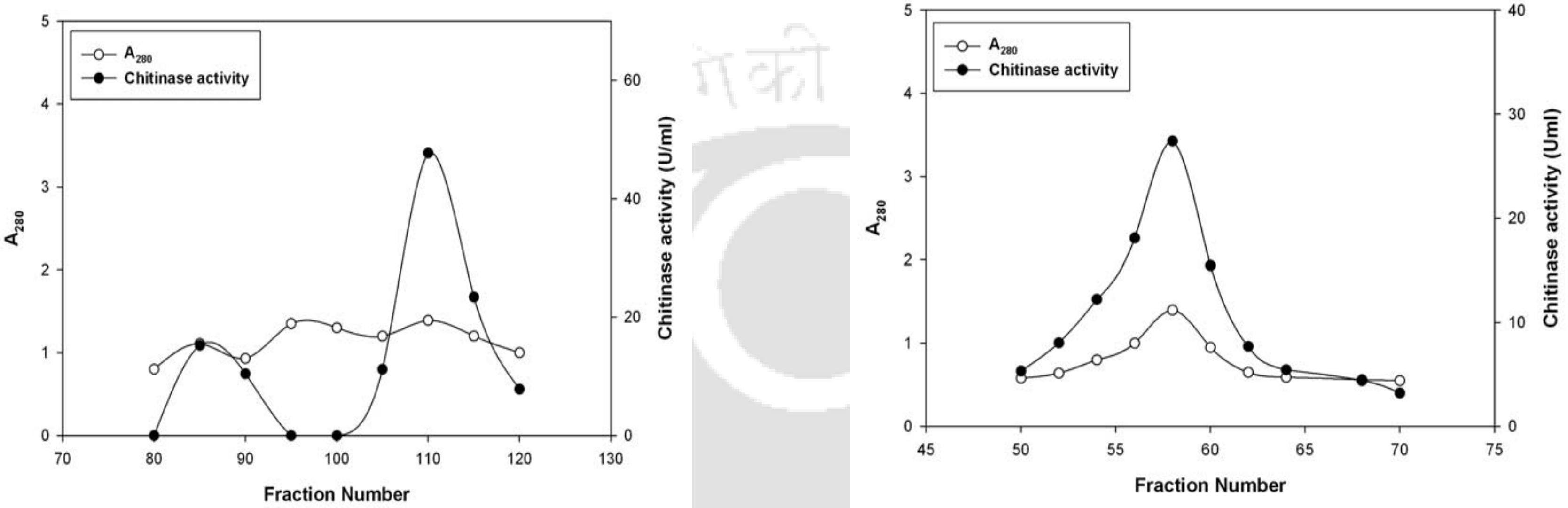
Fig 3.4. Effects of (a) Temperature, (b) pH and (c) inhibitors on purified protease from *M. anisopliae* (UM10)



(a)

(b)

Fig 3.5. (a) SDS-PAGE of purified fractions of protease from *B. bassiana* (UB9): Lane 1 corresponds to mol wt marker, Lane 2 fraction from Gel filtration, Lane 3 Ammonium sulphate ppt. fraction, Lane4 crude extract, (b) SDS-PAGE of purified fractions of protease from *M. anisopliae* (UM10): Lane 1 corresponds to mol wt marker, Lane 2 crude extract, Lane 3 Ammonium sulphate ppt. fraction, Lane4 fraction from Gel filtration



(a) **(b)**

Fig 3.6. Elution profile of chitinase activity (a) *B. bassiana*, (b) *M. anisopliae*

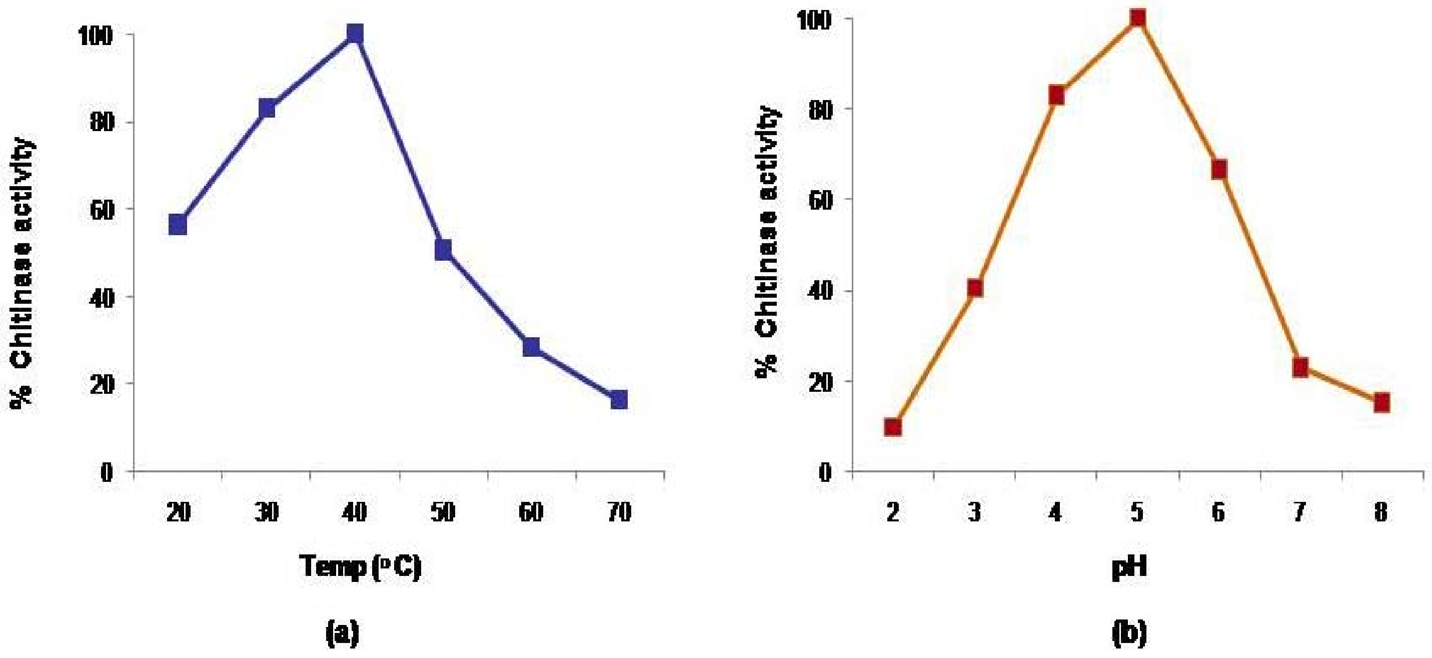
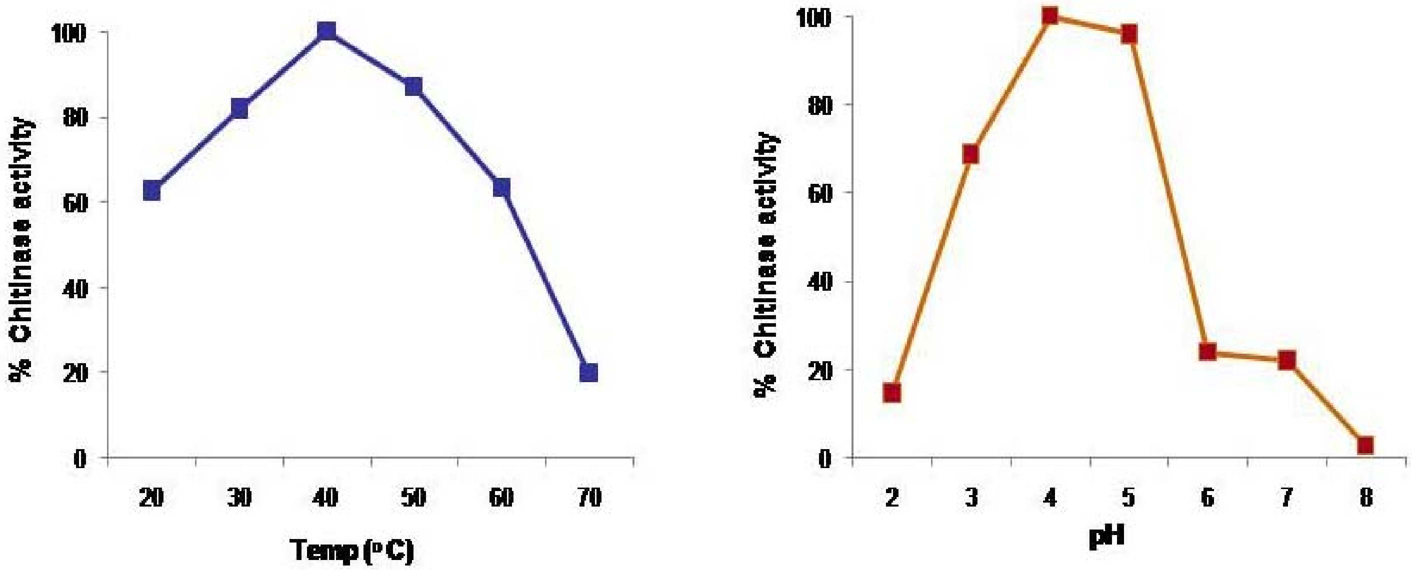


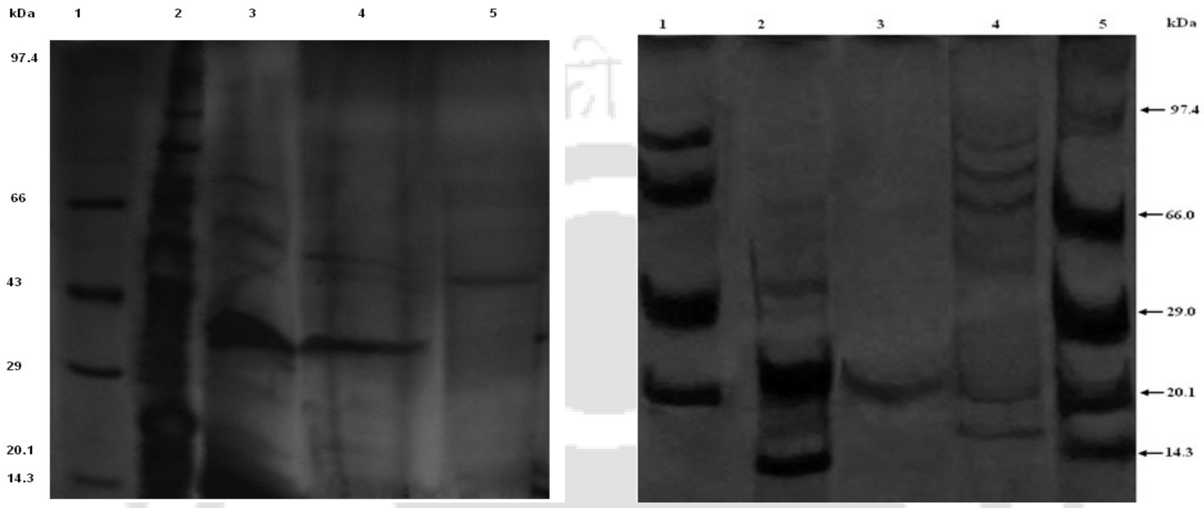
Fig 3.7. Effects of (a) Temperature and (b) pH on purified chitinase from *B. bassiana* (UB9)



(a)

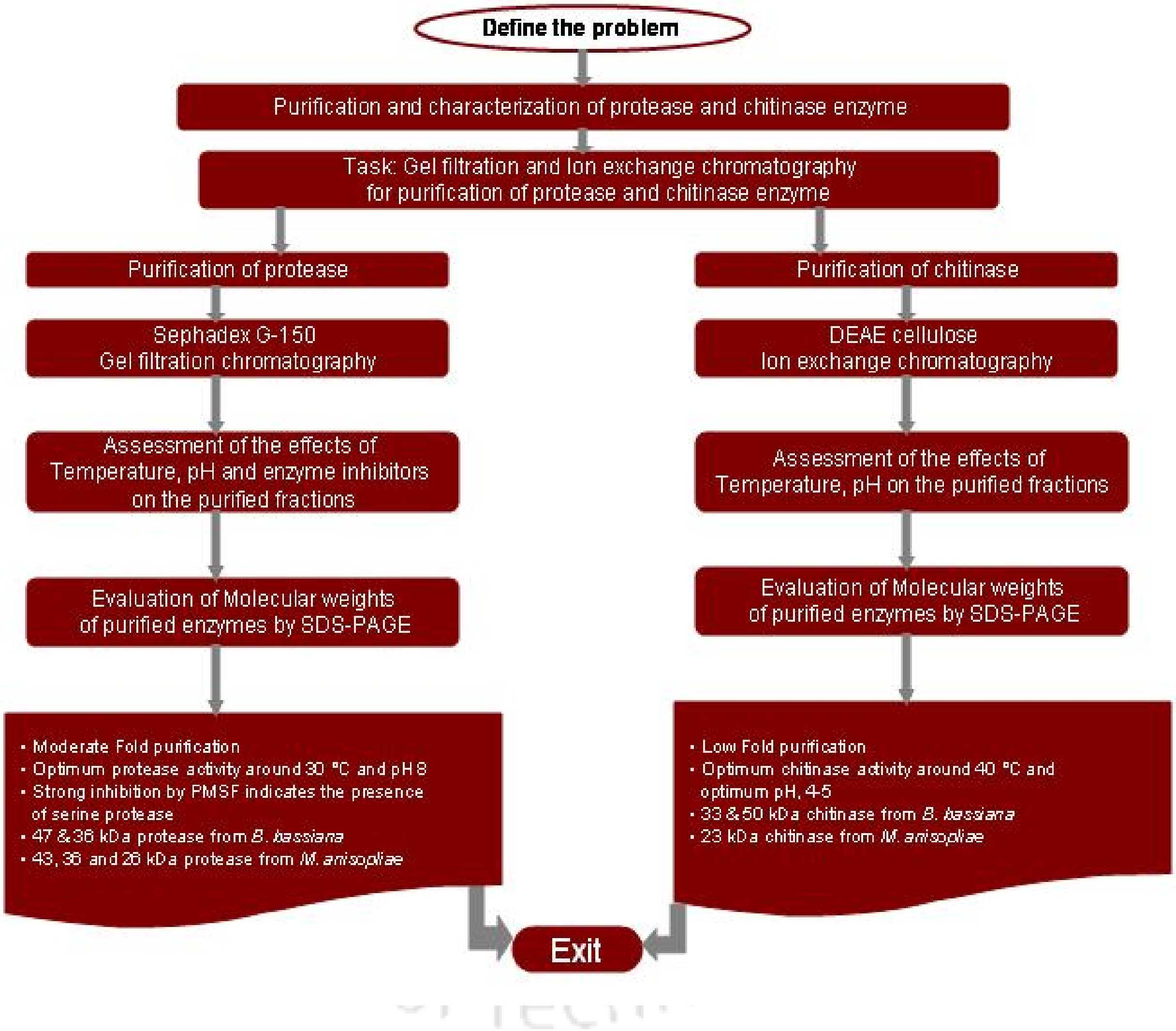
(b)

Fig 3.8. Effects of (a) Temperature and (b) pH on purified chitinase from *M. anisopliae* (UM10)



(a) **(b)**

Fig 3.9. (a) SDS-PAGE of purified fractions of chitinase from *B. bassiana* (UB9): Lane 1 corresponds to mol wt marker; lane2: crude extract, lane 3: ammonium sulfate ppt. Lane 4: DEAE fraction, lane 5: chitin binding proteins, (b) SDS-PAGE of purified fractions of chitinase from *M. anisopliae* (UM10): Lane 1-3 corresponds to chitinase enzyme at different purification step. Lane 1: Crude extract; lane 2: ammonium sulphate ppt. fraction; lane 3: DEAE fraction; lane 4: Chitin binding proteins; lane 5: mol wt marker



Flowchart 3.1. Summary of Chapter 3

4.1 Introduction

Entomopathogenic fungi present a wide spectrum of infectivity and pathogenicity with successful control of various pests. The host pathogen relationship is the key factor to silhouette the organismal diversity despite that the knowledge in evolution of genes responsible for virulence in the pathogen is diminutive. The complex array of enzymes secreted during cuticle penetration and colonization assists to understand molecular parameters of the fungal virulence. Almost all fungal pathogenic genes encoding hydrolytic enzymes are members of gene families which groups genes of common origin that encode products of similar function (Walton, 1996). The functional divergence in genes that arises from gene duplication plays an important role in generating the functional diversification necessary for adaptation (Graur and Li, 2000). Comparison of gene divergence and expression of genetically and ecologically distinct isolates may be beneficial to determine the role of those genes in diversification. Proteases and chitinases are ubiquitous in EPFs which could be used as a model to elucidate the structure-function relationship. Proteases in EPFs are directly related to virulence as isolates with high subtilisin type protease showed high mortality in insects though function of chitinases is not yet apparent (Charnley and St. Leger, 1991). Comparing gene sequences to distinguish the variability among isolates could relate function and phylogeny in terms of molecular adaptation (Golding and Dean, 1998).

This chapter illustrates the study concerning genes of the virulence determinant enzymes. Chitinase and protease gene specific primers (two primer pairs for each gene) were designed based on the conserved sequences. PCR amplified products were sequenced (Xcelris Labs, India) and sequences were analyzed by BLAST (BAST N & BLAST X) and conserved domains in the amplified sequence were detected by CD finder.

4.2 Materials and Methods

Fungal isolates

The fungal isolates, *M. anisopliae* (ARSEF 3295) and *B. bassiana* (ARSEF 2033) were used in this study and the culture was maintained periodically on Sabouraud dextrose agar slants.

Extraction of DNA from fungi

Mycelium was harvested from four day old SD broth by centrifugation at 8000 rpm for 15 min and dried using blotting paper. Mycelium was transferred to a sterile porcelain mortar and crushed with liquid nitrogen. A 10 mg of this crushed mycelium powder was taken into microcentrifuge tube and 500 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 3 % SDS) were added. After incubation at 65 °C for 1 h, 500 µl of TE saturated phenol/ chloroform (1:1) was added and after vigorous vortexing the mixture was centrifuged at 13000 rpm for 20 min. This step was repeated several times to remove all proteins. The supernatant was transferred to a new microcentrifuge tube and 0.1 volumes of 3 M Na acetate and 1 volume of ice cold isopropanol were added. The solution was allowed to stand on ice for 2 h and centrifuged at 13000 rpm for 20 min. DNA pellet was washed with 70 % ethanol, air dried and dissolved in Tris EDTA buffer. RNA was removed by incubating the DNA with 2 µl of 10 mM RNAse A for 30 min at 37 °C. Thereafter the phenol/ chloroform extraction was repeated and DNA pellet was recovered with Na acetate and isopropanol. After washing with 70 % ethanol, the pellet was air dried and dissolved in Tris EDTA buffer and stored at -20 ºC in aliquots.

Primers and PCR

Primers were designed based on the conserved domains of the protease and chitinase enzyme using 10 different fungal protease genes as templates and the same was followed to design the chitinase gene primer (Table 4.1). Enzyme specific DNA sequences were amplified from *B. bassiana* (ARSEF 2033) and *M. anisopliae* (ARSEF 3295) using Polymerase Chain Reaction, 30 PCR cycles consisting of denaturation for 30 sec at 95 °C, annealing for 30 sec at 60 °C and extension for 2 min at 72 °C. PCR products were analyzed on 1 % agarose gel and further submitted for sequencing to Xcelris labs, India.

DNA sequence analysis

DNA sequences of protease and chitinase gene were analyzed by BLAST N searches of NCBI database. Homologous sequences were further analyzed in BLAST X for similar protein sequences and the protein sequences from BLAST X were submitted to CD finder to identify the presence of any conserved domains in that sequence. Furthermore multiple alignments of the DNA sequences were done by Clustal W. Cladograms were constructed using Clustal W which uses neighbor joining method.

4.3 Results

Two gene specific PCR primer pairs were designed that would amplify gene segments in the beginning and at the end of the gene hence if not all, the majority of the DNA sequence of that particular gene will be amplified. The size of the amplified fragment from *B. bassiana* using protease gene specific primers designated as protease primer 1 (PP1) and protease primer 2 (PP2) were 505 bp and 517 bp respectively (Fig 4.1a). A 537 bp and 551 bp fragment was amplified from *M. anisopliae* genomic DNA using PP1 and PP2 respectively (Fig 4.1 b). Later

DNA sequences were obtained from Xcelris labs, India (Fig 4.2). The similarity search revealed that the query sequences from *B. bassiana* (ARSEF 2033) and *M. anisopliae* (ARSEF 3295) were similar to various protease genes belonging to the isolates of *Beauveria* and *Metarhizium* species. BLAST N search of protease gene sequence provided almost 80-100 % sequence similarity of the query sequence with that of the gene sequences stored at NCBI database. Considerable BLAST score generated in the similarity search supports the accuracy of the processes (Table 4.2, 4.3, 4.4 and 4.5). The homologies between the query (protease sequence from *B. bassiana* (ARSEF 2033) and *M. anisopliae* (ARSEF 3295)) and subject sequences (protease sequence stored at NCBI) in terms of protein sequence were confirmed by BLAST X search. Highly similar protein sequences were submitted to CD finder and the results showed the presence of the conserved domains from peptidase superfamily in the query sequences from *B. bassiana* (ARSEF 2033) and *M. anisopliae* (ARSEF 3295). The amplified product using PP1 from *B. bassiana* (ARSEF 2033) showed no conserved domains though 100 % sequence similarity was observed (Table 4.2) while comparing these sequences with the protease gene sequences from *B. bassiana* stored at NCBI database. However, PCR product from *B. bassiana* using PP2 showed conserved domains i.e. actives sites, catalytic triad and calcium binding sites belonging to peptidase superfamily were present in that sequence (Table 4.3). In case of *M. anisopliae* (ARSEF 3295) active sites as well as catalytic triad and calcium binding sites of peptidase were present in the sequences amplified by using both PP1 and PP2 (Table 4.4, 4.5).

The same procedure was followed for chitinase gene isolation, two primers designated as chitinase primer 1 (CP1) and chitinase primer 2 (CP2) were used to amplify the chitinase gene in beginning and at the end leading to the 351 bp and 313 bp amplified fragments for *B. bassiana* and 434 and 438 bp fragments for *M. anisopliae* (ARSEF 3295) (Fig 4.1b). Later DNA sequences were obtained (Fig 4.3). High BLAST score and percent identity was observed for the amplified products while comparing the query sequence with the chitinase sequence stored at NCBI database (Table 4.6, 4.7, 4.8 and 4.9). In case of *B. bassiana*, protein sequence from BLAST X search submitted to CD finder which showed putative active sites of Glycosyl Hydrolase 18 - chitinase like superfamily (Table 4.6 and 4.7). The 434 bp fragment from *M. anisopliae* using CP1 showed high similarity with the database stored sequences and analysis of the results from CD finder indicated the presence of active site, catalytic residue and substrate binding cleft belonging to GH18 chitinase superfamily (Table 4.8). The search for conserved domain in the 438 bp amplified fragment using CP2 from *M. anisopliae* did not show any presence of the chitinase specific conserved domain though 80-100 % sequence similarity was observed (Table 4.9).

The divergence of these enzymes in several filamentous fungi was analyzed (Fig 4.4-4.11). The protease sequences from *B. bassiana* (ARSEF 2033) are closely related to *Pr1A* sequence from *L. lecanii* and *pep C* gene from *A. niger* rather than protease gene sequences from *Metarhizium* and *Beauveria* (Fig 4.4, 4.5). Furthermore, protease sequence from *M. anisopliae* (ARSEF 3295) using PP1 was closely related with the *Pr1A* sequence from *L. lecanii* (Fig 4.6) whereas sequence using PP2 showed no direct relational property with other protease genes (Fig 4.7). Cladogram analysis showed chitinase sequence from *B. bassiana* (ARSEF 2033) shares intimate relation with *chit1* gene from *Metarhizium* and *Beauveria* (Fig 4.8, 4.9). Chitinase sequences from *M. anisopliae* (ARSEF 3295) using both the primers have similarity with the *chit1*, *chi2* and *chi3* gene from *Metarhizium* and *Beauveria* (Fig 4.10, 4.11).

4.4 Discussion

The evolution has resulted in several isoforms of proteases and chitinases in entomopathogenic *B. bassiana* and *M. anisopliae*, the trend not usually observed in saprophytic fungi or plant pathogenic fungi (Siezen and Leunissen, 1997). Entomopathogenic *Verticillium lecanii* expresses large numbers of subtilisin type proteases whereas plant pathogen *Verticillium albo-atrum* does not (St.Leger et al., 1997). The high degree of similarity between these enzymes in different fungal isolates suggests that they may derive from common ancestral genes. Though the entire genes were not isolated in this present study, the partially isolated genes of protease and chitinase from *B. bassiana* and *M. anisopliae* could provide basis for further analysis. Divergent enzymes of *B. bassiana* and *M. anisopliae* are broadly distributed in the phylogenetic tree. Among the isolated protease sequences, most of the sequences showed similarity with the Pr1A protease isozyme that possibly indicates gene multiplicity of that isozyme increasing the chances to isolate the gene easily and frequently. Pr1A is the predominant protein produced by the EPFs and ESTs for *Pr1A* were observed to be 10 times more abundant than the second most expressed sequence, *Pr1J* (St.Leger et al., 1989; Bagga et al., 2004). However, pepC gene from *A. niger* was clustered closely with *B. bassiana* but distantly with *M. anisopliae* which suggests a common ancestral origin of *A. niger* pepC gene with the isolated protease gene of *B. bassiana*. But no such trend was observed for chitinase gene isolation as sequence similarity was observed with *chit1* and *chi2* as well as *chi3* gene. However, the isolated chitinase gene from *B. bassiana* using CP1 and CP2 probably belong to chit1 gene and the closely clustered chit1 genes from both *B. bassiana* and *M. anisopliae* indicates the common ancestral origin of chit1 gene in both species. The chitinase from *M. anisopliae* showed a close evolutionary relation with *M. anisopliae* chi2 and chi3 gene whereas they are distantly related with chiA and ech2 gene from *A. nidulans and H. virens*. The results of the BLAST searches showed high sequence similarity though the query sequence was only partially analogous to the database sequences. Interestingly protease sequences from *B. bassiana* and *M. anisopliae* showed two similar sequences in the same PCR product for instance isolated protease sequence from *M. anisopliae* using PP1 produced similarity of 98-229 bases with the 411-542 bases of *Metarhizium acridum* strain CG191 subtilisin-like protease Pr1A (pr1A) gene and again similar sequences were observed at 291-341 bases with the 642-689 bases of subject sequence suggesting that sequence separating these two parts i.e. base 230-290 may be an intron present in the gene. Most of the isolated sequences of chitinase and protease gene from *B. bassiana* and *M. anisopliae* showed to encode the protein sequences generally present in the active sites or substrate binding cleft which is thought to be highly conserved in particular type of enzymes and fastidiously belongs to certain enzyme superfamilies. In this study the protease sequences from both the isolates are grouped into the Peptidase-S8-S53 superfamily whereas chitinases belonged to GH 18 chitinase superfamily which is in line with the fact that fungal chitinases are grouped into family 18 chitinases rather than family 19 which is exclusively found in plants (Henrissat, 1999; Hamel et al., 1997).

4.5 Inference

Insight to the mechanism of pathogenicity can provide the knowledge for the development of potent mycoinsecticides by identifying the virulence factors or else identifying the genes responsible for virulence and genetically modify the fungi to enhance virulence. Virulence of the fungal isolates *M. anisopliae* (ARSEF 3295) and *B. bassiana* (ARSEF 2033) were studied and described in the next chapter, 5.

Table 4.1. Primers used for isolation of genes

Protease Primers	
Protease Primer1(PP1)	
Forward primer:	AATAGCCCGGCGTTCCGTGGGGATCC
Reverse primer:	GCCAGCTGCAGTGTCGTGGAGGATCC
Protease Primer2 (PP2)	
Forward primer:	CTGCAGGATGGCAACGCCCAGCGTGA
Reverse primer:	CTGCAGGCGAGGCCATTGAGGTGCCA
Chitinase Primers	
Chitinase Primer1 (CP1)	
Forward primer:	GGGGGTTCGAGGATGACACC
Reverse primer:	CTGTTGGCCCTGAGCGTCCG
Chitinase Primer2 (CP2)	
Forward primer:	CTCGTTGGCGTCGACCTGGAT
Reverse primer:	GGGCCTCCCACAACATGGCG

Table 4.2. Results of BLAST analysis of DNA sequence from *B. bassiana* PCR product using PP1

Sequence	Score	Identities	Conserved domains
Beauveria bassiana cuticle-degrading proteinase CDEP-1 (cdep1) gene, cdep1-s allele (gb|AY040532.1|) QS-393-425:SS-405-437	62.1	100%	———
Beauveria bassiana strain MTCC 2028 serine proteinase (Pr1) gene (gb|GU166155.1|) QS-393-425:SS-333-365	62.1	100%	———
Beauveria bassiana cuticle-degrading proteinase (CDEP2) Mrna (gb|EF195164.1|) QS-393-425:SS-268-300	62.1	100%	———
Beauveria bassiana cuticle-degrading protease bassiasin I (bsn1) gene (gb|AF154118.1|) QS-393-425:SS-409-441	62.1	100%	———

Table 4.3. Results of BLAST analysis of DNA sequence from *B. bassiana* PCR product using PP2

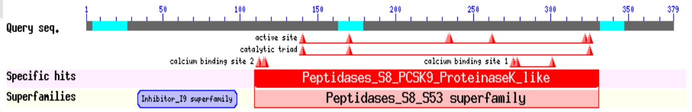
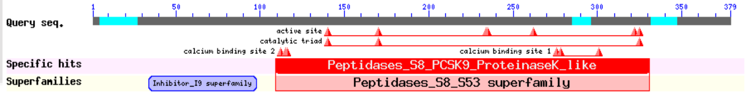
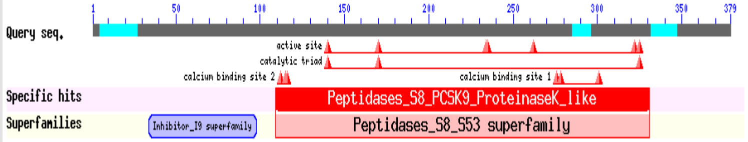
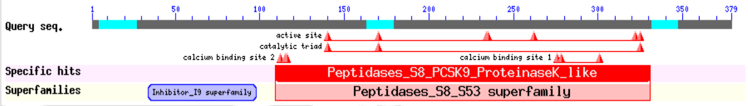
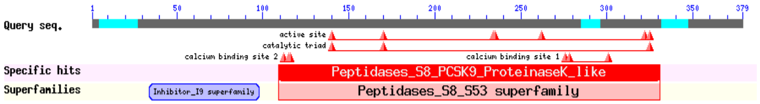
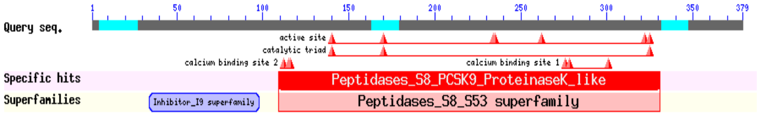
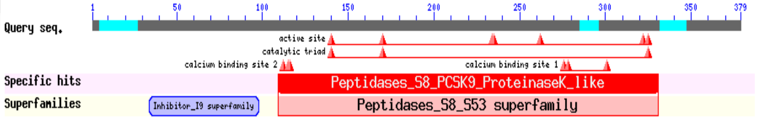
Sequence	Score	Identities	Conserved domains
Beauveria bassiana cuticle-degrading proteinase CDEP-1 (cdep1) gene (gb|AY040532.1|) QS-1-139:SS-762-896; QS-301-483:SS-920-1104	193	93%	
	254	91%	
Beauveria bassiana strain MTCC 2028 serine proteinase (Pr1) gene (gb|GU166155.1|) QS-301-483:SS-848-1032	255	92%	
Beauveria bassiana cuticle-degrading proteinase (CDEP2)mRNA (gb|EF195164.1|) QS-1-139:SS-564-698; QS-301-483:SS-722-906	182	91%	
	248	91%	
Beauveria bassiana cuticle-degrading protease bassiasin I (bsn1) gene (gb|AF154118.1|) QS-1-139:SS-767-901; QS-301-483:SS-925-1109	198	93%	
	248	91%	

Table 4.4. Results of BLAST analysis of DNA sequence from *M. anisopliae* PCR product using PP1

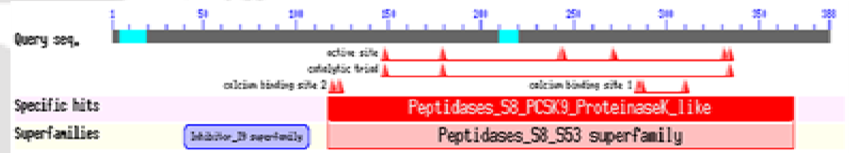
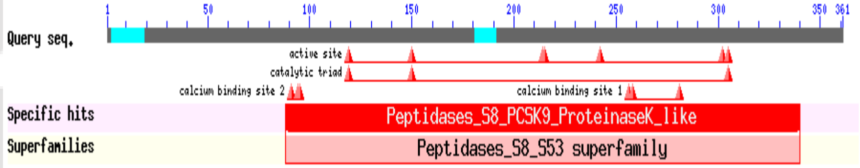
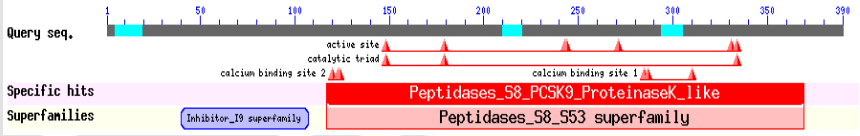
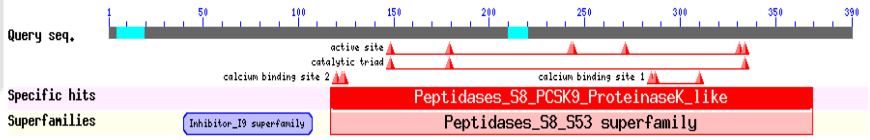
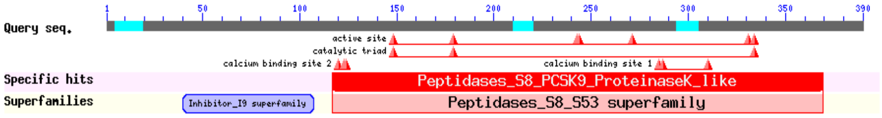
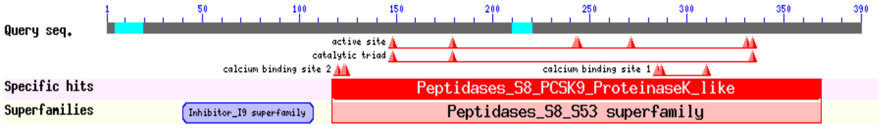
Sequence	Score	Identities	Conserved Domains
Metarhizium anisopliae cuticle-degrading protease mRNA (gb|M73795.1|MEZCDPA) QS-98-241: SS-335-478	161	87%	
Metarhizium anisopliae var. acridum mRNA for Subtilisin-like serine protease PR1A (pr1A gene) (emb|AJ251925.1|) QS-98-237:SS-415-554	132	84%	
Metarhizium acridum strain CG191 subtilisin-like protease Pr1A (pr1A) gene (gb|FJ659172.1|) QS-98-229:SS-411-542; QS-291-341:SS-642-689	156	88%	
	58.4	89%	
Metarhizium anisopliae strain V301 subtilisin-like protease Pr1A (pr1A) gene (gb|FJ659171.1|) QS-98-229:SS-411-542; QS-291-341:SS-642-689	156	88%	
	58.4	89%	

Table 4.5. Results of BLAST analysis of DNA sequence from *M. anisopliae* PCR product using PP2

