BIOCONTROL OF INSECT PESTS IN AGRICULTURE USING CHITINOLYTIC ENZYME COMPLEX OF *MYROTHECIUM VERRUCARIA*

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BY

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Affectionately dedicated to my parents

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Certificate

Certified that the work incorporated in the thesis entitled **"Biocontrol of insect pests in agriculture using chitinolytic enzyme complex of *Myrothecium verrucaria*"** submitted by Mr. S. B. Chavan was carried out under my supervision. Such material as has been obtained from other resources has been acknowledged in the thesis.

M. V. Deshpande
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Declaration by the candidate

I hereby declare that the thesis entitled **"Biocontrol of insect pests in agriculture using chitinolytic enzyme complex of *Myrothecium verrucaria*"**, submitted for the Degree of Doctor of Philosophy to the University of Pune, has been carried out by me at Biochemical Sciences Division, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. M. V. Deshpande. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

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List of abbreviations

Abbreviation	Full form
d	Day
GlcNAc	*N*-Acetylglucosamine
h	Hour(s)
ha	Hactare
L	Liter
µg	Microgram
µl	Microliter
µmol	Micromole
mg	Miligram
ml	Mililiter
mm	Milimeter
min	Minute(s)
M	Molar
mM	Milimole
RH	Relative humidity
rpm	Rotations per minute
RT	Room temperature
v/v	Volume by volume
vvm	Volume/Volume/Minute
w/v	Weight by volume
w/w	Weight by weight

Abstract

The agricultural pests and pathogens known so far include more than 2,000 species of insects and 800 fungi. The major insect pests which cause >30% loss in yield in India are: *Helicoverpa armigera* on pulses, cotton, vegetables and sunflower, *Spodoptera* on vegetables, *Pyrilla* on wheat, rice, millet and sugarcane, recently introduced pest *Ceratovacuna lanigera* (woolly aphids) on sugarcane, mealy bug on cotton (*Phenacoccus gossypiphilous*) and grapes (*Maconellicoccus hirsutus*). While legumes such as groundnut, cowpea, etc. are prone to attack by soil-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani* and others. The development of resistance to chemical pesticides and negative impact of pesticides on the environment has prompted several researchers to evaluate alternative methods for the control of important agricultural pests.

Interestingly, the protective covers of both insects and fungi, respectively viz; cuticle and cell wall share a common structural component chitin. The insect cuticle is composed of wax, lipids, protein and chitin. Chitin, one of the main components comprises of 25-50% of the insect cuticle. The fungal cell wall components comprise of skeletal and matrix constituents. Chitin and R-glucans are the main structural components while S-glucans and mannoproteins are the matrix components. In response to presence of chitin in the cell wall of fungal pathogens plants produce chitinases for their defense. The synergistic and/or consecutive action of different cuticle or fungal cell wall degrading enzymes viz chitinase, protease, lipase and β-1,3-glucanase comprising the chitinolytic enzyme complex are necessary as a killing component against the pests and pathogens. Therefore, while studying the chitinolytic enzyme complex the knowledge of other enzymes involved in cuticle and fungal cell wall degradation is also necessary.

In fungus-insect and fungus-fungus interactions entomopathogenic and mycoparasitic fungi respectively produce chitinolytic enzyme complex. Nahar et al (2008) reported that the reduction in virulence of *Metarhizium anisopliae* after 40 *in vitro* transfers on artificial media was associated with the reduction in extracellular cuticle degrading enzyme activities. Shakeri and Foster (2007) reported that the entomopathogenicity of the mycoparasite *Trichoderma harzianum* towards *Tenebrio molitor* larvae was due to enzymes such as chitinase and protease. Nahar et al (2004a) reported that chitin deacetylase (CDA) and chitosanase facilitated the killing process in *M. anisopliae* towards *H. armigera*.

Myrothecium verrucaria produces chitinase and other enzymes such as lipase, protease and β-1,3-glucanase (Shaikh and Deshpande, 1993). It was reported that both first (I) and fourth (IV) instar *Aedges aegypti* mosquito larvae, were killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa et al 1996). The combined effect of *M. verrucaria* enzymes and cell wall chitin metabolism inhibitors to control pathogenesis caused by a root infecting fungus, *S. rolfsii* on peanut was reported by Patil et al (2001).

An extensive literature survey has been illustrated in the **Introduction (Chapter 1)** on insect pests and fungal pathogens in agriculture, current biological and chemical control strategies, market status of the different chemical and biological control agents, disadvantages and limitations of chemical agents, current and alternative strategies to control plant pathogenic insect and fungal pests and strategic position of enzymes and enzyme inhibitors in a biocontrol scenario have been included.

The **Materials and methods (Chapter 2)** utilized in the course of these investigations include the details of growth and maintenance of *M. verrucaria* and plant pathogenic fungi viz.- *F. oxysporum*, *S. rolfsii*, submerged and solid state fermentation

for enzyme production. Insect rearing and bioassays have been described. Assays for the estimation of total chitinase activity (acid swollen chitin degrading activity), endochitinase, *N*-acetylglucosaminidase (NAGase), CDA, chitosanase, lipase, β-1,3-glucanase, alkaline protease have been described. The biochemical and biophysical techniques including ultra filtration, freeze drying, ammonium sulphate precipitation, solid state NMR, thin layer chromatography, gas chromatography and spray drying have also been described. The field trials conducted to test the efficacy of enzyme preparation to control insect pest such as *C. lanigera* in sugarcane and *H. armigera* in chickpea have been described.

Production of chitinolytic enzyme complex using *M. verrucaria* has been studied under submerged and solid state conditions (Chapter 3). Initially chitinolytic enzyme production was studied using chitin (Sigma Chemical Co, USA) as a carbon source by *M. verrucaria* (Vyas and Deshpande, 1989). Effect of different conditions on chitin metabolizing enzymes (chitinase, endochitinase, NAGase, CDA, chitosanase) and on others, such as lipase, protease and β-1,3-glucanase production was studied. Three different inocula were used for the production of extracellular enzymes. The aerial conidia from 7 d old PDA slants when used as an inoculum the extracellular enzyme production measured as chitinase (1.8±0.2 U/ml) was more than when the blastospores were used as an inoculum (1.4±0.1 U/ml). If the 48 h old mycelial inoculum grown in a chitin medium was used for the production of extracellular enzymes, the chitinase activity obtained after 7 d was 1.7±0.1 U/ml. The differences in the chitinase activities can be attributed to the growth of *M. verrucaria* as a dispersed mycelium or as in a pellet form. The other activities were not significantly affected. The chitin source as well as its purity affected the extracellular enzymes production. The purified chitin (Sigma) supported better growth as well as enzyme production as compared to chitin from other

sources used. The degree of acetylation, crystalline nature, purity and particle size of the three different chitin were studied. The solid state NMR revealed that acetylation of chitin obtained from Sigma USA, Hi-Media India and Shree Agro Products India was > 80%. However, the purity and particle size (<0.2 mm) found to be affected the enzyme production. Chitin from Sigma USA supported maximum production of enzymes.

For the optimization of submerged fermentation in a fermenter operational parameters, such as airflow, agitation, dissolved oxygen (DO), pH and inoculum size need careful consideration (Jsten et al 1996). The effect of airflow (0.5, 1.0 and 1.5 vvm) was investigated for the production of chitinase and other enzymes in 10 L fermenter. It was seen 0.5 vvm airflow was necessary and sufficient for the optimum mixing of the chitin in the fermenter. At higher airflow (1.0-1.5 vvm) the insoluble chitin was found to remain at the top of the fermenter and thus was not utilized by the fungus. The chitinase activities measured with acid swollen chitin were decreased from 0.97±0.07 U/ml to 0.45±0.02 U/ml as the airflow was increased from 0.5 to 1.5 vvm.

Agitation rate is an important parameter as it plays a significant role in determining the productivity by providing mixing; heat and mass transfer during the process. It is also one of the most critical parameters for process scale up. The maximum chitinase activity (1.13±0.06 U/ml) was obtained by *M. verrucaria* after 5 d of incubation with increasing agitation (from 1st to 5th d, respectively; 100, 120, 150, 180 and 200 rpm) and increasing airflow (from 1st to 5th d, respectively; 0.2, 0.2, 0.3, 0.4, 0.5 vvm). However under constant agitation (100, 200 or 300 rpm) condition, chitinase activity was <0.97±0.07 U/ml. Further increase in agitator speed (400 rpm) resulted in decrease in chitinase activity (0.51±0.04 U/ml), which can be due to excessive foaming. The chitin particles entrapped in foam accumulated above the liquid surface making the substrate inaccessible to the growing mycelium of *M. verrucaria*.

Scale-up of chitinase and other enzymes from shake flask to 10 L and 100 L fermenter was carried out with crude chitin from Shree Agro Products for *M. verrucaria*. In shake flasks the chitinase activity obtained after 7 d was 1.04±0.1 U/ml. The similar levels of activity was obtained within 5 d in 100 L fermenter (1.07±0.07 U/ml). The productivities of chitinolytic enzymes (endochitinase, NAGase, CDA, chitosanase) and others enzymes lipase, protease and β-1,3-glucanase also increased by 1.5-2 fold.

The chitinase and other enzymes of *M. verrucaria* were concentrated using different methods such as membrane concentration (Hollow fiber membrane, 5 KD), freeze drying, spray drying and ammonium sulphate (90%) precipitation. Membrane concentration and freeze-drying gave higher yield of enzymes compared to spray drying and ammonium sulphate precipitation. The recovery of all enzymes using membrane concentration and freeze drying were 80-90%, while with spray drying the recovery was 50%. Lower recovery of enzymes by spray drying can be attributed to the heat inactivation of the enzymes during the process.

Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the utilization of solid-state fermentation (SSF) technique to produce several enzymes. In the present studies SSF was carried out using different substrates such as sorghum, wheat, wheat bran, rice, rice bran and bajra. The growth and enzyme production by *M. verrucaria* was supported by sorghum and chitin mixture (3:1). The chitinase production was increased from 1.52±0.1 U/g to 1.62±0.2 U/g with the addition of olive oil along with sorghum and chitin mixture. While the lipase activity was significantly increased from 0.33±0.04 U/g to 0.50±0.04 U/g after 7 d. Different solvents/extractants were tried for the recovery of enzymes from substrate such as, Triton X-100, glycerol 3% (w/v) + ethanol 10% (v/v), acetate buffer (pH 5.0, 50 mM), 1% sodium chloride and Tween 80

(0.1%). Maximum recovery of enzyme was obtained using 1% sodium chloride. The comparative costs for both the fermentation processes were worked out.

Biochemical characteristics and stability of chitinolytic enzyme complex of *M. verrucaria* (Chapter 4) were studied depending on the mode of application. For instance, in case of foliar application the effect of temperature, pH and sunlight (UV) exposure is important. In case of soil application, the adsorption on organic matter, degradation of enzyme mixture by the soil flora add to an instability of the enzyme mixture. Thus the effect of different conditions on enzyme activities of *M. verrucaria* were studied

The optimum temperature for chitinase (acid swollen chitin degrading activity), endochitinase, NAGase and β-1,3-glucanase was 50°C, while lipase and protease showed maximum activity at 37°C. Chitinase, endochitinase, NAGase and β-1,3-glucanase had optimum pH 5.0. Lipase showed maximum activity at pH 7.0 while protease at pH 10.0.

Effect of different conditions such as temperature, pH, light (UV), soil constituents on the stability of chitinase and other enzymes was studied. Stability of *M. verrucaria* enzyme mixture was carried out at 4°C, 25°C and 40°C. Initially the enzyme stability was studied at 40°C with and without polyols such as ethylene glycol, glycerol, xylitol and sorbitol (1-5M). Glycerol (5M) showed maximum protection for chitinase, β-1,3-glucanase and lipase while maximum stability of protease was observed in the presence of sorbitol (5M). Protective effect of polyols increased as the concentration of polyols increased from 1M to 5M. In the presence of glycerol 5M chitinase, endochitinase, NAGase retained 50-52% of initial activity at 40°C after 3 h. While lipase and β-1,3-glucanase retained 56-60% of initial activities. Protease retained 58% activity in presence of sorbitol (5M) and 51% in the presence of 5M glycerol after 3 h at 40°C. In

a lyophilized powder form all enzyme activities were stable at 25°C for 2 months, at 4°C for 1 year and at -20°C for 2 years.

The pH stability of chitinase and other enzymes were carried out by incubating the enzyme preparation at different pH (4 to 10) at 25°C. Chitinase, endochitinase and NAGase were stable in the pH range (5 to 7), with 59-72% residual activities at 25°C after 7 d. These enzymes also retained 50% of initial activities at pH 4 and 8 at 25°C after 7 d. β-1,3-Glucanase was stable in the pH range 4-6, with 70-79% residual activities at 25°C after 7 d. It also exhibited 65% and 48% residual activity at pH 7 and 8. The stability of protease was more in the pH range of 6-10, with 71-79% residual activities at 25°C after 7 d. It also exhibited 60% residual activity at pH 5. Lipase was stable in pH range 5-7, with 65-74% residual activities at 25°C after 7 d. It also exhibited 45% and 43% residual activities at pH 4 and 8, respectively.

During the pot experiment studies, a pot containing soil and peanut seeds (2 no.) was drenched with *M. verrucaria* enzyme mixture. The 100 ml enzyme preparation (containing chitinase, glucanase and protease) was drenched/pot and the adsorption of enzymes on soil organic matter was determined. The soil samples (10g) were taken from different portions of the pot (top 1-3 cm, middle 4-6 cm and bottom 7-9 cm) and enzyme activities were estimated by repeated washing (2-3 times) of the soil with acetate buffer (pH 5.0, 50 mM). Maximum enzyme activities were found to be adsorbed at top portion (50-52%) of the pot, while adsorption in the middle portion was 20-23% and at the bottom 10-17% adsorption was detected. Chitinase, β-1,3-glucanase and protease were stable in the soil with 51-57% residual activity at 25°C after 5 d. In the presence of artificial UV the enzymes retained 85-90% residual activities at 25°C after exposure for 5h. The cumulitive effect of sunlight and temperature on the stability of chitinase and other enzymes of *M. verrucaria* was also studied in presence and absence of glycerol

(1M) for 7 d. The enzyme samples incubated in dark at RT and at 4°C were served as controls. Chitinase and other enzymes retained 41-46% activity in presence of sunlight after 5 d. While in presence of glycerol under sunlight all enzymes showed 60-65% activity after 5 d. The enzymes incubated in dark retained 51-55% activity at RT after 5 d and at 4°C, 88-92% activities were observed

Field performance studies of chitinase and other enzymes of *M. verrucaria* either singly or in combination for the biocontrol of insect pests as well as plant pathogenic fungi were carried out (Chapter 5). To control insect pests such as *C. lanigera*, *H. armigera* and fungal pests like *F. oxysporum* and *S. rolfsii*, an attempt was made to develop an enzyme based biocontrol agent i.e. cuticle degrading and mycolytic enzyme complex of *M. verrucaria*. In case of woolly aphid control, the enzyme mixtures produced by *M. verrucaria, M. anisopliae* and *Bacillus* sp. B1 were used for the bioassay. To study the effect of lipase activity, *per se*, the lyophilized powder (crude culture filtrate) of *M. verrucaria* was diluted with phosphate buffer (pH 6.8, 50 mM) to obtain lipase activities 1-5 U/ml. Lipase activity contributed significantly to the initial hydrolysis of the waxy coating of the aphids and thus exposed the aphids to other hydrolytic enzymes. The mortality of woolly aphids with a commercial lipase preparation was 32% while it was 88% with *M. verrucaria* enzyme preparation (lipase 5U/ml) in 4 d. The *C. lanigera* mortality with enzyme preparation (lipase 1 U/ml) from *M. anisopliae* and *Bacillus* sp. B1 was 59 and 54% respectively. Effect of *M. anisopliae* conidia singly and in combination with *M. verrucaria* enzyme preparation (lipase 1 U/ml) was also studied. The mortality of *C. lanigera* was 66±4% using *M. anisopliae* conidia, however the mortality increased to 96±3% using combined treatment. *Bacillus* sp. liquid culture grown in chitin medium showed 59% mortality of *C. lanigera*

Sugarcane woolly aphid has become a serious pest in Maharashtra from 2002. Woolly aphid multiplies profusely by desaping of the foliage and excretes honeydew like substance on the leaves, which favor the growth of black sooty mould (*Capnodium* sp.), resulting in drying of leaves and ultimately decrease in yield and sugar recovery. The effect of enzyme preparations of *M. verrucaia, M. anisopliae* and *Bacillus* sp. on conidial germination of *Capnodium* sp. was studied. The percent germination of *Capnodium* sp. reduced by 25-35% after 24 h due to the enzyme treatments of *M. verrucaia, M. anisopliae* and *Bacillus* sp. B1

The protective woolly cover of aphids is mainly of lipids. The lipase enzyme in the preparation may be playing a role in the separation and hydrolysis of the waxy mass making the cuticle accessible to degradation by chitinases and proteases. The gas chromatography analysis showed that the waxy mass and cuticular surface lipids of woolly aphid were composed of a mixture of long-chain aldehydes and long-chain alcohols with the major chain lengths being C24, C26 and C28.

Under field conditions, the efficacy of *M. verrucaria* enzyme preparation (lipase 5U/ml) was 61±21%. This can be attributed to the spraying efficiency, stability in the field and other conditions, if any. The efficacy of *M. anisopliae* conidia to control *C. lanigera* was increased to 77±19% with the prior spray of *M. verrucaria* enzyme (lipase 1U/ml). The % efficacies for the biocontrol agents used were *at par* or more than the chemical insecticide, Metasystox. Differences in the population of aphid due to different treatments were recorded up to 28 d. In the demonstration trial due to their synergistic effect *M. verrucaria* enzyme (lipase 1U/ml) and *M. anisopliae* conidia showed the better performance (68±19% efficacy) over farmer's practice (40±18% efficacy, 5% neem seed kernel extract and Metasystox 2 ml/L).

In case of *H. armigera* bioassay, the enzyme mixtures produced by *M. verrucaria* and *M. anisopliae* were used. To study the effect of chitinase activity, *per se*, *M. verrucaria* enzyme preparation was diluted to obtain chitinase activity 1 to 5 U/ml. The other enzyme activities changed proportionately in the preparations. Chitinase activity significantly contributed to the mortality of *H. armigera*. The mortality of *H armigera* (30-89%) increased as the enzyme activity increased (measured as chitinase 1U-5U). Incorporation of glycerol (1M) to *M. verrucaria* enzyme preparation showed increased mortality of *H. armigera*. The mortality of *H. armigera* increased from 53±3% to 71±4% due to the addition of glycerol (1M) to *M. verrucaria* enzyme preparation. This can be attributed to increased enzyme stability due to glycerol. The *M. anisopliae* enzyme preparation also showed 54±3% mortality of *H. armigera.*

Based on the laboratory performance the *M. verrucaria* enzyme preparations were used for the control of *H. armigera* in chickpea field. *M. anisopliae* conidia and a chemical insecticide endosulfan were also used for the control of *H. armigera* in chickpea (Nahar et al 2003; Kulkarni et al 2008). Under field conditions, the efficacies of *M. verrucaria* enzyme preparations (SmF and SSF) measured as chitinase 2 U/ml were 55±23% and 58±20% respectively. This difference can be due to the differences in the proportions of chitinolytic enzymes, protease and lipase in the enzyme mixture obtained in SmF and SSF. The incorporation of glycerol (1M) in enzyme preparation (SmF, chitinase 2 U/ml) of *M. verrucaria* showed increased efficacy (70 ± 19%) against *H. armigera.* The *M. anisopliae* enzyme preparation also showed 52±23% efficacy against *H. armigera*. *M. anisopliae* conidia and endosulfan showed 70-74% efficacy. The percent efficacy was calculated using Henderson and Tilton (1955) formula. Differences in *H. armigera* population due to different treatments were recorded up to 42 d. The percent pod damage of chickpea in the control plot was 40 ± 8% while in all the treated

plots it was 16-28 %. The grain yield of chickpea in control plot was 10±1 q/ha while in all the treated plots it was 15-20 q/ha.