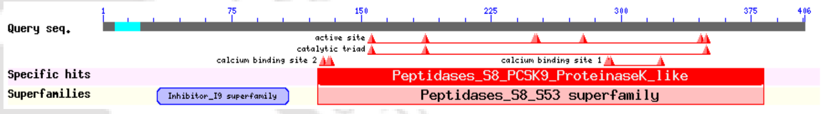
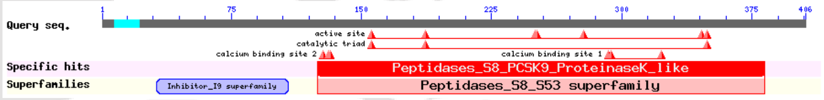
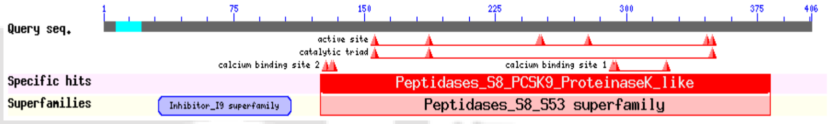
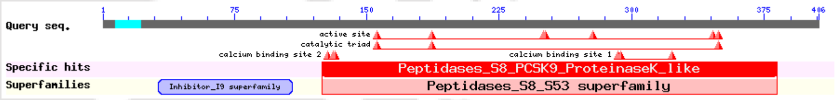
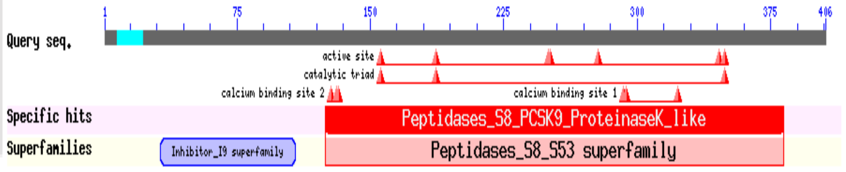
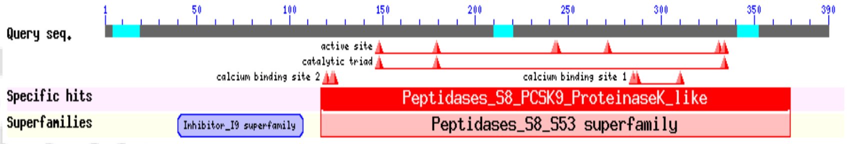
Sequence	Score	Identities	Conserved domains
Metarhizium anisopliae var. acridum mRNA for subtilisin-like protease PR1D (pr1D gene) (emb|AJ431638.1|) QS-86-137:SS-1012-1063; QS-324-376:SS-1063-1114	97.1	100%	
	86.1	97%	
Metarhizium anisopliae var. *anisopliae* pr1D gene for subtilisin-like protease PR1D, exons 1-2 (emb|AJ400706.1|) QS-86-137:SS-1401-1452 QS-324-376:SS-1452-1503	97.1	100%	
	86.1	97%	
Metarhizium anisopliae var. *anisopliae* strain V245 subtilisin-like serine protease PR1A (pr1A) gene (gb|AY389127.1|) QS-38-97:SS-883-931	91.6	95%	
Metarhizium lepidiotae strain CG648 subtilisin-like protease Pr1A (pr1A) gene (gb|FJ659189.1|) QS-27-97:SS-936-1003	89.8	91%	

Table 4.6. Results of BLAST analysis of DNA sequence from *Beauveria bassiana* PCR product using CP1

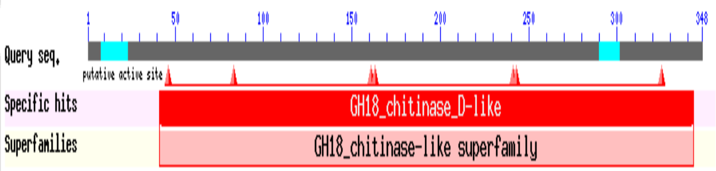
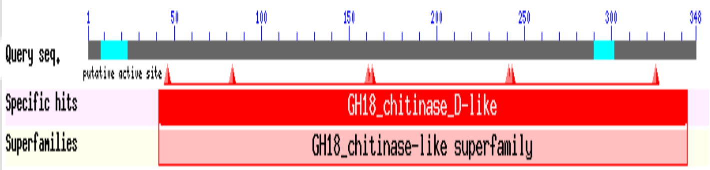
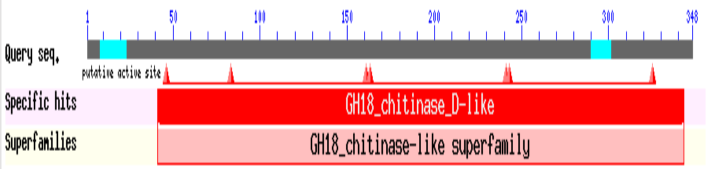
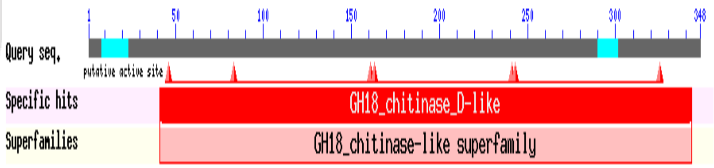
Sequence	Score	Identities	Conserved domains
Beauveria bassiana strain JYBb201-11 chitinase (chit1) gene, complete cds (gb|HQ435871.1||) QS:144-208:SS-146-210	111	97%	
Beauveria bassiana strain MTCC 2028 endochitinase (Ch1) gene, complete cds (gb|GU166156.1|) QS:144-208:SS-146-210	111	97%	
Beauveria bassiana strain NCIM1216 chitinase (chit1) gene, complete cds (gb|EU828354.1||) QS:144-208:SS-146-210	111	97%	
Beauveria bassiana chitinase (chit1) gene, complete cds Length=1669 (gb|AY145440.1|) QS:144-208:SS-611-675	111	97%	

Table 4.7. Results of BLAST analysis of DNA sequence from *Beauveria bassiana* PCR product using CP2

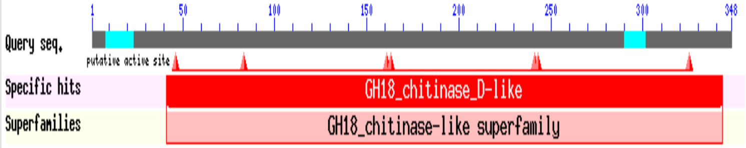
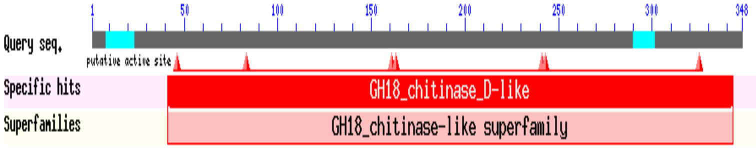
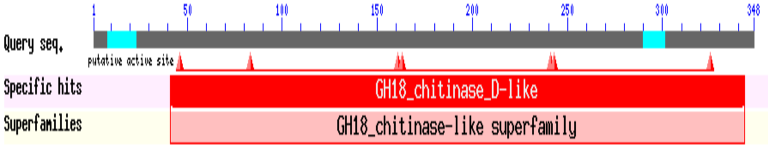
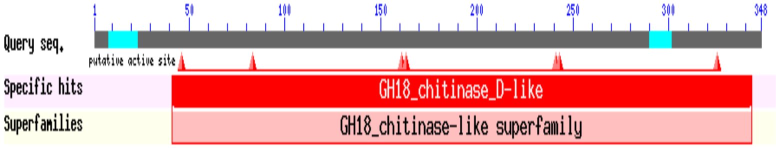
Sequence	Score	Identities	Conserved domains
Beauveria bassiana strain JYBb201-11 chitinase (chit1) gene, complete cds (gb|HQ435871.1||) QS:22-190:SS-727-884	152	85%	
Beauveria bassiana strain NCIM1216 chitinase (chit1) gene, complete cds (gb|EU828354.1||) QS:22-190:SS-727-884	152	85%	
Beauveria bassiana chitinase (chit1) gene, complete cds Length=1669 (gb|AY145440.1|) QS:22-190:SS-1192-1349	152	85%	
Beauveria bassiana strain MTCC 2028 endochitinase (Ch1) gene, complete cds (gb|GU166156.1|) QS:22-190:SS-727-884	141	84%	

Table 4.8. Results of BLAST analysis of DNA sequence from *M. anisopliae* PCR product using CP1

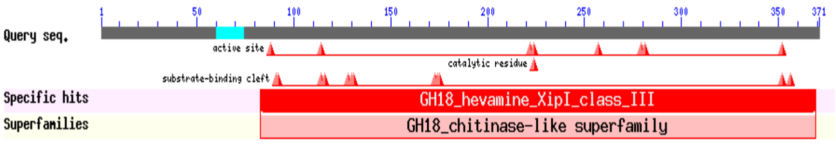
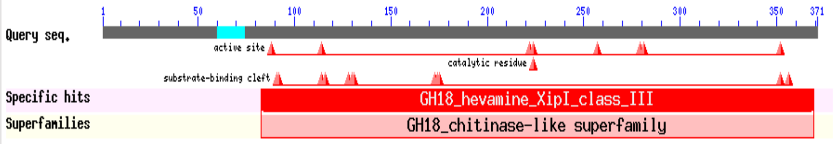
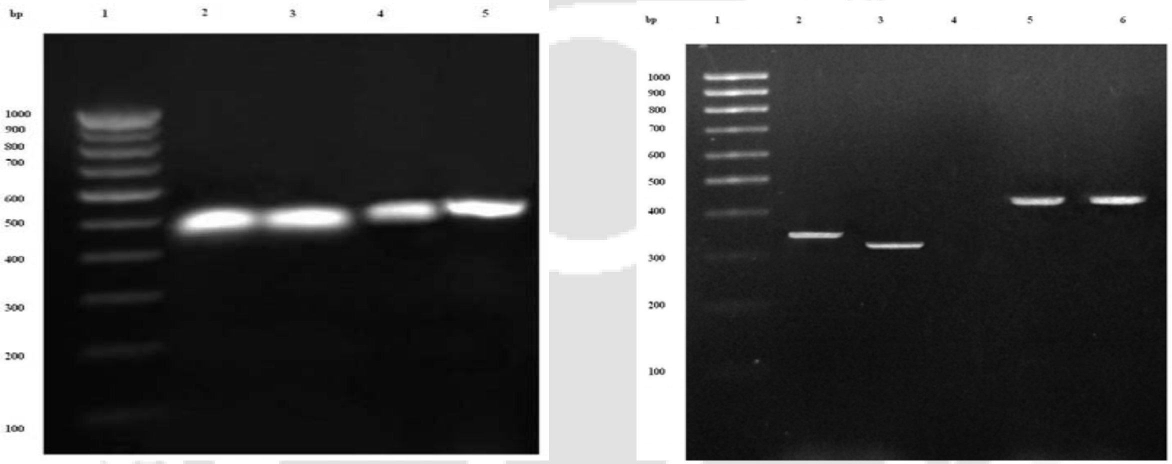
Sequence	**Score**	**Identities**	**Conserved domains**
Metarhizium anisopliae var. anisopliae CHIT30 chitinase (chi3) mRNA, partial cds (gb|AY545982.1|) QS:306-389:SS-271-343	84.2	87%	
Metarhizium anisopliae var. acridum mRNA for putative endochitinase CHI3 (chi3 gene) (emb|AJ293218.1|) QS:306-389:SS-332-404	84.2	87%	

Table 4.9. Results of BLAST analysis of DNA sequence from *M. anisopliae* PCR product using CP2

Sequence	Score	Identities	Conserved domains
Metarhizium anisopliae var. acridum partial mRNA for putative endochitinase CHI2 (chi2 gene) (emb|AJ293217.1|) QS-385-423:SS-958-996	73.1	100%	—
Metarhizium anisopliae var. anisopliae putative chitinase (chi2) gene (gb|DQ011663.2|) QS-75-109:SS-1388-1422	65.8	100%	—
Metarhizium anisopliae var. anisopliae CHIT30 chitinase (chi3) mRNA, partial cds (gb|AY545982.1|) QS:22-51:SS-543-572	56.5	100%	—
Metarhizium anisopliae var. acridum mRNA for putative endochitinase CHI3 (chi3 gene) (emb|AJ293218.1|) QS-22-51:SS-604-633	56.5	100%	—



(a) **(b)**

Fig 4.1. PCR amplification with designed primers, (a): Protease gene (lane 2& 3 *B. bassiana*, lane 4 & 5 *M. anisopliae*) (Lane 1 DNA ladder), (b) Chitinase gene (lane 2& 3 *B. bassiana*, lane 5 & 6 *M. anisopliae*) (Lane 1 DNA ladder)

Sequences: Protease

***B. bassiana* Primer1**

```
  1 NTGTTGCCCT CTAACCTGTG CCTAGGCGGC TCCGGTCGTT GAGCCTGCTC CTATCTCATC
 61 GAGGCCCGCT TCACGCATGG GCAGACTATT GCCGTTCTGA ACATGCNTGA GGACTACACT
121 GACTGACCCT CTCGCTTTGT GCATAACTCC GCATGCACAC CCGCTAAGGA GCTACCATAC
181 ATCGCCTACN AGCGTACTCG CCCGTATTAC GCGCGTCCCG CGTGATGTCA CCCGTGTACC
241 GATCGCGTGG AGTGTGCAAT TATATGCTTT TGTCAAGCTC AAGGACACGG CGACAAGCTT
301 GATGACCCTA CATGGATTCC CGTCGGCCGC ATCCAAGTTG GTACGTCTAT ACAGATGTCT
361 TCCCAACCGG GATACGCGGC CAGTCTCAGT TGATCGAGCA GGATGCCATT GTTAGCATCA
421 ACGCCCCTCC ATCAGCTGGG ATCGGTTTAG GCCACACATT GGCATGGGATATGGCTCGGG
481 TATCGCCCAG GAGCTGTTGC TGGG
```

***B. bassiana* Primer2**

```
  1 GGCGTCCATT TTGGGCGTCA AGGTGCTCGA AGACAGTGGC TCGGGATCGC TCAGCGGCGT
 61 CATGGCCGGA ATGGACTGTG TCGCTAGGGA CCGGAAATCC CGGTGCTCAT GCAGCAAAGG
121 CAAAGTCAGC CAGCATGTCT GTGCCAACAT TGTCAAACCG CCGGTTGTGC ATGCCCGGAC
181 TGTGGACTGG AACTCAACTC ATGTATTGGA TAAAAGCTTA CGCCCTCGAA CGTCTNAACG
241 GTGCACGTGG CATGCAGCTG CATGCTAACG TGCCTGCAGT TACCNTTGGA GGTNNNTACT
301 CCGTGAACAA NGCCNCCGCG CTTCTGCAGG CTTCGGGCGN TTNTGTCGCG GTCGCCGCCG
361 GCAACGACAA TTGCGATGCC GCCACCACCT CGCCCGCCTC GGAGCCGTCC GTCTGCACCG
421 TCGGTGCAAC CGACTCGTCA CCGCCGCTCC AGCTTCTCCA ACTTTGGAAA AGCTTTCGAC
481 ATTGCGTATT CTGGCACCTG GGCCTGGCCT TCTGCCG
```

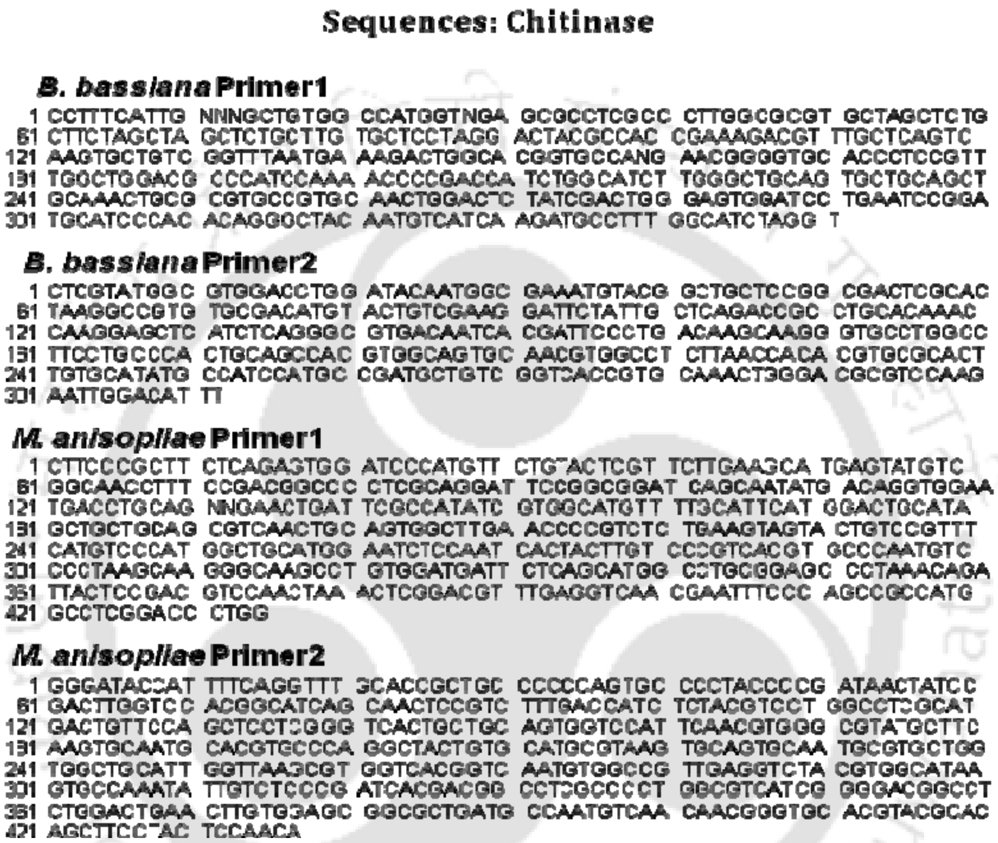
***M. anisopliae* Primer1**

```
  1 TTAGTGGGTC CCGTGGGGCC TTTCGGGTGA ATCGCCCGTA TGGCACTAGT ACGGTCTGCC
 61 CAAATGCCCG GCGTACTCCG TGGGGATCCT CTCCNNTAGA GCGGTGAACC CTGGGGTCTT
121 GCTCGTGTCT CCCATCGCCA GAAGGGAAGC ACCACCTATC GCTACGATGA TACAGCTGGC
181 GCAGGTACTT GCGTATATAT CATTGACACT GGTATTGAGG CCACTCACCA GGAGTTTGAG
241 GCTAACTGGC CGGGCATCAN NTACCTAGTA CCTACTGGCT GGACCGGCTA CAGCGGTCAA
301 ACCTCTGACC TTGGCCACGG CCATGGGACT CACTGCGCTG GAATCTGGCC ATCGGGGCGC
361 TCCCTTATGG ANGCGATGTC AGTCGTCCGA TACAATGTTA NGCGTGCCAA GGAGCTTGGA
421 GATAACTACN NNGGCTGCCC TATCGCGGGT GCCGAACTCT TTACTGTTGG CGGCAAGACC
481 TTCGGTGTCG CCAAGAAGGC TACTATCATT TCGGTTTATT GCGGGGCACC CCCTAGG
```

***M. anisopliae* Primer2**

```
  1 NNCGTTCTGT TGCGGTAGGT GGTATTGCAA CGCCCAGCGG GATGCCCAGA ACACCTCTCC
 61 CGCTTCCGTT GAGCCTTCTG CCTGCACCGT TGGTGCCGTG GACAAGAACT GGAGAATCGT
121 CACGAACTGG CCCAATGTTA CTGGGTCTGG GACTAACTTG TATGAACGCC CACTTCCTAG
181 TTCAATCGGG GATGTCGCTT TGTCGAACTC TGTGGGCAGT CTGAGTACCT ATGATTCAGA
241 TGCATTGCCG TGAGGTCCAC CGTCAGNACT CAGCNNCCCG ATGGGGTGTT GGTGAATTGG
301 ACAACCGCAC GATCTTCCTT ACAGGCCAAG GCTCCAACTA CGGCCCAGTC CTGGATATTT
361 TCGCCCCAGA GCTACGTGCG GCATTCTCTC CACCTGGATC AATGGCCGCA CCAACACGGA
421 CGTGCAAACT GCACATCTCG GGTTCACTCC ATGGCCTCTC CCCACATCTG CGGTCTCCTC
481 GCTTACTTCG AGGCGATACT TCCTTTGTTT GGGTAATGGA AACGGTCTGT TGAGTGCTGC
541 CGTTCTTGCG G
```

Fig 4.2. DNA sequence of protease gene from *B. bassiana* and *M. anisopliae* using PP1 and PP2



Sequences: Chitinase

B. bassiana **Primer1**

```
  1 CCTTTCATTG NNNGCTGTGG CCATGGTNGA GCGCCTCGCC CTTGGCGCGT GCTAGCTCTG
 61 CTTCTAGCTA GCTCTGCTTG TGCTCCTAGG ACTACGCCAC CGAAAGACGT TTGCTCAGTC
121 AAGTGCTGTC GGTTTAATGA AAGACTGGCA CGGTGCCANG AACGGGGTGC ACCCTCCGTT
181 TGGCTGGACG CCCATCCAAA ACCCCGACCA TCTGGCATCT TGGGCTGCAG TGCTGCAGCT
241 GCAAACTGCG CGTGCCGTGC AACTGGACTC TATCGACTGG GAGTGGATCC TGAATCCGGA
301 TGCATCCCAC ACAGGGCTAC AATGTCATCA AGATGCCTTT GGCATCTAGG T
```

B. bassiana **Primer2**

```
  1 CTCGTATGGC GTGGACCTGG ATACAATGGC GAAATGTACG GCTGCTCCGG CGACTCGCAC
 61 TAAGGCCGTG TGCGACATGT ACTGTCGAAG GATTCTATTG CTCAGACCGC CTGCACAAAC
121 CAAGGAGCTC ATCTCAGGGC GTGACAATCA CGATTCCCTG ACAAGCAAGG GTGCCTGGCC
181 TTCCTGCCCA CTGCAGCCAC GTGGCAGTGC AACGTGGCCT CTTAACCACA CGTGCGCACT
241 TGTGCATATG CCATCCATGC CGATGCTGTC GGTCACCGTG CAAACTGGGA CGCGTCCAAG
301 AATTGGACAT TT
```

M. anisopliae **Primer1**

```
  1 CTTCCCGCTT CTCAGAGTGG ATCCCATGTT CTGTACTCGT TCTTGAAGCA TGAGTATGTC
 61 GGCAACCTTT CCGACGGCCC CTCGCAGGAT TCCGGCGGAT CAGCAATATG ACAGGTGGAA
121 TGACCTGCAG NNGAACTGAT TCGCCATATC GTGGCATGTT TTGCATTCAT GGACTGCATA
181 GCTGCTGCAG CGTCAACTGC AGTGGCTTGA ACCCCGTCTC TGAAGTAGTA CTGTCCGTTT
241 CATGTCCCAT GGCTGCATGG AATCTCCAAT CACTACTTGT CCCGTCACGT GCCCAATGTC
301 CCCTAAGCAA GGGCAAGCCT GTGGATGATT CTCAGCATGG CCTGCGGAGC CCTAAACAGA
361 TTACTCCGAC GTCCAACTAA ACTCGGACGT TTGAGGTCAA CGAATTTCCC AGCCGCCATG
421 GCCTCGGACC CTGG
```

M. anisopliae **Primer2**

```
  1 GGGATACCAT TTTCAGGTTT GCACCGCTGC CCCCCAGTGC CCCTACCCCG ATAACTATCC
 61 GACTTGGTCC ACGGCATCAG CAACTCCGTC TTTGACCATC TCTACGTCCT GGCCTCGCAT
121 GACTGTTCCA GCTCCTCGGG TCACTGCTGC AGTGGTCCAT TCAACGTGGG CGTATGCTTC
181 AAGTGCAATG CACGTGCCCA GGCTACTGTG CATGCGTAAG TGCAGTGCAA TGCGTGCTGG
241 TGGCTGCATT GGTTAAGCGT GGTCACGGTC AATGTGGCCG TTGAGGTCTA CGTGGCATAA
301 GTGCCAAATA TTGTCTCCCG ATCACGACGG CCTCGCCCCT GGCGTCATCG GGGACGGCCT
361 CTGGACTGAA CTTGTGGAGC GGCGCTGATG CCAATGTCAA CAACGGGTGC ACGTACGCAC
421 AGCTTCCTAC TCCAACA
```

Fig 4.3. DNA sequence of chitinase gene from *B. bassiana* and *M. anisopliae* using CP1 and CP2

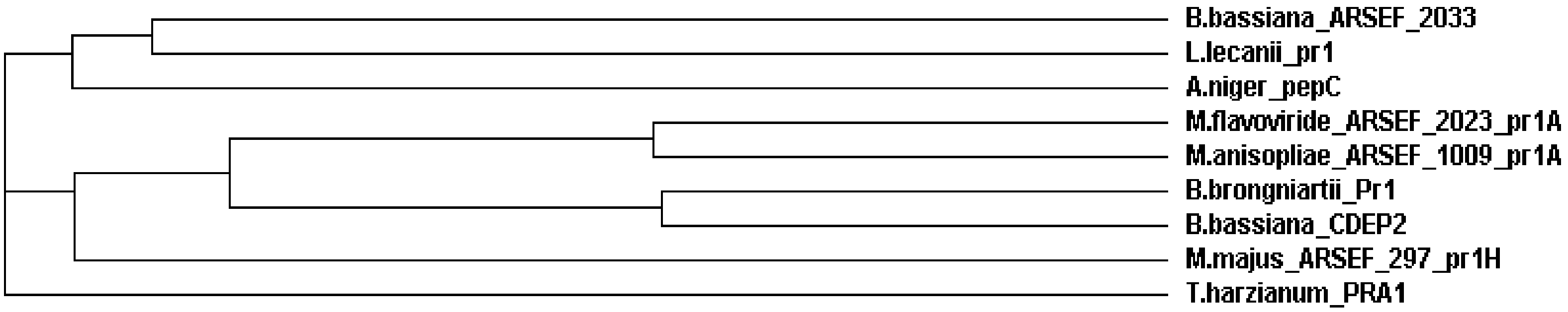


Fig 4.4. Cladogram of protease gene sequences (*B. bassiana* isolate ARSEF 2033 sequence was obtained by PCR using PP1)

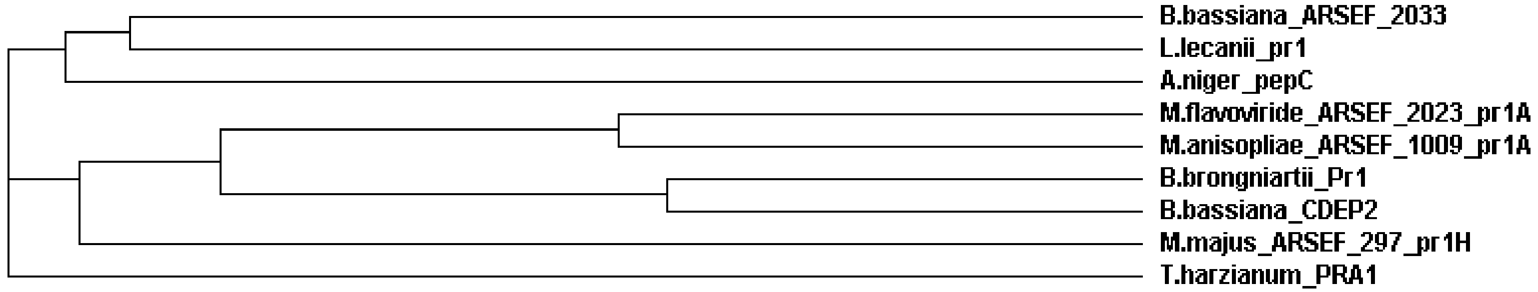


Fig 4.5. Cladogram of protease gene sequences (*B. bassiana* isolate ARSEF 2033 sequence was obtained by PCR using PP2)

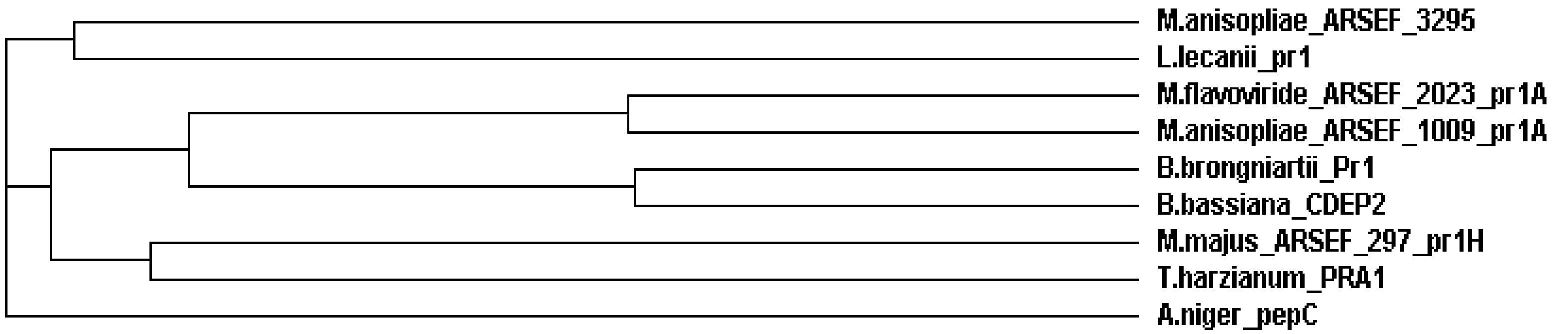


Fig 4.6. Cladogram of protease gene sequences (*M. anisopliae* isolate ARSEF 3295 sequence was obtained by PCR using PP1)

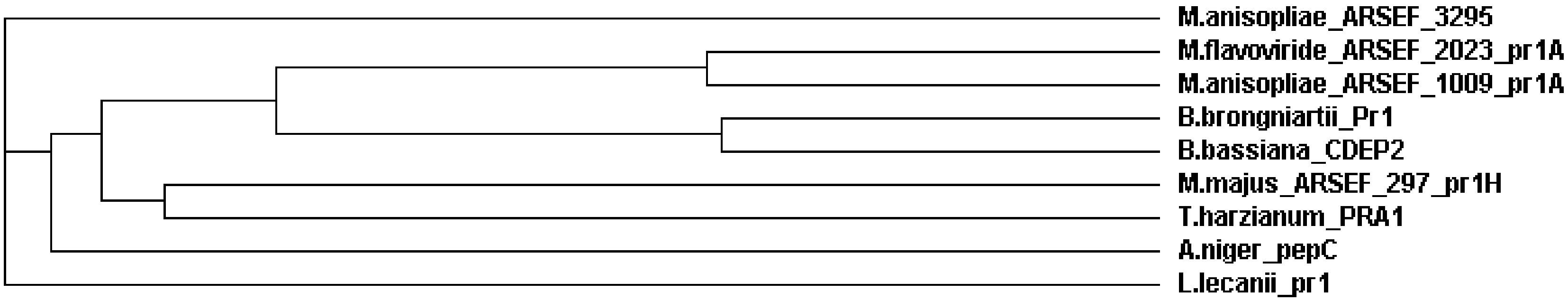


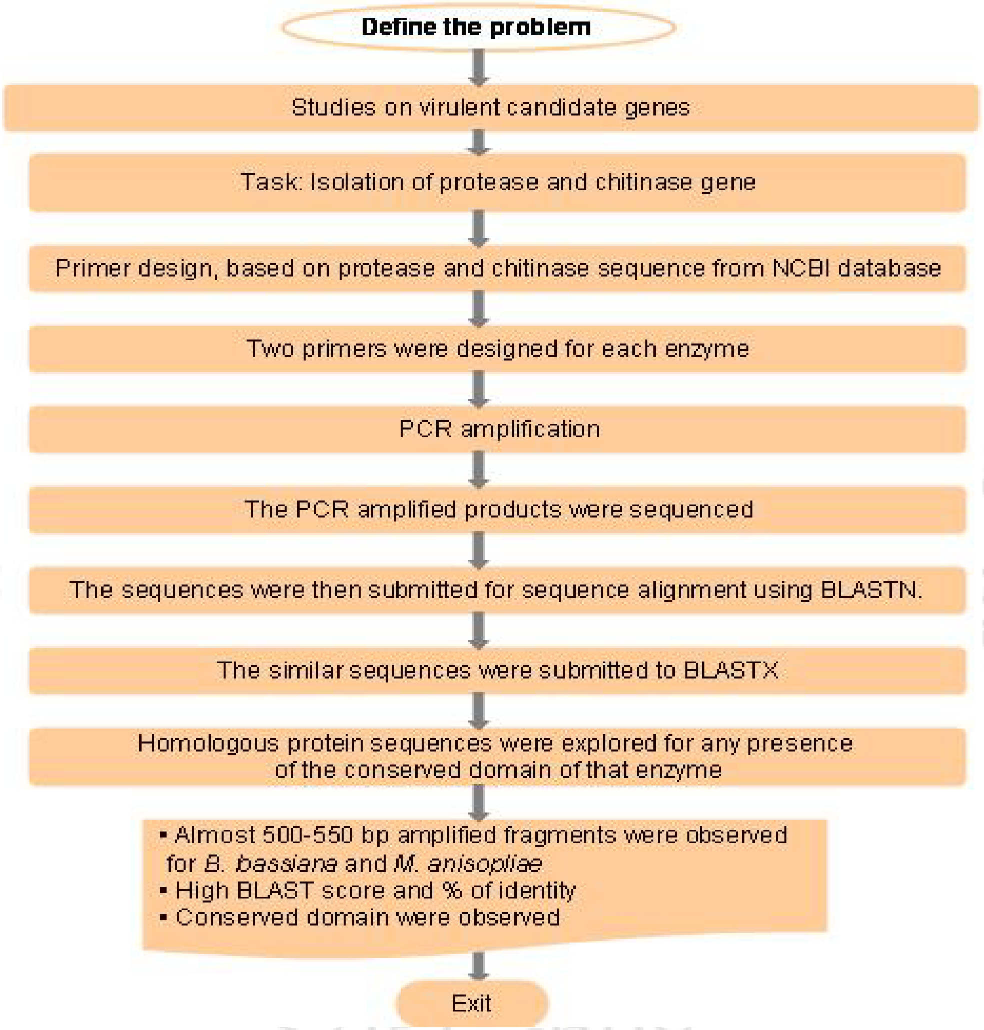
Fig 4.7. Cladogram of protease gene sequences (*M. anisopliae* isolate ARSEF 3295 sequence was obtained by PCR using using PP2)

Fig 4.8. Cladogram of chitinase gene sequences (*B. bassiana* isolate ARSEF 2033 sequence was obtained by PCR using CP1)

Fig 4.9. Cladogram of chitinase gene sequences (*B. bassiana* isolate ARSEF 2033 sequence was obtained by PCR using CP2)

Fig 4.10. Cladogram of chitinase gene sequences (*M. anisopliae* isolate ARSEF 3295 sequence was obtained by PCR using CP1)

Fig 4.11. Cladogram of chitinase gene sequences (*M. anisopliae* isolate ARSEF 3295 sequence was obtained by PCR using CP2)



Flowchart 4.1. Summary of Chapter 4

5.1 Introduction

Helicoverpa armigera popularly known as Cotton-Bollworm is widespread throughout the tropical and subtropical regions. This insect is a moth belonging to order Lepidoptera & family Noctuidae. Polyphagous nature of *H. armigera* is a major threat to a wide range of economically important crops including cotton, corn tomato and legumes (King ABS, 1994). The damaging potential of *H. armigera* thrives on the fact that it prefers to feed and develop in the reproductive structures of crops which are rich in nitrogen (Fitt GP, 1989). *H. armigera* damages cotton blooms which frequently open prematurely and stay fruitless; when the bolls are damaged, some will fall off, and those that remain either fail to produce lint entirely or they produce lint of inferior quality. Resistances to chemical pesticides are frequent features with this notorious pest. It has already developed a high degree of resistance to organophosphates and pyrethroid insecticides (Armes et al., 1996). The alternative to chemical pesticides i.e. entomopathogenic fungi could be used efficiently as mycopesticides are less prone to resistance development.