The performance of enzymes (100 ml preparation/pot, chitinase 50 U, β-1,3-glucanase 7 U and protease 2 U) of *M. verrucaria* and *Bacillus* sp. B1 (chitinase 1 U, β-1,3-glucanase 8 U and protease 27 U) were tested against *S. rolfsii* and *F. oxysporum*. Each treatment was performed for 10 pots with 2 seeds per pot. The germination of peanut seeds was 70±5% in *F. oxysporum* infested pots treated with enzyme mixture of *M. verrucaria*. While 40±5% germination was observed in *F. oxysporum* infested pots treated with enzyme mixture of *Bacillus* sp. B1. The 90±5% germination was seen in a sterile soil while it was only 10±5% in *Fusarium* infested pot drenched with sterile distilled water. In the pots treated with carzim 60±10% germination of peanut seeds in *F. oxysporum* infested soil was seen. The similar results were observed in case of *S. rolfsii* control using above treatments.

The summary and possible recommendations for the use of enzymes in the biocontrol of insect pests and fungal pathogens are given in the **conclusion (Chapter 6)**. The use of enzyme mixture of *M. verrucaria* in IPM with an insect pathogenic fungus *M. anisopliae* will be a useful strategy for the control of an insect pest in field. However, the shelf-life and cost effective production of enzyme mixture of *M. verrucaria* are the major factors of concern.

The **reference** list is given in the **Chapter 7**

Chapter 1

Introduction

1.1 Introduction

One of the eight goals listed by the United Nations Hunger Task Force is related to the conservation of nature and protection of the environment (Sanchez and Swaminathan, 2005). Chemical pesticides (crop protection chemicals) are specifically formulated to be toxic to living organisms and as such they are equally hazardous to humans. The impact of pesticides on the environment is alarming (Litovitz et al 1996). Pesticide use is increasing even in developed countries like the United States. India has 170 million ha of arable land with average pesticide consumption of 0.5 kg/ha. In terms of total consumption, India is placed tenth in the world (Hundal et al 2006). However, the present level should not be equated with low risk to health and environment, given the prevalence of toxic pesticides allowed in India. Interestingly, there is always scope for more pesticide consumption in India in the coming decades owing to the growing population coupled with high demand for food grains and intensification of agriculture under fast changing climate.

The cropping pattern in India is changing, particularly towards cash crops (mainly grapes, pulses, vegetables, spices, cotton and sugarcane). Some of these crops like grapes and sugarcane require as high as 20 sprays in a year. Similarly, cotton needs 12 pesticide sprays per season (Shetty et al 2008). Therefore, large amount of pesticides are being used into such agro-ecosystems in India. For the 21st century nothing more is important than sustainability of the ecosystem. The focus is on intensive pesticide use on crops such as paddy, cotton, sugarcane, pulses, wheat, apple, pomegranate, mango, grapes and vegetables covering different agro-ecological zones in India. The total area under integrated pest management (IPM) is estimated to be only 2% in India. However, in recent years the consumption of biopesticides has increased steadily in many parts of the

country. Interestingly, about 64% of farmers followed at least one of the IPM tools in different agro-ecosystems of India (Shetty et al 2008).

1.2 Crop loss in agriculture due to insect pests and fungal pathogens

India is basically an agricultural country and it has most variable climatic regions owing to its geographic features. Total arable land area is 168 m ha and major part of it is falling under tropical climate and a variety of cereals, oil seeds, pulses, vegetable and horticultural crops are being cultivated. India needs to produce additional 5-6 metric tonne of food grains every year to keep pace with the growth of our population. In realizing this, one of the major problems seems to be the yield losses due to insect and fungal pests. There is an urgent need to assess such losses in order to frame strategies to overcome them**.** Yield loss estimates vary depending on type of cultivar, density of pest population, time of pest attack in relation to crop phenology and cultural practices followed. Another problem is that most of the studies are conducted in small experimental plots at research stations rather than in farmers' fields, which may not give the exact picture of the losses caused. Crops like grapes, cotton, rice, pulses and sugarcane suffered significant yield losses due to insect pests. However, even the limited information available from various sources reveals that crop losses due to insect pests are higher in India than for the other parts of the world (Reddy and Zehr, 2004).

There are several insect pests in India which cause severe loss in different field crops. *Helicoverpa armigera* on pulses, cotton, vegetables and sunflower, *Plutella xylostella* and *Spodeptera litura* on vegetables, woolly aphids (*Ceratovacuna lanigera*) on sugarcane, mealy bugs (*Phenacoccus gossypiphilous* and *Maconellicoccus hirsutus*) on cotton and grapes respectively. The above mentioned crops are also prone to be attacked by plant pathogenic fungi. Rice and wheat can be attacked by *Magnaporthe grisea* and *Puccinia recondiata*, respectively. Pulses by *Fusarium* sp., cotton by

Verticillium dahliae, chillies by *Colletotrichum capsici,* legumes (groundnut and cowpea) by *Sclerotium rolfsii, Fusarium oxysporum, Rhizoctonia solani* and sugarcane by *Colletotrichum falcatum*. All these insect and fungal pests cause severe loss in agricultural crops (**Table 1.1**).

The fungal diseases such as wilt, rots, cankers, rusts, smuts and mildews cause considerable losses in yield of a crop. Infection by fungal pathogens in the field or in post-harvest storage also affects the health of humans and livestock, especially when the contaminating organism produces toxic residues in or on consumable products (Diekman and Green, 1992; Nelson et al 1993).

Sugarcane is a major industrial crop in India and is attacked by several aphid species. Among the different aphids the *C. lanigera* is a serious pest of sugarcane. This species is known from India, Nepal, Bangladesh, East and South Asia, Fiji and Solomon Islands (Joshi and Viraktamath, 2004). *C. lanigera* lives in large colonies, sucks the phloem sap from the leaves and excretes copious honeydew onto foliage leading to the development of sooty mould (*Capnodium* sp.). The direct and indirect damage affects sugarcane yield and quality. In India, the pest was known only from northeast region but recently, it has made its entry into western and southern parts of the country.

Another important insect pest is the gram pod borer *H. armigera.* This pest is cosmopolitan polyphagous pest attacking more than 182 host plants belonging to 47 botanical families in Indian subcontinent and feed on more than 200 plant species (Pawar, 1998). Host species for *H. armigera* come from a broad spectrum of families and include important agricultural crops such as cotton, maize, chickpea, pigeonpea, sorghum, sunflower, soyabean and groundnuts (Cunningham et al 1999). It is one of the most important insect pests in the world due to its mobility, high polyphagy, short life cycle and high reproductive rate (Lawo et al 2008).

Table 1.1 Major insect and fungal pests of different crops

Crop	Insect pests	Maximum crop loss (%)	Fungal pathogens	Maximum crop loss (%)
Cereals				
Rice	Stem borer (*Scirpophaga incertulas*)	48	*Magnaporthe grisea*	21
Wheat	Army worm (*Mythimna separate*)	42	*Puccinia recondiata*	17
Sorghum	Stem borer (*Chilo partellus*)	83	*Ustilago sorghicola*	20
	Woolly aphid (*Melanaphis sacchari*)	78		
Maize	Stalk borer (*Chilo partellus)*	36	*Ustilago maydis*	25
	Shoot fly (*Atherigona soccata)*	61	*Cephalosporium acremonium*	70
			Fusarium sp.	74
			Rhizoctonia solani	53
Pulses				
Pigeonpea	Pod borer (*Helicoverpa armigera*)	100	*Fusarium udum*	24
	Pod webber (*Maruca testulalis)*	60		
Chickpea	Pod borer (*H. armigera)*	70	*Fusarium oxysporum*	24
Oil seeds				
Sunflower	Capitulum borer (*H. armigera)*	60	*F. oxysporum*	50
Peanut	Army worm *Spodoptera litura*	71	*F. oxysporum*	95
			Sclerotium rolfsii,	50
Cash crops				
Cotton	Mealy bug (*Phenacoccus gossypiphilous)*	40	*Verticillium dahliae*	70
	American bollworm (*H. armigera)*	80		
Sugarcane	Woolly aphids (*Ceratovacuna lanigera)*	30	*Colletotrichum falcatum*	20
Grapes	Mealy bug (*Maconellicoccus hirsutus)*	35	*Botrytis cinerea*	50
Vegetables				
Cabbage	Diamond back moth (*Plutella xylostella*)	52	*Rhizoctonia solani*	30
Chilli	Army worm (*S. litura*)	50	*Colletotrichum capsici*	50
Tomato	Fruit borer (*H. armigera*)	61	*F. oxysporum*	44

Compiled from: Sathiah et al 2005; Babujee and Gananamanickam, 2000; Grover and Pental, 2003; Joshi and Viraktmath, 2004; Kumar, 2005; Mallikarjuna et al 2004; Patibanda et al 2002; Rangeshwaran et al 2001; Reddy and Zehr, 2004; Wada et al 2008; Rojo et al 2007; Bejarano-alcazar et al 1997; Yasmin et al 2006; Horinouchia et al 2007; Tripathi et al 2008

Females lay eggs on the flowering and fruiting structures of these crops, where voracious larval feeding leads to substantial economic loss. The ability of ovipositing females to locate and utilize a wide range of hosts from a number of families is one of the major factors contributing to the pest status of this moth (Zalucki et al 1986; Fitt, 1989). However, despite its importance, the host selection behaviour of this moth is still poorly understood (Zalucki et al 1986). The second and older instars enter the pod and feed while concealed inside the pod. When ready for pupation, the fully-grown larva exits the fruit and pupates in the soil. Attempts at preventing the damage to the high value crops including cotton, pulses, cereals, oilseeds and vegetables confer high economic cost in Indian farming. Crop loss to extent of 75-100% in chickpea, 57-80% in cotton, 61% in tomato has been recorded (Sathiah et al 2005).

When viewed with a wide angle, the problem of insect pests and fungal pathogens of crop plants is quite complicated due to heavy losses to agricultural production of the country, directly or indirectly disturbing the national economy. This becomes an important topic of the day for research and needs multidisciplinary approach and efforts to solve it.

1.3 Pest control in agriculture

1.3.1 Chemical pesticides

Different plant protection chemicals are available in India for the control of pests in agriculture, of which insecticides account for 60% followed by fungicides (20%), herbicides (17%) and others (3%) (Shetty et al 2008). On comparing toxicity levels (per unit basis) of various pesticide categories, insecticides and fungicides have several times more toxicity than herbicides.

1.3.1.1 Insecticides

The majority of chemical insecticides consist of an active ingredient and variety of additives which improve the efficacy of its application and action. Chemical insecticides are usually divided into four major classes, the organophosphates, organochlorines, carbamates and the pyrethroids.

The organophosphate insecticides were originally developed during World War II and they work by inhibition of the respiratory enzyme cholinesterase. It was also reported that, organophosphates like, schradans, parathion, sulfenimide and captan selectively prevent chitin formation in insects by inhibiting chitin synthase activity (Deshpande, 1998). Organophosphorous compounds like schradans kill sucking insects like aphids, whitefly and leafhoppers, but caterpillars and beetles that are chewing insects can survive schradans. Monocrotophos and quinalphos are two of the most widely used organophosphate insecticides in India, that together constitute 75% of the total organophosphate insecticide usage in the country. Interestingly, of the total, nearly 85% of quinalphos and 68% of monocrotophos are used solely on cotton in India (Anon, 1997). Resistance to insecticides belonging to organophosphate groups has been reported in the cotton bollworm, *H. armigera* and the leaf worm, *S. litura* in India (Kranthi et al 2001).

Organochlorine (DDT, lindane and dieldrin) insecticides are a class of insecticides characterized by their cyclic structure, number of chlorine atoms and low volatility. These agents were widely used in agriculture for pest control between the 1940s and 1960s. Because of the high persistence of the organochlorine insecticides in the environment (Rombke and Moltmann, 1966) and their subsequent accumulation in the food chain, the use of DDT was banned in the USA and in Europe in 1973, followed by the ban of aldrin and dieldrin in 1975. Most organochlorine insecticides are rarely

used in the developed country; however, they continue to be employed in some developing countries (Purdue et al 2007). Aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, HCB and toxaphene are among the members of the persistent organic pollutants (POPs) group. Due to their toxic, lipophilic and persistent nature, all the POP-pesticides, except DDT, which is under restricted use for disease vector control, are under complete ban in India (Singh et al 2007) for their manufacture, import/export and use. Endosulfan is a broad-spectrum organoclorine insecticide-cum-miticide, which is extensively used on many important crops and use of endosulfan on agricultural crops has been permitted in India. However, the cotton bollworm *H. armigera* exhibited high resistance to endosulfan in Maharashtra, which could have been a result of the indiscriminate use of endosulfan throughout the season (Kranthi et al 2002).

Carbamates are a group of pesticides that contain the –NH(CO)O– functional group. They are widely used in gardens and in agriculture as insecticides, herbicides and fungicides. These are the most widely used pesticides throughout the world since organochlorines have longlasting residue persistence. However, some carbamates, such as carbofuran, are extremely toxic to the central nervous system and they are suspected carcinogens and mutagens. The wide use of carbamates has led to increasing calls to monitor their residues in the environment (Saraji and Esteki, 2008). Thiofanox, carbofuran, pirimicarb, methiocarb, carbaryl, propoxur, desmedipham and phenmedipham are the most widely used carbamates. However, carbaryl, the largest spectrum insecticide with the capacity of controlling 565 species of pests in 141 crops became the most popular insecticide in 1950s (Zang et al 2001). Fadare and Amusa (2003) reported the use of carbaryl for the control of cotton bollworm complex (*H. armigera*, *Earias insulana* Boisd, *Earias biplaga* and *Sylepta derogata*) in Southwestern Nigeria.

Pyrethroids, the most effective and safe natural insecticides derived from flowers of *Pyrethrum cinearaefolium* and their synthetic analogues the pyrethroids (Barlow, 1985). Synthetic pyrethroids are in general, more stable chemically and biochemically than are natural pyrethins. Synthetic pyrethroids have high contact activity and particularly effective against lepidopteran larvae. They are also used to control a wide range of insect pests of agriculture and horticultural crops and for use in the control of insect vectors of diseases (e.g. tsetse fly in parts of Africa).

1.3.1.2 Fungicides

Chemical fungicides are being used for the control of fungal diseases. Benzimidazole fungicides are anti-microtubule toxicants (such as thiabendazole, carbendazim and the related benomyl and thiophanate-methyl) which, were developed in the late 1960s. Benzimidazoles were the first systemic fungicides that exhibited a broad spectrum of activity. Most Ascomycetes and Adelomycetes were sensitive, especially *Botrytis* spp. (Delp, 1995). In *Botrytis cinerea*, these fungicides do not prevent conidial germination, but at low concentrations, they inhibit hyphal growth and cause distortion of germtubes (Leroux et al 1999). Because of their broad spectrum of activity against many foliar diseases and their low cost, they continued to be used in mixtures with other fungicide products. However, resistance to benzimidazole fungicides was observed in different fungi such as *B. cinerea, Penicillium expansum, Sclerotinia homoeocarpa* (Ma and Michailides, 2005) and *Fusarium sambucinum* (Kawchuk et al 1994).

Strobilurins are new class of fungicidal compounds isolated from wood-decaying Basidiomycete species. Strobilurins represent β-methoxyacrylic acid group of natural products, which have become an integral part of disease management programmes (Sudisha et al 2005). Strobilurins have broad-spectrum activity against Ascomycetes, Basidiomycetes, Fungi Imperfecti and Oomycetes. These fungicides have also been used

for the control of fungal isolates resistant to other fungicides with novel mode of action and used in low rates. The mode of action of strobilurins is inhibition of mitochondrial respiration by binding to the ubihydroquinone oxidation centre of the mitochondrial complex (complex III), thereby blocking electron transfer (Herms et al 2003).

Fungicides are essential for maintaining healthy, reliable and high-quality agricultural products. Prior to 1970, nearly all fungicides used for the control of plant pathogens were multi-site inhibitors acting as disease protectants. Despite their widespread use, resistance to these compounds was a rare event. However, since the introduction of the site-specific fungicides in the late 1960s, fungicide resistance in phytopathogenic fungi has become a major problem in crop protection (Ma and Michailides, 2005).

1.3.1.3 Disadvantages of chemical pesticides

The excessive use of chemical pesticides led to several adverse effects. The major problems faced by the agronomists were destruction of natural enemies of pests and the development of resistance in target insect pests or fungal pathogens. Thus, the product life of any pesticide is now reduced to 3-5 years increasing the burden on farmers for pest control as it involves the cost of development of new pesticide molecule (Prasad, 2004). Moreover, the human health effects such as immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer are increasingly linked to the long term, low dose exposure to the toxic residues of pesticides (De Waard et al 1993). The export of several agricultural commodities like cotton, rubber, spices and several fruits like mango, apple, citrus, etc. was affected due to the pesticide residues, for the last few years (Wahab, 2004). The chemical pesticide industry will soon be faced with a scheduled removal of many synthetic pesticides from the marketplace. The European Union has scheduled 56% reduction of synthetic pesticides existing in 1991

and in the US, the Food Quality Protection Act implemented in 1996 requires a re-evaluation of all carbamate and organophosphate insecticides for compliance to new standards. The likely outcome of these actions will be the need for new pesticides that address environmental concerns and meet the needs of crop producers. Biopesticides can meet these challenges (Hynes and Boyetchko, 2006). Thus to reduce the use of chemical pesticides and risk, alternative biological strategies for the control of plant pathogenic fungi and insect pests quickly emerged as an important topic of research in this field.

1.4 Biopesticides

The expected advantages of biopesticides like their specificity, safety to non-target organisms, use in limited amounts, quick decomposition, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides, have led to numerous scientific works on new and safer pesticides, particularly in the last three decades (Rosell et al 2008). Biopesticides are classified by US Environmental Protection Agency (EPA) into three main groups: microbial pesticides, plant pesticides and biochemical pesticides. The molecules which are structurally and/or functionally related to the molecules of the pests can be used as biochemical pesticides which have a nontoxic mode of action. For instance, pheromones are naturally-occurring chemicals that insects use to find mates. Chemically synthesized pheromones can disrupt insect mating by creating confusion during the search for mates or by attracting insects to traps.

Microbial pesticides are naturally-occurring or genetically altered bacteria, fungi, algae, viruses, or protozoans that are used to suppress pests by either producing a toxin specific to the pest, causing disease, preventing establishment of pest microorganisms through competition, or other modes of action. The further discussion will highlight the status of bacteria, viruses, fungi and their products as microbial pesticides.

1.4.1 Microbial control of pests in agriculture

1.4.1.1 Bacteria

The most widely used microbial control agent is *Bacillus thuringiensis* (Bt). Today a number of isolates of the bacterium are commercially produced with activity against insect as well as fungal pests **(Table 1.2)**. As of 1998 about 200 *B. thuringiensis* based products were registered in the United States (Schnepf et al 1998). According to statistics produced by the Institute for the Control of Agrochemicals in the Ministry of Agriculture, more than 300 microbial insecticide products have been registered recently in China, out of which *B. thuringiensis* constitute 276 products (Huang et al 2007). Bt products are primarily used for the control of lepidopteran pests, such as cotton bollworm, *H. armigera*, Asian corn borer, *Ostrinia furnacalis*, rice leaf folder, *Cnaphalocrocis medinalis*, rice skipper, *Parnara guttata*. cabbage worm, *Pieris rapae*, diamondback moth, *P. xylostella*, pine lappet caterpillar, *Dendrolimus punctatus*, and the tea leaf-rollers, *Homona coffearia* and *Adoxophyes orana*. Based on fermentation yields, the annual output of Bt products was about 40,000 metric tons in China (Qiu, 2005), a level that meets domestic and export requirements.

Most of the insecticidal activity of *B. thuringiensis* is associated with the proteinacious toxins. Their mode of action is thought to involve a cascade of events leading to insect death within several hours following ingestion (Hofte and Whiteley, 1989; Gill et al 1992; Knowles, 1994). The largest share of the biopesticide market currently goes to *B. thuringiensis*. Other species of bacteria are used on a much smaller scale for insect control. These include *Serratia entomophila*, the only non-bacillus bacterial microbial insecticide registered for pastureland grass grub control in New Zealand (Klein and Kaya, 1995). *Bacillus sphaericus* is now commercially produced for the control of mosquito and has some advantages over *B. thuringiensis* in that it is more

persistent in polluted habitats and may recycle under certain conditions, but has a narrow host range (Lacey and Undeen, 1986; Charles et al 1996; Nicolas et al 1994).