The effects of entomopathogenic fungi on its insect host could be easily assessed by conducting bioassay. Bioassays can be used to determine and quantify the host-pathogen relationship and have been extensively applied in aspects such as determination of virulence, comparison of virulence among isolates, determination of host range, determination of epizootic potential and studies on the effect of various biotic and abiotic factors. *Beauveria bassiana* & *Metarhizium anisopliae* are both ubiquitous insect pathogenic fungi with a broad host range. *M. anisopliae* has been employed as an effective suppressor of the insect pest including *H. armigera*. Similar reports for *B. bassiana* under laboratory and field conditions established it as a biocontrol agent against *H. armigera* (Sandhu et al., 2001). The objective of this study is to explore the pathogenic potential of *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) against 1st and 2nd instar larvae of *H. armigera.*

5.2 Materials and Methods

Rearing of the insects

H. armigera larvae were collected from the vegetable fields (IARI, New Delhi). The collected larvae reared under laboratory conditions at 28 °C, 65 % RH and 12 h alternate light & dark conditions. 1st and 2nd instar larvae of *H. armigera* were collected from the second generation rearing on artificial diet and used in the experiments.

Fungal isolates

B. bassiana (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) conidia were grown on rice using Solid State Fermentation (SSF) at 28 °C and 50 % initial moisture content.

Bioassays

Three concentrations of conidia suspension were used to evaluate LD_{50} values of *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) against 1st and 2nd instar larvae of *H. armigera.* 1st instar larvae were treated with a lower concentration of conidia such as 1×10^2, 1×10^4, 1×10^6 spores/ ml whereas a higher dose was selected for 2nd instar larvae, 1×10^5, 1×10^7, 1×10^9 spores/ ml. Concentration of conidia was adjusted by serial dilutions in 0.01 % Tween-80 solutions and concentration was determined by Haemocytometer. Bioassays were done by the Diet Surface Technique method (Garcia, 1978). Five replicates were taken for every treatment and 10 larvae were used per replicate which was reared individually in petriplates due to cannibalism. Each replicate contained 3 g of artificial diet which was topically treated with 200 µl spore suspension. Treatment with water was used as a control. The plates were incubated at 28 ± 2 °C and an average relative humidity of 80 ± 2°C was maintained throughout the experiment. The number of dead larvae was recorded every alternate day up to the 6th day. The dead larvae were kept separately in plastic vials to facilitate fungal growth which were observed under the microscope to confirm death due to fungal infection. The LD_{50} and LD_{90} values were calculated based on the observations of the number of dead larvae. Probit analysis was done in DOS operating software. LT_{50} values were calculated for the most significant dose.

Reisolation of fungus and comparison with indigenous isolate

Mycosed cadavers were cut in half and vortexed thoroughly in 0.01 % Tween-80 solution. A 200 µl of this solution was spreaded on SDA plates and fungi were reisolated from the mycosed cadaver of the insect. Features of the Indigenous and Insect passaged conidia were investigated for the virulence determinant chitinase and protease enzyme along with sporulation and germination rate.

5.3 Results

A complete life cycle of *H. armigera* was observed and each instar is shown in Fig 5.1. LD_{50} value for 1st instar larvae was less on day six compared to previous days for *B. bassiana* whereas no such significant difference was observed for *M. anisopliae* between day four and day six (Table 5.1). LD_{90} values of *B. bassiana* were high compared to *M. anisopliae* and a steadfast fiducial limit was observed for both LD_{50} and LD_{90} values of *M. anisopliae* isolate. Higher LD_{50} values were noted for 2nd instar larvae of *H. armigera* after probit analysis for both the isolates

(Table 5.2). A spore load of 0.43× 10^9 spores/ ml appeared to inflict mortality in 50 % of 2nd instar larvae (LD_{50}) using *M. anisopliae* isolate though LD_{90} values were higher (0.57× 10^{12} spores/ ml). LD_{50} and LD_{90} values for 2nd instar larvae on day six were relatively higher in case of *B. bassiana* isolate compared to *M. anisopliae* isolate (Table 5.2).

Considerable mortality was observed in 1st and 2nd instar larvae of *H. armigera* after 144 h (six days) (Table 5.3 & 5.4). Almost 70 % mortality was recorded in 1st instar larvae by using *M. anisopliae* isolate followed by *B. bassiana*, 51 % mortality was recorded at the highest spore load of 1×10^6 spores/ ml. However, mortality in 2nd instar larvae of *H. armigera* was less compared to 1st instar mortality using both the isolates. LT_{50} values confirmed the efficiency of *M. anisopliae* isolate as only 84.75 h were required to facilitate 70 % of insect mortality however more time were taken by *B. bassiana* isolate to kill 50 % of insect, 137.62 h (Table 5.3, 5.4). Same trend in LT_{50} values were also observed for 2nd instar larvae for both the isolates.

Fungus was reisolated from the mycosed cadaver (Fig 5.2). Diminutive increase in chitinase and protease activity was observed in insect passaged *B. bassiana* isolates compared to indigenous one whereas % of germination was high. Significant increase in protease activity and % of germination were exerted by insect passaged conidia of *M. anisopliae* isolates (Table 5.5).

5.4 Discussion

Incorporation of entomopathogenic fungi in the integrated pest management could be beneficial for the economically important crops (Alves, 1998) hence before implementation in pest management, bioassay should be performed to evaluate the efficacy of the pathogen and also to understand the host-pathogen relation. Several aspects must be considered for a effective bioassay such as to ensure that the pathogen has not lost its virulence during subculturing, inoculum is viable and percentage germination is determined, target host is healthy, large enough sample size and enough replicates per treatment (Butt and Goettel, 2000). *B. bassiana* and *M. anisopliae* isolates inflicted mortality in both the larval instars of *H. armigera* at the highest concentration, 1×10^6 and 1×10^9 spores/ ml for 1st and 2nd instar respectively which is in agreement with the findings of Gundannavar et al. (2007). Insect mortality was more in 1st instar larvae compared to 2nd instar. These observations were also endorsed by Gopalkrishnan and Narayanan (1988 and 1989). Sansone and Smith (2001) reported the expected insect mortality of 70-95% in the egg and first instar life stages whereas Berg and Cock (1995) contradicted this observation by reporting high mortality in late stages of *H. armigera* compared to egg and early instars. It is recognized that there is an increasing need for an alternative method to control *H. armigera* as this notorious pest is prone to resistance to chemical pesticides. The significant increase in percentage of germination in both the isolates as well as increased enzyme activity possibly signifies the improved characteristics of the isolates as reports indicates more susceptibility of the insects to insect passaged conidia (Brownbridge et al., 2001; Vandenberg and Cantone, 2004). Shah et al. (2005) observed significantly high virulence of one *M. anisopliae* isolate which was recovered from *T. molitor*, compared to conidia grown in 1 % yeast extract.

Goettel et al. (1990) observed that the isolates most virulent to a host are isolated from the same or related host species. In this study *M. anisopliae* isolate showed higher mortality than *B. bassiana* which can be correlated with the fact that *M. anisopliae* was derived from a lepidopteron host *Anticarsia gemmatalis* whereas Coleopteran insect was host for *B. bassiana*

(Table 5.6). Gupta et al. (1994) demonstrated the relationships between enzyme activities and the virulence of *B. bassiana* towards *Galleria mellonella* L. and *Trichoplusia ni* (Hübner) and also observed the host specificity of fungal isolate which is mainly because cuticle type differs in protein composition and sclerotization and cuticle degrading enzymes are many times induced by degradation products of cuticle components (Charnley, 2003; Paterson et al., 1994).

5.5 Inference

The validity of the bioassay results depends on the bioassay design hence it must be constantly improved for better understanding the host-pathogen interaction. In this study these two entomopathogenic fungi especially *M. anisopliae* performed effectively therefore could be implemented in the IPM though generation studies should be conducted.

Table 5.1. Probit analysis: Pathogenicity of entomopathogenic fungi against 1st instar larvae

Days	LD_{50}	Fiducial Limit	LD_{90}	Fiducial Limit
B. bassiana				
2	0.34×10^{8}	$0.15\times 10^{5} – 0.27\times 10^{16}$	0.92×10^{12}	$0.73\times 10^{10} – 0.61\times 10^{22}$
4	0.23×10^{8}	$0.62\times 10^{5} – 0.54\times 10^{14}$	0.31×10^{12}	$0.43\times 10^{10} – 0.59\times 10^{22}$
6	0.11×10^{7}	$0.44\times 10^{5} – 0.13\times 10^{14}$	0.73×10^{11}	$0.18\times 10^{9} – 0.65\times 10^{20}$
M. anisopliae				
2	0.91×10^{7}	$0.46\times 10^{6} – 0.11\times 10^{14}$	0.79×10^{11}	$0.14\times 10^{9} – 0.24\times 10^{27}$
4	0.82×10^{6}	$0.36\times 10^{6} – 0.65\times 10^{8}$	0.25×10^{10}	$0.22\times 10^{8} – 0.17\times 10^{18}$
6	0.32×10^{6}	$0.12\times 10^{6} – 0.19\times 10^{7}$	0.23×10^{9}	$0.6\times 10^{7} – 0.15\times 10^{14}$

Table 5.2. Probit analysis: Pathogenicity of entomopathogenic fungi against 2nd instar larvae

Days	LD_{50}	Fiducial Limit	LD_{90}	Fiducial Limit
B. bassiana				
2	0.58×10^{10}	$0.58\times 10^{9} – 0.13\times {}^{18}$	0.26×10^{15}	$0.61\times 10^{12} – 0.92\times 10^{24}$
4	0.36×10^{10}	$0.34\times 10^{9} – 0.24\times 10^{17}$	0.42×10^{14}	$0.29\times 10^{11} – 0.60\times 10^{23}$
6	0.12×10^{10}	$0.78\times 10^{8} – 0.34\times 10^{15}$	0.27×10^{13}	$0.53\times 10^{11} – 0.81\times 10^{22}$
M. anisopliae				
2	0.46×10^{11}	$0.89\times 10^{10} - 0.32\times 10^{12}$	0.76×10^{12}	$0.72\times 10^{10} – 0.25\times 10^{18}$
4	0.18×10^{10}	$0.22\times 10^{8} – 0.10\times 10^{11}$	0.65×10^{12}	$0.85\times 10^{10} – 0.95\times 10^{18}$
6	0.43×10^{9}	$0.35\times 10^{7} - 0.23\times 10^{10}$	0.57×10^{12}	$0.63\times 10^{10} – 0.14\times 10^{21}$

Table 5.3. LT_{50} values of *B. bassiana*

Time (h)	**% of Mortality**	**LT_{50} (h)**
	1st instar larvae	
48	24±4.00	137.62
96	40±7.20	
144	51±3.41	
	2nd instar larvae	
48	30±5.05	131
96	44±8.00	
144	52±11.70	

Table 5.4. LT_{50} values of *M. anisopliae*

Time (h)	**% of Mortality**	**LT_{50} (h)**
	1st instar larvae	
48	36±4.4	84.75
96	56±5.3	
144	70±9.9	
	2nd instar larvae	
48	32±1.98	83.57
96	64±8.4	
144	68±4.67	

Table 5.5. Features of Indigenous & Insect passaged conidia

Conidia type	Chitinase (U/ ml)	Protease (U/ ml)	Sporulation (y×10^7) spores/ ml	% of Germination after 12^{th} h
		B. bassiana		
Indigenous Conidia	29.11±5.3^{a}	0.73±0.3^{a}	6.27±0.56^{a}	56.49±1.29^{a}
Insect passaged conidia	30.34±4.1^{a}	0.95±0.07^{a}	6.98±1.2^{a}	59.00±4.4^{b}
		M. anisopliae		
Indigenous Conidia	10.75±3.24^{A}	1.54±0.32^{A}	8.20±5.3^{A}	87.22±3.42^{A}
Insect passaged conidia	11.02±5.4^{A}	2.71±0.21^{B}	9.53±1.11^{B}	90.06±4.59^{B}

Values followed by same lower case alphabets in the column are statistically equivalent according to Tukey's test for *B. bassiana*

Values followed by same upper case alphabets in the column are statistically equivalent according to Tukey's test for *M. anisopliae*

Table 5.6. Relationship of pathogenicity determinants with virulence

Isolates	Geographic origin	Host origin	Chitinase (U/ ml)	Protease (Pr1) (U/ ml)	% of germination after 12th h	% of mortality *H. armigera* (order: Lepidoptera)
UB9 (ARSEF 2033)	USA	*Coccinella Sp.* (order: Coleoptera)	29.11±5.3	0.73±0.3	56.49±1.29	52±11.70
UM10 (ARSEF 3295)	MEXICO	*Anticarsia gemmatalis* (order: Lepidoptera)	10.75±3.24	1.54±0.32	87.22±3.42	68±4.67

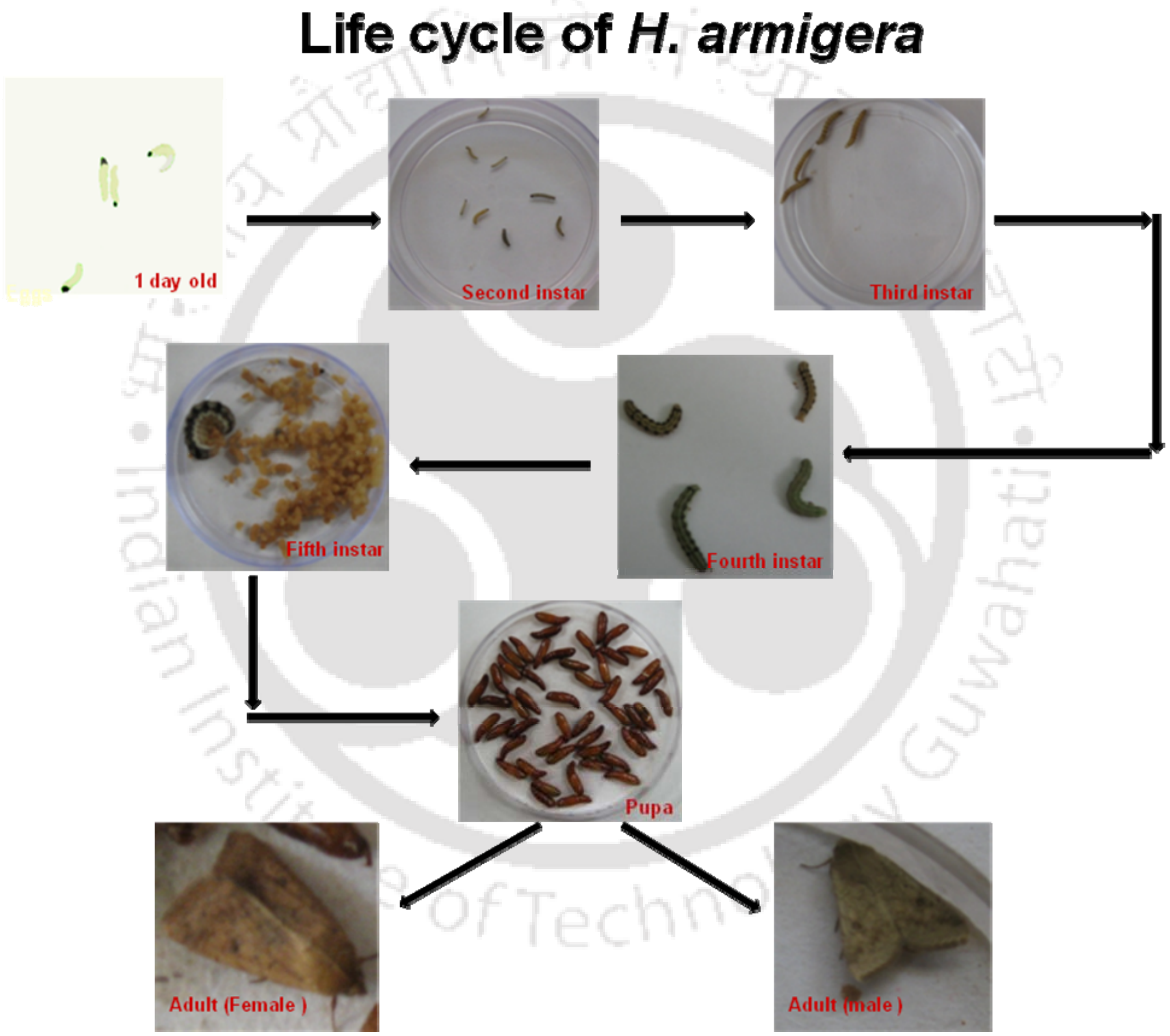
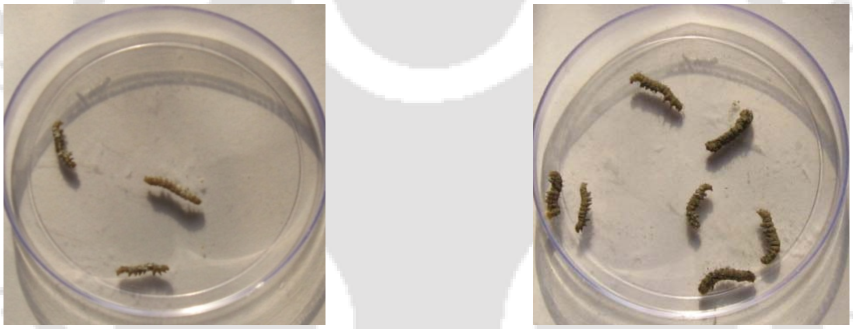
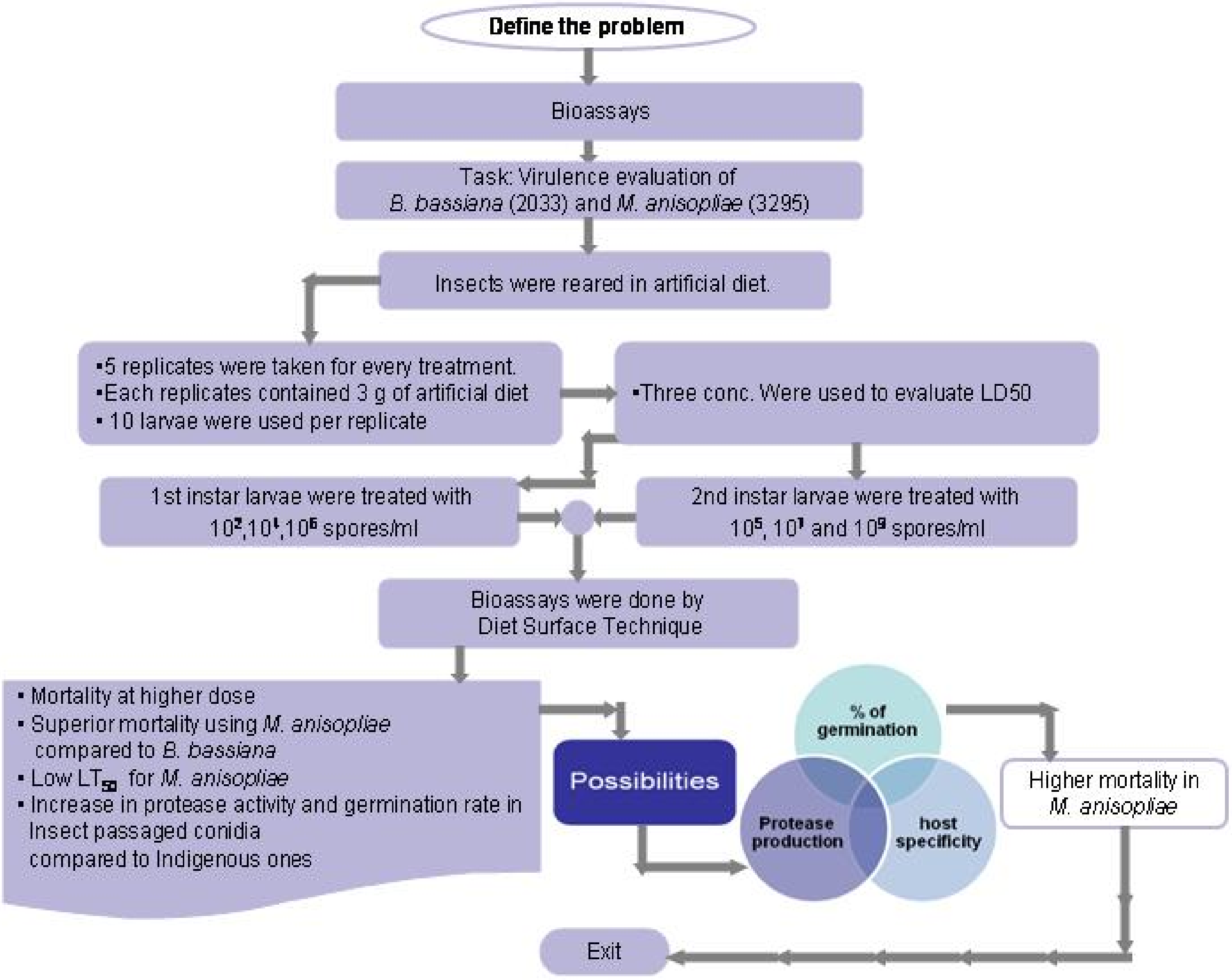


Fig 5.1. Life cycle of *H. armigera*



(a) **(b)**

Fig 5.2. Conidia emerged from 2^{nd} instar larvae of *H. armigera* (a) *B. bassiana*, (b) *M. anisopliae*



Flowchart 5.1. Summary of Chapter 5.

6.1 Introduction:

The role of entomopathogenic fungi as a natural biocontrol agent to reduce the pest population density is being explored currently. Naturally occurring, soil borne hyphomycete, *B. bassiana* and *M. anisopliae* proved to be efficient in the suppression of insect pests (Inglis et al., 2001). Complete or partial removal of cell wall ensuing protoplast and spheroplast generation has a diverse use in the investigation of the structure, biosynthesis of cell wall, and genetic manipulation (Peberdy, 1979a; Peberdy, 1979b; Peberdy, 1994; Ferenczy, 1981). Protoplast isolation and transformation based on protoplasts is being practiced for filamentous fungi (Thakur and Sandhu, 2003; Robinson and Deacon, 2001). Gibberellin producing filamentous fungi was stably transformed with *Aspergillus niger niaD* gene by using protoplasts (Sanchez-Fernandez et al., 1991). Couteaudier et al. (1996) reported that highly virulent hybrid strains of entomopathogenc fungi *B. bassiana* can be obtained by protoplast fusion. It has been reported to obtain highly purified DNA from protoplast in case of *B. bassiana* (Hegedus and Khachatourians, 1996).The formation of protoplast is dependent on factors like culture condition, age of mycelium, preincubation in thiol compounds, choice of osmotic stabilizers and most importantly choice of enzymes and its concentrations. Different optimum conditions are needed for different fungi even for different strains. The effect of mycelium age on protoplast yield was investigated for *Trichoderma longibrachiatum*. Mycelium harvested at 20th h produced high number of protoplasts and a steep decrease in protoplast yield with the use of 24th h old mycelium observed (Anjani Kumari and Panda, 1992). The best protoplast yield was obtained with 18 to 22 h old mycelium for four different *Fusarium* sp. (Vazquez and De Figueroa, 1996). Preincubation of blastospore in thiol compounds was found to be effective in enhancing the protoplast release from *B. bassiana* (Pfeifer and Khachatourians, 1987). Sugars and sugar alcohols were found to be the most effective osmotic stabilizer in the range of 0.3-0.5 M rather than inorganic salts in *Rhizopus niveus* (Yanai et al., 1990). Although, there are reports that 0.6 M NaCl solution was used efficiently for protoplast release from mycelium of *Ozonium* sp. (Zhou et al., 2008). The influence of lytic enzyme digestion time was investigated on protoplast formation of *Rhizoctonia solani*. There was a continuous increase in the protoplast yield giving a peak at 2.5 h with a quick decrease at 3.5 h (Robinson and Deacon, 2001). Enzymatic methods for cell wall degradation are more popular than non enzymatic ones (Chawla, 2002). Enzyme use is dependent on the constituents of the cell wall. Commercial enzyme preparations like Zymolyase produced from *Arthrobacter luteus*, containing β-glucanase and protease activity is commonly used for yeast protoplast isolation but it can be also used for the isolation of protoplasts from the filamentous fungi in combination with other enzyme (Yanagi and Takebe, 1984). Another enzyme preparation used for filamentous fungi was Driselase, containing laminarinase, xylanase, cellulase activity gave best results for *Fusarium graminearum* (Wiebe et al., 1997). The most popular commercial enzyme preparation for the isolation of protoplast from filamentous fungi was Novozyme 234 containing mostly glucanohydrolase activity with small quantity of cellulase and protease (Wiebe et al., 1997; Varma and Kwon-Chung, 1991). Various species of *Aspergillus* released high amounts of protoplasts when *Aspergillus* mycelium is treated with Novozyme 234 and Cellulase CP (Hamlyn et al., 1981). The reversion of protoplast to mycelium by forming cell wall is another important aspect in checking viability of protoplast isolated. Highest regeneration frequency was found to be 0.68 % for taxol producing fungus *Ozonium* sp. (Zhou et al., 2008). A 0.6 M mannitol as osmotic stabilizer produced best results of 5 % protoplast reversion with fungus *Pleurotus sajor-caju* (Lau et al., 1985). Entomopathogenic fungus *Metarhizium flavoviridae* mycelium treated with 5 mg/ ml Novozyme 234 and Cellulase

CP each produced almost 10^8 protoplasts/ ml (Valadares-Inglis and Inglis, 1997). After the production of this effective enzyme preparation discontinued commercially, a requirement for an alternative is raised. This current study demonstrates the optimization of different favorable conditions including the exploration of an effective enzyme preparation to maximize the protoplast release from entomopathogenic fungus *M. anisopliae* and *B. bassiana*. Factors like culture media, age of mycelium, different enzymatic incubation time and combinations, osmotic stabilizer were studied in order to study the effect of these factors on protoplast release.

6.2 Materials and methods

Fungal isolates

The fungal isolates, *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) was procured from ARSEF (USDA-ARS Plant Protection Unit) and the culture was maintained periodically on Sabouraud's dextrose agar slants.

Enzyme combinations

Different combinations of commercial enzyme preparations were tested to optimize the production of protoplast. All the enzymes are prepared in 0.02 M Phosphate buffer (pH 6.2) with 0.7 M KCl. All combinations are detailed in Table 6.1.

Media, mycelium age and incubation time

A biphasic culture method was used for the effective protoplast isolation. 50 ml Sabouraud's dextrose (SD) broth was inoculated with 10^7 spores and incubated for almost 40 h at 28 °C and 180 rpm. A 5 ml of this culture was again inoculated in three different media; Glucose Minimal (GM) medium (4 % Glucose, 0.4 % NH_4NO_3, 0.3 % KH_2PO_4, 0.3 % $MgSO_4$), Yeast Extract Minimal (YM) medium (1 % Yeast Extract, 0.3 % KH_2PO_4, 0.3 % $MgSO_4$), and SD broth (4 % Dextrose, 1 % Yeast Extract, 1 % Peptone), to assess the effect of different media on protoplast isolation and similar culture conditions were maintained for the second phase as well. The mycelium was harvested at three different times viz., 20th, 40th and 60th h to test the effect of mycelial age on protoplast yield. The effect of enzymatic incubation at different incubation intervals of 1 h, 3 h and 5 h was also checked.

Osmotic stabilizer

The ideal osmotic stabilizer, both organic and inorganic, for protoplast release and stabilization was investigated. Two organic stabilizers; Sorbitol and Mannitol; and three inorganic osmotic stabilizers; NaCl, $MgSO_4$ and KCl were used in this study. Concentrations of all the osmoticums were kept constant at 0.7 M. Mycelium preincubation in thiol compounds to increase the protoplast release was checked by incubating the harvested mycelium before enzymatic incubation in preincubation buffer containing 0.01 M β-marcaptoethanol for 1 h at 37 °C (Kawula & Lingg, 1984). After the preincubation, mycelium was washed thrice with the phosphate buffer (pH 6.2) and subjected to enzymatic incubation with 10 mg/ ml Lysing enzyme solution prepared in 0.02 M phosphate buffer with 0.7 M KCl as osmotic stabilizer. Another set of experiment were done with non preincubated mycelium with same buffer and osmotic stabilizer.

Protoplast isolation

Cultured mycelium from different media at different time interval was first washed twice with phosphate buffer (pH 6.2). Enzymes of different concentration and combination were added to the mycelium in the ratio of 50 mg wet mycelium per ml enzyme solution and incubated at 28 °C with gentle shaking. After the incubation the reaction mixture is placed at 4 °C for 15 min to stop the enzyme activity. Protoplasts isolated from fungal mycelium were passed through a glass wool column to eliminate the cell debris and mycelium present and the protoplast were pelleted by the method of Sandhu et al. (2003). Pellet was dissolved in 0.7 M KCl in 0.02 M Phosphate buffer (pH 6.2) and centrifuged at 1920 g for 10 min. Pellet was resuspended in the same osmoticum until further use.

Viability assessment of protoplasts

Protoplasts were treated with Fluorescein diacetate (FDA) to check the viability of the released protoplasts. A 5 mg/ ml FDA stock solution was prepared in acetone and added to the protoplast solution to a concentration of 0.1 %. Fluorescence micrograph was taken after 5 min of incubation at room temperature.

Protoplast regeneration

Serial dilutions of protoplast suspension were made in 0.02 M Phosphate buffer (pH 6.2) with 0.7 M KCl and spread plated on Czapek dox agar medium (Anjani Kumari and Panda, 1993) supplemented with 0.7 M KCl. Approximately 10^4- 10^5 protoplasts were spread plated and plates were incubated for seven days at 28 °C for regeneration of mycelium. Regeneration frequency (RF) on agar plates were calculated using the following equation, RF = (Number of colonies appeared/ Number of protoplast inoculated) × 100.

6.3 Results

Effect of different enzyme combinations

Lysing enzyme preparation alone or combinations with other enzymes were tested for efficient isolation of protoplast. Single use of this enzyme at a concentration of 10 mg/ ml was sufficient to produce approximately 10^8 protoplasts. The combination of 10 mg/ ml Lysing enzyme with 9 mg/ ml Macerozyme seemed to be an effective blend of enzymes, although this enzyme combination showed almost 28 % decrease in the protoplast release when compared to Lysing enzyme alone. Other enzymatic combinations did not seem to increase the protoplast release (Table 6.1). Lysing enzyme concentration of 5 mg/ ml did not produce any protoplast whereas in previous studies it has been reported to be effective for the protoplast release with same concentrations of Novozyme 234. The combination was effective when 5 mg/ ml Lysing enzyme is aided with 0.12 mg/ ml Papain. It is possible that the addition of protease could aid in the protoplast release. Although, the result was not the same when same concentration of Papain, 0.12 mg/ ml, was added to 8 mg/ ml Lysing enzyme, very few protoplasts with large vacuoles were produced.

Increasing the Papain concentration to 0.24 mg/ ml did not facilitate the protoplast generation rather only cell debris were observed this time. It is assumed that protoplasts were lysed due to excessive protease activity. Another amalgamation, containing 4 mg/ ml Macerozyme and 1mg/ ml Pectinase produced protoplast yield of 1.3×10^2/ ml. A comparative study of 10 mg/ ml Lysing enzyme and a combination of 10 mg/ ml Lysing enzyme with 9 mg/ ml Macerozyme showed that Lysing enzyme preparation alone yielded good concentration of protoplasts irrespective of the medium used for mycelium harvesting (Fig 6.1, 6.2). Almost 10^8 protoplasts were obtained each time when mycelium was incubated with this enzyme.

Effect of media, mycelium age and incubation time

The biphasic culture method used in this study ensures the total germination of inoculated spores. The degree of protoplast generation was tested with mycelium from three different media. SD broth and YM medium conferred excellent results among the three media tested. The effect of mycelium age on protoplast production was also investigated. Protoplast release after complete digestion of mycelium collected at 40th h of culture produced a maximum level of protoplast yield compared to 20th and 60th h culture (Fig 6.1. a, c). Optimum enzymatic incubation period was checked by incubating the mycelium for up to 5 h with cell wall lytic enzymes. Higher yields were observed after 3h of incubation, irrespective of the media and culture age used. It was observed that mycelium aging 60 h from GM medium showed a protoplast release of 8.9×10^{7} protoplast/ ml after 5 h of enzymatic digestion (Fig 6.1. b). Mycelium from SD broth produced maximum number of protoplasts at 40th h culture 7.3×10^{8} protoplasts/ ml and hence this medium was selected for further experiments. Prolonged period of enzymatic incubation decreased the protoplast yield as protoplasts were lysed and hence 3 h of enzymatic treatment was used in subsequent studies. Enzyme combination of Lysing enzyme (10 mg/ ml) and Macerozyme (9 mg/ ml), showed high protoplast release at 5 h of enzymatic incubation with 40 h old mycelium from SD broth and on the contrary 60th h old culture yielded high number of protoplast at 1 h incubation time (Fig 6.2a). Enzymatic incubation of mycelium in other two media (GM and YM media) produced optimum protoplast at 3rd h using Lysing enzyme (10 mg/ ml) and Macerozyme (9 mg/ ml) combination regardless of mycelium age. A total of 5.5×10^{8} protoplasts were produced by using 40 h old mycelium from YM medium compared to 1.5×10^{8} protoplasts released from mycelium aging 60 h from same medium (Fig 6.2c).

Effect of different osmotic stabilizer on protoplast release

Among the organic stabilizers, mannitol proved effective in protoplast isolation than sorbitol. Approximately 4.2×10^{8} protoplast/ ml were released when KCl was used as a osmoticum compared to MgSO4 and NaCl which showed almost 66 % and 50 % decrease in protoplast yield respectively (Fig 6.3). Preincubation of mycelium with thiol compound did not show any enhancing effect in protoplast release. Protoplast release with preincubated mycelium in 0.7 M KCl as osmotic stabilizer was 1.9×10^{8} protoplasts/ ml compared to 4.2×10^{8} protoplasts/ ml with non preincubated mycelium in 0.7 M KCl. Hence, 3 h of enzymatic digestion of 40 h old mycelium with 10 mg/ ml Lysing enzyme (Sigma) in 0.7 M KCl was used for protoplast isolation in the subsequent steps (Fig 6.5).

Viability assessment of protoplasts

Protoplast viability was assessed by using Fluorescein diacetate. A comparable observation of fluorescence and phase contrast microscopy are shown in Fig 6.6 (a, b) which demonstrates the viability of almost all fresh released protoplasts. Although a very low amount of protoplasts were viable after three months of storage at 4 °C when sorbitol was used as osmoticum Fig 6.6 (c, d).

Protoplast Regeneration

After 3-4 days of incubation at 28 °C, regenerated colonies appeared on the surface of Czapek-Dox agar. Regeneration frequency was calculated after seven days of incubation at 28 °C. Although, yield of protoplasts was high at 10^8/ ml, regeneration frequency was at 0.81 % and 1.23 % respectively for *M. anisopliae* and *B. bassiana* (Fig 6.7).

6.4 Discussion

Different combinations of commercial enzyme preparations were tested for efficient isolation of protoplast from the mycelium. Previously Novozyme-234 was a popular choice for protoplast isolation (Goettel et al., 1990; Kuklinsky-Sorbal et al., 2004) but currently Lysing enzyme (Sigma) from *Trichoderma harzianum* seems to be efficient in isolation of protoplasts. The most important constraint for protoplast release is the choice of enzyme or enzyme combinations. High protoplast concentrations of 10^8 were obtained by the 10 mg/ ml Lysing enzyme for both the isolates. The result is comparable with Kitamoto et al. (1988) who described the lone use of lysing enzyme from *T. harzianum* causing high protoplast release than any other combination used. Enzyme concentration is important factor in protoplast isolation. Low concentrations of enzyme produce low yield of protoplasts from the hyphal tip and a higher concentration lyses the protoplasts (Prabavathy et al., 2006). Mycelium age is an important factor for protoplast optimization and different filamentous fungi behave differently. Studies on mycelium age of *T. longibrachiatum* at 20th h culture were found effective whereas 60th h culture produced best results in case of plant pathogenic fungi *Rhizoctonia solani* (Anjani Kumari and Panda, 1992; Robinson and Deacon, 2001). Apparently young mycelium is more susceptible to lytic enzymes than conidia which are much more resistant to enzymatic digestion. Mycelium aging 12 to 24 h produced maximum protoplasts for entomopathogenic fungi *B. bassiana* and protoplast yield continuously decreased as the culture sporulated with increasing age (Pfeifer and Khachatourians, 1987). Maximum protoplast release was obtained on the 3rd h of enzymatic incubation. A complete incubation period (1-5 h) and the effect on protoplast release are illustrated in Fig 6.4. Silveira and Azevedo (1986) reported similar time period of enzymatic incubation for maximum protoplast isolation of *M. anisopliae*. Continuous increase in the protoplast yield were recorded when mycelium from an edible fungus was incubated with the lytic enzyme mixture for up to 5 h (Yan et al., 2004). Thiol compounds are assumed to have positive effect on protoplast release. The reduction of disulphide linkages in the cell wall proteins was the concept behind (Anderson and Millbank, 1965). Mycelium pretreated with 0.1 % β-marcaptoethanol resulted in 2 fold increase in the protoplast yield in case of *Fusarium solani* (Elbouami, 2001). But, in the present experiment preincubation of mycelium did not increase the protoplast release. The requirement of osmotic stabilizer for efficient protoplast release is different for all the genera of fungi. A comparison of protoplast yield from different edible fungi revealed that MgSO4, KCl and Sucrose as osmotic stabilizer yielded best results (Chang et al., 1985). Use of inorganic salts as osmotic stabilizers produced a high yield of protoplast than sugar alcohol or sorbitol (Vazquez and De Figueroa, 1996). Among the five osmoticums tested KCl at a concentration of 0.7 M showed optimum release of protoplast with non preincubated mycelium.