Table 1.2 Commercially available bacterial pesticides

Bacteria	Target pest/pathogen	Countries of registration
Fungicides		
Bacillus subtilis	*Rhizoctonia, Fusarium* *Alternaria* and *Aspergillus* root rots and seedling Diseases	Canada, Korea, India, Japan, New Zealand, Switzerland, Mexico, USA
Pseudomonas fluorescens	*Erwinia amylovora* on apple, cherry, almond peach, pear, potato straw-berry, tomato	India, USA
Burkholderia cepacia (formerly *Pseudomonas cepacia*)	*Fusarium, Rhizoctonia*	India, USA
Streptomyces griseoviridis	Soil pathogens *Fusarium, Alternaria* *Rhizoctonia, Phomopsis* *Botrytis* that cause wilt, seed, root and stem rots	Canada, Denmark, Finland, Hungary, Iceland, Italy, Netherlands, Norway Sweden, Switzerland, USA Spain
Insecticides		
Bacillus popilliae	Larvae of Japanese beetle	USA
Bacillus thuringiensis	Several insect species *Helicoverpa armigera* European corn borer *Plutella xylostella* spruce budworm gypsy moth caterpillars	Australia, Canada, France, Greece, India, Italy, Korea, Netherlands, New Zealand, Spain, Turkey, USA
Serratia entomophila	Grass grub (White)	New Zealand
Bacillus sphaericus	Mosquito control	

Compiled from: Arora and Saikia, 2005; Chavan et al 2008; Hynes and Boyetchko, 2006; Rabindra, 2005

Bacteria like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have shown potential for the control of fungal diseases (Handelsman and Stabb, 1996; Ordentlich et al 1987, Weller et al 1985). The *Bacillus* sp. is capable of controlling soil-borne fungal phytopathogens (Ryder et al 1999) and stimulates plant growth (Podile and Dube, 1988; Utkhede and Smith, 1992). Boruah and Dileep Kumar (2003) have demonstrated that wilt disease caused by *R. solani* in brinjal can be controlled by seed coating using *Bacillus* sp. Furthermore, *Bacillus* sp. also exhibited *in vitro* antagonism against other fungal plant pathogens such as *Fusarium solani* and *Fusarium semitectum*. Basha and Ulaganathan (2002) reported the antagonism of *Bacillus* sp. towards *Curvularia lunata*, causative agent of grain mold and leaf spot diseases in dry land crops like sorghum. Also, mixtures such as a combination of *Pseudomonas* strains are effective in siderophore-mediated competition for iron and induction of systemic plant resistance to improve control of *Fusarium* wilt of radish (Boer et al 2003). Recently bacterial strains of *Pseudomonas* and *Bacillus* were found to have biocontrol activity against *Sclerotinia sclerotiorum*, the causal agent of stem rot of canola (Fernando et al 2007).

1.4.1.2 Viruses

A large number of viruses are available as microbial control agents of insects (Payne, 1982). The Baculoviridae viruses have great potential such Nuclear Polyhydrosis Viruses (NPVs) and granuloviruses (Lacey et al 2001). More than 400 insect species, mostly in the Lepidoptera and Hymenoptera, have been reported as hosts for baculoviruses. The NPVs of gypsy moth the *Helicoverpa,* velvet bean caterpillar, *Anticarsia gemmatalis* and others have been applied over fairly large acreages. The NPV of velvet bean caterpillar has been used to treat 1 million ha of soybean fields in Brazil annually (Moscardi and Sosa-Gomez, 2000). Their efficacy, specificity and production of secondary inoculum make baculoviruses attractive alternatives to broad-spectrum

insecticides and ideal components of IPM systems due to no harmful effects on beneficial insects including other biological control organisms (Huber, 1986; Groner, 1990). Two baculoviruses that have relatively broad host ranges against Lepidopteran insects are the NPVs of *Autographa california* (Speyer) (Vail et al 1999) and *Anagrapha falcifera* (Kirby) (Hostetter and Puttler, 1991). The *Ac*-NPV is active against larvae of 43 species in 11 families of Lepidoptera (Vail et al 1999). Some of the drawbacks of the use of entomopathogenic viruses are their relatively slow action compared to that of chemical insecticides, sensitivity to UV light and the requirement for living systems for production.

1.4.1.3 Fungi

1.4.1.3 a Insect pathogenic fungi

Fungi infect a broader range of insects than bacteria and viruses. For instance, they can infect lepidopterans (moths and butterflies), homopterans (aphids and scale insects), hymenopterans (bees and wasps), coleopterans (beetles) and dipterans (flies and mosquitoes). In fact, some fungi such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii* and *Paecilomyces* sp., have broad host ranges that encompass most of the insect groups (Charnley, 1989; Chavan et al 2006). *B. bassiana* has been identified in many insect species in temperate and tropical regions and is used for pest control in eastern Europe and China. *B. bassian*a based mycoinsecticide has been shown to be highly effective against coleopterans. *M. anisopliae* has been most extensively used for the control of spittlebugs on sugar cane in Brazil. *V. lecanii* is a pathogen that has demonstrated better control of green house pests, such as *Myzus persicae* (Sulcer) aphids, on chrysanthemums. A distinct isolate of *V. lecanii* was obtained from whitefly and provided excellent control of greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) and of *Thrips tabaci* Lindeman on cucumber. During 1990s *V. lecanii* was

produced commercially as Vertalec for aphid control and Mycotal for control of whitefly. Now there is a resurgence of commercial interest in its use for control of aphids, whiteflies and thrips because these greenhouse pests have developed resistance to chemical pesticides typically used for their control (Chavan et al 2008). Similarly, fungal pathogen *Entomophaga maimaiga* has been recognized and used in USA as a control for gypsy moth (Elkinton et al 1991). The list of commercially available insect pathogenic fungi in India is given in **Table 1.3**.

The viral and the bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle, which is the outermost covering in insects and fungal cell wall. Moreover, production and application of fungal biomass is easy. The other features are: they do not leave toxic residues, environmentally safe and no known resistance in insect community. This can be attributed to the involvement of multiple factors due to the nature of their mechanism of action. However, research inputs are necessary to make them effective on a wide range of temperature and humidity and to have longer shelf-life.

Three groups of insect pathogenic fungi are observed in the field. Commonly encountered fungi with dry conidia are *Metarhizium, Beauveria, Nomuraea* and *Paecilomyces* which possess lipophilic cell walls. Others though frequently encountered but have less potential are *Verticillium* and *Entomophaga.* Saprophytes *Aspergillus, Fusarium* and *Penicillium* are also sometimes mistaken as entomopathogens. The fungi such as *Metarhizium, Beauveria, Nomuraea, Verticillium* and *Paecilomyces* are the favoured candidates in the area of pest control (Butt et al 2000).

Table 1.3 Commercially available insect pathogenic fungi in India (Compiled from Faria and Wraight, 2007**)**

Species	Trade name	Propagule(s) /Formulation	Target	Supplier and Manufacturer
Beauveria bassiana	BioGuard Rich	Conidia	Coffee berry borer Cotton leaf roller Root grubs, White flies, Aphids, Thrips	Plantrich Chemicals & Biofertilizers Ltd, India
	Bio-power	Conidia / WP	Caterpillars Weevils, Leafhoppers Bugs, Grubs, Leaf-feeding insects	T. Stanes & Company Limited, India
	Racer	Conidia / WP	*Helicoverpa*	Agri Life, India
Metarhizium anisopliae	Bio-magic	Conidia / WP	Root weevils, Plant hoppers, Japanese beetle, Spittlebug and white grubs	T. Stanes & Company Limited, India
	Biomet Rich	Conidia / Liquid suspension	Coconut beetles White grub Termites, leaf miners	Plantrich Chemicals & Biofertilizers Ltd, India
	Pacer	Conidia / WP	Ants that spread mealy bugs Termites, Root grubs	Agri Life, India

Table 1.3 Commercially available insect pathogenic fungi in India (Compiled from Faria and Wraight, 2007**)**

Species	Trade name	Propagule(s)/ Formulation	Target	Manufacturer
Hirsutella thompsonii	Metehit	-	*Calacarus heveae* Acari	Plantrich Chemicals & Biofertilizers Ltd, India
Isaria fumosorosea (Formerly *Pacilomyces fumosoroseus*)	Priority	Conidia/ WP	All mite species	T. Stanes & Company Limited, India
Lecanicillium sp. (Formarlly *Verticillium lecanii*)	Bio-catch	Conidia/ WP	Sucking pests like aphids	T. Stanes & Company Limited, India

Epizootics caused by *Nomuraea rileyi* and *B. bassiana* have been observed on *H. armigera* and *S. litura* in Indian fields as a result the insect population is highly susceptible to these fungi (Uma Devi et al 2003). Different strains of *B. bassiana* were reported for the control of *Locusta migratoria* (Quesada-Moraga and Vey, 2003). The variation in their effectiveness was mainly due to the variation in their virulence and their ability to produce *in vitro* toxic metabolites. The virulence towards insect pest was also found to be affected by the repeated conidial sub-culturing of *M. anisopliae* (Nahar et al 2008).

1.4.1.3 b Mycoparasitic fungi

A wide spectrum of fungi is known to show antagonism among themselves. The potential of *Trichoderma* and *Gliocladium* in controlling plant-pathogenic fungi was reported in the beginning of 20^{th} century (Deshpande, 1999). It was due to the toxic metabolites secreted by these two fungi. Initially parasitic fungus has to locate the host mycelium or spores, using chemical signals originating from the host fungus. There are two types of mycoparasitism. *Trichoderma* exhibits necrotrophic parasitism. It attaches to the host hyphae by coiling, hooks or appressorium-like bodies and penetrates the host by secreting cell wall lytic enzymes. On the other hand, fungi like *Sporidesmium sclerotivorum* show biotrophic mycoparasitism in response to the persistent contact with the host. The haustoria produced in the hyphae of a host *Sclerotinia minor*, trigger the production of cell wall lytic activities by the host itself (Jeffries, 1997).

One of the important aspects in the fungus-insect and fungus-fungus interactions is the possibility of identifying a strain, which has capability to show dual pathogenesis. For instance, Jassim et al (1990) have shown that *T. harzianum,* a mycoparasitic species, can parasitize the elm bark beetle, *Scolytus*. This is mediated through the production of specific hydrolytic enzymes. *V. lecanii*, a pathogen of aphids, can also parasitize rust

fungi. It was suggested that entomopathogenic and mycoparasitic strains within the species can be separated on the basis of morphological and biochemical characteristics (Jun et al 1991). The genus *Verticillium* is sub-divided into five groups, the sections *Verticillium, Nigrescentia, Prostrata, Albo-erecta* and a fifth, residual group (Kouvelis et al 1999). These sections have been defined on the basis of morphological features, particularly those associated with conidial morphology and the verticillate arrangement of phialides on conidiophores. Jun et al (1991) identified five major groups of *Verticillium* on the basis of isoenzyme profiles and physiological activities, although several isolates in their study remained ungrouped.

1.4.1.3 c Fungus-insect and -fungus interaction

As fungi are effective by contact the considerations such as adhesion of an infective propagule to the insect body, entry of the organism in to host and the killing are important in developing effective mycoinsecticide. As mentioned earlier, in case of *Metarhizium*, *Beauveria*, *Nomuraea* and *Verticillium* conidia are the means for attacking an insect population. Pre-infection stages are adhesion and germination of conidia to form germ tubes or appressoria. Entomopathogenic fungi display different strategies in their attachment to insects. Infective propagules are either dry hydrophobic conidia as in case of *B. bassiana, M. anisopliae* and *N. rileyi* or sticky hydrophilic conidia seen in *V. lecanii* and *Hirsutella thompsonii* (Sosa-Gomez et al 1997). The adhesion is either passive (adsorption) or active host-specific (germination and penetration) phenomenon. In many cases, various fungistatic activities have been associated with the cuticle of insects. For example, the presence of antagonistic microbes, chitinases produced during moulting, toxic lipids and cuticle phenolic compounds have been suggested to inhibit fungal growth. For instance, cuticular fatty amides may contribute to *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) in hampering conidial adhesion of

entomopathogenic fungi by decreasing hydrophobicity and static charge (Lord and Howard, 2004).

Once the fungus breaks through the cuticle and underlying epidermis, it may grow profusely in the haemolymph, by forming the hyphal bodies, in which case death is probably the result of starvation or physiological/biochemical disruption brought about by the fungus. The secondary metabolites of the attacking fungus may contribute to the demise of the insect. As a result, one can notice the sporulating mycosed cadavers of the host. This enhances the possibility of horizontal or vertical transmission of the disease within the insect population. Furlong and Pell (2001) studied the horizontal transmission of *B. bassiana* and *Zoophthora radicans* by the diamondback moth. Fundamental to this approach is the efficient horizontal transmission of the pathogen to susceptible individuals within the pest population. Potential routes of infection to susceptible individuals are direct contamination by passive transfer from inoculated adults, indirect contamination by conidia deposited in the crop by inoculated adults and secondary transmission of conidia from the sporulating mycosed cadavers of diseased individuals that die within the crop.

On the other hand fungus-fungus interaction is apparently less complicated. For instance, recent studies of Gao et al (2005) showed the interaction between *Chaetomium spirale* and *R. solani* in dual cultures. They reported that pathogen growth inhibition occurred soon after contact with the antagonist, followed by the overgrowth of *C. spirale* on the colony of *R. solani*. The coiling of *C. spirale* around *R. solani* and intracellular growth of the antagonist in its host occurred frequently. The electron microscopy studies of *Gliocladium roseum* interaction with conidia and germ tubes of *B. cinerea* revealed that infection occurred through direct penetration by hyphal tips of *G. roseum* without formation of appressoria (Li et al 2002).

1.4.1.3 d Killing components

The dual pathogenecity of some fungi towards insects and fungi has been attributed to the hydrolytic enzymes, which facilitate the entry of the pathogen in the respective hosts. The insect cuticle is composed of wax, lipids, protein and chitin. The chitin, a β-1,4-linked *N*-acetylglucosamine polymer, constitute 25-50% of the dry weight of the cuticle. While fungal cell wall components are divided into two groups, such as skeletal and matrix components. Chitin and R-glucans are the main structural components while S-glucans and manno-proteins are the matrix components. Furthermore, chitin is absent in plants and mammals and thus its metabolism too presents an attractive target for the control of plant pathogenic fungi and insects.

The cuticle degrading enzymes (CDEs) mainly include: proteases, lipases and chitinases and enzymes such as chitinases, proteases and β-1,3-glucanases are the main components of the mycolytic enzyme (ME) complex (Deshpande, 1999). The composition of CDE and ME is not drastically different. It has been suggested that among these, chitinases contribute significantly in the degradation of protective covers. Therefore, most of the studies for the production of CDEs and MEs and their use in the field are centered around chitinases. *In vitro* experiments showed that insect pathogens in submerged fermentation produce extracellularly CDEs when locust cuticle was used as a sole carbon source (St. Leger, 1986a). Sequence of appearance of extracellular enzymes in *Metarhizium* and *Beauveria* cultures have been reported. Proteolytic enzymes such as esterase, endopeptidase, aminopeptidase and carboxypeptidase were produced within first 24 h of growth. *N*-Acetylglucosaminidase appeared next and endo-chitinase, which attacks chitin polymer randomly, was produced in significant quantities after 4 days. Lipases were detectable after 5 days (Charnley, 1989). Though chitin forms the main structural mesh, the production of CDEs was in accordance with the cuticular structure.

St. Leger et al (1986a) assigned major role to protease in cuticle degradation by *M. anisopliae*. Nahar et al (2004a) suggested an alternate mechanism of cuticle degradation with the involvement of chitin deacetylase and chitosanase in *M. anisopliae* that show low or delayed chitinase production.

Similarly, in case of mycoparasitic organisms the appearance of extracellular enzymes was found to be corresponding to the main chemical constituents of the fungal cell wall i.e., chitin, glucans and proteins (Goldman and Goldman, 1998). The enzymes appeared sequentially when *T. harzianum* was grown on a mycelium of *R. solani*, an alkaline proteinase was produced first, followed by glucanases and chitinases. Chitinolytic enzymes of *T. harzianum* were found to be inhibitory to a wider range of deleterious fungi than similar enzymes from other sources. A *Fusarium chlamydosporum* strain, a mycoparasite of groundnut rust, *Puccinia arachidis* produces an endochitinase, which inhibits germination of uredospores. This indicates the significant contribution of chitinase in the biocontrol of groundnut rust. *Myrothecium verrucaria* is also known to produce high levels of chitinases, lipase, protease and β-1,3-glucanase (Deshpande, 1986; Shaikh and Deshpande, 1993; Patil et al 2000). This enzyme preparation also showed potential for the control of *S. rolfsii* in peanut (Patil et al 2001).

In addition to the hydrolytic enzymes, fungi secrete number of compounds that exhibit biological activity against pests (Vey et al 2001). The entomopathogenic fungi, *B. bassiana, Beauveria brongniartii, H. thompsonii, M. anisopliae, Paecilomyces fumosoroseus, Tolypocladium* and *V. lecanii* produce different toxins such as, bassianin, beuvericin, cytochalasin C, destruxins, dipcolonic acid, enfrapeptins, hirsutellin A, B, oosporein, swainsinone, etc. (Amiri-Besheli et al 2000; Vey et al 2001). These toxins are not effective to the same extent against different insects. For example, lepidopterean insects are highly susceptible to destruxins produced by *M. anisopliae*. Efrapeptins of

Tolypocladium show insecticidal and miticidal effects against potato beetle, mites, budworm and diamondback moth and others. Oosporein produced by *B. brongniartii* affects enzyme malfunctioning in cockchafer larvae by redox reactions. The hyphomycete, *H. thompsonii* produced an extracellular insecticidal protein, hirsutellin A which was reported to be effective against citrus rust mite, mosquito larvae and others (Vey et al 2001).

Trichoderma and *Gliocladium* sp. produced diverse secondary metabolites with antibiotic properties including polyketides, terpenoids and polypeptides (Vey et al 2001). *T. harzianum* produces a novel trichothecene, *harzianum* A, which is one of the killing components for the biocontrol of plant pathogenic fungi. *Trichoderma viride*, species from '*harzianum'* group produces tricholin, a ribosome-inactivating protein, which is active against *R. solani. Gliocladium* sp. also produces different enzyme inhibitors such as argifin, a potent chitinase inhibitor (Omura et al 2000). Interestingly argifin also retards the moulting process in cockroach larvae that again can be attributed to the inhibition of insect chitinase activity. Nevertheless, antifungal antibiotic production does not sufficiently explain the process of mycoparasitism. Graeme-Cook and Faull (1991) using UV-induced mutants of *T. harzianum* with enhanced antibiotic potential suggested that the activity can not be directly correlated with its potential as a mycoparasitic organism. In other words, the biocontrol efficacy of the mycoparasitic strains is the combination of antibiosis, lysis, competition, mycoparasitism and promotion of plant growth.

1.4.1.4 Microbial enzymes and enzyme inhibitors as biocontrol agents

In many plant species, local invasion of the pathogen induces production of PR-proteins like chitinases, β-1,3- glucanases, proteinases (Kombrink and Somssich, 1995). As pathogenic fungi and insects contain chitin in their protective covers, induction of

chitinases in plants is the main defense responsge. Most of these chitinases are induced in vegetative plant organs by infection but some are also present in seeds. Hadwiger and Beckman (1980) demonstrated that extracts of the pea endocarp contain chitinase and chitobiase activity.