Sugars as osmotic stabilizers tend to increase the regeneration frequency rather than sugar alcohols used as osmoticum in the regeneration medium (Wiebe et al., 1997). The use of sugars as osmotic stabilizers possibly increases the regeneration frequency of *M. anisopliae* protoplasts (Bagalhi, 1987). The regeneration frequency was found to be 0.81 % in Czapek-Dox agar medium supplemented with 0.7 M KCl as osmotic stabilizer. The low regeneration frequency may be due to the presence of anucleated protoplasts (Silveira, 1986). Kitamoto et al. (1988) observed that high proteinase activity present in the enzyme preparation could be a detrimental factor for protoplast regeneration. Efficient and high release of high number of protoplast primarily depends on the physiological stage of mycelium and choice of cell wall degrading enzymes. Protoplasts generating from the terminal region of the hyphae can produce large or small, vacuolated or non vacuolated protoplasts but it may undergo excellent regeneration as this region of hyphae have greater ability to cell wall synthesis (Kim et al., 2000). This current study demonstrated the optimization of different factors for protoplast release and significant protoplast release was obtained using 40th h old mycelium from SD broth and 3 h of enzymatic incubation with Lysing enzyme (10 mg/ ml) (Fig 6.5).

6.5 Inference

The utility of protoplast isolation in transformation studies is explored in the Chapter 7.

Table 6.1. Effect of different enzyme combination on protoplast release

Enzyme combinations	**Remarks**
10 mg/ ml Lysing enzyme (Sigma).	This concentration of this enzyme gave best results through out the experiment. Almost 10^8 protoplasts released each time.
10 mg/ ml Lysing enzyme (Sigma) + 9 mg/ ml Macerozyme	Slight decreased amount of protoplast released than the only use of 10 mg/ ml Lysing enzyme (Sigma); 5.7×10^8 protoplasts/ ml; after 3 h enzymatic digestion with mycelium age of 40 h.
5 mg/ ml Lysing enzyme (Sigma).	Effectively did not produce any protoplast.
4 mg/ ml Macerozyme + 1 mg/ml Pectinase	A smaller amount of protoplast released, 1.3×10^2
8 mg/ ml Lysing enzyme (Sigma) + 0.12 mg/ ml Papain	Very few irregular shaped, big, vacuolated protoplasts released.
8 mg/ ml Lysing enzyme (Sigma) + 0.24 mg/ ml Papain	No protoplasts were observed, only cell debris was found.
5 mg/ ml Lysing enzyme (Sigma) + 0.12 mg/ ml Papain	Almost 3.2×10^6 protoplasts were released.

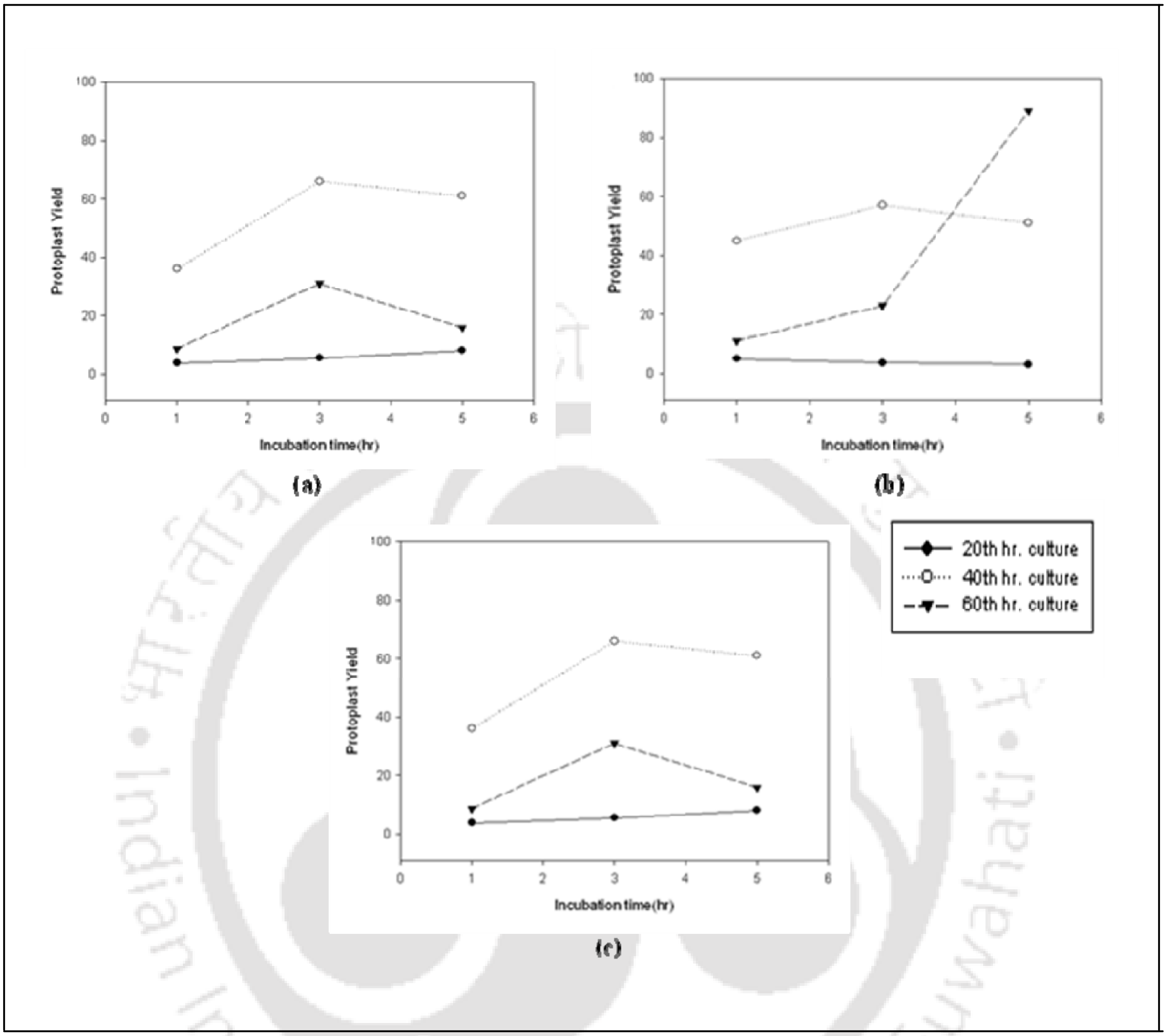


Fig 6.1. Effect of different media, mycelium age and enzymatic incubation period on protoplast release using 10 mg/ ml Lytic enzyme: (a) SD broth, (b) GM medium and (c) YM Medium. (Protoplast yield to be multiplied by 10^7)

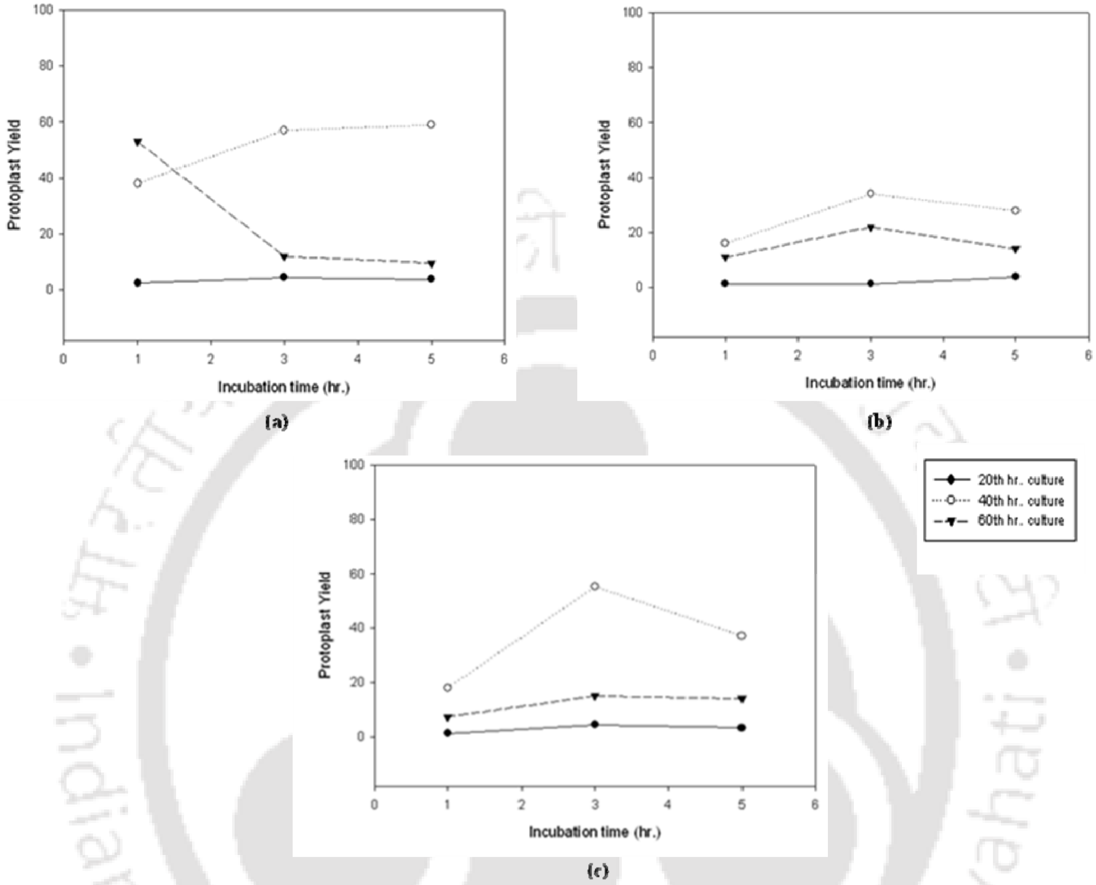


Fig 6.2. Effect of different media, mycelium age and enzymatic incubation period on protoplast release using 10 mg/ ml Lytic enzyme and 9 mg/ ml Macerozyme: (a) SD broth, (b) GM medium and (c) YM Medium. (Protoplast yield to be multiplied by 10^7)

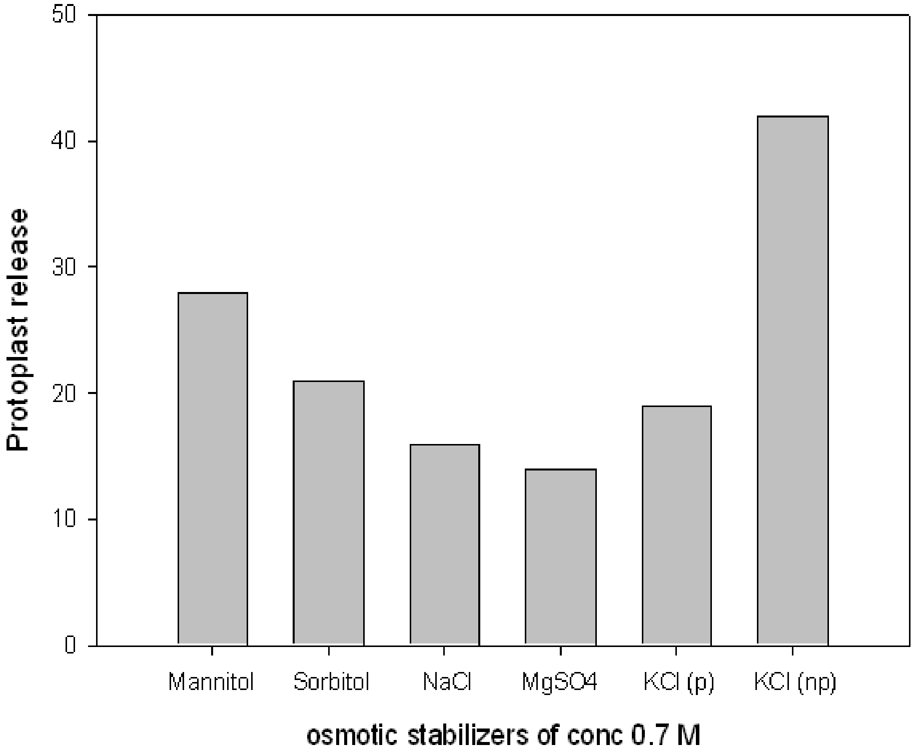
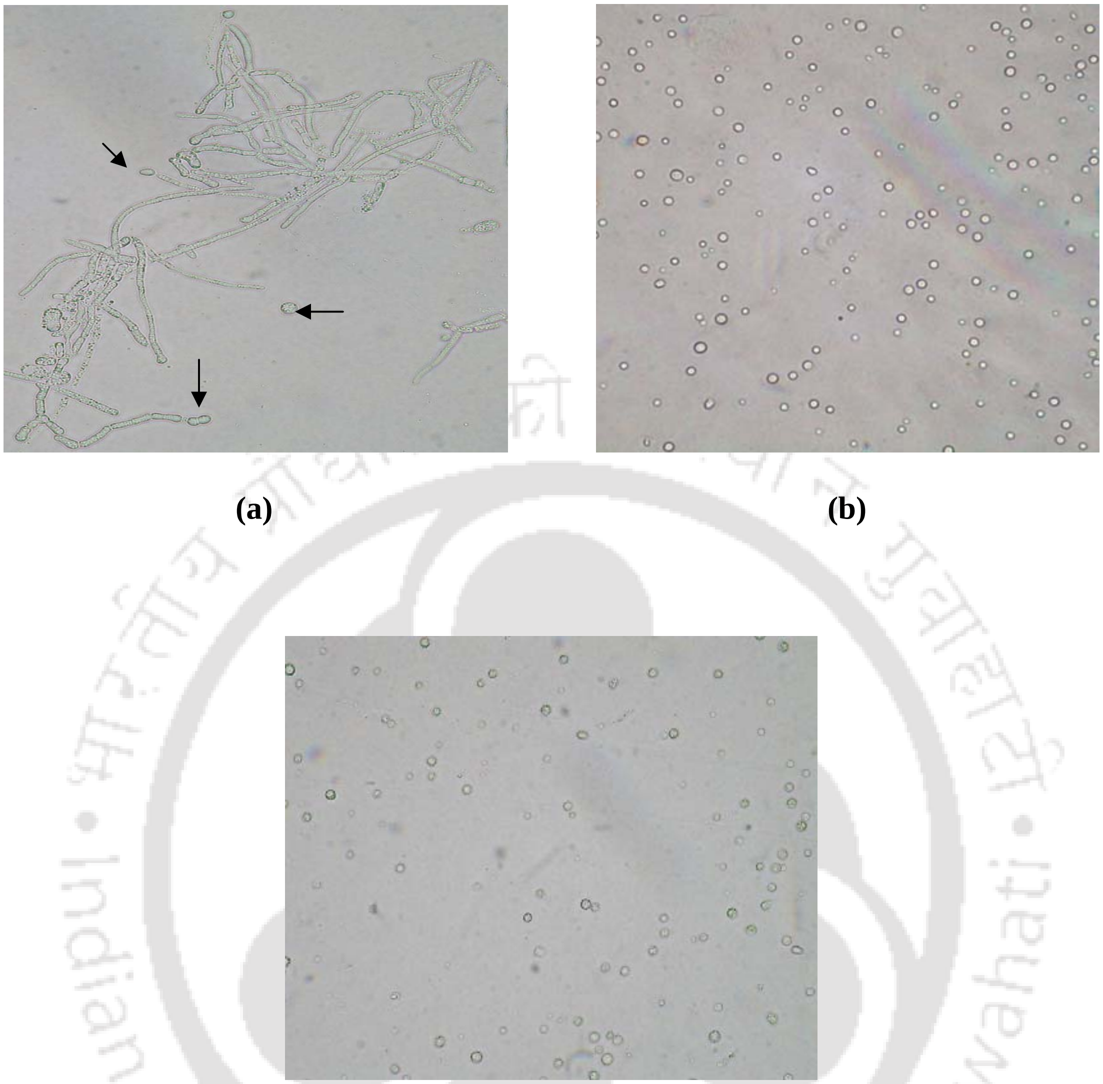


Fig 6.3. Use of different osmotic stabilizer on protoplast release: protoplast released after 3 h enzymatic digestion of 40 h old mycelium. (p= protoplast released from preincubated mycelium in thiol compound, np= protoplast released from mycelium without any preincubation.)



(a) (b)

(c)

Fig 6.4. Protoplast released on different incubation time by using 40 h old culture and 10 mg/ ml Lysing enzyme: (a) after 1 h enzymatic digestion; arrows indicate the release of protoplast from the tip of hyphae, (b) after 3 h enzymatic digestion, (c) protoplast release after 5h enzymatic digestion

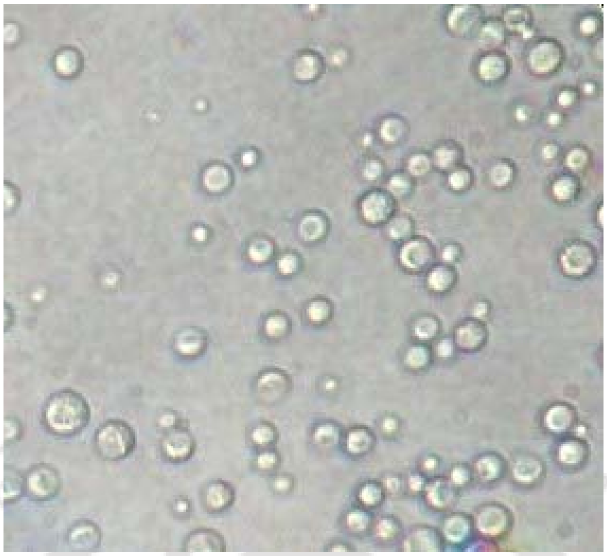
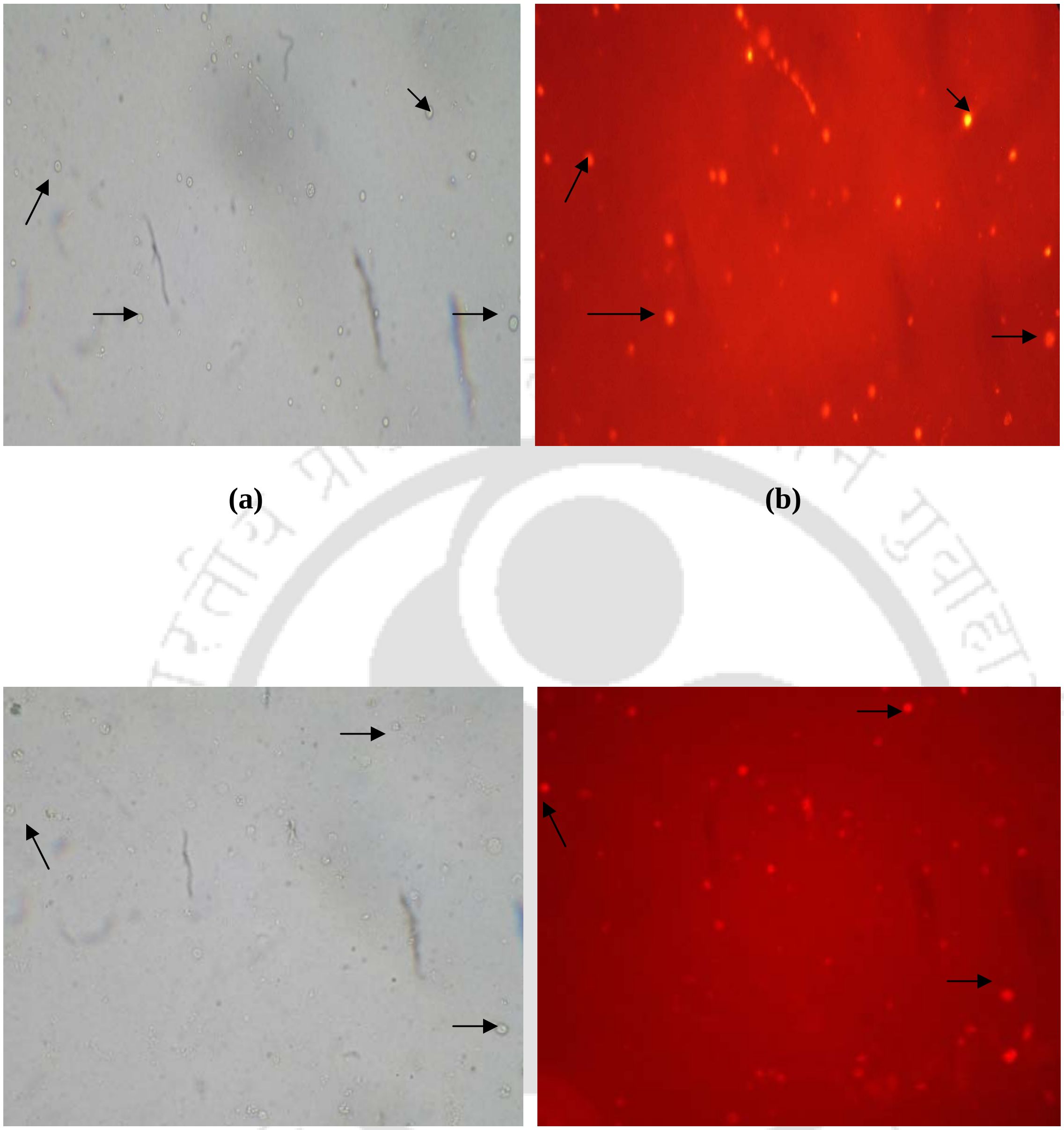


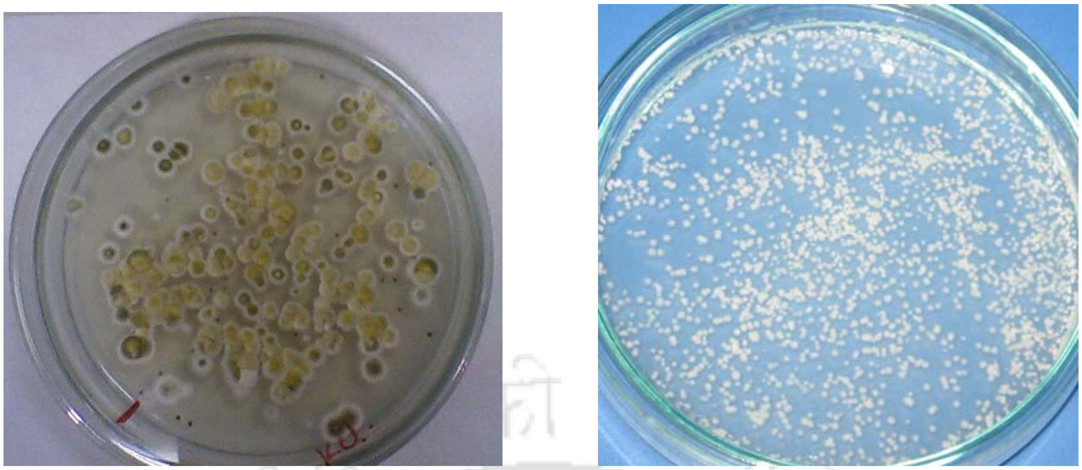
Fig 6.5. Protoplast released after 3 h enzymatic digestion using 10 mg/ ml Lysing enzyme and 0.7 M KCl as osmotic stabilizer



(a) (b)

(c) (d)

Fig 6.6. Protoplast viability was checked using Fluorscein diacetate (FDA): (a) micrograph of fresh isolated protoplasts using 0.7 M KCl, (b) viability of the fresh protoplast using FDA using 0.7 M KCl, (c) and (d) Phase contrast micrograph and FDA treated micrograph of protoplasts after three months of storage at 4 °C using sorbitol as osmoticum



(a) **(b)**

Fig 6.7. Regenerated protoplasts on Czapek-Dox agar supplemented with 0.7 M KCL, (a) *M. anisopliae* (b) *B. bassiana*

7.1 Introduction

The infectivity of the insect pests depends on the stability of a variety of environmental factors. Strain improvement for the purpose of better field persistence is necessary for the survival of the entomopathogenic fungi against harsh field conditions such as high temperature, UV radiation, and application of fungicides or herbicides in the field. Tolerance to such stresses can be achieved by genetically manipulating the fungi. The most adapted method for genetic alteration includes transformation of protoplasts by PEG fusion method. The fundamental process of DNA transfer to cell is the first step of transformation. Mammalian cells employ the endocytic method for the uptake of DNA as *S. cerevisiae* (Khalil et al., 2006; Neukamm et al., 2002; Kawai et al., 2004). The first report of DNA transfer to model fungus *N. crassa* was established utilizing PEG and Ca^{2+} ion (Hinnen et al., 1978; Case et al., 1979). PEG utilization is a frequent feature in transformation protocol. High concentration of PEG is used to facilitate the clumping of the protoplasts and trapping the DNA (Fincham, 1989). The common ingredient of transformation mixture is calcium (Ca^{2+}) ions with exceptions using lithium ions in non-protoplast forming protocols. Various approaches have been investigated to select the transformants. The former version of selecting the transformant colony is to use auxotrophic parental strains. However, this method is time consuming and labor intensive to screen the mutant parental strains. An efficient alternative is the use of dominant selectable resistance markers. Glufosinate ammonium is a broad-spectrum contact herbicide and is used to control a wide range of weeds which inhibits the activity of an enzyme, glutamine synthetase, which is necessary for the production of glutamine and for ammonia detoxification The application of glufosinate leads to reduced glutamine and increased ammonia levels in the plant tissues which is also found to inhibit appresoria formation and conidial germination of fungi. The effectiveness in the dual exploitation of glufosinate ammonium along with the fungi makes it a lucrative option in transformation as it can be used as a dominant selectable marker as well as the recombinant fungus can be used to control pests in combination with this herbicide.

7.2 Materials and Methods

Fungal isolates

The fungal isolates, *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) was procured from ARSEF (USDA-ARS Plant Protection Unit) and the culture was maintained periodically on Sabouraud's dextrose agar slants.

Preparation of fungal protoplasts

Fungal protoplasts were prepared as described in Chapter 6.

Plasmid Propagation and Extraction

The plasmid pBARGEM7-2 (Fig 7.1a) containing bar gene from *S. hygroscopicus* was procured from Fungal Genetics Stock Centre (FGSC) and was multiplied and extracted from *E. coli* DH5α.

Preparation of competent cell

Competent cells were prepared by the method of Chung et al. (1989). *E. coli* DH5α was grown overnight at 37 °C on LB agar plate (Tryptone 10 g/ L, Yeast extract 5 g/ L, NaCl 10 g/ L, pH 7.4, 1.5 % agar; no ampicillin) by streaking. Single colony was picked from LB agar plate and inoculated to 5 ml of liquid LB media and cells were allowed to grow overnight at 37 °C at 200 rpm. A 200 µl of the culture was inoculated in 50 ml of LB media and incubated at 37 °C and 200 rpm until the OD_{590} reaches 0.7. Cells were kept on ice for 20 min and centrifuged at 5000 rpm at 4 °C for 10 min. Cell pellet was gently resuspended in $1/10^{th}$ volume of TSS buffer (LB medium 85 % (v/v), PEG 10 % (w/v), Dimethyl sulfoxide 5 % (v/v), Magnesium chloride 50 mM) and incubated on ice for 20 min. A 200 µl of cells were aliquoted in prechilled microcentrifuge tubes and stored immediately at -20 °C until further use.

*Transformation of plasmid pBARGEM7-2 to E. coli*DH5α

The transformation mixture contained 4 µl of plasmid DNA (pBARGEM7-2) & 100 µl of fresh competent cells. The mixture was incubated on ice for 30 min and after the incubation immediately subjected to heat shock at 42 °C for 60 sec. A 900 µl of pre warmed (37- 42 °C) SOC medium was added to it. Thereafter the mixture was again incubated on ice for 5 min. The mixture was incubated for 1 h at 37 °C and 200 rpm. The transformed colonies were grown and selected in LB agar media containing ampicillin (100 µg/ ml).

Minipreparation of plasmid DNA

Plasmid (pBARGEM7-2) was extracted from transformed *E. coli* DH5α cells by minipreparation of plasmid DNA (Fig 7.1b). A single colony of transformed *E. coli* DH5α was inoculated in 5 ml of liquid LB media supplemented with 50 µg/ ml ampicillin and incubated overnight at 37 °C and 200 rpm. A 2 ml of that culture were taken in a microcentrifuge tube and spinned at 10000 rpm for 1 min, supernatant was discarded. The pellet was suspended in 100 µl of ice cold solution 1 [50 mM glucose, 25 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0)] and incubated for 5 min. A 200 µl of freshly prepared solution 2 [0.2 N NaOH, 1 % (w/v) SDS] was added and mixed thoroughly by inverting the tubes and incubated for 3 min on ice. Thereafter solution 3 [60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml Millipore water] was added and mixed gently and incubated for 5 min on ice. The mixture was centrifuged at 10000 rpm for 15 min at 4 °C and supernatant was transferred to fresh centrifuge tube. A 450 µl of isopropanol was added and tube was kept at -20 °C. The quality of the plasmid DNA was checked on 1 % agarose gel.

Transformation of plasmid pBARGEM7-2 to fungal protoplasts

A modified method of Herrera-Estrella et al. (1990) was adapted for transformation experiments. Transformation mixture consisted of 200-300 µl protoplast solution (6×10^7 protoplasts/ ml) in 10 ml centrifuge tube along with 15 µg of plasmid pBARGEM7-2. Poly ethylene glycol (PEG 8000; Sigma), 25 % (w/v), in 10 mM Tris-HCL (pH 7.5) containing 50 mM $CaCl_2$ was added and tubes were incubated for 30 min on ice. A 2 ml of PEG solution was added again and mixed gently. After 5 min of incubation at room temperature the solution was diluted by adding 4 ml 0.7 M KCL and 50 mM $CaCl_2$. A 200 µl of diluted solution is plated on SDA containing 200 µg/ ml of glufosinate ammonium as minimum inhibitory concentration of glufosinate ammonium was determined to be 200 µg/ ml.

Selection and Stabilization of Transformants

Stable transformants were selected after repeated sub-culturing for four generations by transferring colonies to glufosinate ammonium containing SDA plates and allowing the fungus to grow until sporulation.

Extraction of DNA

Mycelium was harvested from four day old SD broth by centrifugation at 8000 rpm for 15 min and dried using blotting paper. Mycelium was transferred to a sterile porcelain mortar and crushed with liquid nitrogen. 10 mg of this crushed mycelial powder was taken into a microcentrifuge tube and 500 µl of lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 3 % SDS) were added. After incubation at 65 °C for 1 h, 500 µl of TE saturated phenol/ chloroform (1:1) was added and after vigorous vortexing the mixture was centrifuged at 13000 rpm for 20 min. This step was repeated for several times to remove all proteins. Supernatant transferred to a new microcentrifuge tube and 0.1 volumes of 3 M Na acetate and 1 volume of ice cold isopropanol added. The solution allowed to stand on ice for 2 h and centrifuged at 13000 rpm for 20 min. DNA pellet was washed with 70 % ethanol, air dried and dissolved in Tris EDTA buffer. RNA was removed by incubating the DNA with 2 µl of 10 mM RNAse A for 30 min at 37 °C. Thereafter the phenol/ chloroform extraction was repeated and DNA pellet was recovered with Na acetate and isopropanol. After washing with 70 % ethanol, air dried and dissolved in Tris EDTA buffer and stored at -20 ºC in aliquots.

Confirmation of the transformation

DNA was isolated by the above described method. Molecular confirmation was achieved by a confirmation PCR using Primers, specifically designed for bar gene based on the conserved domains of the bar gene product.

Forward primer	GGCGGGCTATGCGTATGCGA
Reverse primer	GCAATCACCGCAATCATCTGG

In order to achieve biochemical confirmation, native & transformed isolates were grown in Czapek-Dox broth supplemented with glufosinate ammonium (100 µg/ ml) and the culture supernatants were analyzed by SDS-PAGE

Features of the original and transformed isolate

Virulence determinant enzymes, Chitinase and Protease; percentage of germination along with sporulation was studied for both the Native isolates and Transformed ones.

7.3 Results

Minimum inhibitory concentration of glufosinate ammonium for *M. anisopliae* & *B. bassiana* was similar, 197 & 200 µg/ ml respectively. No growth was observed in the Czapek-Dox media supplemented with 200 µg/ ml of glufosinate ammonium for the untransformed isolates while substantial growth was observed for transformed isolates (Fig 7.2). Initial transformation experiment resulted in 4-5 transformants/ µg of DNA whereas almost 8-7 transformants/ µg of transforming DNA were achieved in the following repeat experiment. The glufosinate ammonium resistant transformed colonies appeared between 5-7 days. PCR was performed to confirm the insertion of bar gene at molecular level. A primer pair was designed for bar gene based on the conserved domains of the bar gene product, Phosphinothricin Acetyl

Transferase (PAT). A 185 bp amplified fragments were observed for both *B. bassiana* and *M. anisopliae* confirming the integration of the bar gene in fungal DNA (Fig 7.3a). The bar gene product PAT protein was detected in the culture supernatants of the transformed isolates. Native and transformed isolates were grown in Czapek-Dox media supplemented with 100 µg/ ml glufosinate ammonium. SDS-PAGE analysis revealed a 22-23 kDa band for transformed isolates whereas no such protein is present in case of native isolates (Fig 7.3b). Several factors were investigated to study the impact of gene insertion. Comparison of virulence determinant chitinase and protease enzyme activity in native and the transformed isolates didn't show any significant difference for both *M. anisopliae* & *B. bassiana* (Table 7.1). Sporulation decreased diminutively for transformed isolates but no change in germination potential was observed.

7.4 Discussion

Minimum inhibitory concentration (MIC) could vary with different isolates (Valadares-Inglis and Inglis, 1997), MIC of glufosinate ammonium differed diminutively for these two isolates in this study. Transformation frequency was low in this experiment which is supported by the findings of Bernier et al. (1989), only 4 transformants were obtained using 50 µg of DNA whereas Goettel at al. (1990) reported 9 transformants using the same amount of DNA by PEG/ Ca^{2+} method. The traditional method to transform DNA to fungi is PEG/ Ca^{2+} method. Although argued to be less efficient than other DNA transfer methods such as electroporation and biolistic technique (St. Leger et al., 1995), it is the most easiest, reliable and economical way to transfer DNA. Various factors are associated with this method such as concentration of PEG and suitable osmotic stabilizer (Valadares-Inglis and Inglis, 1997). Protoplast quality is another important factor affecting transformation in fungi (Goettel et al., 1990) moreover strain specific factors could also effect the transformation. The gene specific PCR confirmed the insertion of the bar gene at molecular level whereas detection of PAT protein in the culture supernatants of the transformed isolates confirms the insertion at the enzyme level. It is important to explore the affects of gene insertion as homologous recombination of the gene could possibly integrate in the sites of pathogenically important genes or other genes which regulates those genes thus affecting the virulence directly or indirectly. No significant difference in the enzyme activities, sporulation & germination percentage suggests no disruption of pathogenically important genes due to transformation.

7.5 Inference

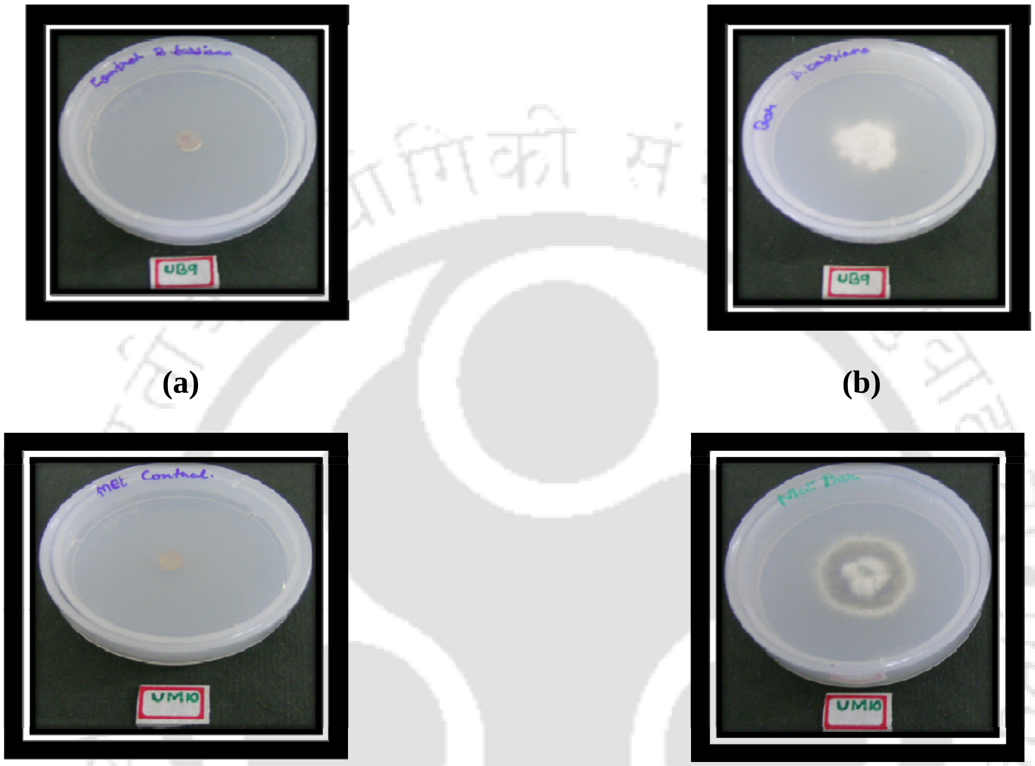
The suitability of entomopathogenic fungus for commercial purpose leads to the improving characteristics for better performance and stability in the environment. Developing techniques for using the fungus along with herbicides was being attempted in this study as a result these transgenic fungi with improved qualities generates a wider concern in these fungi as environmentally friendly means for IPM that in turn generates interests to produce these fungi in a cost effective way. Chapter 8 deals with the mass production techniques with both *B. bassiana* and *M. anisopliae* isolates.

Table 7.1. Various virulence determinant factors of native and transformed isolates

Isolate type	**Chitinase activity (U/ ml)**	**Protease activity (U/ ml)**	**Sporulation ($y \times 10^7$) spores/ ml**	**% of germination after 12th h**
B. bassiana				
Native isolate	36.29	0.94	6.39	55.99
Transformed isolate	36.11	0.96	5.22	56.06
M. anisopliae				
Native isolate	7.65	0.57	8.24	85.92
Transformed isolate	7.84	0.55	7.91	85.57

(a) (b)

Fig 7.1. (a) plasmid pBARGEM7-2 , (b) The extracted 4.9 kb Plasmid from *E. coli* DH5α



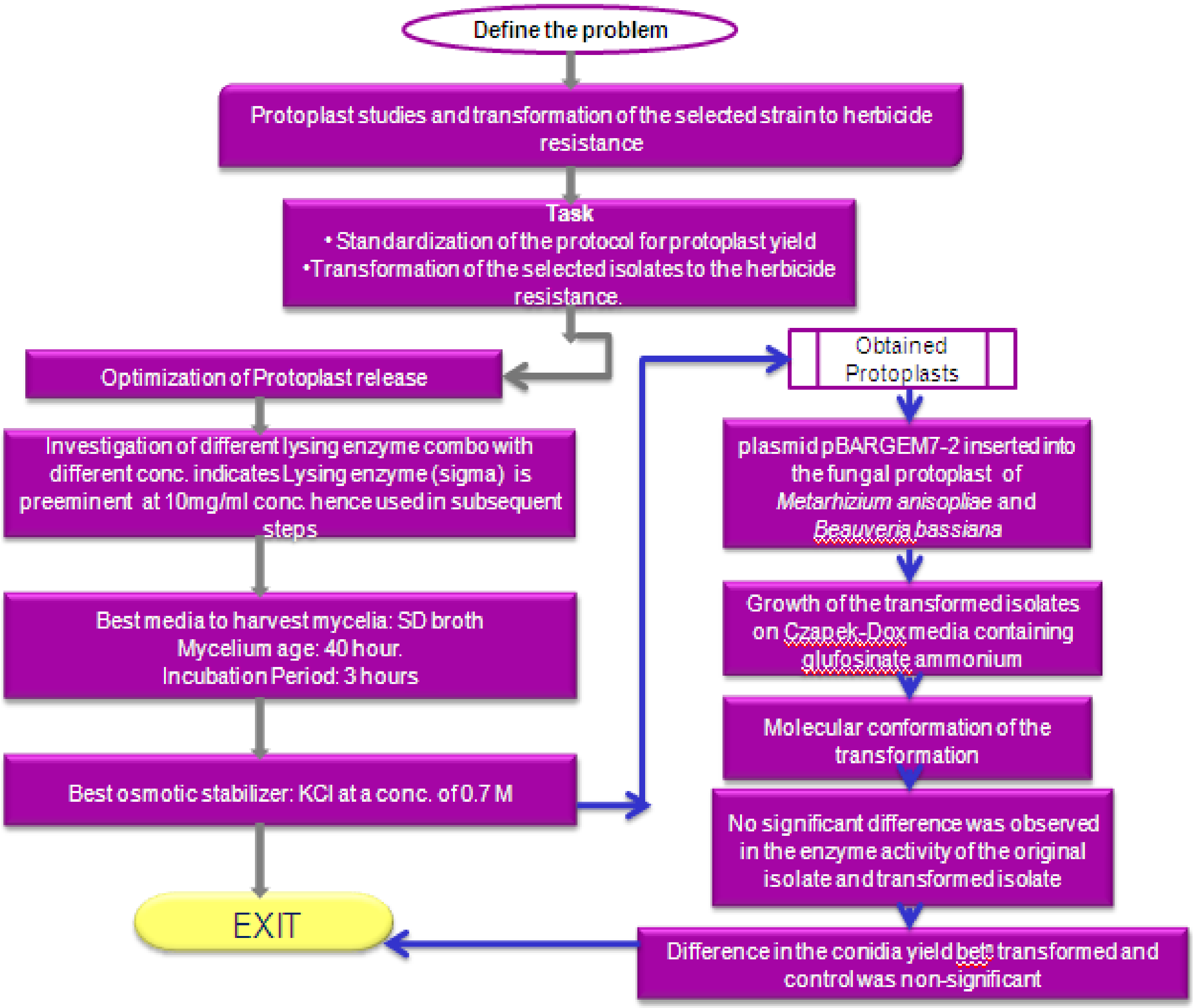
(a) (b)

(c) (d)

Fig 7.2. Growth of (a) untransformed, (b) transformed *B. bassiana*; (c) untransformed, (d) transformed *M. anisopliae* on Czapek-Dox media containing 200 µg/ ml glufosinate ammonium

(a) (b)

Fig7.3: Transformation confirmation. (a) Lane 1 consists of DNA Ladder, Lane 3 and 4 are the PCR products from *B. bassiana* and *M. anisopliae* using the designed primer. (b) Lane 1 & 2: native isolates of *B. bassiana* (UB9) & *M. anisopliae* (UM10) Lane 3 & 4: transformed isolates of *M. anisopliae* & *B. bassiana*



Flowchart 7.1. Summary of Chapter 6 and 7

8.1 Introduction

The past decade has experienced unprecedented increase in the research and commercial development of entomopathogenic fungi as a biopesticide, which directed the focus on *Beauveria bassiana* and *Metarhizium anisopliae,* soil borne entomopathogenic fungi, due to their broad host range. The potential of entomopathogenic fungi in pest management has been well documented. Considerable interest was generated when it was established that production of 10^{13} conidia, costs the same as chemical insecticides used per hectare. Therefore, high production efficiency leads to the development of consciousness to mass produce conidia in a cost effective way (Wraight et al., 2001). Automated technologies for solid state production of conidia have been developed in several countries to achieve this level of production efficacy (Feng et al., 1994). Significant efforts have been made to establish *B. bassiana* and *M. anisopliae* as a commercial mycopesticide by using solid State Fermentation (SSF) as a suitable method for mass production (Shah and Pell, 2003). Solid-state fermentation (SSF) can be defined as growth of a microorganism on solid substrate in the absence or near absence of free liquid, but the substrate must possess enough moisture to support fungal growth (Moo-Young et al., 1983). The solid material can serve as a carbon and nutrient source promoting fungal growth or it can act as an inert matrix which is supplemented with carbon and nitrogen sources (Pandey, 2003). Conidia production of entomopathogenic fungi by SSF on whole grains is being practiced in Latin America for a long time (Grimm, 2001) which involves a biphasic system where a liquid culture is used as an inoculum for the solid substrate (Mendonca, 1992) and the most popular solid substrate for conidia production of entomopathogenic fungi is rice, due to its complex nutritional source (Posada-Flórez, 2008; Ye et al., 2006). Bioprocesses involving agro industrial residues proved to be advantageous as it processes the waste resourcefully and provides alternative substrates leaving whole grains for animal consumption (Soccol and Vandenberghe, 2003). Different economical nutritive and non nutritive substrates are being explored as solid media for SSF (Jenkins et al., 1998). It involves a lower operating cost and a copious prospect of processing agro-industrial residues like declassified potato flour, rice flour as solid substrate for conidia production of entomopathogenic fungi *B. bassiana* (Soccol et al., 1997).The optimal conditions for solid-state fermentation includes many variables such as substrate, moisture content, and concentration of initial inoculum and length of time for fermentation (Masangkay et al., 2000). Water is considered as appropriate humidifying agent. Grajek and Gervais (1987) reported the efficiency of water for supporting conidia formation on wheat bran, rather than other liquid mineral medium. Yeast extract is another major factor influencing fungal growth due to its complex nutritional source of nitrogen and vitamins (Im et al., 1988).

The application of different optimization techniques in fermentation is used to determine the most suitable reaction conditions for instance pH, temperature and specially medium component concentrations which maximize or minimize economically or technologically important process variables namely, product concentration, yield, selectivity and raw material costs (Weuster-Botz, 2000).

The Plackett-Burman fractional factorial design allows the identification of the main effects of a large number of variables with relatively few experiments and is the most effective tool for screening independent variables. Four physical factors namely, moisture content, pH, inoculum age and inoculum volume and five additional nitrogen sources namely, yeast extract, beef extract, peptone, tryptone and urea were used in this study. This step is generally followed by a process optimization tool such as Response Surface Methodology (RSM). Response Surface Methods are empirical statistical tools that are being used for modeling and analyzing problems where the response is influenced by several independent variables and the aim is to maximize the process variables to get an optimum response (Montgomery, 2001). The experimental runs of the Central Composite Design (CCD) serve as inputs in determining the mathematical model that correlates physical and media parameters with the product yield.

Machine learning can overcome the problems of biological systems on chemical and physical basis which arise due to its non-linear nature of biochemical interaction networks (Franco-Lara et al., 2006). It can provide proper modeling of biological systems as MLT can mimic the biological processing of information efficiently. Machine learning is concerned about the design and development of algorithms such as artificial neural networks; a Bayesian Network and Decision table which evolves based on the input data and recognizes the patterns so that it can efficiently provide predictive information on unseen data. Supervised machine learning algorithms properly predict the class of unlabelled instances given a data set of labeled instances prior to it. Here we report the application of Decision Tables (DT); simple hypothesis based supervised learning algorithms in predicting the output of fungal conidia on different substrates.

This current study illustrates screening of different physical and media factors through Plackett-Burman design followed by optimization of screened factors by Response Surface Methodology using four different substrates i.e. Rice, Sorghum, Rice+ Rice bran and Sugarcane bagasse + BFW to observe the effect of each substrate and their correlation with the conidial yield. Moreover this study addresses the efficiency evaluation of statistical method (RSM) and non statistical information processing paradigm (MLT) that were applied to the optimization study for the first time in literature.

8.2 Experiment 1: Initial study on mass production of conidia from *B. bassiana* (ARSEF 2034)

An initial study on mass production of conidia was conducted using one *B. bassiana* isolate in order to establish a basis for further experiments. The optimization of two important factors; moisture content and yeast extract concentration for conidial production through SSF of entomopathogenic fungus *B. bassiana* (ARSEF 2034). Both the variables were tested for four different substrates i.e. rice, crushed sorghum, rice+ wheat bran and crushed sorghum + rice bran to observe the effect of each substrate and their correlation on the conidial yield.

8.2.1 Materials and Methods

Fungal culture

The fungal isolate, *Beauveria bassiana* (ARSEF 2034) was used in this experiment and the culture was maintained periodically on Sabouraud dextrose agar slants.

The *B. bassiana* isolate was cultured in Sabouraud dextrose broth (4 % Dextrose, 1 % Peptone and 1 % Yeast Extract). Approximately 10^7 conidia/ ml were inoculated and mycelium cultivated for three to four days at 28 °C and 180 rpm.

Media preparation and Solid State Fermentation

Rice and Sorghum were used as single substrates and two mixed substrates were used; Rice + Wheat bran and Sorghum + Rice bran, mixed in equal amounts. Sixty grams of a substrate were weighed and taken into polyethylene bags and supplemented with appropriate amounts of water (20-80 %) and yeast extract (1-2 %) for different substrates (Table 8.1). Aeration was provided by incorporating small cotton plugged PVC pipes (10 cms). The bags were then autoclaved at 121 °C (15 psi) for 20 min. All the substrates were inoculated with 5 ml of cultivated mycelium in SD broth and substrate clumps if any were broken before inoculation to provide more surface area for fungal growth. Bags were then incubated for 15 days at 28 °C. All the experiments were carried out in triplicates. Subsequently, after 15 days of incubation, 1 g of each solid fermented substrate were mixed with 5 ml of sterile 0.01 % Tween-80 solution and vortexed thoroughly. The spore concentrations were determined using Haemocytometer and this was repeated thrice for each bag and the average counts were taken into consideration and the conidial yield determined.

Experimental design and optimization

Central composite design is widely used statistical design for fitting second order models. In this experiment 2^2 central composite design with 5 center points , 4 cube points and 4 axial points was used ($\alpha = 1.414$) giving 11 trials per substrate (Table 8.1).

8.2.2 Results and Discussion

The experimental results of SSF on different substrates are shown in Tables 8.2, 8.3 and 8.4. Single substrate rice at 40 % moisture content yielded the second best conidial yield of 16.2×10^9 conidia/ gm (Table 8.2). The initial moisture content is argued to be most critical parameter for product formation in fungi (Nampoothiri et al., 2004). A reduced amount of moisture content (20 %) was optimum for substrate sorghum and the conidial yield was 9.63×10^9 conidia/ gm although yeast extract concentration was higher compared to other three substrates. On the contrary, mixed substrate sorghum + Rice bran showed poor sporulation at 20

% moisture content and required moisture content level of 40 % for maximum conidial yield of 6.25 ×10^9 conidia/ gm and the yeast extract concentration was 1.5 % (Table 8.3). The requirement of moisture content for conidiation was different for each substrate which was also documented by Deshpande et al. (2001) as conidia production of *N. rileyi* on wheat required more moisture for conidiation compared to barley, rice and sorghum. The mixed substrate, Rice + wheat bran was the most effective substrate for conidia production by SSF. A conidial yield of 28.80 ×10^9 conidia/ gm was obtained at 35 % moisture content and 1.5 % yeast extracts (Table 8.4). Among the cereals tested, crushed sorghum supplemented with 1 % yeast extract produced 13.68 ×10^8 conidia/ gm and rice bran was the best agro waste for conidia production. In this study, yeast extract concentration ranging from 1-2 % was sufficient to support the vegetative growth and yielded excellent sporulation. Yeast acts as a source of N_2 & vitamin and it was observed to be necessary for the mycelial growth of the entomopathogenic fungi (Im et al., 1988). The responses of the statistical design were fitted into the second order polynomial equation (equation ii). The significance and adequacy of the model was checked by Fishers F-test value of Analysis of Variance (Table 8.5). The model F value for mixed substrate Rice + Wheat bran was 10.91 and low probability value of 0.010, accounts for the high significance of the model. There is only 1.01 % chance that model F value this large could occur due to noise. The model F and P values for other substrates also correspond to high significance of the respective models (Table 8.5). For the model using Rice as a solid substrate, only linear term B i.e. yeast extract concentration and squared terms, A^2 (moisture content × moisture content) and B^2 (yeast extract × yeast extract) are significant (Table 8.6). For substrate sorghum, linear term A and interaction term AB are the significant model terms only; this implies that moisture content and interaction between moisture content and yeast extract have significant effect on response i.e. conidial yield. In case of other two mixed substrates Sorghum + Rice bran and Rice + Wheat bran; linear term A i.e. moisture content, squared term A^2 (moisture content × moisture content) and squared terms A^2, B^2 were significant model terms respectively. Although yeast extract is essential in the vegetative growth of fungus but except for substrate Rice, it did not exert any significant effect except for the interaction terms. The Coefficient of determination (R^2) for mixed substrate Rice + Wheat bran was 0.9160, which indicates almost 92 % variability in the response which could be explained and only 8 % of total variations could not be explained by the model. The coefficient of determination (R^2) for Rice, Sorghum, and Sorghum + Rice bran was 0.9291, 0.9134, and 0.9021 respectively which describes goodness of fit of the model (Table 8.7). The optimized values of the two variables on different substrate are detailed in Table 8.8. The response surface methodology (RSM) is preferred statistical technique with the advantages such as rapid and reliable optimization of process parameters at varying concentration that in turn save time, chemical and manpower (Gohel et al., 2006). Three dimensional response surface plot of conidial yield for substrate rice shows that decreasing both the variables increases the response i.e., conidial yield, giving highest response value at 1% yeast extract concentration (Fig 8.1a). The contour plot with minimax approach or saddle point for substrate crushed sorghum shows that simultaneous increase or decrease of both the variables leads to a decrease in the response whereas decreasing one variable and increasing the other at the same time increases the response variable (Fig 8.1b). For the substrate crushed sorghum + rice bran, continuous increase in both factors increases the response, showing optimum conidial yield at 40% moisture content and 1.5% yeast extract (Fig 8.2a). Mixed substrate rice + wheat bran showed maximum conidial yield almost at design center point i.e. at 35% of moisture content and 1.5% of yeast extract concentrations (Fig 8.2b).

8.2.3 Inference

Optimization studies help in understanding interaction among the various factors and in calculating the optimal concentrations and conditions for improved production of conidia. This study suggests the importance of optimization of process conditions which was investigated in detail in the next experiment, Experiment 2. The significant factors for conidia production from *M. anisopliae* (ARSEF 3295) and *B. bassiana* (ARSEF 2033) were screened out using Plackett-Burman design and further variables were analyzed using CCD design (Table 8.9, 8.17).

8.3 Experiment 2: Mass production of conidia from *M. anisopliae* (3295) and *B. bassiana* (2033)

8.3.2 Materials and Methods

Fungal culture

The fungal isolates, *M. anisopliae* (ARSEF 3295) and *B. bassiana* (ARSEF 2033) were used in this experiment. The culture was maintained periodically on Sabouraud dextrose agar slants. The isolate was cultured in Sabouraud dextrose broth [Dextrose (4 %), Peptone (1 %) and Yeast Extract (1 %)]. Approximately 10^7 conidia were inoculated into 50 ml SD broth and mycelium cultivated for three to four days at 28 °C and 180 rpm.

Media preparation and Solid State Fermentation

Rice and Sorghum were used as single substrates and two mixed substrates were used; Rice + Rice bran and Sugarcane bagasse + Biscuit factory waste (BFW), mixed in equal amounts. Each polyethylene bags contained Sixty grams of a substrate were weighed and taken into. For PB design bags were supplemented with appropriate amounts of water and various nitrogen supplements and adjusting the proper pH based on the experiment design for different substrates. In case of RSM experiments bags were supplemented with water and yeast extract (For *B. bassiana* only) according to CCD design. Aeration was provided by incorporating small cotton plugged PVC pipes (10 cms). The bags were then autoclaved at 121 °C (15 psi) for 20 minutes. All the substrates were inoculated with cultivated mycelium in SD broth; inoculum volume and age were adjusted according to the PB and CCD design (Table 8.9, 8.17). Substrate clumps were broken before inoculation to provide more surface area for fungal growth. Bags were then incubated for 15 days at 28 °C. All the experiments were carried out in triplicates. Subsequently, after 15 days of incubation 1 g of each solid fermented substrate was mixed with 5 ml of sterile 0.01 % Tween-80 solution and vortexed thoroughly. The spore concentrations were determined using Haemocytometer and this was repeated thrice for each bag and the average counts were taken into consideration and the conidial yield determined.

Experimental design and optimization

Plackett-Burman design

Nine independent factors were screened in eleven combinations at two levels, maximum (+) and minimum (-) according to the Plackett-Burman design (Plackett-Burman, 1944) (Table

8.9, 8.17). Four physical factors viz; Moisture content, pH, volume of inoculum and age of inoculum and five additional nitrogen sources viz; Yeast extract, Beef extract, Peptone, Tryptone and Urea were used and all experiments were performed in triplicates.

The Plackett-Burman design is based on the first order model

$$Y = a_0 + \sum a_i X_i \tag{1}$$

where Y is the response (conidial yield), a_0 is model intercept and a_i is linear coefficient and X_i is the level of independent variable.

The effect of each variable was calculated by

$$E_{xi} = \sum (Y_{i+} - Y_{i-}) / N \tag{2}$$

where Ex_i is the effect of the variable, Y_{i+} and Y_{i-} is the conidial yield from the trials at the maximum and minimum level of that variable. N is the number of experiments carried out.

Central composite design

A central composite design generally consists of 2^n factorial design, where n is the number of variables used in the experiment with 2n axial runs and n center runs that gives a total of N = 2^n + 2n + n runs. In this experiment 2^2 central composite design with 5 center points , 4 cube points and 4 axial points was used (α = 1.414) giving 11 trials per substrate.

The test variables were coded according to the following equation,

$$x_i = (X_i - X^{\circ}_i) / \Delta X \tag{3}$$

where, x_i is the coded variable and X_i is the natural value of independent variable, X°_i is the value of the variable at the center point and ΔX is the step change value. The details of the range and levels of each variable for each substrate are given in Table 8.9 and 8.17.

A second order polynomial equation was then fitted to the response data.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\Sigma\, \beta_{ij} X_i X_j + \varepsilon \qquad (4)$$

Where Y is the response variable, β_0 is the intercept term, β_{ii} is the squared effect and β_{ij} is the interaction between X_i and X_j. The goodness of fit of the model is explained by the Coefficient of determination R^2, which measures the amount of reduction in the variability of response Y in a model (Montgomery, 2001). All the statistical calculations, design and graphical analysis were done by Minitab statistical software (release15) and Design Expert (version 7.1.5).

Decision table

A Decision Table (DT) stores the input data in condensed form based on a selected set of attributes and uses it as a lookup table when making predictions. Each entry in the table is associated with class probability estimates based on observed frequencies. The key to learning a DT is to select a subset of highly discriminative attributes (Hall and Frank, 2008). A decision table is composed of four quadrants; the upper left and right quadrant contains the conditions and condition rules of alternatives. The lower left quadrant contains the actions to be taken and the lower right quadrant contains the action rules. The simplest way to create a decision table is to list down all the causes, calculate the number of possible combinations, fill columns with possible combinations, reduce test combinations and add effects to the table. Most of the decision table use fuzzy logic or probabilistic representation for condition alternatives. All the modeling and predictive calculations were done in WEKA 3.6; a machine learning software.

8.3.3 Results

The mycelial growth on the different substrates was visible from the fifth day onwards and sporulation started on seventh day after incubation at 28 $^{\circ}$C (Fig 8.3). A two level Plackett Burman design were set in two separate experiments to screen out significant sporulation promoting physical factors (Table 8.10, 8.18) as well as supplementary nitrogen sources (Table 8.11, 8.19).

B. bassiana

The positive effect of the moisture content in all four substrates was established whereas age of the inoculum indicated negative impact on the conidial yield suggesting lower values of these factors augment the yield (Table 8.10). Volume of the inoculum and pH demonstrated negative effects on substrates Rice, Sorghum and Rice+ rice bran however an exception was observed for substrate Sugarcane bagasse + BFW indicated positive effects on substrate. Analysis of the PB design confirmed the significant factor for conidial yield was yeast extract which showed positive effects for all four substrates for *B. bassiana* (Table 8.11). But surprisingly no conidial yield was observed for substrate Sorghum using five nitrogen supplements consequently the reason was investigated by OVAT experiment which showed no conidial yield when urea was used as nitrogen supplement for substrate sorghum.

Optimization of three screened out factors; Moisture content and Inoculum age and Yeast extract concentration were carried out designing a CCD (Table 8.9) and response is further analyzed by RSM. The predicted response along with the experimental data on the four substrates presented in tables (Table 8.12, 8.13) reveal a close correspondence between the values. Substrate Rice yielded highest conidial yield of 11.32 $\times 10^9$ conidia/ gm at 50 % moisture content and 6 days old inoculum although yeast extract concentration was high 3.68 % whereas conidial yield on sorghum was favored by lesser concentration of yeast extract 2 %. The same trend was also observed for substrate bagasse with a maximum yield of 5.76 $\times 10^9$ conidia/ gm at 50 % moisture content, 6 days old inoculum and 2 % yeast extract concentration. Contour plots and 3-D response surface plots were used to determine the relationship between three predictor variables as well as the optimum moisture content and inoculum age.

A second order polynomial equation (equation ii) was fitted with the responses from the design. The significance and adequacy of the model was checked by Fishers F-test value of Analysis of Variance (Table 8.14). The F value is the variance ratio between group and within group variability (S_B^2 / S_W^2) that actually measures the ability of factors to describe the variation in the data about its mean. The model F value for the four substrates viz, Rice, Sorghum, Sugarcane bagasse+ BFW and Rice + Rice bran are quite high as 7.12, 17.00, 14.46 and 59.15 respectively implying significant models moreover low p value (p value$\leq$ 0.05)of these models also confirms the significance of the models. The p value is the probability value that is associated with the F Value i.e. it is the probability of getting an F Value of this size if the term did not have an effect on the response. The significance of the linear, squared and interaction terms were also determined by F and p values (Table 8.14). Larger the F value and lower the p value ($p<0.1$), more significant are the coefficients (Khuri and Cornell, 1987). The squared terms, A^2 (moisture content × moisture content), B^2 (Inoculum age× Inoculum age) and C^2 (yeast extract × yeast extract) are the significant model terms for all substrates tested. Contour plots and three dimensional response surface plots of four substrates demonstrate the relationship between the predictor variables and its effects on the response (Fig. 8.4, 8.5). The Coefficient of determination (R^2) describes how efficiently variability in the response can be explained by the model (Table 8.14). For substrate Rice, Sorghum, Sugarcane bagasse + BFW and Rice + rice bran; 87 %, 94 %, 93 % and 98 % variability in the response is explained by the model respectively.

Function Decision Table is used for Machine Learning and the predictive analysis is presented in the respective tables for each substrate along with observed responses and predicted response from RSM analysis (Table 8.12, 8.13). Various functions have been tested for the best predictive response and lowest Root Mean Squared Error (RMSE) in MLT. It was found that Gaussian processes and Radial basis function networks provided more satisfying results than Multilayer perceptron and isotonic regression; however classifier DT outperformed them all. A genetic search technique was adopted by the DT for condition alternatives. The genetic search is defined by the crossover probability of 0.6 with population size of 20. The comparison between the two methods was expressed in terms of the experimental and the predicted yield plots (Fig. 8.6) which undoubtedly reveals the supremacy of the MLT over RSM. A generalized model was then investigated which could be used to fit the data from the four substrates into a single model i.e. one most exclusive feature of the MLT. The model was supervised with the training data from the RSM experiment on a single substrate and then the model was tested on the data from other three substrates. Among the four sets of data from four substrates; the dataset from Sorghum was used for training the model as it gave the best fit. The data from the other three substrates were supplied as a test set and a good correlation coefficient and low RMSE was observed in each case (Table 8.15). Optimization studies help in understanding interaction among the various factors and in calculating the optimal concentrations and conditions for improved production of conidia. In case of *B. bassiana* superior conidial production was observed for the substrate rice, 12.06×10^9conidia/ gm at 50.33 % moisture content, 6.27 days old inoculum and 2.23 % yeast extract concentration (Table 8.16). Optimized condition for *B. bassiana* conidia production on substrate sorghum are 52.53 % moisture content, 5.67 days old inoculum and 1.86 % yeast extract concentration whereas for substrate Sugarcane bagasse + BFW the optimum conditions were 50.82 % moisture content and inoculum age of 5.62 days along with 2.07 % yeast extract concentration yielding 6.22×10^9conidia/ gm. However, the optimum moisture content for the Rice+ rice bran was higher than other substrates (69.70 %) and inoculum age was 4.74 days but low yeast extract concentration was required 1.97 %.

M. anisopliae

The statistical analysis from the Plackett Burman experiment revealed that moisture content and age of the inoculum are the significant factors influencing conidial yield in *M. anisopliae* (Table 8.18 and 8.19). Analysis of the effects of each factor along with P value for substrate Sugarcane bagasse + BFW showed positive effect for moisture content along with pH suggesting that higher values of these factors augment the yield whereas volume of inoculum and inoculum age had negative effect over the response. (Table 8.18). The same trend was also observed for substrate Rice + rice bran. However, all the factors showed positive effect towards response when Sorghum was used as a solid substrate. But as usual no conidial yield was observed for substrate Sorghum using the five nitrogen supplements. Therefore a One Variable at a Time (OVAT) experiment was set up to find out if any of the nitrogen sources were responsible for this peculiar behavior. No conidial yield was observed using urea as nitrogen source. For all the nitrogen sources used with all the substrates, the P value observed was > 0.05 indicating nitrogen sources not significant to screen out (Table 8.19). The positive effect of the moisture content in all four substrates was established whereas age of the inoculum indicated negative impact on the conidial yield suggesting lower values of these factors augment the yield.

Optimization of two significant factors from Plackett-Burman design; Moisture content and Inoculum age were carried out using CCD and response further analyzed by RSM (Table 8.20). The predicted response from the model reveals a close correspondence with the experimental data on the four substrates. Substrate rice at 50 % moisture content with inoculum age of six days yielded the best conidial yield of 7.35×10^9 conidia/ gm (Table 8.20). The same trend was observed for other substrates, giving highest conidial yield of 3.28×10^9 conidia/ gm , 3.29×10^9 conidia/g and 3.81×10^9 conidia/ gm for substrate sorghum, Sugarcane bagasse + BFW and + rice bran respectively. 3D plots of conidial yields for the substrates shows that conidial yield was highest at the design center points i.e. at 50 % moisture content and inoculum age of six days (Figure 8.7).

The responses of the statistical design were fitted into the second order polynomial equation (equation 2). The model F value for the four substrates were quite high as 17.87, 25.38, 23.39 and 56.93 respectively implying significant models, moreover low p value (p value$\leq$ 0.05) of these models further confirm the significance of the models. The significance of the linear, squared and interaction terms were also determined by F and p values (Table 8.21). Humidity (A) and inoculum age (B) both were observed to be significant model terms for substrate

Sorghum and Rice+ rice bran. The squared terms (A^2 and B^2) were found to be significant model terms for substrate Rice and Sugarcane bagasse + BFW along with main effect A for substrate rice and interaction term AB for substrate Sugarcane bagasse + BFW. The coefficient of determination (R^2) for Rice, Sorghum, and Sugarcane bagasse + BFW and Rice + rice bran was 0.8754, 0.9104, 0.9032 and 0.9589 respectively which describes goodness of fit of the model. The Coefficient of determination (R^2) describes how efficiently variability in response can be explained by the model. For substrate Rice, Sorghum, Sugarcane bagasse + BFW and Rice + rice bran; 88 %, 91 %, 90 % and 96 % variability in response is explained by the model respectively.

The predictive analysis by the Machine Learning Technique which uses the function Decision table is presented in the respective tables for each substrate along with observed responses and predicted response from RSM analysis (Table 8.20). The results showed a closer proximity between the observed response and MLT predicted response than the RSM predicted values. A genetic search technique was adopted by the DT for condition alternatives. The genetic search is defined by the crossover probability of 0.6 with population size of 20. The correlation coefficient from MLT was much higher for each substrate than RSM and RMSE was also quite lower than RSM (Table 8.21). The comparison between the two methods was expressed in terms of the experimental and the predicted yield plots (Fig. 8.8) which showed the superiority of the MLT over RSM. A generalized model was then investigated which could be used to fit the data from the four substrates into a single model i.e. one most exclusive feature of the MLT. The model was supervised with the training data from the RSM experiment on a single substrate and then the model was tested on the data from other three substrates. Among the four sets of data from four substrates; the dataset from Rice+ rice bran was taken for training the model as it gave the best fit. The data from the other three substrates were supplied as a test set and a good correlation coefficient and low RMSE was observed in each case (Table 8.22). The superior predictive capability of the MLT can surely be attributed to its ability to approximate the non-linearity of the system better than RSM. The best conidial yield of 7.76×10^9 was recorded for substrate Rice at 58.56 % moisture content and inoculum age of 5.51 days for *M. anisopliae* (Table 8.23).

8.3.4 Discussion

Conidia production can vary depending upon the substrate and the organism. Bharati et al. (2007) investigated the conidial yield of *M. anisopliae* on different crushed cereals and agro wastes and observed that among the cereals tested, crushed sorghum was the best substrate for conidia production whereas rice was found to be the most suitable substrate for mass production of *Beauveria brongniartii* conidia with highest sporulation of 10^9 recorded at 50 % moisture content (Hadapad and Zeditz, 2006). In this study rice showed the preeminent conidia yield for both *B. bassiana* and *M. anisopliae.* The nutritional requirements promoting vegetative growth and sporulation are well documented in entomopathogenic fungi (Hallsworth and Magan, 1996). Surprisingly, no additional nitrogen source proved to be efficient in producing high conidial yield for *M. anisopliae*; this could be due to the fact that solid substrates used for mass production of conidia already contain enough nitrogen supplements. However the requirement of yeast extract in *B. bassiana* conidiogenesis indicates the dependency of vegetative growth of the fungi for a complex nutritional source of nitrogen and vitamins (Im et al., 1988). Highest conidial yield was observed for *M. anisopliae* when 6 day old inoculum was used on all the four substrates. Gutarra et al., (2007) observed that inoculum age has a significant influence on the *Penicillium simplicissimum* lipase production by SSF though Survase et al. (2008) observed that increasing the seed culture age does not contribute in increasing the product formation. Mature culture (9 days old) produced reduced conidia yield on the solid substrate accordingly highest conidial yield was observed using 6 days old inoculum for the substrates Rice, Sorghum and Sugarcane bagasse + BFW excluding substrate Rice+ rice bran where maximum conidiation was observed using 3 day old culture for *B. bassiana*. Although pH and inoculum volume have been reported to be important factors in fermentation (Survase et al., 2008), in our study P value analysis suggests that only moisture content and inoculum age are the two significant factors while yeast extract was another additional significant factor in case of *B. bassiana* (P value$\leq$ 0.05). Low moisture content resulted in poor nutrient diffusion whereas high moisture content can lead to particle clumping and reduced gas transfer leading to the reduced response (Moo-Young et al., 1983) therefore optimum moisture content should be identified. Highest conidial yield was observed at center point of the design for both the fungi however high moisture content (70 %) and low yeast extract concentration (1 %) along with 3 days old inoculum favors the high conidial yield of 7.53×10^9 conidia/ gm on substrate Rice+ rice bran for *B. bassiana*.