Number of soil bacteria also produce chitinolytic enzymes. Bacteria, which secrete a complex of mycolytic enzymes, are considered to be possible biological control agents of plant diseases. *Bacillus* sp. is known as antagonists of many mycelial fungi (Dass and Teyegaga, 1996; Podile and Prakash, 1996; Seifert et al 1987). It is also known that *Bacillus* sp. strains are able to secrete a number of hydrolytic enzymes, including chitinases, laminarinases and cellulases (Bertagnolli et al 1996; Nielsen and Sorensen, 1997; Takayanagi et al 1991; Yabuki et al 1988). However, the antagonistic role of hydrolases of *Bacillus* against fungi is not yet understood. It is often supposed that chitinase and laminarinase are important for the antagonism but possibly also other hydrolases are important for the lysis of fungal cell walls (Nielsen and Sorensen, 1997).

Ordentlich et al (1988) attributed slower disease development by *S. rolfsii* on bean seeds in presence of *Serratia marcescens* to its high chitinase activity. Furthermore, Shapira et al (1989) cloned *S. marcescens* chitinase gene in *Escherichia coli* and the chitinase preparation obtained was found to be effective in reducing disease incidence caused by *S. rolfsii* in beans and *R. solani* in cotton under greenhouse conditions. *Streptomyces violaceusniger* was reported to be antagonistic to many plant pathogenic fungi (Trejo-Estrada et al 1998). The antagonistic activity was attributed to the production of enzymes like chitinase and glucanase, and antifungal compounds, such as AFA (Anti-*Fusarium* activity), nigericin and geldanamycin.

The mycoparasitic and entomopathogenic fungi produce chitinases for invasion and as one of the host killing components (Higuchi et al 1998; Kang et al 1998;

Mathivanan et al 1998). Chitinolytic enzymes of *T. harzianum,* a most studied mycoparasitic fungus, were found to be inhibitory to a wider range of deleterious fungi than similar enzymes from other sources (Lorito et al 1993). While mycoparasitism of pathogenic fungi involved volatile/ non-volatile antibiotics and hydrolytic enzymes such as glucanases, proteinases and chitinases (Ridout et al 1988). For instance, *T. harzianum* was reported to produce mainly fungal cell wall degrading enzymes, which degraded the cell wall of a root pathogen *S. rolfsii* (Elad et al 1984). It has been reported that in a fungus-insect interactions production of cuticle-degrading enzymes such as endo-chitinase, chitin deacetylase, chitosanase, alkaline protease and lipase, play a significant role (Chavan et al 2006; Nahar et al 2004a; 2008; St. Leger et al 1986a). Recently, it was reported by Shakeri and Foster (2007) that the entomopathogenicity of a mycoparasitic *T. harzianum* towards *T. molitor* larvae was due to enzymes such as chitinase and protease as well as the antibiotic, peptaibol. In addition to above role, Nahar et al (2004a) suggested that chitin deacetylase from *M. anisopliae* may have a dual role in modifying the insect cuticular chitin for easy penetration as well as for altering its own cell walls for defence from insect chitinase. Several studies utilizing *B. bassiana*, *N. rileyi* and other fungi suggested that virulence towards insect was correlated, at least in part, with chitinase activity (El-Sayed et al 1989; 1993; St. Leger et al 1996). The studies by Binod et al (2007b) showed that the culture filtrate containing chitinase from *T. harzianum* was capable of negatively affecting growth and metamorphosis of *Heliothis* larvae, while St. Leger et al (1986b) reported that extracellular proteases and chitinases produced by *M. anisopliae, M. flavoviride* and *B. bassiana* were important for insect penetration.

Several microbial chitinases, with β-1,3-glucanase, have been formulated with propane -2-ol and polyoxyethylene lauryl ether and sprayed on rice fields to control rice blight caused by *Pyricularia oryzae* (Tanaka et al 1970). Microbial chitinases have

attracted attention as one of the potential candidates in biological control, and especially as synergizers of *B. thuringiensis* strains during biopesticidal action. Crude chitinase preparations from Bt enhanced the insecticidal activity of Bt against the spruce budworm and *P. xylostella* (Brar et al 2008; Smirnoff, 1973; 1974). Chitinase was also used under field conditions for the control of *Didymella applanata,* a fungal pathogen of raspberry midge blight in Siberia (Shternshis et al 2002a). The cell wall degrading enzymes produced by *Trichoderma* sp. increased the efficacy of bacterium, *Pseudomonas* sp., which produced syringomycins against *B. cinerea* and other phytopathogenic fungi. A combined effect of mycolytic enzyme mixture of *M. verrucaria* and the cell wall chitin metabolism inhibitors to control the growth of a root infecting fungus, *S. rolfsii* on peanut was reported by Patil et al (2001). It has also been seen that both first (I) and fourth (IV) instar larvae of a mosquito, *Aedes aegypti*, can be killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa et al 1996).

1.5 Production of chitinolytic enzymes

Microbial production of chitinase has captured the worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also for the lacuna of an effective production method. Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation and fed-batch fermentation. Generally, chitinase produced by microorganisms is inducible in nature (Dahiya et al 2006). Entomopathogenic fungi in submerged fermentation produce extracellularly proteases, lipases and chitinases when locust cuticle is used as a sole carbon source (St. Leger, 1986a). Most of the fermentations with the biocontrol organisms include chitin as the inducer to increase the levels of chitinases. (Vyas and Deshpande, 1989; Domnas and Warner, 1991) Chitin also supported production of other cell wall-degrading enzymes such as protease, β-1,3-glucanase and β-1,6-glucanase in *T.*

harzianum and *M. verrucaria* (Deshpande, 1999). Most of the cell wall degrading enzymes, including endo-β-1,6 and β-1,3-glucanases in *T. harzianum,* have been found to be produced on the autoclaved mycelia of *B. cinerea* when used as a sole carbon source (De La et al 1995). Bidochka and Khachatourians (1988) reported that *B. bassiana* strain required gelatin for protease production and on glucose medium it did not produce any proteolytic activity. However, some strains of *B. bassiana* produced proteases when grown on glucose medium (Gupta et al 1992; Samuels and Paterson, 1995). Similar to glucose, *N*-acetylglucosamine also regulated the chitinase and protease production (Deshpande, 1999). The chitinolytic enzyme production in fermenters by different bacteria and fungi is given in **Table 1.4.**

Several microorganisms, including bacteria such as *Bacillus licheniformis* (Takayangi et al 1991), *Bacillus pabuli* (Frandberg and Schnurer, 1994), *B. thuringiensis* (Thamthiankul et al 2001), *S. marcescens* (Young et al 1985a; 1985b), *Nocardia orientails* (Usui et al 1984), *Streptomyces cinereoruber* (Tagawa and Okazaki, 1991), *Streptomyces lydicuis* (Mahadevan and Crawford, 1997), *Vibrio alginolyticus* (Ohishi et al 1996) and many species of fungi such as: *M. verucaria* (Vyas and Deshpande, 1989), *Stachybotrys elengans* (Tweddell et al 1994), *T. harzianum* (Felse and Panda, 1999), *T. viride* (Rogalski et al 1997), *V. lecanii* (Fenice et al 1998b) were reported to produce high levels of extracellular chitinolytic enzymes.

Table 1.4 Brief summary of chitinolytic enzyme production in fermenter

Microorganisms	Fermentation condition	Substrate	Reference
Bacteria			
Paenibacillus sp.	Volume: 3 L, pH = 6.4 Temperature: 34.5°C Aeration: 3 vvm Agitation: 200 rpm Fermentation time: 72 h	Crab shell chitin	Kao et al 2007
Pantoea dispersa	Volume: 5 L, pH = 6.4 Temperature: 30°C Airflow: 0.5 vvm Agitation: 300 rpm Fermentation time: 88 h	Crab shell chitin	Gohel et al 2007
Bacillus lichiniformis	Volume: 3 L, pH = 7.0 Temperature: 50 °C Airflow: 1 vvm Fermentation time: 48 h	Colloidal chitin	Takayanagi et al 1991
Nocardia orientalis	Volume: 1 L, pH= 5.0 Temperature: 28°C Fermentation time: 96 h	Colloidal chitin	Usui et al 1984
Serratia marcesscens	Volume: 0.75 L, pH= 8.0 Temperature: 30°C Fermentation time: 100 h	Chitin and glucose	Young et al 1985a, 1985b
Serratia marcesscens	Volume: 6 L, pH= 8.5 Temperature: 30°C Airflow: 1 vvm	Purified chitin	Khoury et al 1997
Streptomyces cinereoruber	Volume: 20 L, pH= 6.8 Temperature: 30°C Fermentation time: 96 h Airflow: 1 vvm Agitation: 500 rpm	*Aspergillus niger* cell wall	Tagawa and Okazaki, 1991
Vibrio alginolyticus	Volume: 20 l, pH= 7.0 Temperature: 37°C	Squid chitin	Ohishi et al 1996

Table 1.4 Brief summary of chitinolytic enzyme production in fermenter

Microorganisms	Fermentation condition	Substrate	Reference
Fungi			
Trichoderma viride	Volume: 5 L, pH= 7.0-8.0 Temperature: 40°C	Chitin	Rogalski et al 1997
Trichoderma harzanium	Volume 2 L, pH= 4.9 Temperature: 30°C Fermentation time: 120 h Airflow: 1.5 vvm Agitation: 224 rpm	Chitin	Felse and Panda, 1999; 2000a
Verticillium lecanii	Volume 3 L, pH= 4.0 Temperature: 24°C Fermentation time: 144 h Airflow: 0.6 vvm Agitation: 150 rpm	Chitin	Liu et al 2003
Talaromyces emersonii	Volume: 8 L, pH= 5.0 Temperature: 45°C Fermentation time: 240 h Airflow: 1 vvm Agitation: 200 rpm	Chitin	McCormack et al 1991

Solid-state fermentation (SSF) has emerged as an appropriate technology for the management of agro-industrial residues and production of industrial enzymes on large-scale (Nampoothiri et al 2004). Most of the studies on chitinase production have been carried out in submerged fermentation (SmF), although some attempts have been made in more recent times on solid-state fermentation. SSF offers several economic advantages over conventional SmF such as use of simple growth and production media comprising agro-industrial residues, simple processes, use of low volume equipment (lower cost), and are yet effective by providing high product titres (Binod et al 2007a; Viniegra-Gonzalez et al 2003; Lonsane et al 1985). Among the various groups of microorganisms used in SSF, the filamentous fungi are the most widely exploited because of their ability to produce a wide range of extracellular enzymes. SSF offers a cost effective technique

for the production of chitinase (Pandey et al 1999; Sandhya et al 2005a). Several reports reveal that fungi are excellent producers of extracellular chitinases, mainly species of genera *Aspergillus* and *Penicillium* (Binod et al 2005; 2007a).

Different types of substrates, which contain chitin, have been tried for the production of chitinase, which included fungal cell walls, crab and shrimp shells (Tagawa and Okazaki, 1991; Wang and Chang, 1997; Casio et al 1982). The use of *Trichoderma* sp. in SSF for the production of lytic enzymes such as cellulase and chitinase has tremendous impact for an industrial scale production (Felse and Panda, 2000b; Chahal, 1985; Sim and Oh, 1990).

1.6 Industrially important issues for biocontrol agents

1.6.1 Downstream processing

In order to become an efficient commercial product, a biocontrol agent must withstand post fermentation processing. The main objective of a good harvesting technique is to minimize number of unit operations involved in the process, reducing overall process and validation costs. This is a key issue for the development of any biopesticide. Further, it should be stable during storage and retain its activity under field conditions. The main unit operations involved as well as critical condition affecting stability of biocontrol agent is as follows (**Table 1.5**).

1.6.2 Formulation

The problem of stability of biopesticides both during storage and after application have stalled biopesticide development to a great extent. At this juncture, formulation development can play a key role addressing four major objectives which can serve as benchmarks for success: (1) stabilize bioagent during distribution and storage (2) aid handling and application of the product (3) protect agent from adverse environmental factors (4) enhance activity of bioagents in field. Principally, a formulation comprises of

an active ingredient (fungi, bacteria, virus or microbial product) and additives to give shape to biopesticides. Commercial biopesticides must be economical to produce, have persistent storage stability, high residual activity, be easy to handle, mix, and apply, and provide consistently effective control of the target pest (Brar et al 2006). Hence, formulation, a mandatory prerequisite for all biopesticides, bridges fermentation and field application.

Table 1.5 Main post fermentation processing steps for a commercial biocontrol agent

Product goal	Unit operations involved	Factors affecting stability
Separation	Centrifugation, filtration pumping, milling	Shear, retention time, heat
Stabilization	Concentration, granulation drying, encapsulation	Heat, shear, mixing osmotic stress
Shelf-life	Formulation, packaging storage	Temperature, time moisture
Survival	Dispersion, adhesion	Environmental stresses (temperature, UV, soil constituents etc)

The characteristics and composition of biopesticidal formulations vary with type of habitat (foliage, soil, water), pathogen (type, characteristics, regeneration mechanism), rheology of technical material (viscosity, particle size, density), insect species (feeding habits, feeding niche, life cycle), mode of action (oral/contact), host-pathogen environment interactions (behavioral changes; resistance; stability), mode of application (aerial; land) and application rate (L/ha and kg/ha).

1.6.2.1 Adjuvants/additives

Adjuvants are chemically and biologically active compounds that can alter the formulation physics and kill the targeted species without harming other insects (i.e. enhance its selectivity) and reduce the effective biopesticide dose required (Rodham et al 1999). Adjuvants may be added to a biocontrol agent to enhance activity by: (1) prolonging water retention to overcome dew period requirements, (2) adding nutrients to maintain fungal viability and stimulate spore germination, penetration and infection, (3) modifying leaf wettability to improve spore deposition and retention on sprayed leaves and (4) mixing with proper fillers for extended shelf-life (Zhang et al 2003). The term adjuvant includes a wide range of compounds such as surfactants, stickers, inert carriers, anti-freezing compounds, humectants, sunscreen agents, anti-evaporation agents and micronutrients (Prasad, 1993). Details of different adjuvant/ additives used in biopesticide formulations with their functions and examples are illustrated in **Table 1.6.** An adjuvant may have one or more functions. For example, the Tween and Tergitol NP series are primarily surfactants that reduce surface tension and increase leaf-surface wettability and fungal distribution, whereas gelatin and sorbitol have adhesive, humectant and some nutritional properties. Emulsifiers, stickers, spreaders and other adjuvants and additives aid in application, dispersal and adhesion of the microbes on plant surfaces and protect the microbes from adverse environmental conditions, such as desiccation, unfavorable pH and UV radiation (Shieh, 1995).

Table 1.6 Different types of adjuvants/additives used in biopesticide formulations (Compiled from Brar et al 2006)

Adjuvants/additives	Function(s)	Example(s)
Dispersant	Dispersion of formulation into dispersant medium	Amylose, Aluminium silicate, Sodium starch glycolate
Surfactants and wetters	Enhance the emulsifying, dispersing, spreading, sticking or wetting properties of the biopesticide (includes spray modifiers)	Ethoxylates (Tween/Triton series), polyethylene glycol
Stickers and spreaders	Adhesion of pesticides onto the foliage protecting from rain wash-off and spreading evenly for maximum coverage	Gelatin; gums, molasses, vegetable gels, skimmed milk, vegetable oils, waxes, water-soluble polymers
Drift control agents/anti-evaporants/ humectant	Reduce spray drift, which most often results when fine (<50 μm diameter) spray droplets are carried away from the target area by breezes, including those caused by the vehicle carrying the spray equipment and control of foam while mixing	Glycerol, Polysaccharides, sorbitol, sucrose, molasses, polyglycol, and certain types of gums
Thickening agents	Modify the viscosity of spray solutions and are used to reduce drift, particularly for aerial applications	Water swellable polymers producing a particulate solution hydroxyethyl celluloses and polysaccharide gums
pH Buffers	Improve the dispersion or solubilization in the formulation, control its ionic state and increase adjuvant compatibility	Sodium phosphate Potassium phosphate
UV radiation screens	Protect from the deleterious effect(s) of sunlight by forming a protective layer on the formulations	Congo red, folic acid, lignin, molasses

Table 1.6 Different types of adjuvants/additives used in biopesticide formulations (Compiled from Brar et al 2006)

Adjuvants/additives	Function(s)	Example(s)
Synergists	Multiple modes of action; generally complements various formulation components	Sorbitol, sorbic acid, Tinopal sodium phosphate, silicate, protease inhibitors, oleic acid, linoleic acid
Anti-microbial agents	Suppresses the growth of other microorganisms, retaining formulation purity	Sorbic acid, propionic acid, crystal violet
Carriers	Aid in delivery of formulation to target	Alginate, carrageenan, peat, diatomaceous earth
Binders	For binding the particulates in granules together	Gums, molasses, PVP, resins
Suspending agents	Keep the formulation in suspension	Sorbitol, soya polysaccharides, starch glycolates, sucrose
Attractants	Act as baits to attract target pests	Pheromones, cucurbitacin and various alkaloids, plastisol (PVC and cotton seed oil)

1.6.3 Shelf-life

Shelf-life is one of the major concerns of biopesticide industry due to its inevitable comparison with the chemical industry. It was observed that shelf life of biocontrol formulations containing living propagules of fungi vary depending upon the strain as well as the type of fungal propagule used in the formulation. Ideally stability for 18 months at 20°C, is suitable for the further commercialization (Couch and Ignoffo, 1981; Deshpande, 2005).

In case of fungal enzymes stability that need to be addressed depend on the mode of application. For instance, in case of foliar application the effect of temperature, pH and sunlight (UV) exposure is important. The activity of an enzyme in soil depends on intra- and extracllular enzymes. The intracellular enzymes associated with living cells are more stable as compared to dormant cells and cell debris. It was also reported that free enzymes are less stable as compared to adsorbed/immobilized enzymes (Burns, 1982).

1.6.4 Application

Application of biocontrol agent must be easy, economical, effective, timely to the appropriate site of action and compatible with current agronomic practices as well as equipments. Biocontrol products can be applied to plant roots, wounds and foliage by drenchmg, dipping, or spraying. A broad range of spray application equipment and techniques is available for applying chemical pesticides, including high volume (1000 L/ha), medium volume (350 L/ha), low to very low volume (3 L-50 L/ha), ultra low volumes (0.5 L-3 L/ha), controlled droplet application and electrostatic spraying (Auld, 1992). If biocontrol agents are to be applied using the same techniques, formulations must have the necessary physical properties. Steinke and Akesson (1993) found that surface tension and viscosity of the suspension to be sprayed are important factors in reducing droplet size and maintaining the necessary dispersion and control of droplets.

Density of the suspension was not an important factor. Successful application of biocontrol agents using different spray techniques has been achieved. For example, Bt-based products have been applied to numerous crops using conventional ground or aerial spraying methods. Highly concentrated ultra-low volume liquid formulations of Bt-based products have also been used to control insect pests on such crops as cotton and banana (Shieh, 1995) and to control spruce bud worm over large areas of coniferous forests (Bryant, 1994; Shieh, 1995). A low-volume electrostatic rotary atomizer has been used to apply *V. lecanii* to successfully control the aphid *Aphis gossypii*. In addition, ultra low volume equipment, such as spinning disk sprayers, is commonly used for application of baculoviruses in forests (Cory and Bishop, 1995). Nahar et al (2003; 2004b) used ultra low volume sprayer for application of *M. anisopliae* for the control of *H. armigera* in chickpea and pigeon pea. Recently, Kulkarni et al (2008) reported knapsack sprayer for the application of *M. anisopliae* for the control of *H. armigera* in chickpea.

Shternshis et al (2002b) applied the test composition containing *Mamestra brassicae* NPV (MbNPV) (1×10^7 polyhedra/ml), *B. thuringiensis galleriae* (2.5×10^7 spores/ml) and chitinase (0.5 mU/ml) at an application rate of 300 L/ha using hand sprayer for the control of lepidopteran cabbage pests, *Mamestra brassicae*, *P. xylostella* and *Pieris brassicae.* While Patil et al (2001) used *M. verrucaria* enzyme preparation containing chitinase, β-1,3-glucanase and protease for soil irrigation to control *S. rolfsii* a root pathogen of peanut. Recently, Binod et al (2007b) showed that the culture filtrate containing chitinase from *T. harzianum* is capable of negatively affecting the growth and metamorphosis of *H. armigera* larvae. In this case the feeding and topical (applied on the thorax back of the larvae) application of chitinases were tested for *H. armigera* control.