Artificial learning is based on the function used to guide the learning process which ultimately constructs an adaptable model. In this study function Decision Table was used for Machine Learning. A closer proximity was observed between the experiential response and MLT predicted response than the RSM predicted ones. Results from the regression analysis unambiguously place MLT ahead of RSM as the correlation coefficient from MLT was much higher for each substrate than RSM and RMSE was also quite low than the latter one. The superior predictive capability of the MLT can surely be attributed to its ability to approximate the non-linearity of the system better than RSM.

8.6 Inference

The development of fermentation and formulation process must be a coordinated effort in order to reduce costs and maximize product efficacy. The widespread acceptance and use of mycoinsecticides will depend on improvements in the development of formulations that will enhance virulence, extend the shelf life of the pathogen, improve efficiency of application, and prolong field persistence hence Chpater 9 addresses the issues of formulation of conidia.

Table 8.1. The variables (Moisture content & yeast extract) and their levels for the CCD design experiment (Experiment 1)

Symbols	Independent variables	Substrates	Range and Levels				
			-α	**-1**	**0**	**+1**	**α**
A	Moisture content	Rice	31.72	40	60	80	88.28
		Sorghum	11.72	20	40	60	68.28
		Sorghum + Rice bran	11.72	20	40	60	68.28
		Rice + Wheat bran	13.79	20	35	50	56.21
B	Yeast Extract Concentration	Rice	0.79	1	1.5	2	2.21
		Sorghum	0.79	1	1.5	2	2.21
		Sorghum + Rice bran	0.79	1	1.5	2	2.21
		Rice + Wheat bran	0.79	1	1.5	2	2.21

Table 8.2. Experimental design with observed and predicted values for the production of *B. bassiana* conidia using Rice as substrate (Experiment 1)

Trials	Variables		Substrate	
	Moisture Content (%)	Yeast Extract (%)	Rice	
			Observed Response ($Y \times 10^9$)	Predicted Response ($Y \times 10^9$)
1	60.00	1.50	7.95	8.43
2	60.00	1.50	8.15	8.43
3	80.00	1.00	15.90	15.60
4	60.00	0.79	15.60	16.60
5	40.00	1.00	16.20	15.20
6	88.28	1.50	12.80	12.10
7	60.00	1.50	9.20	8.43
8	80.00	2.00	8.93	10.20
9	40.00	2.00	13.20	13.80
10	31.76	1.50	14.00	14.40
11	60.00	2.21	13.00	11.80

Table 8.3. Experimental design with observed and predicted values for the production of *B. bassiana* conidia using Sorghum and Sorghum + Rice bran as substrates (Experiment 1)

Trials	**Variables**		**Substrates**			
	Moisture Content (%)	**Yeast Extract (%)**	**Sorghum**		**Sorghum+ Rice bran**	
			Observed Response ($Y \times 10^9$)	**Predicted Response ($Y \times 10^9$)**	**Observed Response ($Y \times 10^9$)**	**Predicted Response ($Y \times 10^9$)**
1	40.00	0.79	7.43	7.89	4.98	5.01
2	40.00	1.50	7.80	7.70	6.25	6.16
3	60.00	1.00	7.40	7.25	5.73	5.93
4	40.00	2.21	8.28	8.11	5.78	4.97
5	60.00	2.00	5.40	5.69	4.18	4.97
6	11.72	1.50	8.05	8.50	2.43	2.21
7	40.00	1.50	8.00	7.70	5.98	6.16
8	20.00	2.00	9.63	9.49	3.18	3.73
9	68.28	1.50	5.73	5.56	5.83	5.27
10	40.00	1.50	7.30	7.70	6.25	6.16
11	20.00	1.00	8.20	7.62	2.88	2.84

Table 8.4. Experimental design with observed and predicted values for the production of *B. bassiana* conidia using combined substrate Rice + wheat bran (Experiment 1)

Trials	Variables		Substrate	
	Moisture Content (%)	Yeast Extract (%)	Rice + Wheat bran	
			Observed Response ($Y\times10^9$)	Predicted Response ($Y\times10^9$)
1	50.00	1.00	19.50	17.90
2	35.00	1.50	26.50	27.00
3	35.00	0.79	17.10	17.30
4	13.79	1.50	17.50	15.60
5	35.00	1.50	25.80	27.00
6	56.21	1.50	15.50	17.50
7	20.00	1.00	14.40	15.60
8	20.00	2.00	15.90	17.30
9	35.00	1.50	28.80	27.00
10	50.00	2.00	19.00	17.60
11	35.00	2.21	18.20	18.20

Table 8.5. ANOVA table: Effect of the parameters on conidial yield (Experiment 1)

Substrate	Source	SS (Sum of square)	MS (Mean of Square)	F (Freedom)	P (Probability)
Rice	Model	93.51	18.70	13.11	0.007
	Residual	7.13	1.43		
	Total	100.64			
Sorghum	Model	12.63	2.53	10.55	0.011
	Residual	1.19	0.24		
	Total	13.83			
Sorghum + Rice bran	Model	18.78	3.76	9.22	0.015
	Residual	2.04	0.41		
	Total	20.82			
Rice + Wheat bran	Model	2.20	0.44	10.91	0.010
	Residual	0.20	0.04		
	Total	2.41			

Table 8.6. Model coefficients estimated by multiple linear regressions for the production of *B. bassiana* conidia with various substrates (Experiment 1)

Substrate	Terms	Coefficient	T-value	P value
Rice	Constant	8433333333	12.242	0.000
	A	-783382034	-1.857	0.122
	B	-1701449990	-4.033	0.010
	A^2	2408645833	4.797	0.005
	B^2	2864895833	5.706	0.002
	AB	-992500000	-1.664	0.157
Sorghum	Constant	7700000000	27.253	0.000
	A	-1038871933	-6.005	0.002
	B	79010191	0.457	0.667
	A^2	-333750000	-1.621	0.166
	B^2	148750000	0.722	0.502
	AB	-857500000	-3.505	0.017
Sorghum + Rice bran	Constant	6.15833	16.712	0.000
	A	1.08229	4.796	0.005
	B	-0.01483	-0.066	0.950
	A^2	-1.20729	-4.495	0.006
	B^2	-0.58229	-2.168	0.082
	AB	-0.46250	-1.449	0.207
Rice + Wheat bran	Constant	27033333333	23.295	0.000
	A	671446609	0.945	0.388
	B	319454365	0.450	0.672
	A^2	-5235416667	-6.190	0.002
	B^2	-4660416667	-5.510	0.003
	AB	-500000000	-0.498	0.640

Table 8.7. Results of regression analysis for the production of *B. bassiana* conidia with various substrates (Experiment 1)

Substrate	**Regression equation (in terms of coded factors)**	**R^2**	**Adjusted R^2**	**Adequate precision (signal to noise ratio>4)**
Rice	$Y=8.43-0.78A-1.70B-0.99AB+2.41A^2+2.87B^2$	0.93	0.86	9.23
Sorghum	$Y=7.70-1.04A+0.079B-0.86AB-0.33A^2+0.15B^2$	0.91	0.83	10.87
Sorghum + Rice bran	$Y=6.16+1.08A-0.015B-0.46AB-1.21A^2-0.58B^2$	0.90	0.80	8.37
Rice + Wheat bran	$Y=27.03+0.67A+0.32B-0.50AB-5.24A^2-4.66B^2$	0.93	0.83	7.69

Table 8.8. Optimized Solid State Fermentation variables for the *B. bassiana* conidial yield with different substrates (Experiment 1)

Substrate	**Optimized variables**		**Experimental conidial yield ($Y\times10^9$)**	**Predicted conidial yield ($Y\times10^9$)**
	Moisture Content (%)	**Yeast Extract (%)**		
Rice	40	1	16.2	15.2
Sorghum	20	2	9.63	9.49
Sorghum + Rice bran	40	1.5	6.25	6.16
Rice + Wheat bran	35	1.5	28.80	27.00

Table 8.9. The variables and their levels for Plackett-Burman and CCD design experiment: *B. bassiana*

Variables	**Abbreviation**	**Low level(-)**	**High level(+)**
Moisture content (%)	A	20	80
pH	B	5	7
Volume of inoculum (ml)	C	5	10
Inoculum age (Days)	D	3	9
Yeast extract (%)	E	1	3
Beef extract (%)	F	1	3
Peptone (%)	G	1	3
Tryptone (%)	H	1	3
Urea (%)	I	1	3

CCD design

Symbols	Independent variables	Substrates	Range and Levels				
			-α	-1	0	+1	α
A	Moisture content (%)	Rice	7.57	20	50	80	92.43
		Sorghum	7.57	20	50	80	92.43
		Sugarcane bagasse + BFW	7.57	20	50	80	92.43
		Rice + Rice bran	7.57	20	50	80	92.43
B	Inoculum age (Days)	Rice	1.76	3	6	9	10.24
		Sorghum	1.76	3	6	9	10.24
		Sugarcane bagasse + BFW	1.76	3	6	9	10.24
		Rice + Rice bran	1.76	3	6	9	10.24
C	Yeast extract (%)	Rice	0.32	1	2	3	3.69
		Sorghum	0.32	1	2	3	3.69
		Sugarcane bagasse + BFW	0.32	1	2	3	3.69
		Rice + Rice bran	0.32	1	2	3	3.69

Table 8.10. Experimental design and results of the Plackett-Burman design (Physical factors): *B. bassiana*

Trials	Moisture content (%)	Inoculum age (Days)	Volume of inoculum (ml)	pH	Response($Y \times 10^9$)			
					Rice	Sorghum	Sugarcane bagasse + BFW	Rice + Rice bran
1	80	9	5	8	3.4	3.73	2.02	2.33
2	20	3	10	8	2.28	3.36	2.19	1.5
3	20	9	10	5	1.24	2.84	1.33	1.3
4	20	3	5	8	3.79	3.53	2.51	1.7
5	80	9	10	5	3.33	2.98	2.42	1.7
6	80	3	5	5	4.01	6.68	3.05	5
7	20	9	5	5	2.37	2.04	1.07	1.8
8	20	3	5	5	3.62	2.61	1.12	2
9	80	3	10	8	4.83	4.49	2.86	2.6
10	20	9	10	8	2.28	2.2	1.3	1.1
11	80	3	10	5	5.59	4.55	2.54	2.95
12	80	9	5	8	3.25	3.06	2.72	1.9
		ANOVA results			Substrates			
effects	1.4717	-1.375	-0.1483	-0.055	Substrate: Rice			
P value	0.008	0.011	0.722	0.895				
effects	1.485	-1.395	-0.205	-0.2217	Substrate: Sorghum			
P value	0.019	0.025	0.689	0.665				
effects	1.015	-0.5683	0.025	0.345	Substrate: Sugarcane bagasse + BFW			
P value	0.004	0.048	0.919	0.189				
effects	1.18	-0.9367	-0.5967	-0.6033	Substrate: Rice + Rice bran			
P value	0.015	0.039	0.152	0.148				

Table 8.11. Experimental design and results of the Plackett-Burman design (additional Nitrogen sources): *B. bassiana*

Trials	**Yeast extract (%)**	**Beef extract (%)**	**Peptone (%)**	**Tryptone (%)**	**Urea (%)**	**Response**		
						Rice	**Sugarcane bagasse + BFW**	**Rice + Rice bran**
1	1	1	1	3	3	3.01	2.5	1.88
2	1	3	1	1	1	3.45	1.19	1.79
3	1	3	3	3	1	2.56	2.48	1
4	3	3	1	3	1	4.07	3.52	3.1
5	1	1	3	3	3	2.99	1.95	2.42
6	3	1	3	3	1	5.04	3.66	2.89
7	1	3	3	1	3	4.75	3.7	2.87
8	3	1	3	1	1	2.39	2.74	2.36
9	3	1	1	1	3	5.44	3.59	3.05
10	3	3	1	3	3	4.97	2.99	3.25
11	1	1	1	1	1	3.21	3.46	2.94
12	3	3	3	1	3	2.48	2.32	2.03
			ANOVA results					
effects	1.7667	-0.5833	0.33	-0.4333	-0.2867	Substrate: Rice		
P value	0.002	0.126	0.354	0.235	0.416			
effects	1.29	0.01333	0.35667	0.17333	0.09667	Substrate: Sugarcane bagasse + BFW		
P value	0.004	0.963	0.247	0.556	0.74			
effects	1.1033	-0.2433	-0.0667	-0.1867	0.31	Substrate: Rice + Rice bran		
P value	0.002	0.304	0.768	0.421	0.202			

Table 8.12. Experimental design with observed and RSM and MLT predicted values for the production of *B. bassiana* conidia using Rice as substrate

Trials	Variables			Substrate: Rice			Substrate: Sorghum		
	Moisture Content (%) (A)	Inoculum age (Days) (B)	Yeast extract (%) (C)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)
1	50	6	2	11.03	10.9135	11.048	9.59	9.45602	9.179
2	50	6	2	11.09	11.3201	11.048	9.5	9.75442	9.179
3	30	9	1	8.97	8.5277	8.97	6.98	6.84901	7.05
4	50	6	2	11.1	9.7239	11.048	9.54	8.85883	9.179
5	50	6	3.681793	11.32	8.9831	11.32	7.37	7.23156	9.179
6	30	9	3	8.65	8.9831	8.65	7.12	7.23156	7.05
7	70	9	3	7.77	8.8051	7.77	6.38	7.93906	6.405
8	70	3	3	7.89	8.2476	7.89	7.79	7.30321	7.655
9	83.63586	6	2	7.52	6.7266	7.52	7.63	6.39895	7.63
10	50	11.04538	2	8.72	8.1489	8.72	7.02	7.64348	7.02
11	16.36414	6	2	6.65	8.9831	6.65	5.76	7.23156	5.76
12	70	9	1	8.82	8.9212	8.82	6.43	7.43888	6.405
13	50	6	2	10.99	10.7822	11.048	9.58	8.76912	9.179
14	50	6	0.318207	7.93	9.0729	7.93	8.63	8.60585	9.179
15	50	6	2	11	8.9831	11.048	9.66	7.23156	9.179
16	50	0.954622	2	8.55	8.9831	8.55	6.88	7.23156	6.88
17	70	3	1	8.76	9.2089	8.76	7.52	7.71011	7.655
18	50	6	2	11.08	10.1782	11.048	9.56	8.96338	9.179
19	30	3	3	7.67	8.9831	7.67	6.75	7.23156	6.995
20	30	3	1	7.89	8.9245	7.89	7.24	7.85028	6.995

Table 8.13. Experimental design with observed and RSM and MLT predicted values for the production of *B. bassiana* conidia using as substrate

Trials	Variables			Substrate: Sugarcane bagasse + BFW			Substrate: Rice + Rice bran		
	Moisture Content (%) (A)	Inoculum age (Days) (B)	Yeast extract (%) (C)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)
1	50	6	2	5.75	5.74643	5.546	6.87	6.88388	6.755
2	50	6	2	5.75	5.74643	5.546	6.88	6.88388	6.755
3	30	9	1	4.23	4.20659	4.31	4.68	4.74151	4.6
4	50	6	2	5.73	5.74643	5.546	6.9	6.88388	6.755
5	50	6	3.681793	5	4.98139	5.546	6.32	6.3713	6.755
6	30	9	3	4.39	4.15869	4.31	4.52	4.71691	4.6
7	70	9	3	4	4.32571	4.11	6.78	6.8052	6.615
8	70	3	3	4.53	4.58881	4.335	7.33	7.19947	7.43
9	83.63586	6	2	4.33	3.88337	4.33	7.2	7.28949	7.2
10	50	11.04538	2	4.54	4.43838	4.54	6.03	5.73009	6.03
11	16.36414	6	2	3.29	3.68657	3.29	3.29	3.29811	3.29
12	70	9	1	4.22	4.34861	4.11	6.45	6.6148	6.615
13	50	6	2	5.74	5.74643	5.546	6.9	6.88388	6.755
14	50	6	0.318207	4.9	4.86854	5.546	6.4	6.4463	6.755
15	50	6	2	5.74	5.74643	5.546	6.87	6.88388	6.755
16	50	0.954622	2	4.72	4.77155	4.72	5.55	5.94751	5.55
17	70	3	1	4.14	4.40671	4.335	7.53	7.26407	7.43
18	50	6	2	5.76	5.74643	5.546	6.9	6.88388	6.755
19	30	3	3	4.59	4.49679	4.61	4.56	4.32619	4.63
20	30	3	1	4.63	4.33969	4.61	4.7	4.60579	4.63

Table 8.14. ANOVA table: Effect of the parameters on *B. bassiana* conidial yield

Substrate	Source	SS (Sum of square)	MS (Mean of Square)	F (Freedom)	P (Probability)	Terms	F-value	P value	Regression equation (in terms of coded factors)	Correlation coefficient		RMSE	
										RSM	MLT	RSM	MLT
Rice	Model	39.00	4.33	7.12	0.0025	A	0.28	0.6088	Y=+11.05+0.11A +0.17B +0.24 C-0.27AB -0.17 AC-0.035BC-1.40A^2-0.85 B^2 -0.50C^2	0.8650	0.9999	0.78	0.024
						B	0.63	0.4463					
	Residual	6.09	0.61			C	1.26	0.2872					
						AB	0.92	0.3593					
						AC	0.39	0.5457					
						BC	0.016	0.9015					
						A^2	46.32	< 0.0001					
						B^2	17.14	0.0020					
						C^2	5.94	0.0351					
sorghum	Model	29.89	3.32	17.00	< 0.0001	A	3.78	0.0806	Y =+9.57+0.23A-0.16B-0.16C -0.33A B+0.071AC +0.039BC-1.03A^2-0.94B^2-0.57C^2	0.9386	0.9236	0.44	0.2833
						B	1.74	0.2166					
	Residual	1.95	0.20			C	1.90	0.1986					
						AB	4.36	0.0634					
						AC	0.21	0.6582					
						BC	0.061	0.8092					
						A^2	77.75	< 0.0001					
						B^2	64.70	< 0.0001					
						C^2	23.57	0.0007					
Sugarcane bagasse+ BFW	Model	9.32	1.04	14.46	0.0001	A	0.65	0.4378	Y=+5.75+0.05A-0.099 B+0.034 C+0.019A B+6.250E-003 AC-0.051BC-0.69A^2-0.40B^2-0.29C^2	0.9287	0.9453	0.27	0.231
						B	1.87	0.2012					
	Residual	0.72	0.072			C	0.21	0.6530					
						AB	0.039	0.8469					
						AC	4.365E-003	0.9486					
						BC	0.29	0.5998					
						A^2	96.81	< 0.0001					
						B^2	32.79	0.0002					
						C^2	16.98	0.0021					
Rice + Rice bran	Model	25.75	2.86	59.15	< 0.0001	A	397.62	< 0.0001	Y =+6.88+1.19A-0.065B-0.022 C-0.20A B+0.054A C+0.064BC-0.56A^2-0.37B^2 - 0.17C^2	0.9816	0.9901	0.22	0.1608
						B	1.18	0.3029					
	Residual	0.48	0.048			C	0.14	0.7157					
						AB	6.37	0.0302					
						AC	0.48	0.5051					
						BC	0.67	0.4314					
						A^2	94.17	< 0.0001					
						B^2	40.68	< 0.0001					
						C^2	8.41	0.0158					

Table 8.15. Results of the Training and test data set for a robust generalized model: *B. bassiana*

Training set:	**Correlation coefficient**	**Root mean squared error**
Sorghum	0.9236	0.2833
	Test set:	
Rice	0.8172	1.5825
Sugarcane bagasse+ BFW	0.8946	3.1097
Rice+ rice bran	0.6029	1.9991

Table 8.16. Optimized Solid State Fermentation variables for the *B. bassiana* conidial yield with different substrates

Substrate	**Optimized variables**			**Experimental conidial yield ($Y \times 10^9$)**	**Predicted conidial yield ($Y \times 10^9$)**
	Moisture Content (%)	**Inoculum age (Days)**	**Yeast extract (%)**		
Rice	50.33	6.27	2.23	12.06	11.0839
Sorghum	52.53	5.67	1.86	10.57	9.608
Sugarcane bagasse + BFW	50.82	5.62	2.07	6.22	5.75504
Rice + rice bran	69.70	4.74	1.97	7.79	7.55008

Table 8.17. The variables and their levels for Plackett-Burman and CCD design experiment: *M. anisopliae*

Variables	**Abbreviation**	**Low level(-)**	**High level(+)**
Moisture content (%)	A	20	80
pH	B	5	7
Volume of inoculum (ml)	C	5	10
Inoculum age (Days)	D	3	9
Yeast extract (%)	E	1	3
Beef extract (%)	F	1	3
Peptone (%)	G	1	3
Tryptone (%)	H	1	3
Urea (%)	I	1	3

CCD design

Symbols	Independent variables	Substrates	Range and Levels				
			-α	-1	0	+1	α
A	Moisture content (%)	Rice	7.57	20	50	80	92.43
		Sorghum	7.57	20	50	80	92.43
		Sugarcane bagasse + BFW	7.57	20	50	80	92.43
		Rice + Rice bran	7.57	20	50	80	92.43
B	Inoculum age (Days)	Rice	1.76	3	6	9	10.24
		Sorghum	1.76	3	6	9	10.24
		Sugarcane bagasse + BFW	1.76	3	6	9	10.24
		Rice + Rice bran	1.76	3	6	9	10.24

Table 8.18. Experimental design and results of Plackett-Burman experiment (Physical factors): *M. anisopliae*

Trials	Moisture content (%)	pH	Volume of inoculum (ml)	Inoculum age (Days)	Response ($Y\times10^9$)			
					Rice	Sorghum	Sugarcane bagasse + BFW	Rice + Rice bran
1	80	9	5	8	2.30	2.600	1.50	1.51
2	20	3	10	8	4.25	1.100	0.90	2.30
3	20	9	10	5	3.85	1.900	0.35	1.30
4	20	3	5	8	4.95	1.250	1.15	2.10
5	80	9	10	5	2.15	2.500	1.00	1.70
6	80	3	5	5	3.25	1.600	2.00	3.10
7	20	9	5	5	2.05	0.650	1.00	1.20
8	20	3	5	5	4.65	0.195	1.00	1.95
9	80	3	10	8	2.75	1.000	1.75	2.70
10	20	9	10	8	4.30	1.050	1.00	1.30
11	80	3	10	5	2.85	1.700	1.85	2.40
12	80	9	5	8	2.50	2.800	1.60	2.85
		ANOVA results			Substrates			
effects	-1.375	0.375	0.075	-0.925	Rice			
P value	0.009	0.362	0.851	0.047				
effects	1.00917	0.20917	0.02583	0.77583	Sorghum			
P value	0.017	0.539	0.939	0.048				
effects	0.7167	0.1167	-0.2333	-0.3667	Sugarcane bagasse + BFW			
P value	0.001	0.426	0.135	0.033				
effects	0.685	0.185	-0.1683	-0.7817	Rice + Rice bran			
P value	0.024	0.463	0.503	0.013				

Table 8.19. Experimental design and results of Plackett-Burman experiment (additional Nitrogen sources): *M. anisopliae*

Trials	**Yeast extract (%)**	**Beef extract (%)**	**Peptone (%)**	**Tryptone (%)**	**Urea (%)**	**Response ($Y\times10^9$)**		
						Rice	**Sugarcane bagasse + BFW**	**Rice + Rice bran**
1	1	1	1	3	3	3.00	1.60	0.75
2	1	3	1	1	1	2.75	2.05	0.85
3	1	3	3	3	1	3.20	1.85	2.35
4	3	3	1	3	1	3.70	2.00	2.1
5	1	1	3	3	3	4.75	1.95	1
6	3	1	3	3	1	4.05	1.95	2.3
7	1	3	3	1	3	3.05	1.90	1.5
8	3	1	3	1	1	2.30	1.65	2.35
9	3	1	1	1	3	4.65	1.60	2.3
10	3	3	1	3	3	1.50	1.80	2.25
11	1	1	1	1	1	2.65	1.00	0.6
12	3	3	3	1	3	5.05	1.40	2.4
ANOVA results						Substrates		
Effects	-0.2417	-0.8917	0.5583	0.3417	-1.025	Rice		
P value	0.684	0.166	0.361	0.568	0.12			
Effects	-0.0417	0.025	0.175	-0.0083	-0.1583	Sugarcane bagasse + BFW		
P value	0.852	0.911	0.446	0.97	0.488			
Effects	0.225	-0.2083	0.475	-0.425	-0.6417	Rice + Rice bran		
P value	0.609	0.635	0.299	0.348	0.175			

Table 8.20. Experimental design with observed, RSM and MLT predicted values for the production of *M. anisopliae* conidia using various substrates

Trials	Variables		Rice			Sorghum		
	Moisture Content (%)	Inoculum age (Days)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)	Observed Response ($Y\times10^9$)	RSM Predicted Response ($Y\times10^9$)	MLT Predicted Response ($Y\times10^9$)
1	50	6	7.00	7.25	7.25	3.27	3.19	3.20
2	50	6	7.35	7.25	7.25	3.10	3.19	3.20
3	50	6	7.34	7.25	7.25	3.28	3.19	3.20
4	20	3	4.35	3.93	4.35	1.70	1.99	1.70
5	50	6	7.25	7.25	7.25	50	6	3.2
6	50	6	7.29	7.25	7.25	50	6	3.14
7	80	9	5.30	5.43	5.30	2.60	2.51	2.60
8	80	3	5.55	6.04	5.58	3.03	2.97	2.93
9	50	10.24264	4.70	5.12	4.70	2.58	2.49	2.57
10	92.42641	6	6.00	5.50	5.93	2.25	2.39	2.25
11	20	9	4.5	3.71	4.71	1.28	1.54	1.28
12	7.573593	6	2.00	2.00	2.80	1.35	1.00	1.48
13	50	1.757359	5.80	5.80	5.69	3.25	3.13	3.17
Trials	**Variables**		**Sugarcane bagasse + BFW**			**Rice + rice bran**		
1	50	6	3.26	3.27594	3.277	3.75	3.77203	3.764
2	50	6	3.29	3.27594	3.277	3.81	3.77203	3.764
3	50	6	3.27	3.27594	3.277	3.72	3.77203	3.764
4	20	3	0.85	0.41921	1.054	1.51	1.35111	1.742
5	50	6	3.27	3.27594	3.277	3.8	3.77203	3.764
6	50	6	3.29	3.27594	3.277	3.78	3.77203	3.764
7	80	9	0.83	0.97486	0.83	0.575	0.87056	0.575
8	80	3	2.01	1.56188	1.999	1.875	1.74799	1.875
9	50	10.24264	2	1.72358	2	2.875	2.50302	2.846
10	92.42641	6	1	1.15519	1.113	0.82	0.73017	0.82
11	20	9	1.85	2.01219	1.85	2.9	3.17367	2.9
12	7.573593	6	0.95	1.08069	0.95	2.125	2.07819	2.214
13	50	1.757359	0.45	1.0127	0.618	1.6	1.83514	1.704

Table 8.21. ANOVA table showing the effect of the parameters on conidial yield and Results of regression analysis for the production of *M. anisopliae* conidia with various substrates

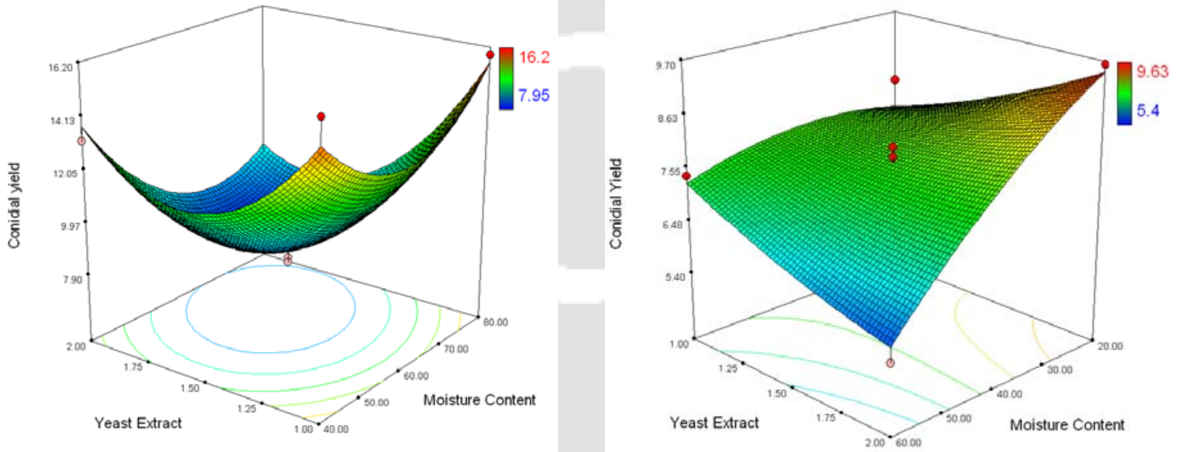
Substrate	Source	SS (Sum of square)	MS (Mean of Square)	F (Freedom)	P (Probability)	Terms	F-value	P value	Regression equation (in terms of coded factors)	Correlation coefficient		RMSE	
										RSM	MLT	RSM	MLT
Rice	Model	28.06077	5.612154	17.86862	0.0007	A	23.33308	0.0019	$Y=7.25+0.96A-0.21B-0.10AB-1.55A^2-0.92B^2$	0.88	0.99	0.56	0.10
						B	1.090939	0.3310					
	Residual	2.198552	0.314079			AB	0.127357	0.7317					
						A^2	53.07565	0.0002					
						B^2	18.86935	0.0034					
Sorghum	Model	6.315306	1.263061	25.37627	0.0002	A	38.64595	0.0004	$Y=3.20+0.49A-0.23B+0.00AB-0.75A^2-0.19B^2$	0.91	0.99	0.22	0.07
						B	8.178477	0.0243					
	Residual	0.348413	0.049773			AB	0	1.0000					
						A^2	78.73525	0.0001					
						B^2	5.27712	0.0552					
Sugarcane bagasse+ BFW	Model	14.48564	2.897127	23.38854	0.0003	A	0.044804	0.8384	$Y=3.28+0.026A+0.25B-0.54AB-1.08A^2-0.95B^2$	0.90	0.99	0.35	0.08
						B	4.085215	0.0830					
	Residual	0.867087	0.12387			AB	9.591546	0.0174					
						A^2	65.41417	0.0001					
						B^2	51.13898	0.0002					
Rice + Rice bran	Model	16.80073	3.360145	56.92875	< 0.0001	A	30.67031	0.0009	$Y=3.77-0.48A+0.24B-0.67AB-1.18A^2-0.80B^2$	0.96	0.99	0.24	0.08
						B	7.589985	0.0283					
	Residual	0.413166	0.059024			AB	30.64913	0.0009					
						A^2	165.4319	0.0001					
						B^2	75.85517	0.0001					

Table 8.22. Results of the Training and test data set for a robust generalized model: *M. anisopliae*

Training set	**Correlation coefficient**	**Mean absolute error**	**Root mean squared error**
Rice + Rice bran	1	0.0043	0.0074
Test set			
Rice	0.7184	3.7008	3.8499
Sorghum	0.7638	0.741	1.0801
Sugarcane bagasse + BFW	0.9161	0.6123	0.6993

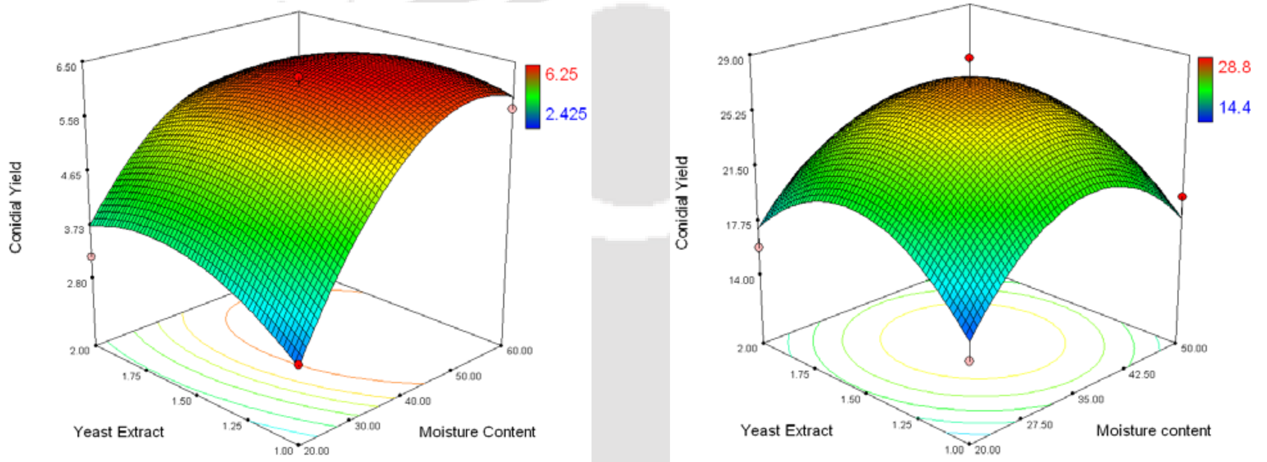
Table 8.23. Optimized Solid State Fermentation variables for the *M. anisopliae* conidial yield with different substrates

Substrate	**Optimized variables**		**Experimental conidial yield ($Y\times10^9$)**	**Predicted conidial yield ($Y\times10^9$)**
	Moisture Content (%)	**Inoculum age (Days)**		
Rice	58.56	5.51	7.76	7.41
Sorghum	57.85	4.09	3.62	3.34
Sugarcane bagasse + BFW	50.50	6.26	3.48	3.29
Rice + rice bran	40.88	6.90	4.01	3.87



(a) (b)

Fig 8.1. Response surface and Contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B. bassiana* using (a) Rice, (b) sorghum as substrate. (Conidial Yield represented to be multiplied by 10^9)



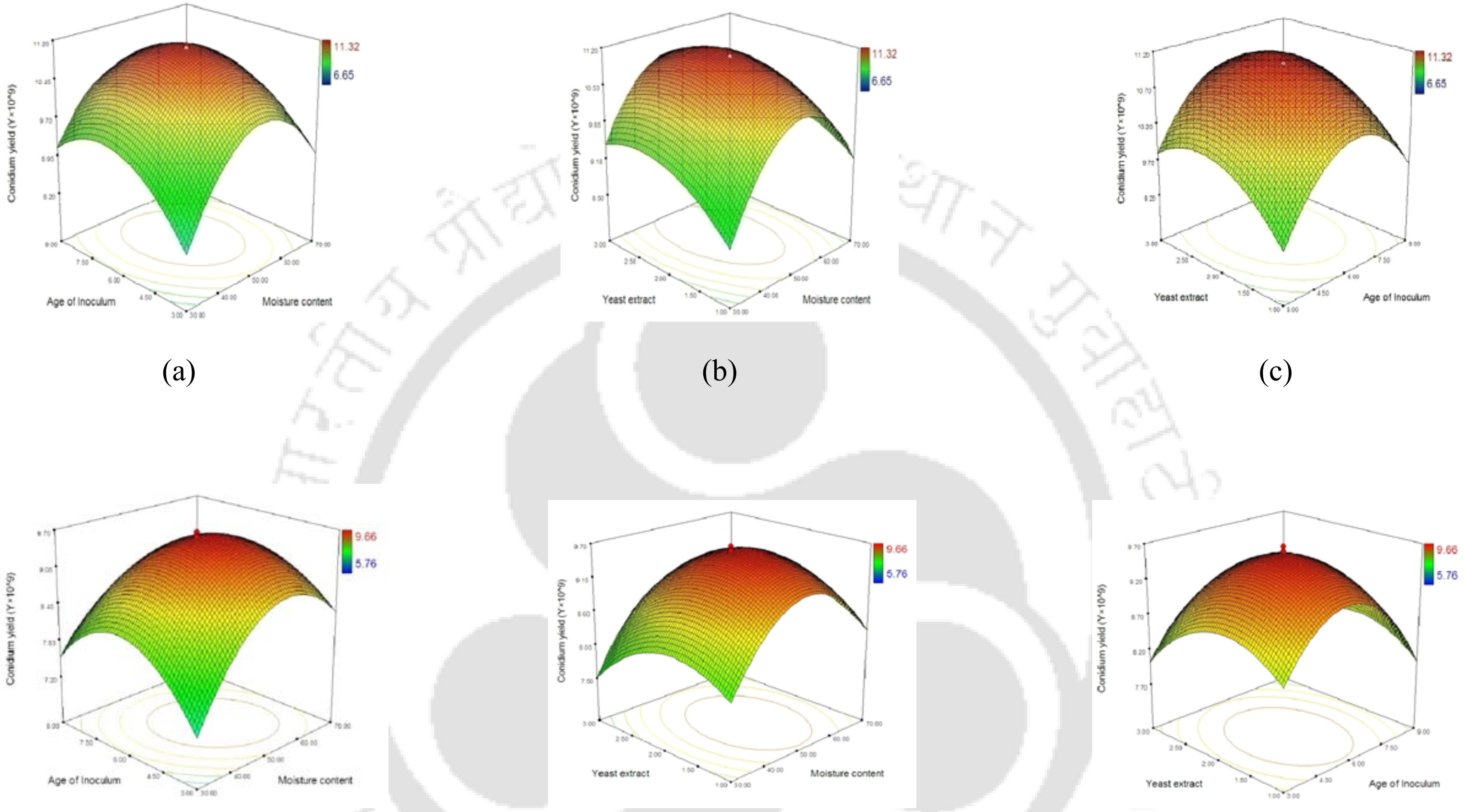
(a) (b)

Fig 8.2. Response surface and Contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B. bassiana* using (a) Sorghum+ Rice bran, (b) Rice+ Wheat bran as substrate. (Conidial Yield represented to be multiplied by 10^9)

Mass production of conidia in poly propylene bags using Rice as solid substrate

Conidia production on rice after fermentation

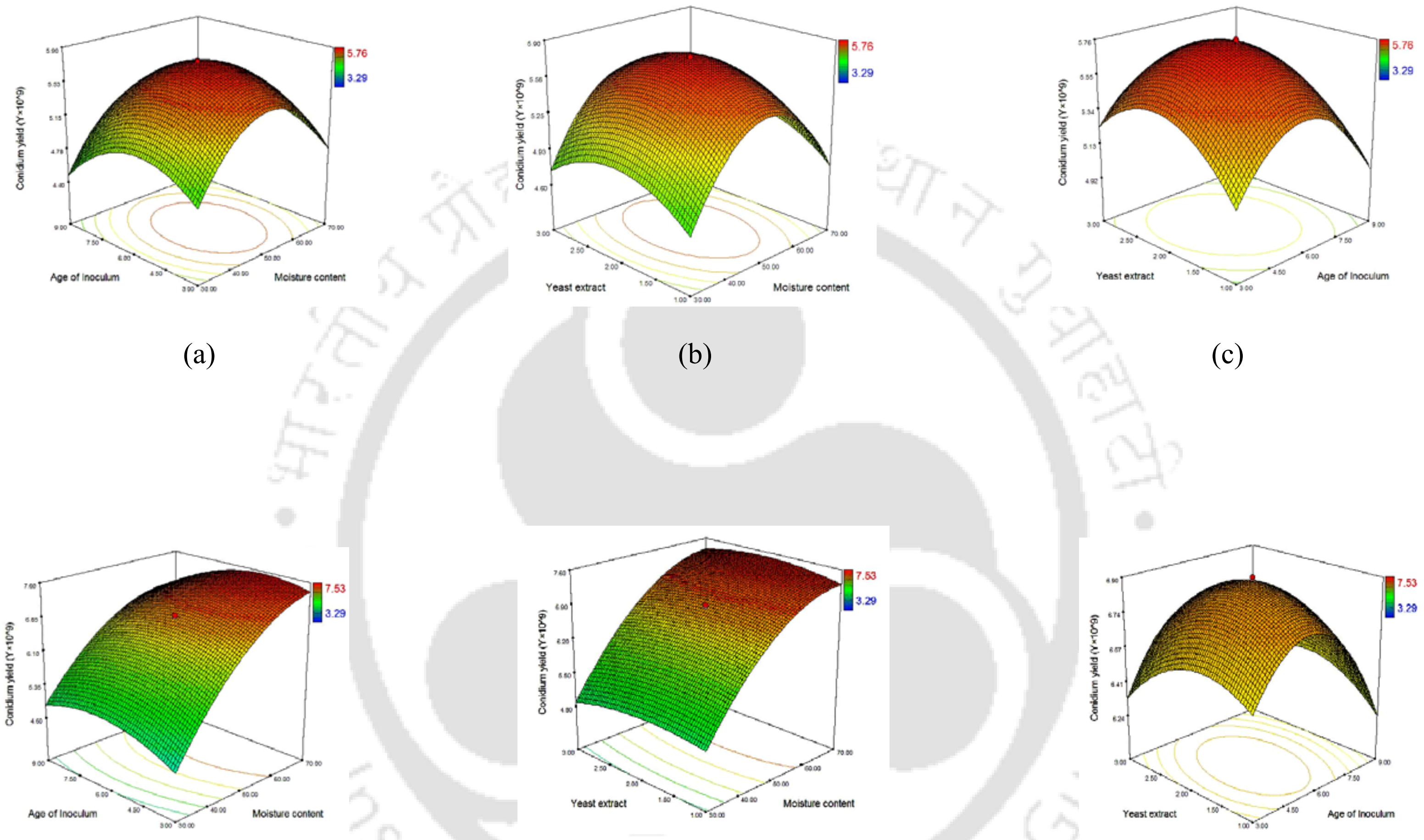
Fig 8.3. Mass production of *B. bassiana* and *M. anisopliae* conidia



(a) (b) (c)

(d) (e) (f)

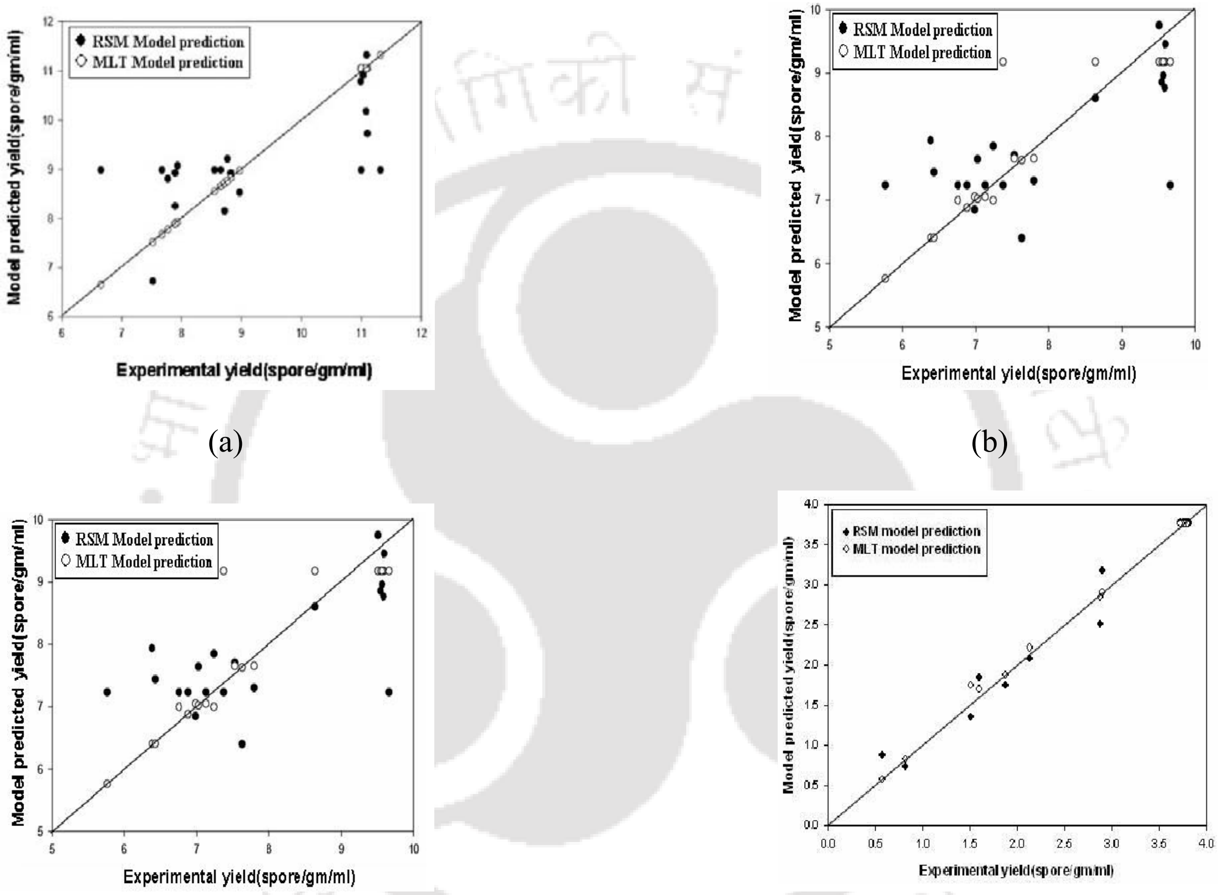
Fig 8.4. Response surface and Contour plots: Response surface and Contour plot showing the effect of moisture content, inoculum age and yeast extract concentration on the conidial yield of *B. bassiana* using (a), (b) and (c) rice as substrate, (d), (e) and (f) Sorghum as substrate, (Conidial Yield represented to be multiplied by 10^9)



(a) (b) (c)

(d) (e) (f)

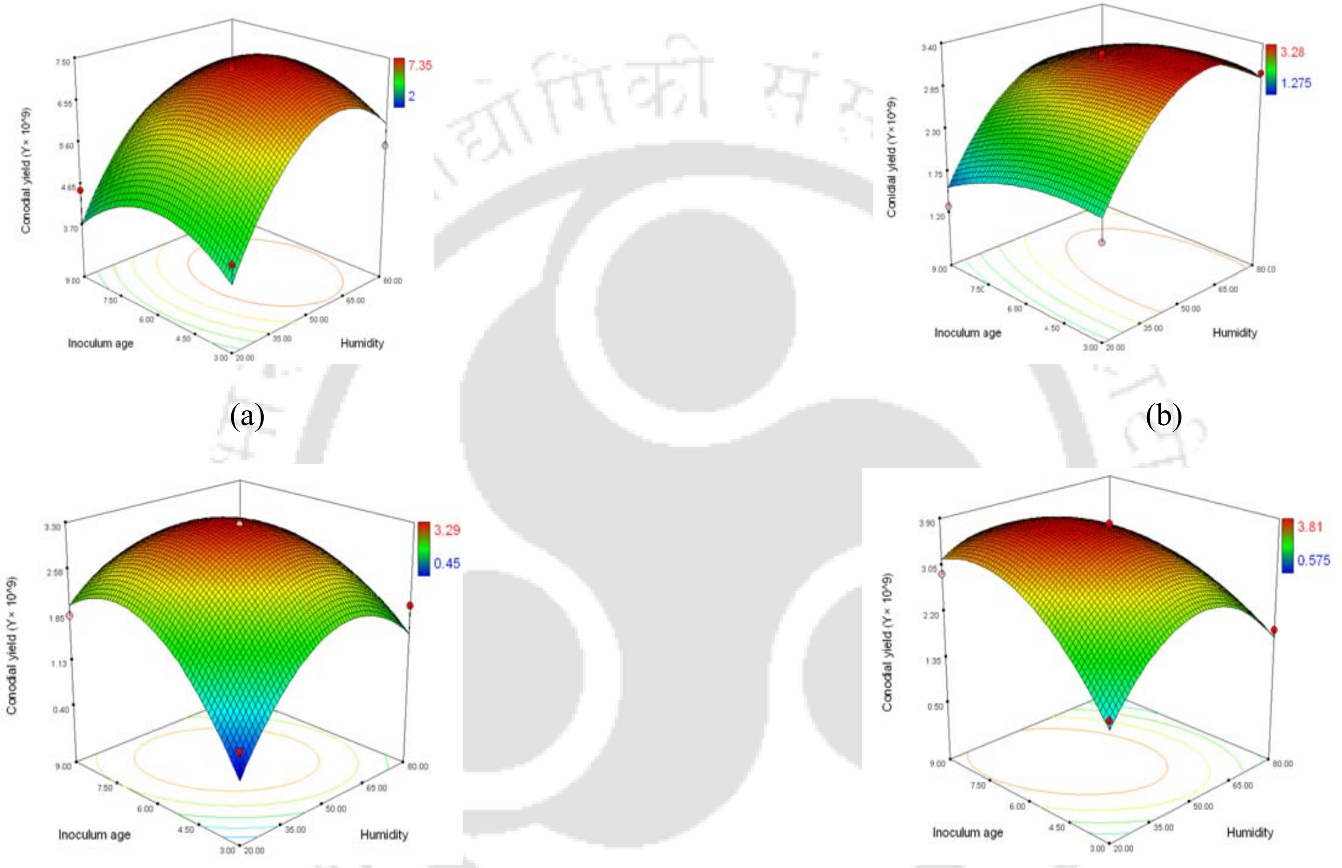
Fig 8.5. Response surface and Contour plots: Response surface and Contour plot showing the effect of moisture content, inoculum age and yeast extract concentration on the conidial yield of *B. bassiana* using (a), (b) and (c) Sugarcane bagasse+ BFW as substrate, (d), (e) and (f) Rice+ rice bran as substrate(Conidial Yield represented to be multiplied by 10^9)



(a) (b)

(c) (d)

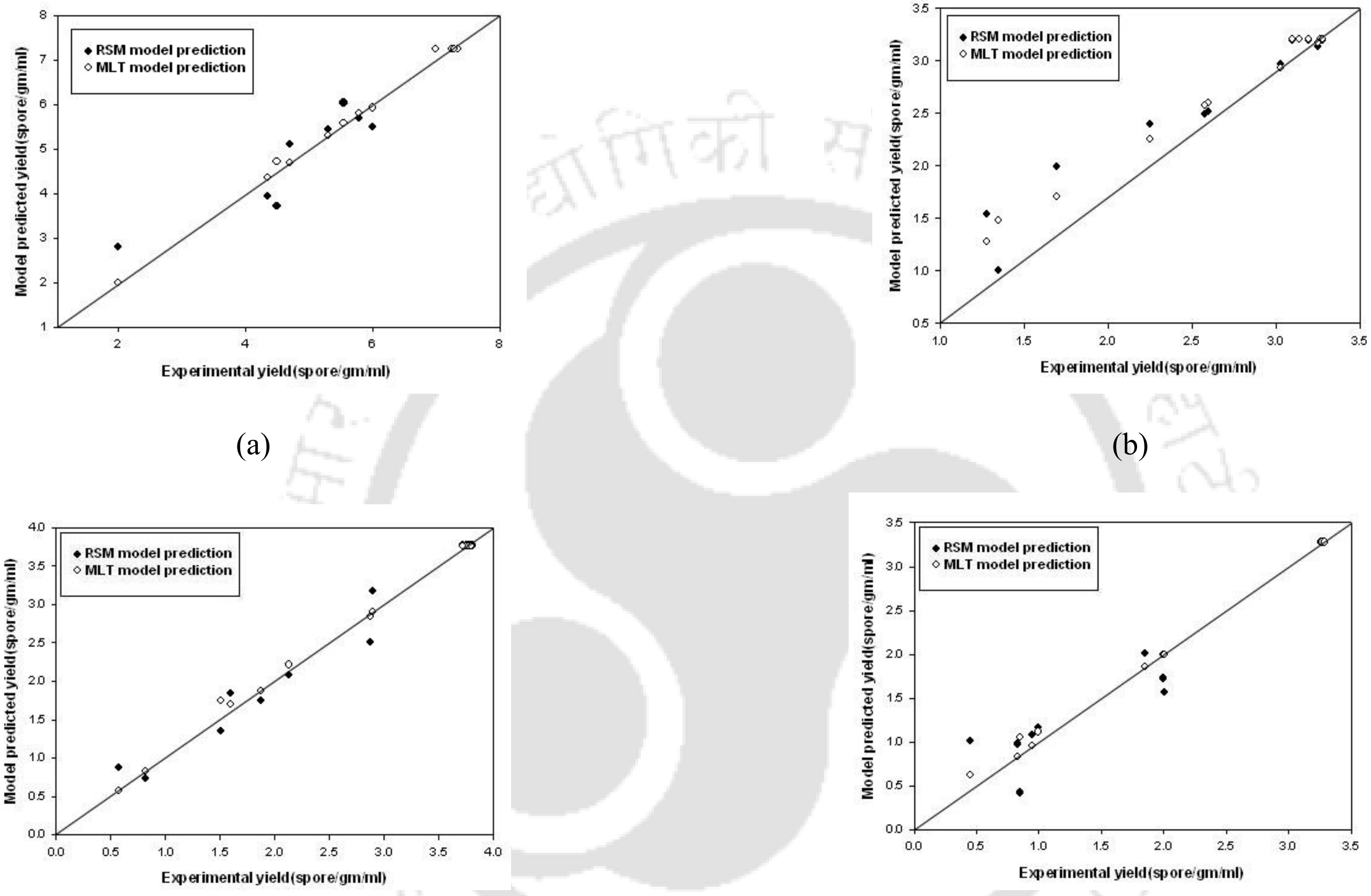
Fig 8.6. Comparative prediction analysis of the RSM and MLT models: *B. bassiana*: Experimental vs. Predicted yield of MLT and RSM models. (a) Substrate Rice, (b) Substrate Sorghum, (c) Substrate Sugarcane bagasse+ BFW, (d) Substrate Rice + rice bran



(a) (b)

(c) (d)

Fig 8.7. Response surface and Contour plot show the effect of Humidity and inoculum age on the conidial yield of *M. anisopliae* using (a) rice as substrate, (b) Sorghum as substrate, (c) Sugarcane bagasse+ BFW as substrate, (d) Rice+ rice bran as substrate (Conidial Yield represented to be multiplied by 10^9)



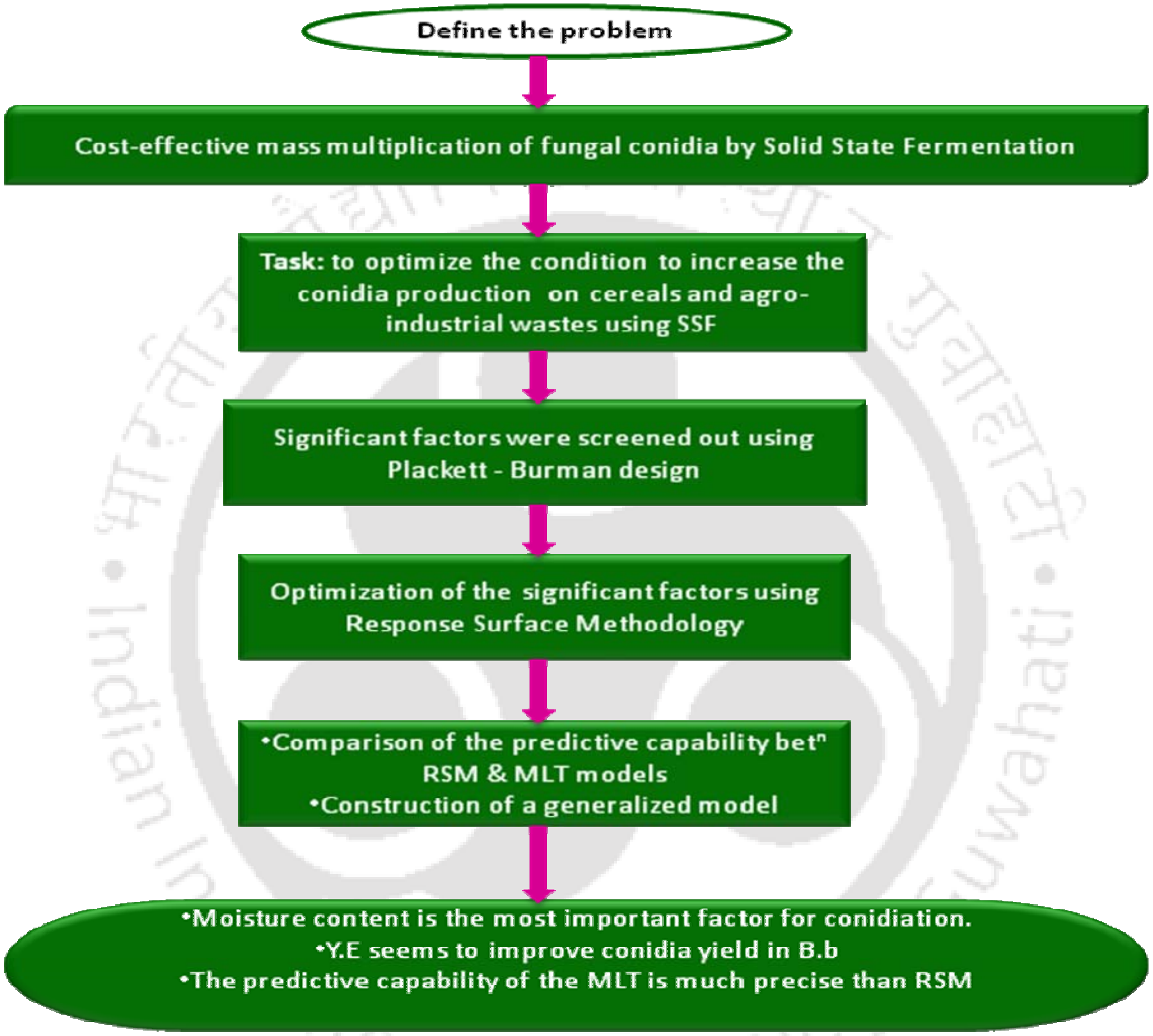
(a)

(b)

(c)

(d)

Fig 8.8. Experimental vs Predicted yield of MLT and RSM models: *M. anisopliae*. (a) Substrate Rice, (b) Substrate Sorghum, (c) Substrate Sugarcane bagasse+ BFW, (d) Substrate Rice + rice bran



Flowchart 8.1. Summary of Chapter 8

9.1 Introduction

A major constraint in developing fungal biocontrol agent is poor long term storage stability. The fundamental challenge to develop a suitable formulation is the active ingredient, conidia, a living organism that has to be kept in a relatively inactive but viable state while in storage while it should resume its normal metabolic process upon applying to the target site. Maintenance of conidial viability is an important aspect as fungal infectivity and persistence of the inoculum in the environment after field application largely depend on the formulation. Formulations of conidia could be achieved by mixing conidia with matrices to retain its viability and increase shelf life. Formulation of the mass produced conidia addresses several major issues of viability of fungi, dispersibility, suspendibility and necessarily compatibility with other agricultural products required for crop protection. Stability of conidia at room temperature for 18-24 months is still a cynical goal. Eleven different technical grade active ingredients or formulation types have been identified till date, with technical concentrates (fungus-colonized substrates) (26.3 %), wettable powders (20.5 %) and oil dispersions (15.2 %) being most common and approximately 43 % of all products were developed by South American companies and institutions (de Faria and Wright, 2007). Dry formulations are already available in the market e.g., BioBlast, Green-Muscle, MetaGuard etc. for field applications on insects. Dry clay based formulations enhance spore survival for its desiccation properties and the same property is also applicable to oil-water emulsions (Wright et al., 2001). Oil-based formulations show promise for the control of pests and it is assumed that oils reduce the dependency on saturated conditions normally required for successful infections (Bateman et al., 1993). It is well documented that effectiveness is increased with the addition of oils (Moore and Prior, 1993), which probably prevents conidial desiccation, helping adhesion and spreading the inoculum on the host body (Vimala Devi and Prasad, 1996) and they are effective for ultra low volume applications (Wraight et al., 2001). Moore et al. (1995) found that oil formulations of dried conidia showed longer viability when stored at relatively low temperatures, 10-14 °C. Reports of granular formulation of yeast developed for biocontrol of postharvest diseases are stable for 12-18 months when stored at cold, vacuum and dry conditions (Wright et al., 2001), which indicates the temperature dependency of viability during storage. Cottage industries could use cold storage temperature which is economically feasible means of stabilizing fungal bodies and also greatly extending the shelf life of conidial formulations. The control of target pests within a distinct habitat requires different methods of application. Therefore, formulations must satisfy different modes of delivery. Further, the widespread acceptance and use of mycoinsecticides will depend on improvements in the development of formulations that will enhance virulence, extend the shelf life of the pathogen, improve efficiency of application and prolong field persistence (Butt et al., 1998).

9.2 Materials and Method

Fungal isolates

The fungal isolates, *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) were procured from ARSEF (USDA-ARS Plant Protection Unit) and the cultures were maintained periodically on Sabouraud’s dextrose agar slants.

Conidia production

The fungal isolates were cultured in Sabouraud's dextrose broth (4 % Dextrose, 1 % Peptone, and 1 % Yeast Extract). Approximately 10^7 conidia were inoculated and mycelium cultivated for few days (according to optimized Inoculum age for each isolate specific to substrate rice) at 28 °C and 180 rpm. A 5 ml aliquot of cultivated mycelium in SD broth was used as inoculum for autoclaved rice supplemented with optimized water. Optimized concentration of yeast extract (2.23 %) was used for the culture of *B. bassiana* and incubated for 15 days at 28 °C. Conidia were collected by sieving the fermented substrate through a sieve.

Formulation

Conidia were formulated using dry matrices (Diatomaceous earth, Kaolinite and Fullers earth), oils (Coconut oil, Mustard oil and Soybean oil), oil and water emulsions and conidial entrapment by Na-alginate beads. Conidia were air dried for 3-4 h at room temperature. Air dried conidia and dry matrices were mixed in 1:1 ratio, for other formulations 0.1 g of air dried conidia was mixed with 1 ml of oils and oil-water emulsions and vortexed thoroughly. Conidia entrapment was achieved by suspending conidia at a concentration of 1×10^6 in a 1.5 % solution of sterile Na-alginate. The conidia suspension was slowly dropped into 0.1 M $CaCl_2$ solution through a 22-gauge needle. Beads were allowed to polymerize for 15 min, then dried in sterile airflow for 30 min. All the formulations were prepared in two sets (triplicate each) and stored at 28 °C and 4 °C respectively. Conidial viability was checked by the agar-microscope slide assay at the end of each month and this practice continued for six months (Cano et al., 1988). Conidium was considered viable when germ tube length is approximately equal to its width. Numbers of viable or non viable conidia were assessed after 12 h of incubation. Unformulated conidia, stored at different temperatures, served as control.

9.3 Results

Following mass production the process proceeds to the formulation area which is considered to assess the shelf-life of the mass produced conidia and increase the shelf life during storage with proper formulations. No significant differences were observed for conidia formulated in dry carrier materials which were stored at 28 °C and 4 °C for a period of one month (Fig 9.1). Dry carrier materials were efficient to stabilize conidia at 28 °C although for a short time period of 2 months compared to storage temperature of 4 °C (Fig 9.2). Among the different formulations tested dry carrier materials performed better for both the isolates especially Kaolinite clay. More than 75 % of conidia were viable after 3 months of storage at 4 °C for Kaolinite formulations (Fig 9.3). Alginate encapsulation was regarded as the best formulation method for *B. bassiana* conidia as it retained almost 80 % of conidial germination even after three months of storage (Fig 9.3). Soybean oil and water formulation also showed improved efficacy towards conidial viability as 72 and 88 % of conidia were viable after three months of storage at 4 °C for *B. bassiana* and *M. anisopliae* respectively and only 22 % and 30 % of viable conidia were observed after six months of storage at 4 °C for *B. bassiana* and *M. anisopliae* respectively (Fig 9.3, 9.4). Almost 34 % germination was observed for *M. anisopliae* conidia formulated in Mustard oil and water emulsion (Fig 9.4b). Sequential decrease of viability was observed for conidia stored at 28 °C, starting from one month of storage to three months of storage when no germination was observed for formulated as well as non formulated conidia.

9.4 Discussion

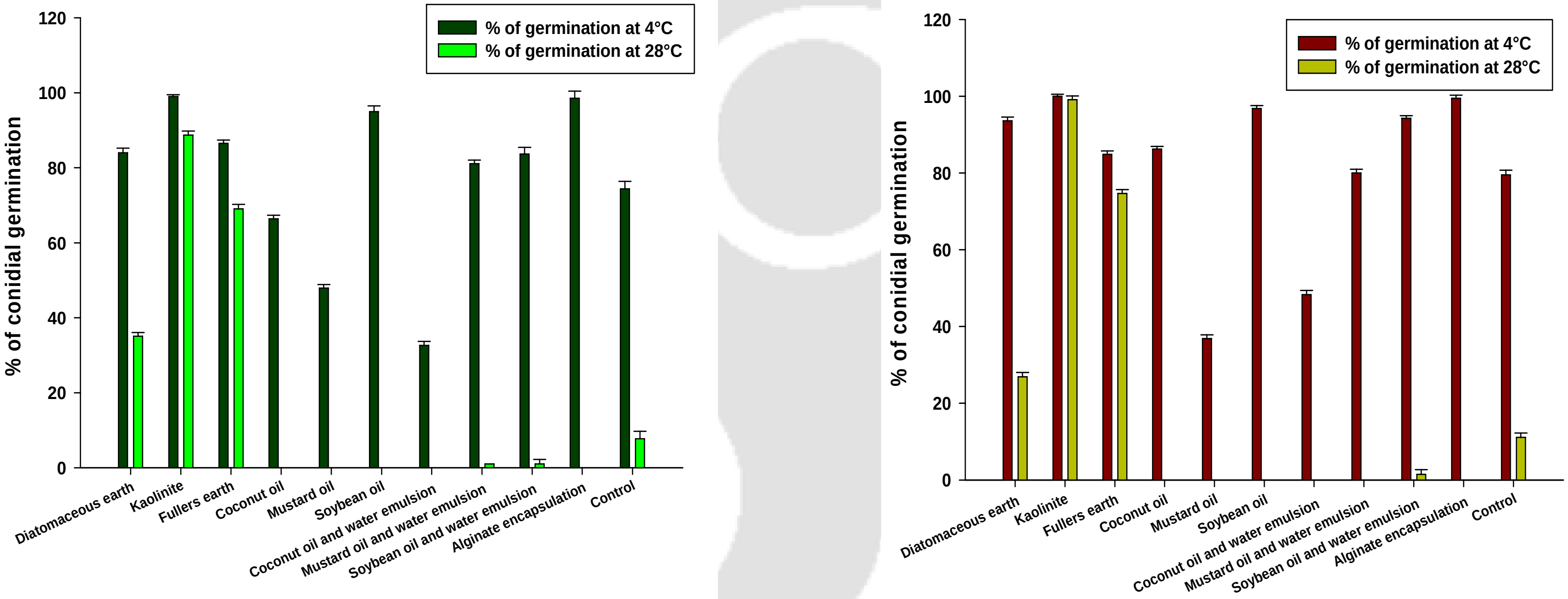
It is imperative to assess the shelf-life of the mass produced conidia and increase the shelf life during storage with proper formulations. Dry carrier materials, oils and oil-water emulsions are generally used for formulating the conidia. Storage temperature along with storage time must be well characterized to formulate conidia. Sandhu et al. (1993) observed that favorable relative humidity (RH) and temperature could prolong the viability of *B. bassiana* conidia and virulence for 24 months. Storage temperature plays an important role in increasing the shelf life of the formulations whereas appropriate carrier materials could improve the stability under storage by preventing desiccation and maintaining proper RH. Daoust et al. (1983) suggested the added weightage of temperature over RH as longevity of the conidia increased at nearly all RH values when temperature was declined from 37 °C to 4 °C. Dry carrier materials, oils and oil-water emulsions are generally used for formulating the conidia. A study indicated that talc and lignite as carrier material were suitable for formulation with a shelf life of 4-9 months respectively (Srikanth et al., 2006). In this study, almost 70-75 % germination was observed using dry carrier materials at storage temperature of 4 °C after three months of storage kaolinite especially showed almost 80 % of conidial viability (Fig 9.3 a, b). No significant difference was found in the conidial viability with the formulated and the control (non-formulated) conidia at storage temperature of 4 °C after one month, although a major difference was observed after three months (Fig 9.1- 9.3). Differences in viability with respect to storage time were observed in *Metarhizium*. Reinecke et al. (1990) showed enhanced shelf-life of 8-18 months when stored at low temperature and in vacuum whereas Wandersch et al. (1990) studied a dry granular formulation of *M. anisopliae* with a shelf-life of 6 months. In this investigation only 30- 40 % of conidia were viable after 6 months of storage. Conidial viability was high for dry formulations after one month of storage at 28 °C. Ward and Roberts (1981) documented *B. bassiana* formulation in kaolinite clays remained stable for almost 12 months at 26 °C although Daoust et al. (1982) were unable to improve the conidial viability of *M. anisopliae* stored at 20 °C. Shi et al. (1988) reported similar stability of this clay formulation for a storage time of over 48 weeks, however no germination was observed for formulated conidia stored at 28 °C after three months of storage in this study.

Oil formulations are believed to increase the viability of the conidia by preventing desiccation (Moore and Prior, 1993) and helping adhesion and spreading the inoculum on the host body (Vimala Devi and Prasad, 1996). Among the three oils tested, soybean oil showed promising results which showed almost 90 % germination at 12th h, even after three months of storage at 4 °C, however, oil-water emulsions did not perform well during prolonged storage except for soybean oil-water emulsion.

The hydrophobic characteristic of the aerial conidia makes it compatible with oils for superior spray carriers. For safety concerns, oil formulations are preferred to eliminate dust hazards associated with dry conidia. Vegetable oils such as sunflower oil and canola oil are more acceptable for its organic label. The contrary opinion prefers granular formulation for the easy incorporation of other necessary materials such as UV protectants and nutrients in the formulation. Granules can reach deep into the soil which can in turn target the soil inhabitating insects.

9.6 Inference

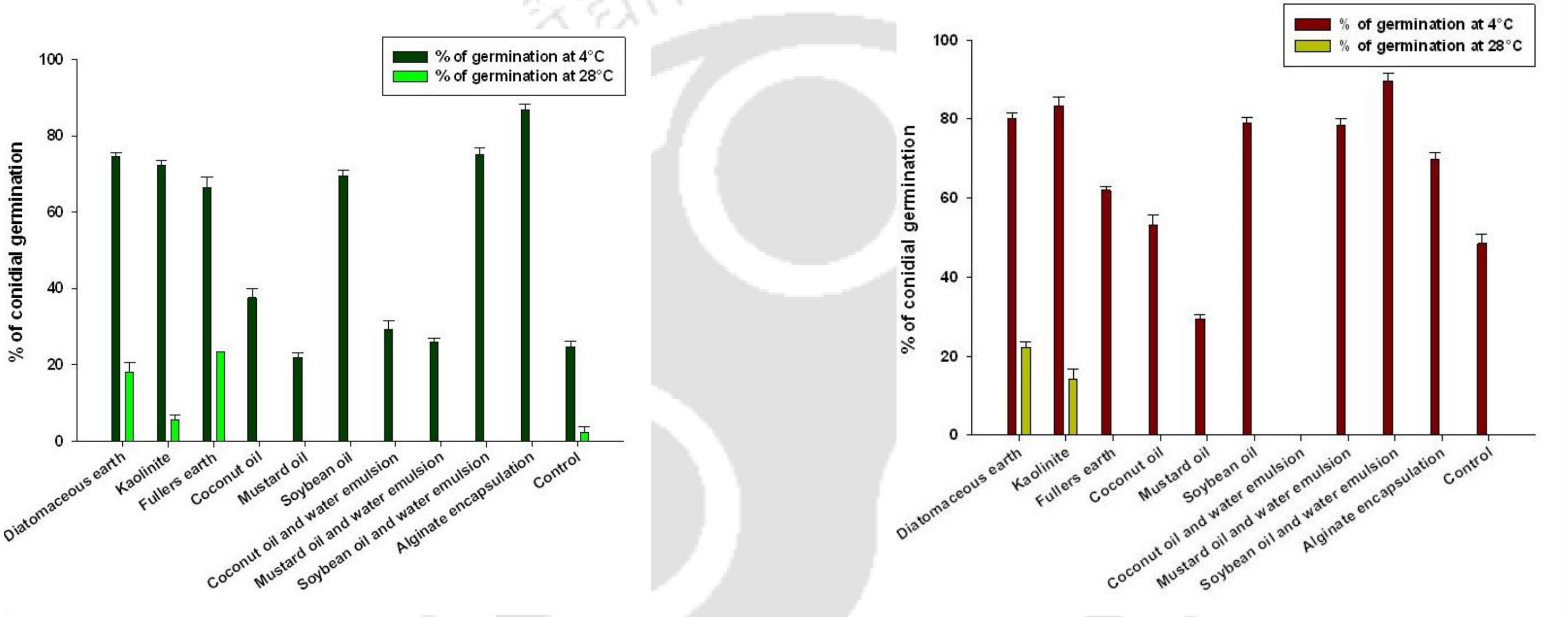
Large scale utilization and successful commercialization is not easy for entomopathogenic fungi. Substantial progress for development of fungal BCAs has directed to the stabilization of the product consequently enhancing shelf life. In this study, dry formulations especially kaolinite clay and emulsion of soybean oil and water comparably performed better furthermore field trials should be conducted for the better understanding of the formulation performance.