1.6.5 Risk assessment

Biocontrol agents may represent an acceptable and effective means of disease management as they may control pathogens and pests resistant to chemical pesticides and reduce the possibility of development of further resistance. However, biocontrol agents may also pose risks to the non-target organisms. Brimmer and Boland (2003) and Cook et al (1996) reviewed the potential non-target effects of fungal biocontrol agents including allergenicity, toxicity and pathogenecity. Finally, the potential of a biocontrol agent to infect organisms other than the target pests is of major concern.

The assessment of risk posed by the secreted metabolites is one of the major hurdles in the registration and subsequent commercialization of fungal biocontrol agents (Strasser et al 2000). There is a concern that toxic fungal metabolites may enter the food chain and pose a risk to humans and animals. Usually, the detailed information on the pathological and toxicological risks of biocontrol agents to man and other non-target organisms is provided but most often with assays being conducted under unnatural conditions. Strasser et al (2000) emphasized the need to develop the tools and methodologies that will identify and detect fate of toxic fungal metabolites in the environment.

1.6.6 Market status of biocontrol agents for global agricultural use

Biopesticide usage is increasing, from 1.4% in 1995 (Gaugler, 1997) to 2.9% in 2001 (Hynes and Boyetchko, 2006) and sales of synthetic pesticides are decreasing. The worldwide sale of synthetic pesticide was reduced by 12% from the previous 5 years (Hynes and Boyetchko, 2006). This reduction may in part be due to the loss of product effectiveness because of development of resistance by pest populations to synthetic pesticides and/or a desire to replace existing chemicals with ones that have greater target specificity, but otherwise are environmentally benign and display lower mammalian

toxicity. Current sales of biopesticides are approximately $350-400 million, much less than the predictions of 10% of synthetic pesticide sales made in the early 1980's (Cross and Polonenko, 1996; Gaugler, 1997). This percentage is expected to grow to around 4.2 % by 2010 (Thakore, 2006). However, in defense, this estimation was made before the complexities and implications of fermentation, formulation, application and regulatory challenges were fully understood. Earlier Uri (1999) indicated that the growth of biopesticide use is difficult to predict because of regulatory procedures and unknown biopesticide production costs relative to synthetic pesticides. Nevertheless, research on biopesticides by public and private sectors continues to be supported for the potential of this technology which can offer crop protection.

1.6.7 Patenting, registration and commercialization

Before the commercial exploitation of a microbial pesticide, its legal protection as a biotechnological invention can be assured by means of a patent. A patent is a temporary privilege for industrial or commercial exploitation given by the administration to the owner for 20 years after the application date, according to a series of claims. Biopesticide patents are considered biotechnological inventions because they include microbial products and processes. However, a patent is not an authorization for commercial use (Montesinos, 2003).

In India there is registration committee constituted under the Insecticides Act 1968. Every microbial pesticide, which is used, distributed, sold needs official registration of the product with the Central Insecticide Board. The regulatory requirements for microbial pesticides were simplified to promote their fast commercialization but with maintenance of quality standards (Baptiwale, 2004). The registration depends on specific rules within each country. In the United States, the register is authorized by theOffice of Pesticide Programs in the EPA. In the European

Union, the register is kept by the Directorate of the Consumer Health Protection and is regulated by Directive 91/414/CEE, which was been amended specifically for biopesticides by Directive 2001/36/EC (Montesinos, 2003).

1.7 Future considerations for fungal enzymes as a biocontrol agent

The plant-pathogenic fungi and insects share a common structural component, chitin, as well as some of the insects like woolly aphids, mealy bugs and white flies secrete woolly mass as their protective covers. Therefore, the fungal enzymes consisting mainly of lipase, chitinase, protease, β-1,3-glucanase can prove their potential in controlling pathogenic pest. Similarly, the seed dressing or soil application of microorganisms producing these enzymes extracellularly and/or their soil application may contribute significantly in controlling root infecting pathogens.

The progress in biotechnology of chitinase research is truly remarkable and attracting worldwide attention. Chitinases have wide applications in food, pharmaceuticals, agricultural and as fine chemicals for academics. Some of these applications prefer one or two selected components of chitinases while others require mixtures of chitinases and other enzymes for maximum benefit. With the advancement in biotechnology especially in the area of metagenomics, proteomics, bioinformatics and the availability of sequence data have opened a new era of enzyme applications in many industrial processes and has led to speculation and anticipation of their enormous commercial potential in biotechnology and research.

1.8 Present investigations

Objectives of the present investigation are as follows

1. Production of chitinolytic enzyme complex using *Myrothecium verrucaria*
    a. Optimization of submerged fermentation
    b. Optimization of solid state fermentation
2. Biochemical characteristics and stability of chitinolytic enzyme complex of *Myrothecium verrucaria*
3. Proof of concept

    Field performance studies of chitinases and other enzymes of *Myrothecium verrucaria* either singly or in combination for the biocontrol of insect pests as well as plant pathogenic fungi

Chapter 2

Materials and Methods

2.1 Materials

Table 2.1 The materials and chemicals used in the present studies

Chemicals, Materials	Source
N-Acetylglucosamine (GlcNAc), bovine serum albumin, chitin, chitosan, ethylene glycol chitin, glycol chitosan, glucosamine, gum arabic, laminarin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, tyrosine, lipase, xylitol, dialysis tubings, 3-methyl -2- benzothiazoline hydrazone (MBTH)	Sigma Chemical Co., USA
Ultra filtration membrane, Whatmann filter paper No 1.	Milipore Molsheim, France

All other chemicals used were of analytical grade, procured from s.d.fine-Chem Ltd., India; Sisco Research Laboratoies Ltd., India; Hi-media, India; Loba Chemie, India

2.2 Organisms

The organisms used during the course of this work are listed in Table 2.2.

Table 2.2 List of organisms used

Organism(s)	Purpose
Myrothecium verrucaria	Chitinase production
Fusarium oxysporum, Sclerotium rolfsii, Capnodium sp.	Plant pathogens
Metarhizium anisopliae	Entomopathogenic fungus
Bacillus sp. B1	Antagonist

2.3 Media

The media used during the course of this work are listed in Table 2.3

Table 2.3 Media

Name	Constituents (g/L)
Potato dextrose agar (PDA)	Potato, 200; glucose, 20; agar, 20; pH, 5.5-6.0.
Yeast extract peptone glucose medium (YPG)	Yeast extract, 3.0; peptone, 5.0; glucose, 10.0; pH, 5.0-5.5.
Chitin medium	KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract; 0.5; bacto-peptone, 0.5; chitin, 5.0; trace metal solution, 1ml of (mg/ml): $FeSO_4$, 5.0; $MnSO_4$, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0; pH, 6.0.
Medium for blastospore production	Sucrose, 20.0; $(NH_4)_2SO_4$, 4.62; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CaCl_2$, 0.5; pH, 4.0.
Nutrient agar	Beef extract, 3.0; NaCl, 5.0; peptone, 5.0; agar, 20.0; pH, 7.0.

2.4 Maintenance

M. verrucaria (MTCC 5191), *F. oxysporum, S. rolfsii* NCIM 1084, *M. anisopliae* (MTCC 5190) and *Capnodium* sp. were maintained on potato dextrose agar slants. *Bacillus* sp. B1 was maintained on nutrient agar. The fungal cultures were incubated at 28°C with 70-80% RH for 7 d, while *Bacillus* sp. B1 was incubated at 28°C for 4 d. All the isolates were routinely subcultured after 1 month. The mother cultures were maintained at 8°C until used.

2.5 Production of chitinolytic enzyme using submerged fermentation

2.5.1 Shake flask studies

M. verrucaria, M. anisopliae and *Bacillus* sp. B1 were grown in a medium containing chitin (0.5 %) as a carbon source for chitinolytic (chitinase, endochitinase, *N*-acetylglucosaminidase (NAGase), chitin deacetylase, chitosanase) and other enzyme (lipase, protease and β-1,3-glucanase) production as described earlier (Vyas and

Deshpande, 1989). The flasks were incubated at 28°C for 7 d for *M. verrucaria* while *M. anisopliae* and *Bacillus* sp. B1 were incubated at 28°C for 4 d. The conidial inocula for *M. verrucaria* and *M. anisopliae* was 1×10^7 conidia/100 ml medium and for *Bacillus* the inoculum was 1×10^7 cfu/100 ml medium. Effect of different inocula such as mycelia and blastospore on chitinase and other enzyme production was also studied. The mycelia (10% v/v) and blastospores (1×10^7) were inoculated independently in 100 ml chitin medium.

The effect of different chitin sources such as purified chitin (Sigma Chemical Co. USA), practical grade chitin (Hi-Media, India) and crude chitin (Shree Agro Products, India) on enzyme production was also studied. All the experiments were carried out in 500 ml flasks containing 100 ml medium. Enzyme production was carried out in three different flasks and enzyme assays were carried out in triplicate for each flask. The experiment was conducted three times on different occasions using freshly prepared medium. The culture supernatants were collected by centrifugation at 5000×g for 10 min. The culture filtrate was lyophilized in dry powder form and stored at -20°C until use.

2.5.2 Fermenter studies

2.5.2.1 Effect of different airflow and agitation on chitinase and other enzyme production using *M. verrucaria*

The experiments were carried out in a lab scale 10 L fermenter (Navin Process Systems Ltd, Pune) with two ruston type impeller of 75 mm diameter. In the initial phase, fermenter was operated to optimize airflow and agitation for the production of enzymes by *M. verrucaria*. The effect of airflow (0.5, 1.0 and 1.5 vvm) was investigated for the production of enzymes in 10 L fermenter. Enzyme production was studied with

increasing agitation (from 1st to 5th d, respectively; 100, 120, 150, 180 and 200 rpm) and increasing airflow (from 1st to 5th d, respectively; 0.2, 0.2, 0.3, 0.4, 0.5 vvm). The enzyme production under constant agitation (100, 200 or 300 rpm) conditions with 0.5 vvm airflow was also studied.

The enzyme production was carried out in a chitin medium at 28°C for 5 d. The *M. verrucaria* mycelial inoculum was developed in a chitin medium by inoculating 1×10^7 conidia/100 ml medium. The inoculum flask was incubated at 28°C for 3 d. The 10% mycelial inoculum (v/v) was used for the enzyme production in fermenter. The fermentations were carried out at 28°C for 5 d. The effect of different chitin (purified chitin, practical grade chitin and crude chitin) on enzyme production was also studied in 10 L fermenter under optimized airflow and agitation.

The scale-up study for enzyme production was carried out in 100 L fermenter (at Hi-Tech Bio Sciences, Pune, India) with two ruston type impeller of 108 mm diameter. Enzyme production was studied with increasing agitation (from 1st to 5th d, respectively; 60, 60, 70, 80, 80 rpm) and increasing airflow (from 1st to 5th d, respectively; 0.2, 0.2, 0.3, 0.4, 0.5 vvm). Crude chitin was used as a substrate for enzyme production. The fermentations were carried out at 28°C for 5 d.

2.6 Optimization of solid state fermentation

2.6.1 Growth conditions

Sun-dried sorghum and crude chitin (3:1) mixture was used as substrate for solid state fermentation. The 25 g substrate in 250 ml flask was soaked overnight with 12.5 ml distilled water at 4°C. The flask containing soaked substrate was sterilized at 15 psi pressure for 45 min. After cooling, the substrate was inoculated with mycelial inoculum (2.5 ml) developed in chitin medium. The fermentation was carried out at 28°C for 7 d. After fermentation the enzyme was extracted using 1% NaCl from the

substrate. The enzyme from 25 g substrate was extracted with 125 ml NaCl at 180 rpm for 30 min. After extraction, the enzyme was separated by centrifugation at 10,000 × g for 10 min. The culture filtrate was lyophilized to the dryness and stored at -20°C until used.

2.7 Downstream processing of chitinolytic enzyme complex of *M. verrucaria*

Attempts were made to concentrate cell free broth by ultrafiltration using hollow fiber polysulfone membrane of 5 KD cut off. Other methods include ammonium sulphate (90 %) precipitation, spray drying using Buchi 190 Mini Spray Dryer and freeze drying using DW6 Freeze Dryer (Heto Holten Lab Equipment, Denmark). The concentrated enzyme preparations were stored at -20°C until use.

2.8 Biochemical characteristics and stability of chitinolytic enzyme complex of *M. verrucaria*

2.8.1 Optimum temperature and temperature stability

The optimum temperature of chitinases (chitinase, endochitinase, NAGase) and other enzymes (lipase, β-1,3-glucanase and protease) of *M. verrucaria* was determined by estimating the enzyme activity at different temperatures (30, 37, 45, 50, 55, 60, 70°C). The reduction of chitinase and other enzyme activity was measured by incubating the enzyme in acetate buffer (pH 5.0, 50 mM) at 4°C, 25°C and 40°C. The effect of different polyols (ethylene glycol, glycerol, xylitol and sorbitol; 1M-5M) on the temperature stability was also studied. At regular intervals, aliquots of the enzyme were removed and the residual enzyme activities in the samples were then estimated at 50°C for chitinase and glucanase while protease and lipase at 37°C. The enzymes without polyols were used as control.

2.8.2 Optimum pH and pH stability

The optimum pH of chitinase and other enzymes was measured by estimating the enzyme activity at different pH (pH 4.0 - 10.0). The pH stability of the enzymes was measured by incubating crude enzyme mixture at 25°C for 7 d in a buffer of desired pH. The following buffer systems were used: acetate buffer (pH 4.0-5.0, 50 mM), phosphate buffer (pH 6.0-7.0, 50 mM), Tris-HCl buffer (pH 8, 50 mM) and carbonate bicarbonate buffer (pH 9.0-10.0, 50 mM). At regular intervals, aliquots of the enzyme were removed and the residual enzyme activities in the samples were then estimated at 50°C for chitinase and glucanase while protease and lipase activities at 37°C.

2.8.3 Light (UV) stability

The crude culture filtrate of *M. verrucaria* containing chitinases and other enzymes in glass petriplate was irradiated with 12- W electric UV lamp from 20 cm distance at 25°C for 5 h. The enzyme sample kept in glass plate with lid at same distance was used as control. After every hour aliquots of the enzymes were removed.

The cumulitive effect of sunlight and temperature on the stability of chitinase and other enzymes of *M. verrucaria* was also studied in presence and absence of glycerol (1M) for 7 d. The enzyme samples incubated in dark at RT and at 4°C were served as controls. The residual chitinase, endochitinase, NAGase and β-1,3-glucanase activities in the samples were then estimated at 50°C, while protease and lipase at 37°C.

2.8.4 Adsorption and stability of enzymes in presence of soil constituents

During the pot experiment studies, a pot containing soil and peanut seeds (2 no.) was drenched with *M. verrucaria* enzyme mixture. The 100 ml enzyme preparation was drenched/pot (containing chitinase 50 U, β-1,3-glucanase 7 U and protease 2 U) and the adsorption of enzymes on soil organic matter was determined. The soil samples (10g) were taken from different portions of the pot (top 1-3 cm, middle 4-6 cm and bottom 7-9

cm) and enzyme activities were estimated by repeated washing (2-3 times) of the soil with acetate buffer (pH 5.0, 50 mM). In case of stability of enzymes, the 10 g soil samples were removed every day from top, middle and bottom of the pot. The residual chitinase and glucanase activities in the samples were then estimated at 50°C, while protease at 37°C.

2.9 Germination of conidia of *Capnodium* sp.

The effect of hydrolytic enzymes of *M. verrucaria, M. anisopliae* and *Bacillus* sp. B1 on conidial germination of sooty mould (*Capnodium* sp.) was studied. To 1ml *Capnodium* sp. conidial suspension (1×10^7 conidia/ml), 1ml of crude culture filtrate (filter sterilized) from *M. verrucaria, M. anisopliae* and *Bacillus* sp. grown in chitin medium was added and kept at 25°C for 1 h. The % viability was studied by the dilution plate method using PDA in triplicate. The plates were incubated at 28±2°C and 70-80% RH for 24 h. The counting of germ tubes was carried out randomly in 10 different fields. The experiment was conducted 3 times. The acetate buffer (pH 5.0, 50 mM) was used as a control.

2.10 Enzyme assays

2.10.1 Chitinase assay

Total chitinase activity was estimated using acid-swollen chitin as a substrate, as described earlier (Kulkarni et al 2008). To prepare acid-swollen chitin, 10 g chitin (purified powder from crab shells, Sigma) was suspended in 300 ml *O*-phosphoric acid (88% w/v) and left at 4ºC for 1 h with occasional stirring. The mixture was poured into ice-cold distilled water (4L) and left for 30 min. The swollen chitin was repeatedly washed with ice-cold distilled water, followed by a wash with 1% (w/v) $NaHCO_3$ solution to adjust the pH to 7. The swollen chitin was then dialyzed at 4ºC against

distilled water. After homogenization in a Waring blender for 1 min, the concentration of acid swollen chitin was adjusted to 7 mg/ml by adding 50mM acetate buffer, pH 5.

The reaction mixture containing 1 ml 0.7% swollen chitin, 1 ml of acetate buffer (pH 5.0, 50 mM) and 1 ml of suitably diluted enzyme solution was incubated at 50ºC for 1h. The *N*-acetylglucosamine (GlcNAc) residues produced were estimated colorimetrically at 585 nm with p-dimethyl amino benzaldehyde (DMAB) as described by Reissig et al (1955). One international unit was defined as the activity which produced 1µmol of GlcNAc per min.

2.10.2 Endochitinase assay

Endochitinase activity was estimated using ethylene glycol chitin as a substrate, as described earlier (Vyas and Deshpande, 1989). The reaction mixture containing 0.5 ml 1% ethylene glycol chitin in acetate buffer (pH 5.0, 50 mM) and 0.5 ml of enzyme solution was incubated at 50ºC for 30 min. The *N*-acetylglucosamine (GlcNAc) residues produced were estimated colorimetrically at 540 nm by Somogyi method (1952). One international unit was defined as the activity which produced 1µmol of GlcNAc per min.

Endochitinase activity was also estimated using 4-methylumbelliferyl- N', N'', N'''-triacetylchitotrioside (4-MU-$(GlcNAc)_3$) as a substrate, as described earlier (Ghormade et al 2000; Nahar et al 2008). The reaction mixture containing 20 µl (4-MU-$(GlcNAc)_3$), 130 µl acetate buffer (pH 5.0. 50 mM) and 50 µl of enzyme solution was incubated at 50ºC for 30 min. The reaction was stopped by adding 2.3 ml of 0.2 M Na_2CO_3. The fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer, using excitation at 360 nm and emission at 445 nm. One international unit was defined as the activity which produced 1µmol of 4-methyl umbelliferone per min.

2.10.3 *N*-Acetylglucosaminidase assay

The *N*-acetylglucosaminidase activity was determined according to Nahar et al (2008). The activity was carried out by measuring the amount of *p*-nitrophenol released in a reaction mixture containing 0.9 ml of 1 mg/ml of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in acetate buffer (pH 5.0, 50 mM) and 0.1ml of enzyme, incubated at 50°C for 30 min. The reaction was terminated by adding 2 ml of 0.2 M Na_2CO_3. One international unit was defined as enzyme activity that produced 1μmol of *p*-nitrophenol per min.

The *N*-acetylglucosaminidase activity was also estimated using 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide (4-MU-GlcNAc) as a substrate (Ghormade et al 2000; Nahar et al 2008). The reaction mixture containing 20 μl (4-MU-GlcNAc), 130 μl acetate buffer (pH 5.0. 50 mM) and 50 μl of enzyme solution was incubated at 50ºC for 30 min. The reaction was terminated by adding 2.3 ml of 0.2 M Na_2CO_3. The fluorescence was measured in a Perkin-Elmer fluorescence spectrophotometer, using excitation at 360 nm and emission at 445 nm. One international unit was defined as the activity which produced 1μmol of 4-methyl umbelliferone per min.

2.10.4 Chitin deacetylase assay

Chitin deacetylase activity was measured according to Nahar et al (2004a), using acetylated ethylene glycol chitosan as a substrate, prepared according to the method of Araki and Ito (1975). For preparation of the substrate, ethylene glycol chitosan (40 mg) was treated at 4ºC with 400 mg of $NaHCO_3$ and 200 μmol of acetic anhydride in a total volume of 4.5 ml and kept at 4ºC. After 24 h, 200 μl of acetic anhydride was added and the mixture was allowed to stand for further 24 h at 4ºC. After thorough dialysis, the

product, acetylated ethylene glycol chitosan (1 mg/ml) was used as a substrate for the assay of CDA.

The assay for CDA was carried out according to Kauss and Bausch (1988) with 100 µl of sodium tetraborate buffer (50 mM, pH 8.5), 100 µl of 1 mg/ml acetylated ethylene glycol chitosan and 50 µl enzyme incubated at 37ºC for 30 min. The reaction was terminated with addition of 250 µl of 5% (w/v) $KHSO_4$. For color development, 250 µl of 5% (w/v) $NaNO_2$ was added and allowed to stand for 15 min, and then 250 µl of 12.5% (w/v) ammonium sulfamate ($N_2H_6SO_3$) was added. After 5 min, 250 µl freshly prepared 0.5% (w/v) 3-methyl-2-benzothiazoline hydrazone (MBTH) was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled under tap water and 250 µl of freshly prepared 0.5% (w/v) $FeCl_3$ was added and estimated spectrophotometrically at 650 nm. One unit of enzyme released 1 µmol of glucosamine from acetylated ethylene glycol chitosan per min

2.10.5 Chitosanase assay

Chitosanase activity was estimated using acid-swollen chitosan as a substrate (Kulkarni et al 2008). For preparation of acid-swollen chitosan, crystalline chitosan (Sigma chemical Co., USA) was swollen with 10N HCl. The pH of the swollen chitosan was adjusted to 7 with 1N NaOH. Repeated washing of swollen chitosan was carried out with ice-cold distilled water by centrifugation at 10,000 g for 10 min. The swollen chitosan was dialysed at 4ºC against distilled water. After homogenization in a Waring blender for 1 min, the concentration of swollen chitosan was adjusted to 10 mg/ml by adding acetate buffer (50 mM pH 5).

The assay mixture containing 1 ml acid-swollen chitosan (10 mg/ml), 1 ml of acetate buffer (50 mM pH 5) and 1 ml suitably diluted enzyme was incubated at 50ºC for 1 h. The amount of glucosamine produced was determined using the method of Good and

Bessman (1964). One unit of enzyme produced 1µmol of glucosamine equivalents per min.

2.10.6 Protease assay

Protease activity was measured using Hammerstein casein as a substrate (Kulkarni et al 2008). The reaction mixture contained 100 µl of suitably diluted enzyme solution, 1 ml casein (1%) and 0.9 ml carbonate-bicarbonate buffer, pH 9.7. Enzyme reaction was carried out at 37ºC for 20 min and terminated by the addition of 3 ml trichloroacetic acid (TCA) (2.6 ml of 5% TCA + 0.4 ml of 3.3N HCl). The absorbance of the TCA soluble fraction was measured at 280 nm. One unit of enzyme liberated 1 µmol of tyrosine per min.

2.10.7 β-1,3-Glucanase assay

The extracellular β-1,3-glucanase was estimated using laminarin as substrate (Vyas and Deshpande, 1989). An aliquot of 0.5 ml enzyme solution was mixed with 0.5 ml of 1% laminarin prepared in acetate buffer (50 mM pH 5) and incubated at 50ºC for 30 min. Reducing sugars were estimated as glucose equivalents as described by Somogyi (1952). One international unit was defined as the enzyme activity that produced 1 µmole of glucose per min

2.10.8 Lipase assay

Lipase activity was determined as described by Pignede et al (2000). The substrate emulsion was prepared with olive oil (20 ml) and gum arabic (165 ml, 10% w/v, Sigma). The reaction mixture contained 1ml enzyme, 5 ml substrate emulsion, and 2 ml of phosphate buffer (50 mM, pH 6.8) and was incubated at 37ºC for 1 h under shaking (80 rpm). The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty

acids released with 50 mM NaOH. One unit of lipase is the amount of enzyme that released 1 µmol of fatty acids per min.

2.10.9 Protein estimation

Protein was estimated according to Lowry et al (1951) using bovine serum albumin as a standard.

2.11 Hydrolysis of waxy coating of *Ceratovacuna lanigera*

The waxy mass present on *C. lanigera* was removed using soft brush from the cuticle. The separated waxy mass (1 g) was treated with *M. verrucaria* enzyme preparation (Lipase 50 U) at room temperature (25°C) for 24h. The volume of reaction mixture was adjusted to 10 ml with phosphate buffer (pH 6.8, 50 mM). The hydrolysed waxy mass was dissolved in 2:1 chloroform: methanol (150 ml). The reaction mixture was refluxed for 30 min and was filtered. The total lipids were analyzed by capillary gas chromatography-mass spectrometry (CGC-MS) on a Hewlett-Packard quadrupole system equipped with an auto injector and a temperature and pressure programmable cool on column injection port. The injection port was connected to a 1 m retention gap connected to a 12 m × 0.2 mm capillary column of cross-linked dimethyl silicone Ultra 1. The column was programmed from 150° to 320°C at 4°C/min and then held at 320°C for about 200 min. The mass spectra were interpreted as described by Nelson et al (1997). The similer protocol was used for cuticular wax analysis of *C. lanigera*.

2.12 NMR spectroscopy

The degree of acetylation of crude chitin, practical grade chitin and purified chitin (particle size > 0.2 mm) was determined by NMR (Nuclear Magnetic Resonance) spectroscopy. All the NMR spectra were taken on a Bruker DR×500 FT- NMR spectrophotometer operating at the Larmor frequency of 500 and 125 MHz for ^{13}C as described earlier (Rajamohanan et al 1996).

^{13}C Solid- state NMR experiments were carried out at ambient probe temperature (20 - 22°C), using a Hartmann-Hahn cross - polarization scheme at an applied field of 40 kHz, combined with Magic Angle Sample Spinning (CP/MASS). The optimum mixing time of 1ms and a 5 second recycle delay were used. The spinning speed was kept at 10 kHz in order to minimize the interfering effects of spinning side bands. Free induction decays were collected in a 50 - kHz spectral window and were apodized with a 20 - Hz line broadening to increase the S/N. The decays were analyzed by online software. The chemical shifts were referenced to an external sample of spinning adamantine, with its high frequency signal taken as 37.8 ppm with respect to TMS. Quadrature phase cycling was incorporated in the pulse sequence to eliminate baseline and intensity artifacts.

2.13 Proof of concept

2.13.1 Biocontrol of *C. lanigera*

2.13.1.1 Laboratory bioassay

Bioassay was carried out with 3rd instar *C. lanigera* (woolly aphid). In each set of experiments, sugarcane leaf discs (5cm×5cm) with 100 woolly aphids were used for treatment. The enzyme mixture of *M. verrucaria* measured as lipase activity (1-5 U/ml), was used for the bioassay. Similarly crude culture filtrates (lipase 1U/ml) of *M. anisopliae* and *Bacillus* sp. were used for the bioassay. The commercial lipase preparation (2U/ml, from *Candida rugosa*, Sigma Chemical Co. USA), *Bacillus* sp. B1 liquid culture (lipase 0.1 U + 1×10^7 cfu/ml) grown in chitin medium, *M. anisopliae* conidia singly (1×10^7 conidia/ml) and in combination with *M. verrucaria* enzyme preparation (lipase 1 U/ml) was also used for the bioassay study against woolly aphid. In case of combined treatment *M. verrucaria* enzyme preparation sprayed first and spraying of *M. anisopliae* conidia was carried out after 1 h. Spraying of all the treatments was carried out using an atomiser (hand-spraying device for laboratory bioassay). The

experiments were conducted in completely randomized block design (CRBD) with each treatment containing the set of 100 aphids repeated three times. The same experiments were repeated further two times. After treatment, sugarcane leaf discs were kept in sterile petri plates containing moist filter paper and were incubated at 25±2°C with 70-80% RH for 4 d. The mortality was recorded using a dissection microscope at 4x-64x magnification. The mortality was corrected over control by Abbott's (1925) formula.

2.13.1.2 Field experiments

Effect of enzyme preparation of *M. verrucaria,* liquid culture of *Bacillus* sp. B1, *M. anisopliae* conidia, singly and in combination with *M. verrucaria* enzyme preparation against *C. lanigera* in sugarcane (variety CO86032) fields was studied. The metasystox treatment and plots treated with water containing 0.1% Tween 80 were used as controls. Scientific trials were conducted at the Sugarcane Research Station, Mahatma Phule Agriculture University for the two successive years. The experimental layout was randomised complete block design (RCBD) having six treatments including control plots with three replications for each treatment. The plot size was 5m×5m.

The enzyme preparation of *M. verrucaria* (lipase 5U/ml), *M. anisopliae* conidia (5×10^{12} conidia/ha in water containing 0.1% (w/v) Tween 80), *Bacillus* sp. B1 (liquid culture grown in a chitin medium containing 5×10^{12} cfu/ha), metasystox (2ml/L) and combined effect of *M. verrucaria* enzyme preparation (Lipase 1U/ml) with *M. anisopliae* conidia (5×10^{12} conidia/ha in water containing 0.1% (w/v) Tween 80) were used for the control of *C. lanigera* in sugarcane. In case of combined treatment *M. verrucaria* enzyme preparation sprayed first and spraying of *M. anisopliae* conidia was carried out after 1 h. The dose for all the above treatments was 500 L/ha. All the treatments were sprayed with a hand operated knapsack sprayer. Spraying in control plots was carried out

with water containing 0.1% Tween 80. The spraying was carried out 2 times with 14 d interval between 1600 h-1800 h IST with precautions such as monitoring wind direction.

Three sugarcane plants per plot (three leaves/plant, 2.5 cm^2/leaf) were randomly selected and tagged for recording observations. Live aphids were counted before and after each treatment on the 3^{rd}, 7^{th}, 10^{th} and 14^{th} d. The percent efficacy was calculated for each treatment according to Henderson and Tilton (1955).

In the demonstration trial performance of *M. anisopliae* conidia singly and in combination with *M. verrucaria* enzyme preparation (lipase 1U/ml) was evaluated against *C. lanigera* in sugarcane fields using farmer's practice as a control. The farmer's practice included first spray of neem seed kernel extract (5%) in water and a second spray of Metasytox 2ml/L. The dose for all the above treatments was 500 L/ha. Spraying of all the treatments and necessary precautions were taken as per scientific trial.

Field experiment was conducted at Tulapur, Pune during 2006-2007. The plot size was 0.4 ha. Sugarcane plants (20 plants/plot; three leaves/plant, 2.5 cm^2/leaf) were randomly selected and tagged for recording observations. Counting of live aphids and percent efficacy of all the treatments was calculated against woolly aphids.

2.13.2 Biocontrol of *H. armigera* by chitinase and other enzymes of *M. verrucaria*

2.13.2.1 Insect rearing and bioassay

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

Enzyme preparations of *M. verrucaria* and *M. anisopliae* were used for the laboratory bioassay against *H. armigera*. *M. anisopliae* conidia (1×10^7 conidia/ml) and *M. verrucaria* enzyme preparation (Chitinase 2 U/ml) mixed with glycerol (1M) were also used in the bioassay against *H. armigera.* A set of 30 larvae with 3 replication were used for the bioassay. Larvae were dipped individually in 10 ml enzyme solution for 5 seconds. The experiment was conducted 3 times using freshly prepared enzyme solution. After treatment, each larva was individually transferred to a separate sterile vial containing moist Whatman No. 1 filter paper and a piece of disinfected okra. The diet was changed every day and the larvae were kept at 25±2°C with 65±5% RH and 16L: 8D for 14 d. The data on percent mortality from three experiments were pooled to get an average values. The mortality was corrected by Abbott's (1925) formula.

2.13.2.2 Field experiments

Performance of enzyme preparations of *M. verrucaria* (SmF and SSF, chitinase 2 U/ml) and *M. anisopliae* (SmF) were studied against *H. armigera* in chickpea (variety: Vishal) fields. *M. verrucaria* enzyme preparation mixed with glycerol (1M) was also used for experiment. Other treatments includes, *M. anisopliae* conidia (5×10^{12} conidia/ha in water containing 0.1% Tween 80), chemical insecticide endosulfan (2ml/L) and the plots sprayed with water containing 0.1% Tween 80 were used as control. The dose for all the treatments was 500L/ha. The spraying was carried out 3 times with 14 d interval with a hand operated knapsack sprayer between 1600 h-1800 h IST. Precautions such as monitoring wind direction as necessary were taken.

Field experiments were conducted at the Agriculture University, Rahuri, Ahmadnagar, Maharashtra (19°20' N and 74°35' E) for two successive years during *Rabi* season, (October-March) 2005-2006 and 2006-2007. The experimental layout was a RCBD with seven treatments having three replications. The plot size was 5m×5m. The

crop was sown during the first fortnight of October and was raised following normal agronomical practices.

Five plants per plot were randomly selected and tagged for recording observations. Live larvae were counted before and after treatment on the 3rd, 7th and 10th day. The percent efficacy was calculated for each treatment according to Henderson and Tilton (1955). The observations on pod damage were recorded on five randomly selected plants at the time of harvest by counting the total number of healthy and damaged pods. The grain yield of chickpea was calculated from individual plots of the treatment.

2.13.3 Biocontrol of fungal pathogens by chitinolytic enzymes *M. verrucaria*

The root pathogens, *S. rolfsii* and *F. oxysporium* were grown in YPG medium (100 ml), under shaking (180 rpm) at 28°C for 72 h. The soil: sand (2:1) mixture (25 kg) was sterilized by autoclaving. The soil-sand mixture was allowed to cool and infested with the mycelial mass (1000 ml) of *S. rolfsii* and *F. oxysporium*. Peanut seeds (2 nos.) were sown per pot (10 pots/treatment) containing 200 g soil. The effect of enzymes (100 ml preparation/pot, chitinase 50 U, β-1,3-glucanase 7 U and protease 2 U) of *M. verrucaria* and *Bacillus* sp. B1 (chitinase 1 U, β-1,3-glucanase 8 U and protease 27 U) against *S. rolfsii* and *F. oxysporum* were studied. The results were compared with chemical fungicide, carzim (0.8 % w/v) treatment. Sterile soil and pathogen infested soil were also kept for comparison. The drenching of enzyme preparation was done at an interval of 7 d (0, 7 and 14th d) while on other days the enzyme treated pots were irrigated with with sterile distilled water (100 ml/pot). All other pots were irrigated with sterile distilled water (100 ml/pot) every alternate day. Plants were allowed to grow up to 21 d. Percent peanut seed germination was recorded.

Chapter 3

Production of chitinolytic enzyme complex using *Myrothecium verrucaria*

A 3 Optimization of submerged fermentation

A 3.1 Introduction

Chitinases (EC 3.2.1.14) are known to catalyze the hydrolysis of chitin to its monomer *N*-acetyl-β-D-glucosamine. Chitin forms the exoskeleton of many invertebrates and is one of the major components of fungal cell wall (Shaikh and Deshpande, 1993). The hydrolytic property of chitinases makes them an attractive alternative as an environmentally safe biocontrol agent. Extensive research on role of chitinolytic enzymes in fungal and insect pathogenesis have been reported (Budi et al 2000; Kulkarni et al 2008; Nahar et al 2004a; Nahar et al 2008; Patil et al 2000; Patil et al 2001; St. Leger et al 1986a; 1996). Traditionally selected strain of *S. marcescens* was used for the production of chitinolytic enzymes (Green et al 2005). Among fungi, *T. harzianum* was reported to be a main source for large-scale production of chitinases (Donzelli et al 2005; Nampoothiri et al 2004). *M. verrucaria* (Vyas and Deshpande, 1989), *Penicillium* sp. (Fenice et al 1998a; Binod et al 2005), *V. lecanii* (Liu et al 2003; Matsumoto et al 2004) and *B. bassiana* (Suresh and Chandrasekaran, 1999) were also reported as potent producers of chitinolytic enzymes. Various studies reported the optimization of nutritional parameters in shake flask studies (Donzelli et al 2005; Felse and Panda, 1999; Sandhya et al 2004). However, very few studies were aimed at understanding the effect of different process parameters on fungal chitinase production at fermenter level (Felse and Panda, 2000a; Liu et al 2003).

M. verrucaria produces extracellularly chitinolytic enzymes (chitinase, endochitinase, NAGase, CDA and chitosanase) as well as other enzymes such as lipase, protease and β-1,3-glucanase in appreciable quantities (Deshpande, 1986; Shaikh and Deshpande, 1993; Patil et al 2000). In this study effect of different nutritional and

biophysical conditions on enzyme production in shake flask, 10 L fermenter and 100 L fermenter were carried out.

A 3.2 Results and Discussion

A 3.2.1 Shake flask studies

A 3.2.1.1 Effect of different inocula

Initially chitinolytic enzyme production was studied using purified chitin (Sigma Chemical Co, USA) as a carbon source by *M. verrucaria* (Vyas and Deshpande, 1989). Effect of different inocula on chitinases and other enzymes production was studied. Aerial conidia, blastospores and mycelia were used as an inoculum for the production of extracellular enzymes using *M. verrucaria*. The aerial conidia from 7 d old PDA slants produced higher chitinolytic activities (chitinase, 1.8±0.2 U/ml) compared to blastospore inoculum (chitinase, 1.4±0.1 U/ml). However, other enzyme activities such as lipase, protease and β-1,3-glucanase were not significantly affected (**Table 3.1a**). The chitinase activities using mycelial inoculum (48 h old) grown in chitin medium and potato dextrose broth were 1.7±0.1 U/ml and 1.61±0.15 U/ml respectively (**Table 3.1b**). In the present investigation similer enzyme production was observed using conidial and mycelial inoculum, however slight reduction in chitinolytic enzyme production was observed using blastospores as an inoculum (**Table 3.1a and Table 3.1b**). The use of mycelia of *V. lecanii* grown in chitin containing medium as inoculum was reported to significantly improve *N*-acetylhexosaminidase production as compared to conidial inoculum due to the earlier adaptation of the fungus to media supplemented with chitin, rapid growth initiation and early entry of the fungus into enzyme production phase (Matsumoto et al 2004).

Table 3.1a Production of chitinase and other enzymes using conidia and blastospores of *Myrothecium verrucaria* as an inoculum

Enzymes	Activity U/ml after 7 d	
	Conidia	Blastospores
Chitinase	1.8±0.2	1.4±0.07
Endochitinase	0.5±0.05	0.43±0.02
NAGase	6.0±0. 43	5.0±0.36
CDA	$1\pm0.1\times10^{-3}$	$1\pm0.1\times10^{-3}$
Chitosanase	1.6 ±0.10	1.42±0.12
Lipase	1.58±0.08	1.49±0.08
Protease	0.013±0.002	0.011±0.001
β-1,3-Glucanase	0.19±0.02	0.18±0.01
*Protein	0.67±0.07	0.60±0.03

All the activities were mentioned as U/ml; Conidia and blastospore inoculum: 1×10^{7} cells/100 ml medium *Protein was expressed in mg/ml; CDA- Chitin deacetylase, NAGase- N-acetylglucosaminidase

Table 3.1b Production of chitinase and other enzymes using mycelial inoculum of *Myrothecium verrucaria*

Enzymes	Mycelial inoculum from	
	Chitin medium	Potato dextrose broth
Chitinase	1.7±0.10	1.61±0.15
Endochitinase	0.50±0.03	0.46±0.02
NAGase	5.9±0.1	5.6±0.4
CDA	$1\pm0.1\times10^{-3}$	$0.92\pm0.1\times10^{-3}$
Chitosanase	1.58±0.1	1.49±0.14
Lipase	1.58±0.08	1.53±0.04
Protease	0.012±0.002	0.014±0.001
β-1,3-Glucanase	0.19±0.01	0.20±0.02
*Protein	0.65±0.04	0.63±0.06

All the activities were mentioned as U/ml; Mycelial inoculum: 10% v/v; *Protein was expressed in mg/ml; CDA- Chitin deacetylase, NAGase- N-acetylglucosaminidase

Size of inoculum is also an important biological factor, which determines biomass production in fermentation. The *M. verrucaria* mycelial inoculum (10% v/v) led to higher production of the chitinolytic enzymes (chitinase 1.7±0.1 U/ml). While in the presence of 2.5-7.5% (v/v) mycelial inoculum, lower enzyme production (chitinase 1.18±0.11 to 1.54±0.11 U/ml) was observed. Slight reduction in enzyme production (1.58±0.12 U/ml chitinase) was also observed when increased (12.5% v/v) inoculum was used. Similerly, maximum production of lipase, protease and glucanase was observed using 10% mycelial inoculum. Sandhya et al (2005b) used *Aspergillus oryzae* conidial inoculum (0.5-4 ml, 8×10^8 conidia/ml) for protease production in submerged as well as in solid state fermentation. There was a significant increase in enzyme production with an increase in inoculum size up to an optimum level after that enzyme yield was reduced. In SmF, maximum enzyme yield was obtained when medium was inoculated with 3 ml conidial suspension. While in SSF maximum enzyme production was obtained when medium was inoculated with 1 ml inoculum. At lower inoculum levels, the yield was very low. The decrease seen with larger inoculum sizes could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture (Sandhya et al 2004). Hence, a balance between the proliferating biomass and available material will yield maximum enzyme production.