(a)

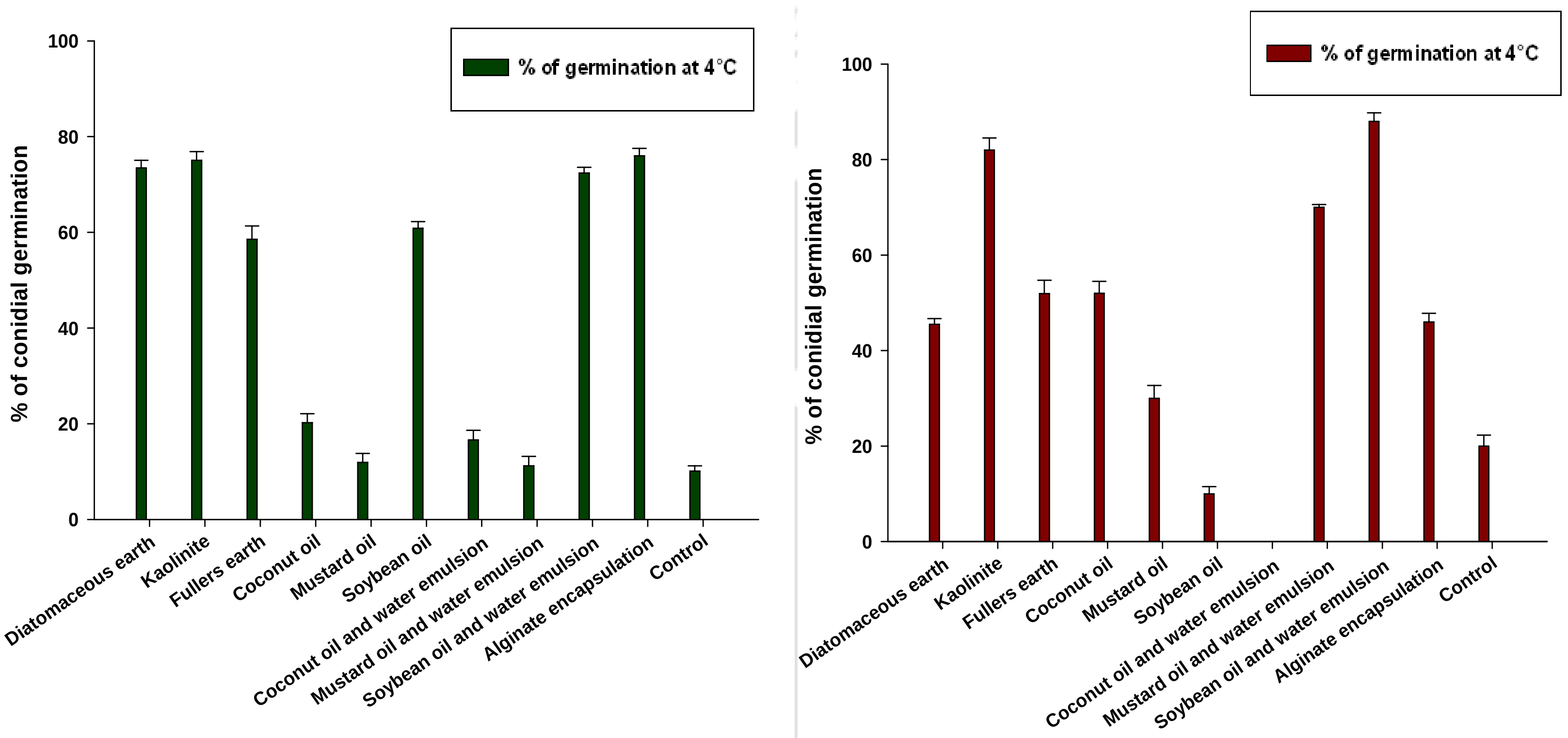
(b)

Fig 9.1. % of (a) *B. bassiana* and (b) *M. anisopliae* conidial germination after one month of storage at different temperatures



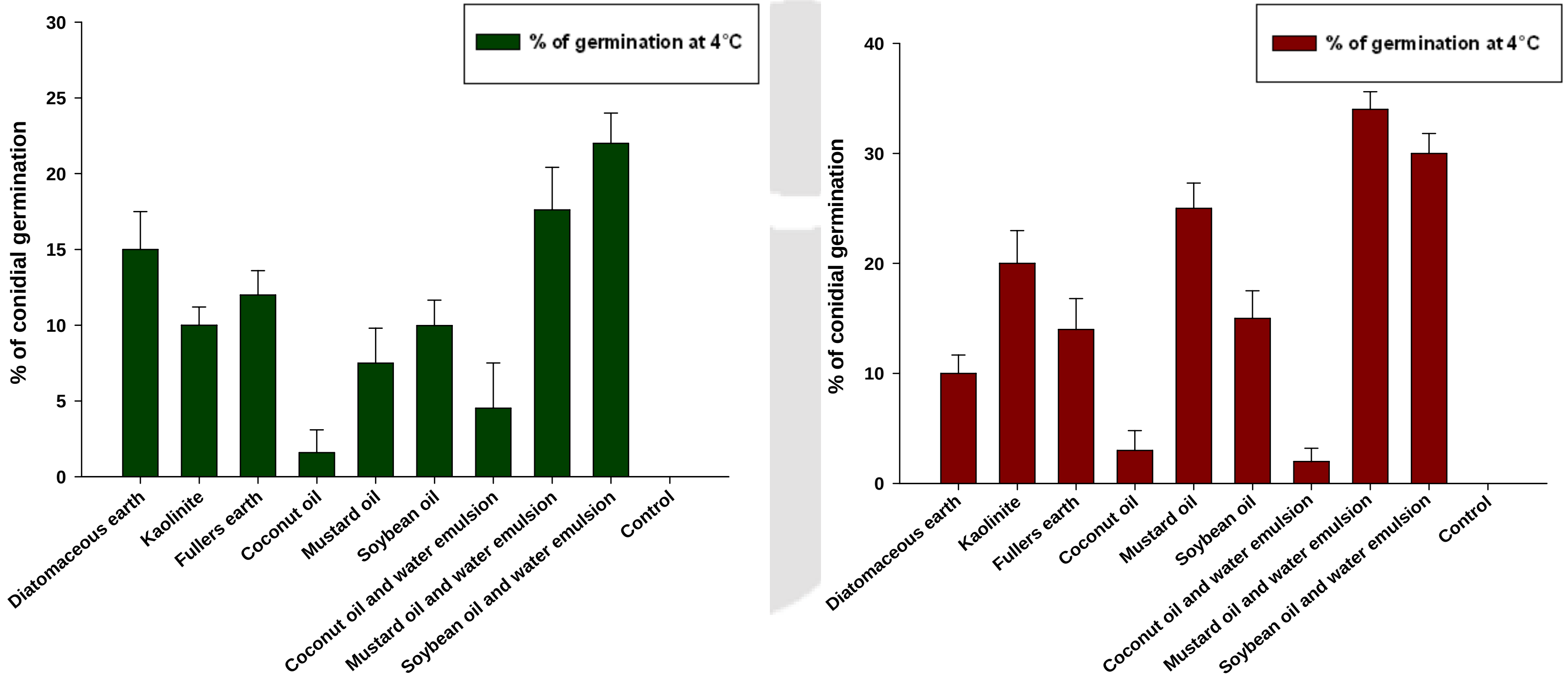
(a) **(b)**

Fig 9.2. % of (a) *B. bassiana* and (b) *M. anisopliae* conidial germination after two months of storage at different temperatures



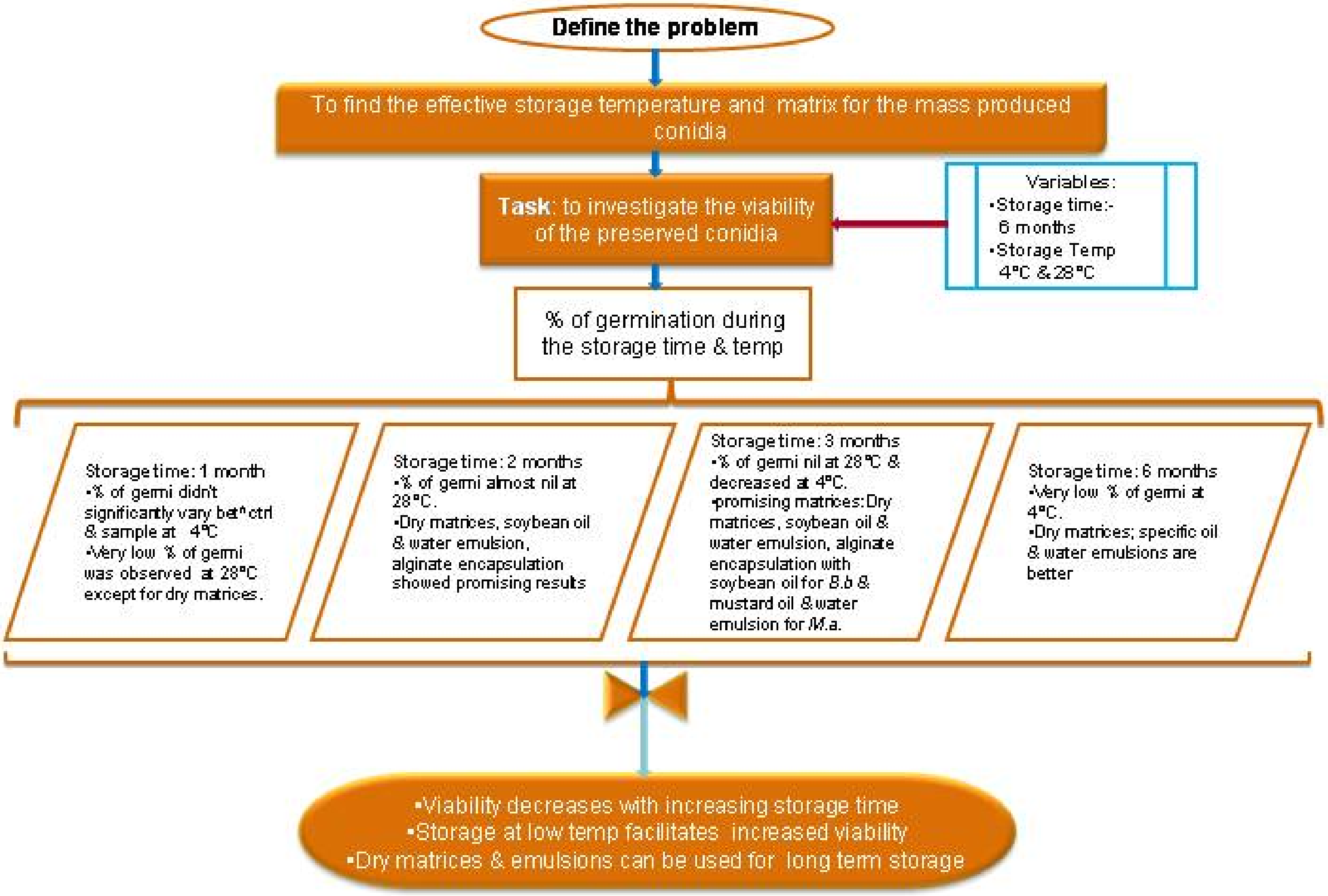
(a) (b)

Fig 9.3. % of (a) *B. bassiana* and (b) *M. anisopliae* conidial germination after three months of storage at different temperatures



(a) **(b)**

Fig 9.4. % of (a) *B. bassiana* and (b) *M. anisopliae* conidial germination after six months of storage at different temperatures



Flowchart 9.1. Summary of Chapter 9

Compatibility of entomopathogenic fungi with neonicotinoid insecticide Acetamiprid

Introduction

Entomopathogenic fungi *B. bassiana* and *M. anisopliae* are two most widely used fungi against a wide range of insects. However, occasionally inconsistent results were observed during field applications due to harsh environmental conditions and in addition fungi cannot replace the need for chemical insecticides in all agro ecosystems. The contrary opinion would debate on reduction of pesticide use and increased pesticide resistance among the pest population. Hence, strategies should be employed to implement EPFs and chemical pesticides as an effective solution in sustainable pest management. Synergistic effect of imidacloprid along with EPFs was observed for the control of a coleopteran insect *Diaprepes abbreviatus* in soil (Quintela and McCoy, 1998). Similar results were also obtained by Purwar and Sachan (2006). Farenhorst et al. (2011) observed higher impact in mosquito population when a combination of fungi and permethrin were used. The population of this soil inhabitating fungus should be conserved to get an effective suppression of insect pests. However, incompatible pesticides possibly inhibit the development and reproduction of these pathogens affecting IPM (Malo, 1993; Duarte et al., 1992; Anderson & Roberts, 1983) though use of insecticide may not significantly reduce fungi population in a treated area and therefore has potential to be used with EPFs for effective pest management (Mietkiewski et al, 1997; Saito T, 1988).

In this study a neonicotinoid insecticide acetamiprid was used which has systemic activity intended to control sucking insects on crops such as leafy vegetables, grapes and cotton. The objective of this study is to asses the in-vitro fungitoxic effect of acetamiprid on fungi since combining both biological & chemical control features could be useful in Integrated Pest Management (IPM) as it could target a wide range of insects.

Materials and Methods

Fungal isolates

The fungal isolates, *B. bassiana* (ARSEF 2033) & *M. anisopliae* (ARSEF 3295) was procured from ARSEF (USDA-ARS Plant Protection Unit) and the culture was maintained periodically on Sabouraud's dextrose agar slants.

Media and culture condition

Acetamiprid used in this experiment is a commercial product (acetamiprid 20 %; Crystal Phosphates Ltd.) Three concentrations of this insecticide were used in this study such as the field recommended conc. 0.2 g/ L (1×), 0.1× (10 times lower than field recommended concentration) and 10× (10 times excess concentration). Conidia suspension was made in 0.02 % Tween-80 solution using 7-10 days old cultures on SDA plates. A 200 µl of this conidia suspension were spread plated on SDA medium. After 3 days of incubation at 28 °C, 5 mm agar disc were taken with the help of cork borer and placed on fresh SDA medium supplemented with different concentrations of the insecticide. The insecticide was added to the medium only after autoclaving when temperature was lowered to 40-45 °C. Controls of each isolate were maintained on SDA medium without the insecticide. Five replicates were maintained for each isolate and each concentration. Radial growth of the fungal colony was measured every alternative day up to 8th day.

Results

Resistance against the insecticide was observed to be high for both isolates. Significantly reduced growth was observed only at high concentration of insecticide (10×) though insecticide tolerance was higher for *M. anisopliae* isolate compared to *B. bassiana* (Fig 1, 2). Radial growths in the other two concentrations (1× and 0.1×) were comparable with the control and interestingly *B. bassiana* isolate showed increased growth at 0.1 × concentrations whereas *M. anisopliae* at 1× concentration compared to control. Variation among different insecticide concentrations were analyzed by one way ANOVA (Dunn's test) (Sigma Stat 3.5). Significant difference ($p< 0.05$) was observed in 0.1× and 10× treatments against the control for *M. anisopliae* while no significant difference was observed in case of *B. bassiana* treatments against control (Table 1, 2).

Discussion

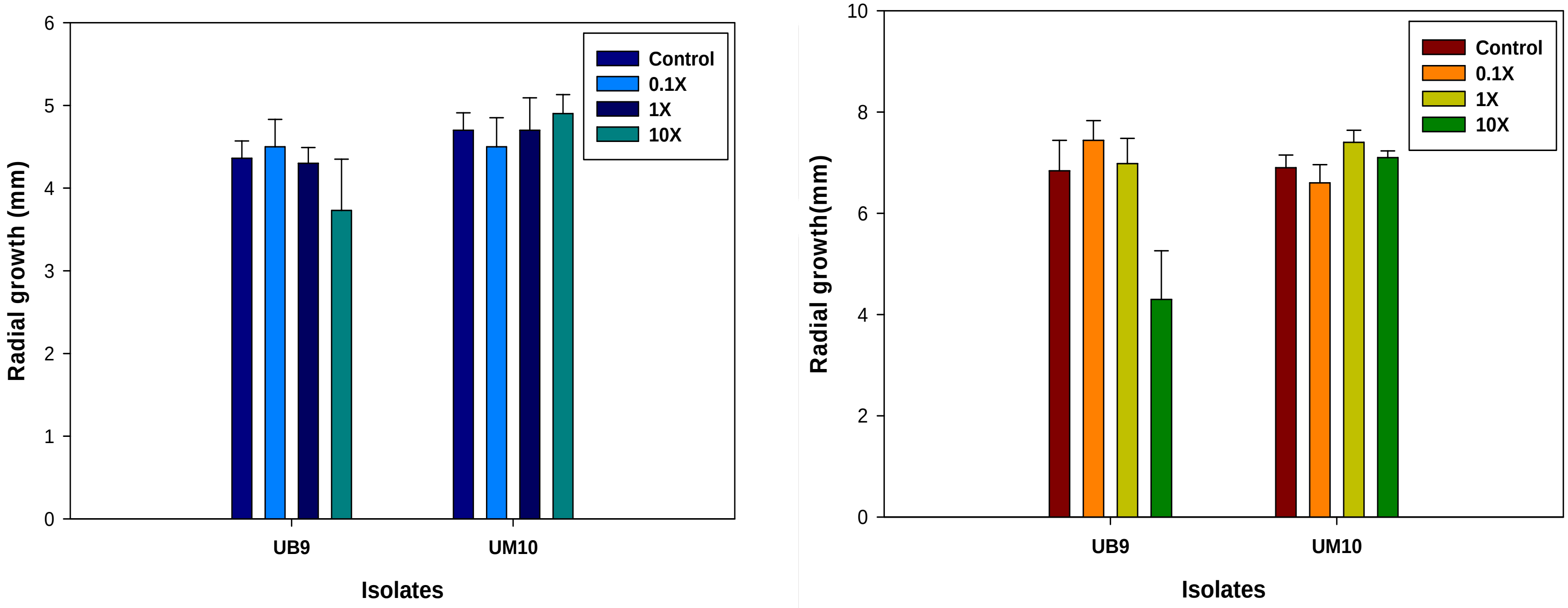
Although it is not always possible to predict the outcome when *in-vitro* study is applied to the field (Alves et al., 1998), it could give a notion about the compatibility of these fungi especially when they are applied with agrochemicals. The compatibility of *Beauveria bassiana* with insecticides was evaluated by Anderson and Roberts (1983). In an investigation to integrate the use of both biological and synthetic chemical insecticides against the Colorado potato beetle, the aim was to reduce the input of the chemical counterpart (Anderson and Roberts, 1983). The efficacy evaluation of the combined treatment was based on the criterion of spore germination on which compatibility was to be set (Anderson and Roberts, 1983), because of the importance of viable, germinating spores in the infection process. Findings were that synthetic insecticides, and in particular pyrethroids, inhibited germination of *B. bassiana* within 4 hours when applied as a tank mix, although there were some differences in the levels of inhibition. The association of delmithrin with *M. anisopliae* has been studied to control a pyrethroid resistant *Boophilus microplus* tick. High mortality was observed when delmithrin was applied along with *M. anisopliae* rather when chemical used alone (Bahiense, 2006). James and Elzen (2001) observed an antagonistic effect of *B. bassiana* and imidacloprid applied against *B. argentifolii* whereas Bednarek et al. (2004) reported inhibition of fungal growth by insecticide curbosulfan but growth was stimulated by carbofuran though pathogenicity was not affected in both the cases. The compatibility studies of these fungi with acetamiprid demonstrated a peculiar feature in fungal growth. Both the isolates indicated increased growth at 0.1× and 1× concentrations of acetamiprid. Neves et al. (2001) also confirmed this unusual characteristic in growth for *Paecilomyces* sp. Similar results were also reported by Paris et al (1975). The reason for this peculiar behavior could be due to the fungal physiological resistance to the pesticide and its ability to degrade the chemical to use it as a secondary nutrient or it could be an effort for reproduction. However, growth inhibition of soil fungi by insecticides contradicts these findings (Cowley and Lichtenstein, 1970). There are certain advantages of using biological control agents over their chemical counterparts mainly because of less or no residue problem associated with biological control. In several occasions pest eradication using EPFs are not successful due to environmental factors such as low humidity, solar radiation short shelf life or improper formulation. This present study showed that the growth of *B. bassiana* and *M. anisopliae* isolates were compatible with the insecticide, acetamiprid, therefore this insecticide and fungi in combination could be recommended for the implementation in the IPM program though after the field study.

Table 1. Results of ANOVA: *B. bassiana*

Comparison	Difference of Ranks	Q (test statistic value)	Significant (P<0.05)
0.1 × vs. Control	15.656	1.848	No
1 × vs. Control	3.313	0.391	No
10× vs. Control	2.563	0.302	No

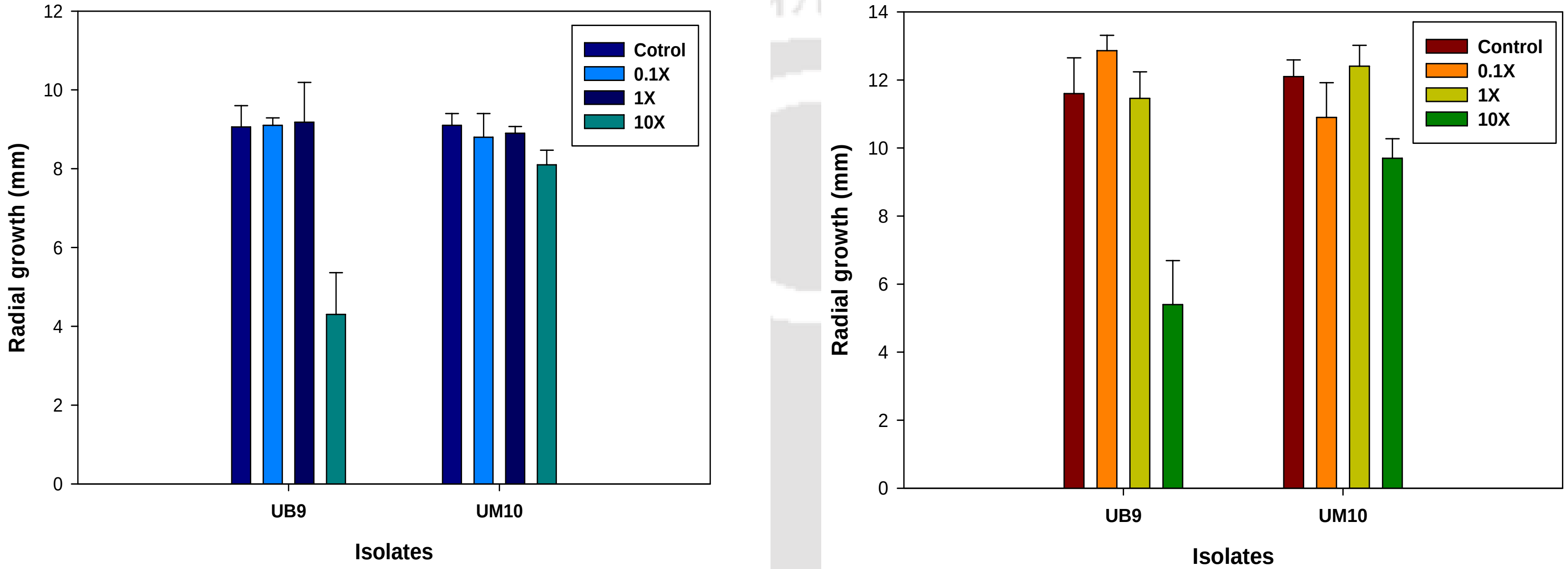
Table 2. Results of ANOVA: *M. anisopliae*

Comparison	**Difference of Ranks**	**Q (test statistic value)**	**Significant (P<0.05)**
0.1× vs. Control	18.667	3.266	Yes
1× vs. Control	17.167	3.004	Yes
10× vs. Control	4.667	0.816	No



(a) **(b)**

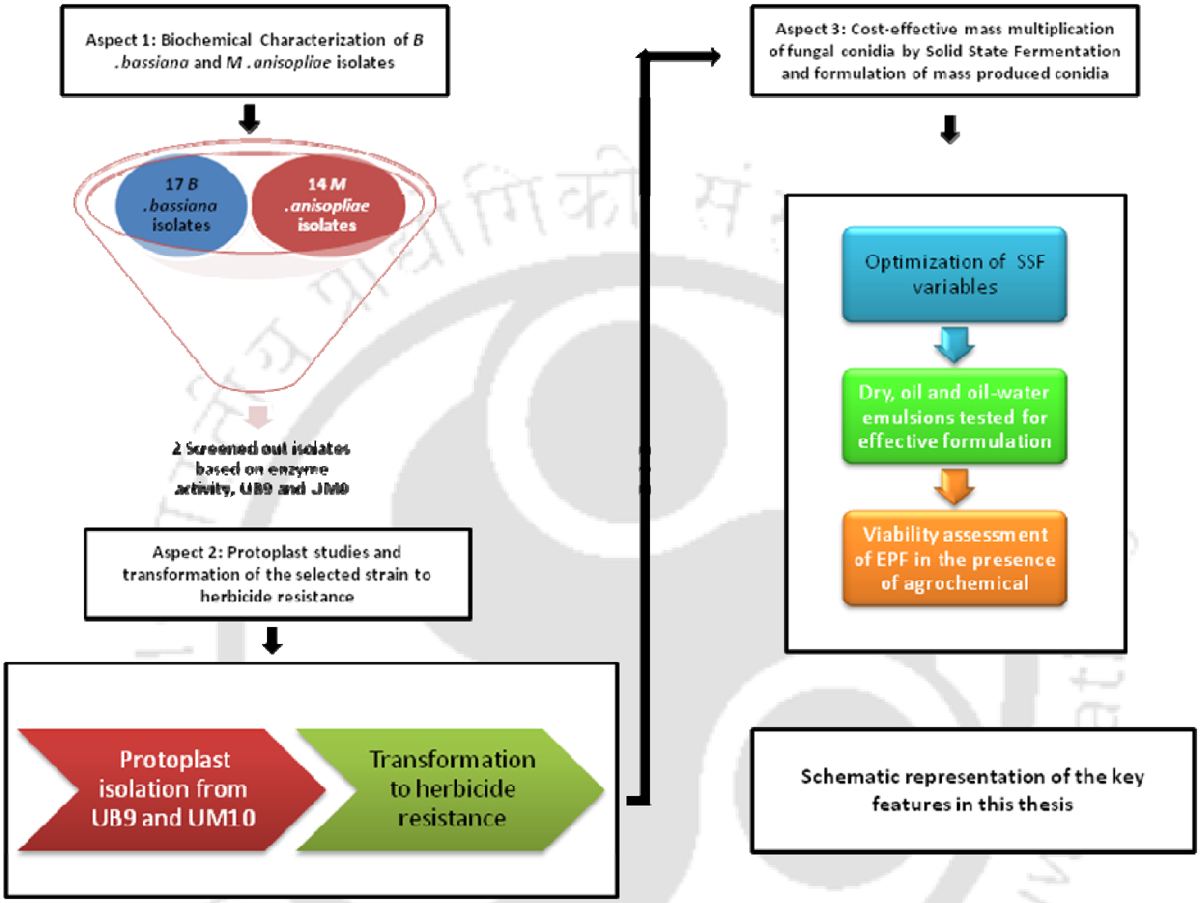
Fig 1. Radial growth of *B. bassiana* and *M. anisopliae* isolates on different concentrations of the insecticide. (a) Day 2, (b) Day 4



(a) (b)

Fig 2. Radial growth of *B. bassiana* and *M. anisopliae* isolates on different concentrations of the insecticide. (a) Day 6, (b) Day 8

10.1 Summary of the research



Schematic representation of the key features in this thesis

10.2 Salient features of this research

- No major difference is observed in growth sporulation and germination among the *B. bassiana* and *M. anisopliae* isolates.
- Chitinase secretion is repressed in the presence of glucose in the media.
- Chitinase and exo chitinase activity is high for *B. bassiana* isolates compared to *M. anisopliae*.
- Protease activity is high in *M. anisopliae* compared to *B. bassiana* isolates.
- Chitin and Casein significantly induce Pr1 activity in both *B. bassiana* and *M. anisopliae* isolates.
- pH increases during Pr1 production.
- No significant difference in Pr2 activity.
- Lipase activity was observed to be high in *B. bassiana* isolates.
- UB9 (ARSEF 2033) and UM10 (ARSEF 3259) were inferred as the best isolates based on the enzyme activity.
- A 23 kDa chitinase was purified from *M. anisopliae.*
- One 33 kDa chitinase along with a 50 kDa isoform was purified from *B. bassiana.*
- 47 kDa protease along with 36 and 17 kDa proteases were purified from *B. bassiana*.
- 43 kDa protease along with 36 & 26 kDa proteases were purified from *M. anisopliae*
- PCR amplified products showed significant similarities with Chitinase and Protease gene.
- UM10 (ARSEF 3295) is more effective against UB9 (ARSEF 2033) against Cotton Ball worm.
- KCl is the best osmoticum for protoplast yield along with 10 mg/ ml Lysing enzyme (Sigma).
- No change of enzyme activity upon transformation with plasmid pBARGEM7-2

- Moisture content and Inoculum age are most significant variables for mass production of conidia by SSF.
- Predictive capability of the Machine Learning Techniques (MLT) is more accurate than Response Surface Methodology (RSM).
- Dry matrices are better for conidia formulation.
- Conidial viability during storage is strictly dependent on storage temperature.
- Neonicotinoid insecticide Acetamiprid is compatible enough to implement it in IPM along with *B. bassiana* (UB9) & *M. anisopliae* (UM10).

10.3 Future prospects

- Isolates were screened out on the basis of virulent enzyme production hence these robust isolates could be further exploited in terms of improved performance via genetic engineering. In this present research we have attempted to isolate the chitinase and protease gene from *M. anisopliae* and *B. bassiana* though only partially isolated, efforts could be directed to entire isolation of the genes and inserting extra copies of the gene to enhance virulence.
- Several traits could be incorporated in fungi for improved efficacy in field conditions. Herbicides and fungicides along with other chemical pesticides are often required to control plant diseases. Fungi were transformed to herbicide resistance in this research furthermore resistance to fungicides or other agrochemicals could be incorporated to append dynamic features in fungi.
- Optimization of the variables effecting conidia production was achieved in this present study in an effort to maximize the conidia yield. Important constraints are being addressed for the commercial development of EPFs as mycoinsecticides through

formulation followed by mass production. The concern to develop a suitable formulation technique provided the impetus to analyze various materials to increase the efficacy of the conidia however the performance of the conidia formulations under field conditions should be assessed repeatedly to ensure efficacy and reproducibility of the mycoinsecticides.

Research Output

List of Publications

Published/accepted

1. **Priyanka Dhar** and Gurvinder Kaur. Cuticle-degrading proteases produced by *Metarhizium anisopliae* and their induction in different media. *Indian Journal of Microbiology*. DOI: 10.1007/S 12088-011-0098-1, Published online, 21st January, 2011. (Publisher: Springer)

2. **Priyanka Dhar** and Gurvinder Kaur. Effects of different carbon and nitrogen sources in the induction and repression of chitinase enzyme from *Beauveria bassiana* isolates. *African Journal of Biotechnology*, 9(47), 8092-8099, 2010. (Publisher: Academic Journals).

3. **Priyanka Dhar** and Gurvinder Kaur. Response surface Methodology for optimizing process parameters for the mass production of *B.bassiana* conidiospores. *African Journal of Microbiology Research*, 2010. (Accepted) (Publisher: Academic Journals).

4. **Priyanka Dhar** and Gurvinder Kaur. Production of cuticle degrading protease by *Beauveria bassiana* and their induction in different media. *African Journal of Biochemistry research*, 4 (3), 65-72, 2010.(Publisher: Academic Journals)

5. **Priyanka Dhar** and Gurvinder Kaur. Optimization of process parameters for the mass production of *Beauveria bassiana* conidiospores under solid state fermentation. *Proceedings of the International conference on food security and environmental sustainability*, pp. 1-10, 2009.

6. **Priyanka Dhar** and Gurvinder Kaur. Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Metarhizium anisopliae* isolates. *Annals of Microbiology*, 59 (3) 545-551, 2009. (Publisher:Springer)

7. **Priyanka Dhar** and Gurvinder Kaur. Optimization of different factors for efficient protoplast release from entomopathogenic fungus *Metarhizium anisopliae*. *Annals of Microbiology*, 59 (1) 183-186, 2009. (Publisher:Springer)

8. **Priyanka Dhar** and Gurvinder Kaur. Compatibility of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* with neonicotinoid insecticide Acetamiprid. *Journal of Entomological Research*, 33(3): 195-202, 2009. (Malhotra Publishing House, New Delhi) **Print ISSN:** 0378-9519 **Online ISSN:** 0974-4576

Manuscripts under preparation:

1. Efficiency evaluation of artificial intelligence based optimization compared to response surface methodology for mass production of *Metarhizium anisopliae* conidiospores in solid-state fermentation.
2. Efficiency evaluation of artificial intelligence based optimization compared to response surface methodology for mass production of *B.bassiana* conidiospores in solid-state fermentation.
3. Purification, characterization of virulence determinant enzymes Chitinase, Protease and studies on virulent candidate genes for correlational analysis with virulence potential in *M.anisopliae* isolate.
4. Purification, characterization of virulence determinant enzymes Chitinase, Protease and studies on virulent candidate genes for correlational analysis with virulence potential in *B.bassiana* isolate

Conferences/ Symposia

International

1. **Priyanka Dhar** and Gurvinder Kaur (**2010**). A study on the virulence of the entomopathogenic fungi in relation to two major virulent determinant enzymes Chitinase and Protease at the Genetic and Enzymatic level. 9th International Mycological Congress, 1st – 6th August 2010, Edinburgh, United Kingdom
2. **Priyanka Dhar** and Gurvinder Kaur (**2009)**. Optimization of process parameters for the mass production of *Beauveria bassiana* conidiospores under solid state fermentation. International conference on food security and environmental sustainability, December, 17-19, Indian Institute of Technology Kharagpur, proceedings, pp. 1-10

3. **Priyanka Dhar** and Gurvinder Kaur **(2009)**. Production of cuticle - degrading proteases by *Beauveria bassiana* and their induction in different media. International Conference on Emerging Trends of Biotechnology, Dec 4-6, 2009, Banaras Hindu University, India. pp.206

4. **Priyanka Dhar** and Gurvinder Kaur (**2008**). Optimization of Solid State Fermentation variables for mass production of *Beauveria bassiana* conidia using Response surface methodology. International Congress on Bioprecesses in Food industries (5th Convention of Biotech Research society of India, November 6-8, 2008 Osmania university, Hyderabad, India. p.114)

5. **Priyanka Dhar** and Gurvinder Kaur **(2008**). Optimization of different factors for efficient protoplast release from entomopathogenic fungus *Metarhizium anisopliae*. International Symposium on microbial Biotechnology: Diversity, Genomics and Metagenomics (49th annual conference of Association of Microbiologists of India), November 18-20, 2008, University of Delhi, India. p.326)

6. **Priyanka Dhar** and Gurvinder Kaur **(2007)** characterization of *Metarhizium anisopliae* isolates for extracellular chitinolytic activity. 48th annual conference of Association of Microbiologists of India), December 18-20, 2007, IIT Chennai

Grants

Received full financial support from **Department of science and Technology (DST),** Govt. of India, under the International Travel Support Scheme for attending an international conference, "**9th International Mycological Congress**" on 1st – 6th August 2010 at Edinburgh, United Kingdom.

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