A 3.2.1.2 Effect of different chitin

M. verrucaria was grown in a medium containing chitin from different sources, crab shell chitin (Sigma and Hi-Media) and prawn shell chitin (Shree Agro Products, Ratnagiri, Maharashtra) for enzyme production. The purified chitin (Sigma) supported better growth as well as enzyme production compared to chitin from other sources used. Maximum chitinase production was achieved using purified chitin (1.7±0.1 U/ml) than practical grade chitin (Hi-Media) (1.6±0.1 U/ml) and crude chitin (Shree Agro Products)

(1.0±0.1 U/ml). Higher lipase production was also achieved using purified chitin than practical grade chitin and crude chitin. However, the protease production was found to be more in crude chitin than other chitin. The β-1,3-glucanase and protein production was similar for all the chitin (**Table 3.2**).

Table 3.2 Chitinases and other enzyme production in shake flask using chitin from different sources by *Myrothecium verrucaria*

Enzyme	**Activity (U/ml)**		
	Crude chitin	Practical grade chitin	Purified chitin
Chitinase	1.0±0.10	1.6±0.07	1.7±0.10
Endo-chitinase	0.30±0.01	0.44±0.02	0.50±0.03
Endo-chitinase (**a**)	0.19±0.01	0.26±0.01	0.27±0.01
NAGase	3.80±0.10	5.75±0.05	5.90±0.10
NAGase (**a**)	1.03± 0.02	1.31±0.01	1.36± 0.02
CDA	0.85×10^{-3}±0.04	0.91×10^{-3}± 0.1	1.00×10^{-3} ±0.04
Chitosanase	0.87±0.01	1.50±0.10	1.58±0.08
Lipase	0.33±0.04	0.42±0.04	1.58±0.08
Protease	0.30±0.01	0.15±0.01	0.012±0.002
β-1,3-glucanase	0.19±0.01	0.20±0.01	0.19±0.01
*Protein	0.62±0.05	0.63±0.06	0.65±0.04

Endochitinase and Endochitinase (**a**) were estimated using ethylene glycol chitin and 4-methylumbelliferyl-*N*',*N*'',*N*'''-triacetylchitotrioside (4-MU-$(GlcNAc)_3$) as a substrate respectively. NAGase and NAGase (**a**) were estimated using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) as a substrate respectively. Fermentation was carried out at 28°C for 7 d as described under materials and methods. *Protein was expressed in mg/ml

A 3.2.1.3 Effect of crude chitin concentration

Different concentrations of crude chitin were used for chitinase and other enzyme production. In the present studies maximum production of chitinase and other enzymes by *M. verrucaria* was observed using 1.5% (w/v) chitin after 7 d (**Table 3.3)**. Chitinase is produced as an inducible enzyme with chitin or its degradation products acting as inducers. In most cases chitin concentration in the range of 1-2% was found to be suitable for chitinase production (Sandhya et al 2004). Similarly, maximum chitinase production by *Talaromyces emersonii* was also observed using 1-2% (w/v) chitin (McCormack et al 1991). According to Gupta et al (1995) *Streptomyces viridificans* produced maximum chitinase activity using 1.5% (w/v) colloidal chitin at 30°C after 6 d of fermentation. Similarly, maximum chitinase production by *T. harzianum* was also observed using 1.5% (w/v) colloidal chitin after 4 d (Sandhya et al 2004). The possible reason, which has been so far unsubstantiated, could be that the signal for induction of synthesis of chitinase could be resulted from the physical contact between the cell surface and the insoluble substrate, as has been proposed for cellulase biosynthesis (Berg and Petterson, 1977).

Table 3.3 Effect of crude chitin concentration on chitinase and other enzyme production under submerged fermentation by *Myrothecium verrucaria*

Chitin	**Activity (U/ml)**			
(%)	**Chitinase**	**Lipase**	**Protease**	**β-1,3-Glucanase**
0.1	0.30±0.03	0.08±0.04	0.08±0.01	0.05±0.01
0.5	1.02±0.06	0.33±0.08	0.31±0.03	0.21±0.03
1.0	1.45±0.07	0.33±0.04	0.43±0.02	0.23±0.01
1.5	1.52±0.04	0.35±0.02	0.46±0.01	0.24±0.02
2.0	1.33±0.05	0.30±0.02	0.40±0.02	0.18±0.03

Fermentation was carried out at 28°C for 7 d as described under material and method

A 3.3 Enzyme production in 10 L fermenter by *M. verrucaria*

A 3.3.1 Effect of airflow

For the optimization of submerged fermentation in a fermenter, operational parameters such as airflow, agitation, dissolved oxygen (DO), pH and inoculum size need careful consideration (Jsten et al 1996). The effect of airflow (0.5, 1.0 and 1.5 vvm) was investigated for the production of chitinase and other enzymes in 10 L fermenter. It was seen that 0.5 vvm airflow was necessary and sufficient for the optimum mixing of the chitin in the fermenter. At higher airflow (1.0-1.5 vvm) the insoluble chitin was found to surface out at the top of the fermenter and thus was not effectively accessible for the fungus. More foaming was also observed at higher airflow. The chitinase activity measured with acid swollen chitin decreased from 0.97 ± 0.07 U/ml to 0.45 ± 0.02 U/ml as the airflow was increased from 0.5 to 1.5 vvm. Similar trend was observed for protease, lipase and β-1,3-glucanase production by *M. verrucaria* **(Fig. 3.1a)**. Gohel et al (2007) also studied the effect of different airflow (0.25 to 1.5 vvm) on production of chitinases (total chitinase, endochitinase, chitobiase), proteases and β-1,3-glucanase by *Pantoea dispersa* in bench-top 5 L fermenter. An airflow of 0.5 vvm was favourable for the production of enzymes. Increasing the airflow above 0.5 vvm resulted in a drastic decrease in enzyme and biomass production, which might have been due to increased shear stress. Above results suggests that enzyme production and cell growth are sensitive to the airflow. Mild conditions of agitation and airflow (300 rpm and 0.04 vvm) were also found to be suitable for maximum chitinase production by *S. marcescens* (Khoury et al 1997).

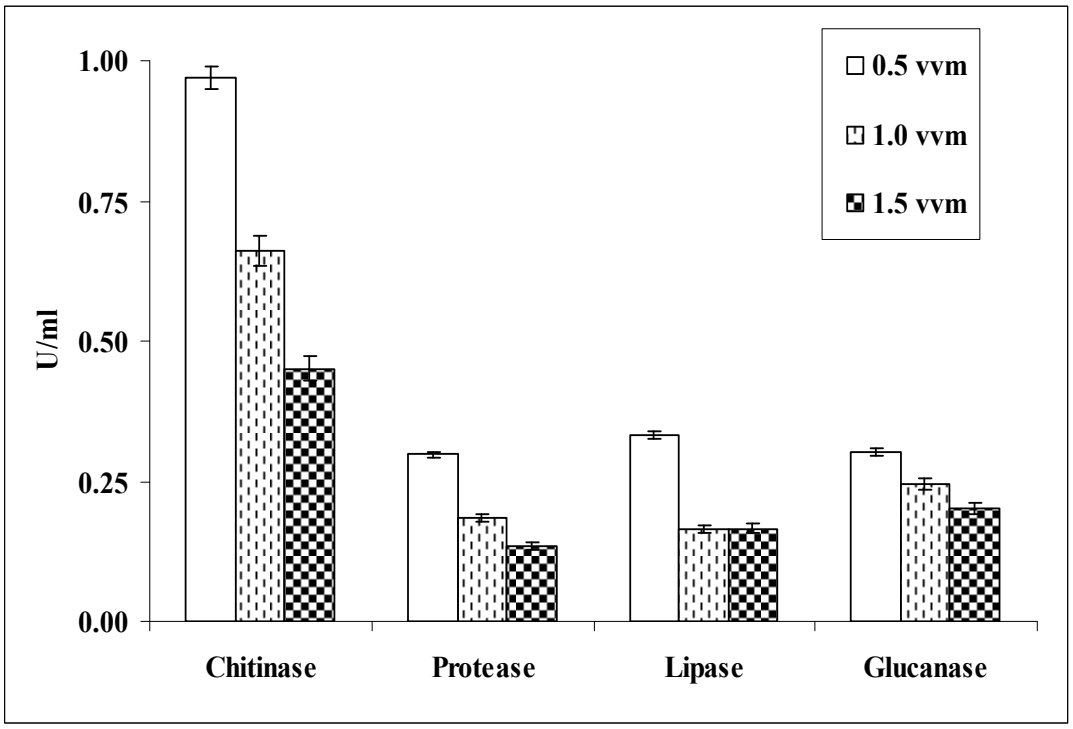

Fig. 3.1a Effect of airflow on chitinase and other enzyme production in 10 L fermenter by *Myrothecium verrucaria*

In the present investigation enzyme production was corelated with mycelial biomass and DO level. Mycelial biomass (wet wt) of *M. verrucaria* was increased for initial 3 d while it was decreased during the 4th and 5th d of fermentation. At low airflow (0.5 vvm), as the substrate was more accessible more biomass (62 g/L wet wt) was observed after 3 d, while at 1.0 and 1.5 vvm biomass production was less i.e. 48 g/L wet wt and 40 g/L wet wt, respectively (Fig **3.1** b). This can be attributed to improper mixing of the substrate at higher airflow.

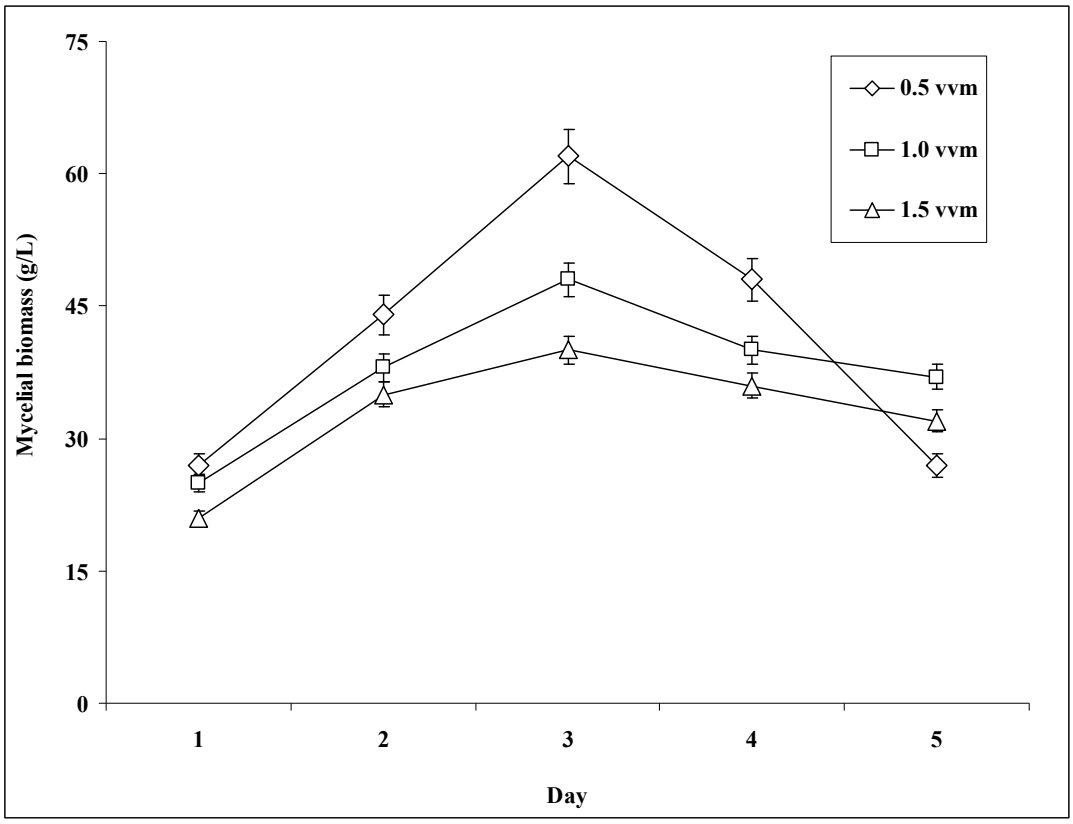

Fig. 3.1b Effect of airflow on mycelial biomass of *Myrothecium verrucaria* in 10 L fermenter. The biomass was determined by separating mycelial mass from the fermented broth (100 ml) by centrifugation at 10,000 × g for 20 min

The DO level during the fermentation was initially decreased for 3 d while it was again increased for 4th and 5th day of the fermentation. At low airflow (0.5 vvm), DO levels were between 42-68% saturation while at 1.0 and 1.5 vvm the DO levels were 52-81% and 57-91% respectively (**Fig. 3.1 c**). During the fermentation for chitinase production using *V. lecanii*, the DO level was decreased rapidly during the first 2 d. It appeared that the DO level was lowered down to negligible on 3rd and 4th d then switched back to 90% level at the time of harvest (6th d) (Liu et al 2003).

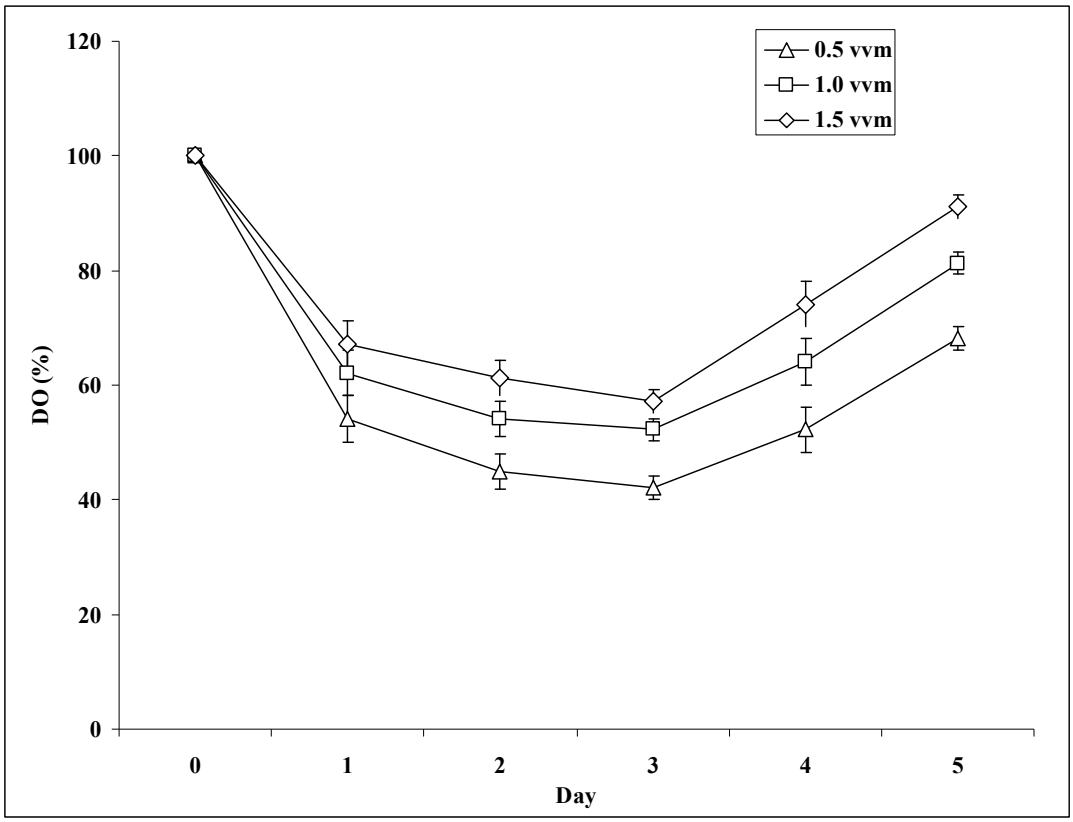

Fig. 3.1c Effect of airflow on % dissolve oxygen saturation in 10 L fermenter by *Myrothecium verrucaria*

A 3.3.2 Effect of agitation

Agitation rate is an important parameter as it plays a significant role in determining the productivity by providing mixing, mass and heat transfer during the process (Jsten et al 1996). Also, it is one of the most critical parameters used for process scale up (Felse and Panda, 2000a; Liu et al 2003). The agitator speed depends upon the resistance of the organism to shear, its morphological state, the nutrient composition, pH and many other conditions (Felse and Panda, 2000a). The maximum chitinase activity (1.13 ± 0.06 U/ml) was obtained by *M. verrucaria* after 5 d of incubation with increasing agitation (from 1st to 5th d, 100, 120, 150, 180 and 200 rpm respectively) and increasing airflow (from 1st to 5th d, 0.2, 0.2, 0.3, 0.4, 0.5 vvm respectively). However under constant agitation (100, 200 or 300 rpm) conditions, chitinase activity was <0.97 ± 0.07

U/ml. Further increase in agitator speed (400 rpm) resulted in decreased chitinase activity (0.51 ± 0.04 U/ml) **(Fig. 3.2)**.

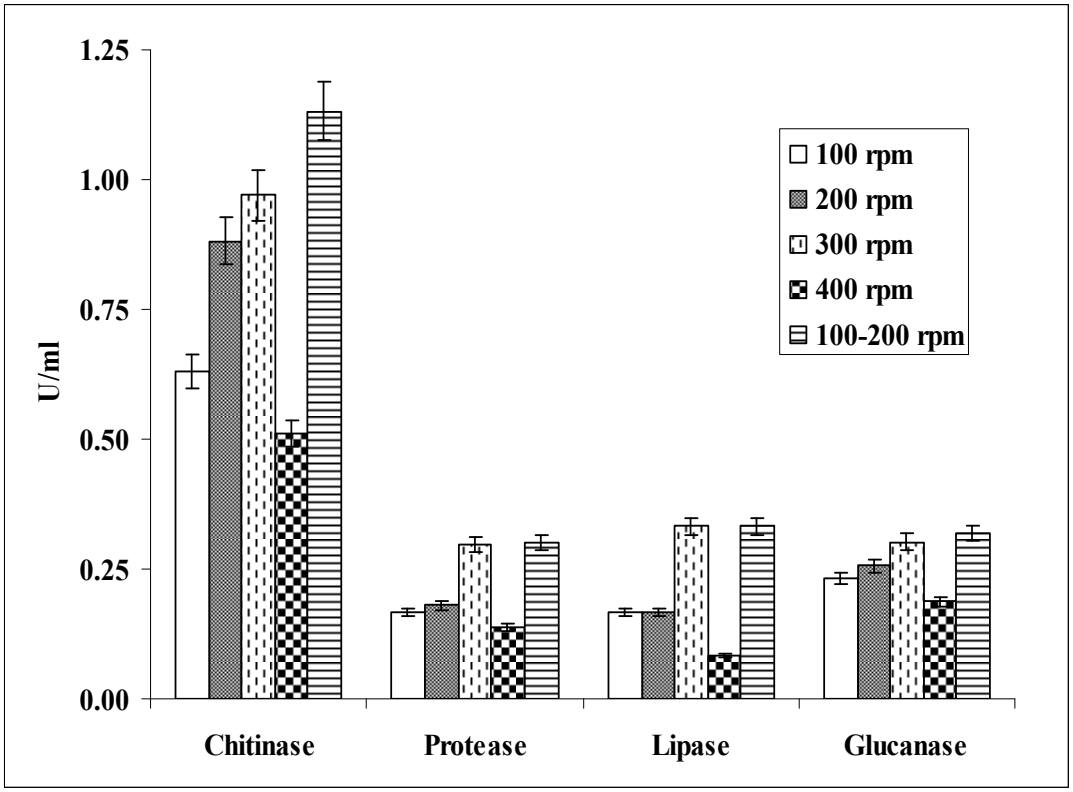

Fig. 3.2 Effect of agitation on enzyme production in 10 L fermenter by *Myrothecium verrucaria*

Maximum chitinase production by *T. harzianum* was reported at an agitator speed of 224 rpm (Felse and Panda, 2000a). While the optimum agitator speed for the chitinase production by *Penicillium janthinellum* was observed to be 500 rpm (Fenice et al 1998a). The highest chitinase production by *V. lecanii* was observed with the agitator speed of 150 rpm. Gohel et al (2007) also studied effect of different agitation (100, 200, 300, 400, 500 and 600 rpm) on production of chitinases (total chitinase, endochitinase, chitobiase), proteases and glucanase by *P. dispersa* in 5 L bench-top fermenter. An agitation of 300 rpm was favourable for the production of enzymes and biomass. All these studies indicate that the production of chitinase was facilitated with certain critical agitator speed specific for a particular isolate. The lower production of chitinases observed with agitator

speeds lower than the critical agitator speed was attributed to the insufficient oxygenation (Fenice et al 1998a) and incomplete mixing and/or mass transfer resistance (Felse and Panda, 2000a). While decrease in chitinase production at agitator speeds higher than critical agitator speed was ascribed to the higher shear stress experienced by the fungus (Fenice et al 1998a; Liu et al 2003).

A 3.3.3 Effect of different chitin

Comparative evaluation of different chitin on chitinase and other enzyme production was studied in 10 L fermenter. Higher production of chitinases (chitinase, endochitinases, NAGase, CDA, chitosanase) and lipase was achieved using purified chitin (Sigma) than practical grade chitin (Hi-Media) and crude chitin (Shree Agro Products). However, the protease production was found to be more in crude chitin than other chitin. Crude chitin is rich in protein. This could be reason for higher protease activity. The β-1,3-glucanase production was similar for all the chitin (**Table 3.4**). In 10 L fermenter the productivities of chitinase and other enzymes were higher as compared to shake flask for all chitin. Gohel et al (2007) studied utilization of various chitinous sources for production of chitinases (total chitinase, endochitinase, chitobiase), proteases and β-1,3-glucanase by *P. dispersa* in 5 L bench-top fermenter. Different chitinous sources, such as crustacean waste chitin, solid crustacean wastes, dried fungal mycelia of *Fusarium* sp. and *Macrophomina phaseolina* were compared with commercially available Sigma and matsyafed chitin (prepared from shells of shrimp and crab), for the production of enzymes. Chitin particle of similar size was used during the fermentation process. Higher enzyme production was obtained with Sigma chitin compared to matsyafed chitin and crustacean waste chitin. However, dried fungal mycelia of *Fusarium* sp. and *M. phaseolina* did not lead to high levels of enzyme production.

Table 3.4 Chitinases and other enzyme production in 10 L fermenter using different chitin by *Myrothecium verrucaria*

Enzyme activities	**Activity U/ml**		
	Crude chitin	Practical grade chitin	Purified chitin
Chitinase	1.13±0.06	1.88±0.08	1.95±0.05
Endochitinase	0.32±0.01	0.48±0.01	0.50±0.02
Endochitinase (**a**)	0.21±0.01	0.27±0.01	0.29± 0.01
NAGase	3.92±0.09	5.81±0.07	6.06±0.06
NAGase (**a**)	1.12±0.02	1.37±0.02	1.43±0.01
CDA	$0.8\pm0.02\times10^{-3}$	$0.91\pm0.03\times10^{-3}$	$0.95\pm0.04\times10^{-3}$
Chitosanase	0.91±0.02	1.55±0.05	1.61±0.04
Lipase	0.33±0.08	0.42±0.04	0.83±0.04
Protease	0.30±0.02	0.04±0.01	0.01±0.001
β-1,3-glucanase	0.32±0.03	0.229±0.02	0.20±0.02
*Protein	0.60±0.03	0.64±0.04	0.62±0.05

Endochitinase and Endochitinase (**a**) were estimated using ethylene glycol chitin and 4-MU-$(GlcNAc)_3$ as a substrate respectively. NAGase and NAGase (**a**) were estimated using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide and (4-MU-GlcNAc) as a substrate respectively. Fermentation was carried out at 28°C for 5 d as described under material and method. *Protein was expressed in mg/ml

A 3.3.4 Scale-up of enzyme production from shake flask to 100 L fermenter

Scale-up of production of chitinase and other enzymes by *M. verrucaria* from shake flask to 10 L and 100 L fermenter was carried out using crude chitin as a sole carbon source. In shake flask the chitinase activity was 1.04 ± 0.1 U/ml after 7 d. The similar levels of activity was obtained in 10 L and 100 L fermenter (1.07 ± 0.07 U/ml) within 5 d **(Fig. 3.3)**.

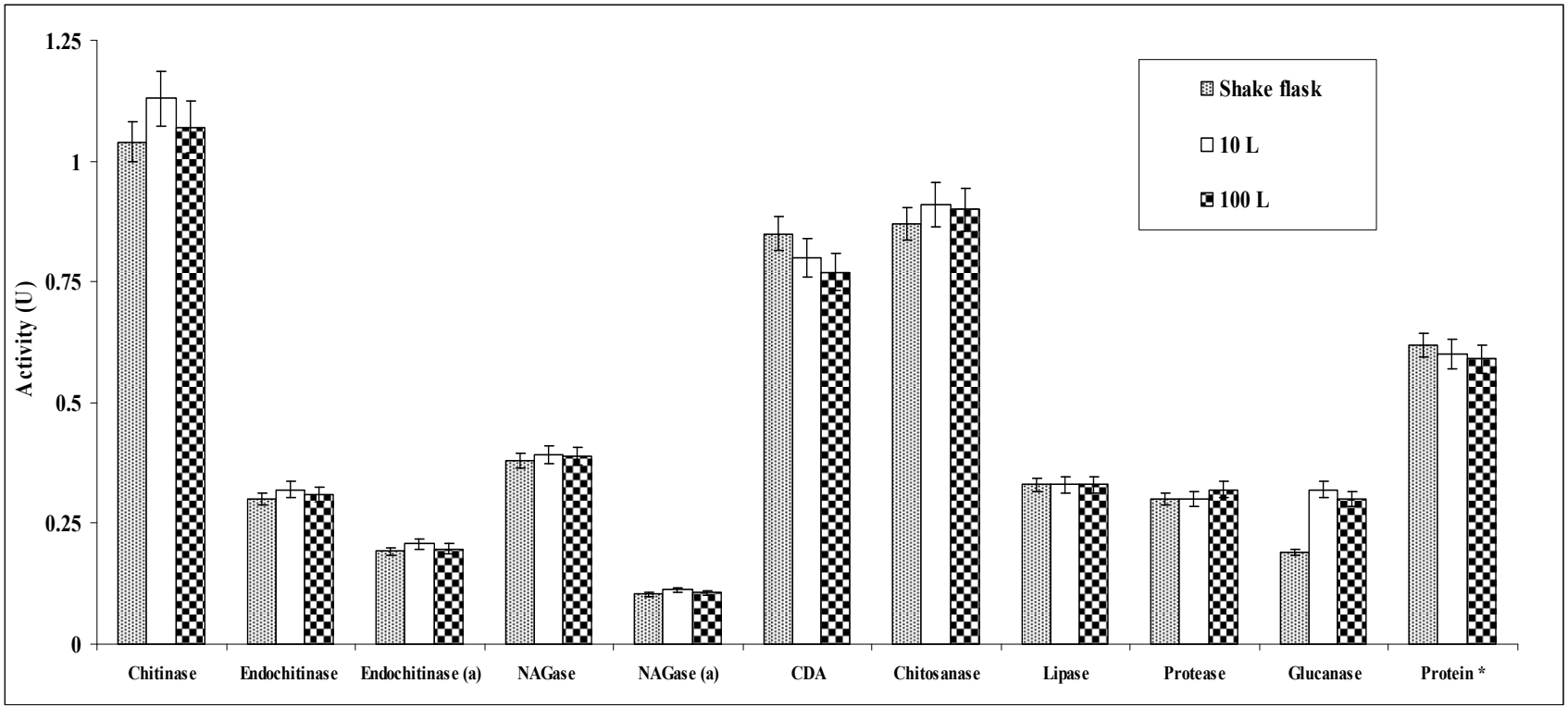

Fig 3.3 Scale-up of enzyme production from shake flask to 100 L fermenter using crude chitin as a carbon source by *Myrothecium verrucaria*; All the enzyme activities were expressed as U/ml while NAGase- *N*-acetylglucosaminidase was expressed as U/0.1 ml while CDA- chitin deacetylase was expressed as U/L; *Protein was expressed in mg/ml. Endochitinase and Endochitinase (**a**) were estimated using ethylene glycol chitin and 4-MU-$(GlcNAc)_3$ as a substrate respectively. NAGase and NAGase (**a**) were estimated using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide and (4-MU-GlcNAc) as a substrate respectively. Fermntation in shake flask and fermenter was carried out at 28°C for 7 d and 5 d respectively.

Similarly, maximum chitinase activity by *P. dispersa* in shake flask was obtained after 6 d while in fermenter the same level of activity was obtained after 3 d (Gohel et al 2007). The productivities (U/D) of chitinolytic enzymes (endochitinase, NAGase, CDA, chitosanase) and other enzymes such as lipase, protease and β-1,3-glucanase by *M. verrucaria* also increased by 1.5-2 fold in fermenter.

In the present studies, the extracellular protein production in shake flask, 10 L and 100 L fermenter were 0.62±0.05 mg/ml, 0.60±0.03 mg/ml and 0.59±0.04 mg/ml respectively. It was also observed that as the pH of the broth increased the enzyme production was found to be increased. The pH of the broth was increased from 6.0 to 7.24 in shake flask, while 6.0 to 7.35 and 7.27 in 10 L and 100 L fermenter, respectively.

A 3.4 Effect of particle size of chitin on chitinase production

It was observed that smaller particle size enhanced the production of chitinase by *M. verrucaria*. Mixed chitin (without sieving) from all the sources showed significant difference in chitinase production. However, when similar particle size of all chitin used in the medium, the difference in chitinase production was less (**Table 3.5**).

Table 3.5 Effect of particle size of chitin on chitinase production by *Myrothecium verrucaria*

Different	Chitinase activity (U/ml)		
	Particle size		
Chitin	<0.2 mm	0.2-0.6mm	Mixed
Purified chitin	1.91±0.09	1.80±0.2	1.80±0.2
Practical grade chitin	1.81±0.09	1.64±0.1	1.60±0.1
Crude chitin	1.63±0.04	1.41±0.1	1.00±0.1

Chitin particles were separated using sieves of different sizes (<0.2 mm, 0.2-0.6mm) and particle size of chitin was determined using micrometry

The particle size of purified chitin was smaller as compared to practical grade and crude chitin. It was also observed that the major portion of particle size was 0.2-0.6 mm for all chitin. The purified chitin showed 88% particles with size of 0.2-0.6 mm. While practical grade and crude chitin showed 72% and 55% particles, respectively, with size of 0.2-0.6 mm (**Fig. 3.4**) Substrate particle size is most critical factor for microbial growth and activity. Smaller substrate particles provide larger surface area. However, too small substrate particles may result in substrate aggregation which may interfere with microbial respiration, and thus may result in poor growth. At the same time, larger particles provide better respiration efficiency (due to increased inter-particle space) but provide limited surface for microbial attack (Pandey et al 2000). Thus, it would be necessary to have suitable particle size for a particular process.

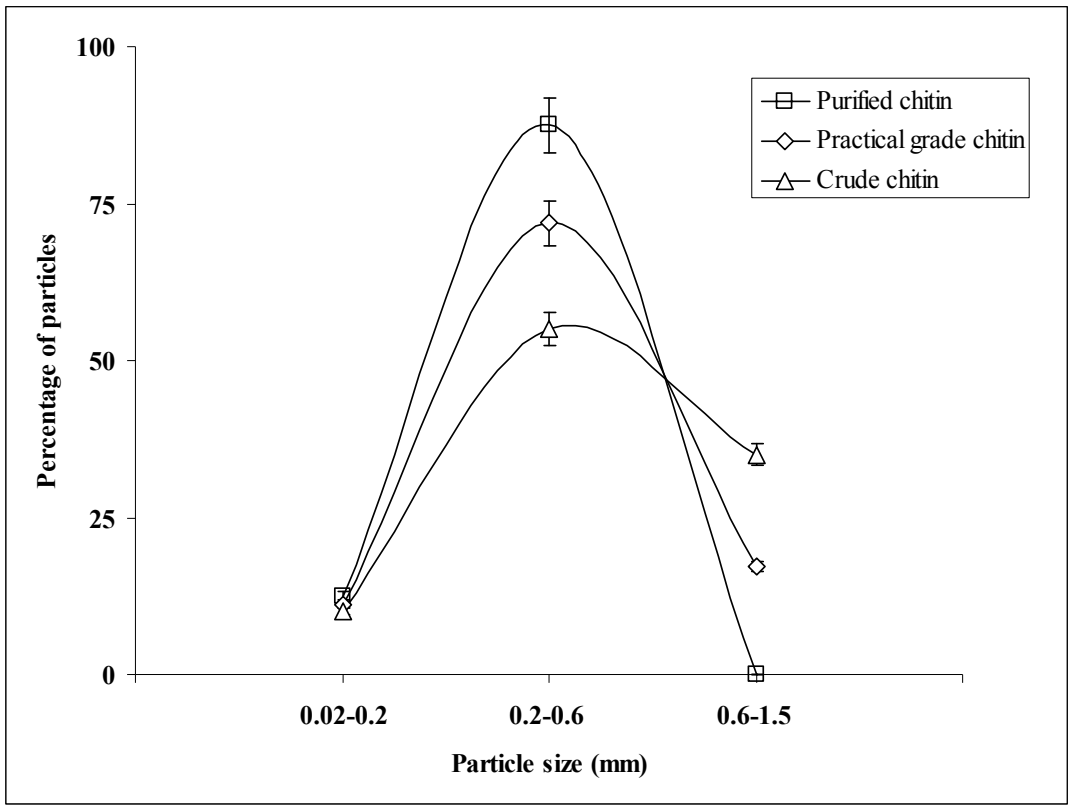

Fig. 3.4 Particle size and % distribution of small (0.02-0.2 mm), medium (0.2-0.6 mm) and large (0.6-1.5 mm) chitin particles obtained from different sources such as purified chitin (Sigma), practical grade chitin (Hi-Media) and crude chitin (Shree Agro Products)

A 3.5 Determination of degree of acetylation of chitin

The degree of acetylation (DA) is the share of nitrogen sites occupied by acetyl groups (each nitrogen atom can react with one acetyl group). The DA is hardly ever 100% in commercially available chitin since chitin purification involves alkali treatment for protein removal, also resulting in a lower DA. Values of about 90% degree of acetylation are typical for chitin. An extra reaction is necessary to reach 100% DA. Reaction with acetic anhydride in a dry solvent (e.g. methanol) results in N-acetylation (Van de Velde and Kiekens, 2004). The solid state NMR reveled that acetylation level of purified chitin, practical grade chitin and crude chitin were > 80% (**Fig. 3.5)**.

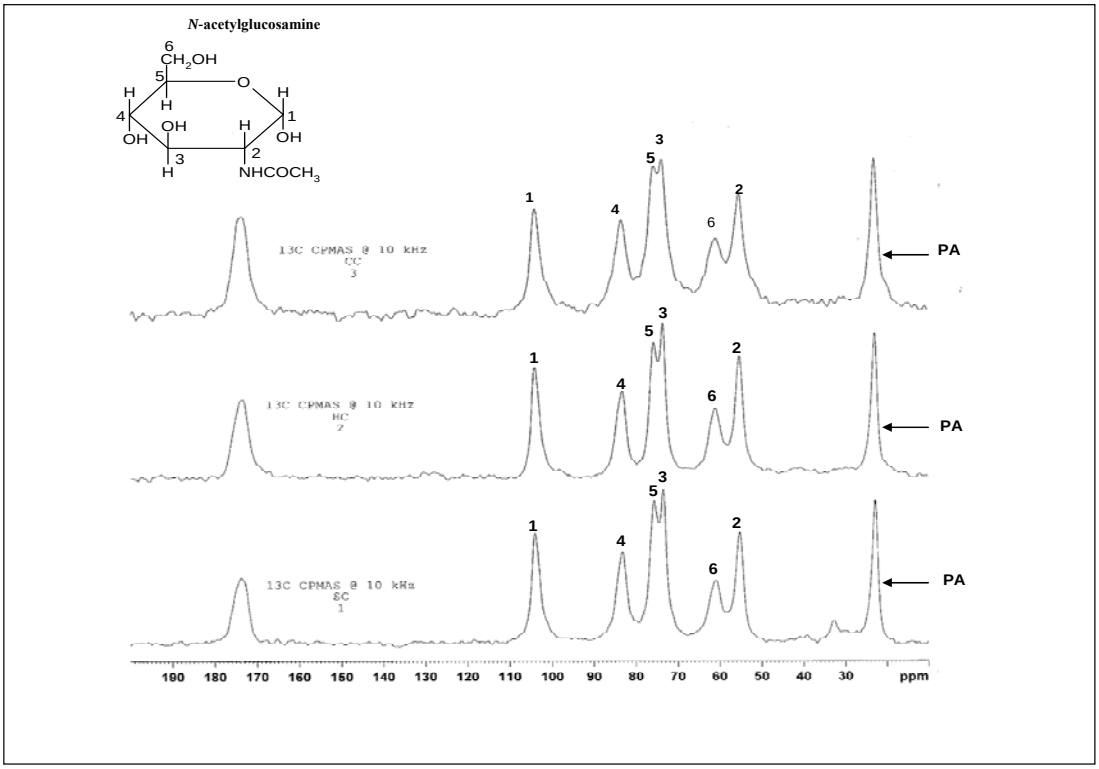

Fig. 3.5 Determination of degree of acetylation of chitin from different sources using ^{13}C CP/MASS NMR Spectroscopy; CC- Crude chitin (Shree Agro Products); HC- Practical grade chitin (Hi-Media); SC- purified chitin (Sigma). PA: Peak area

A 3.6 Estimation of chitinase activity using different acid swollen chitin

The purity of all chitin was checked by estimating chitinase activity on respective acid swollen chitin. As the concentration of acid swollen chitin increased, the chitinase activity increased. Chitinase activity using purified acid swollen chitin (7 mg/ml) was higher (1.55±0.06 U/ml) than practical grade (1.42±0.06 U/ml) and crude chitin (1.18±0.04 U/ml) (**Table 3.6**). Difference in the chitinase activity can be attributed to purity of chitin, degree of acetylation, crystallinity, particle size and removal of more oligomers during washing.

Table 3.6 Effect of different chitin on chitinase activity of *Myrothecium verrucaria*

Chitin	**Chitinase activity (U/ml)**		
mg/ml	**Purified**	**Practical grade**	**Crude**
1	0.46±0.02	0.37±0.02	0.30±0.03
4	1.10±0.05	0.85±0.05	0.61±0.02
7	1.55±0.06	1.42±0.06	1.18±0.04
10	1.80 ±0.02	1.58±0.05	1.31±0.03
15	1.85±0.05	1.61±0.09	1.37±0.06
20	1.81±0.05	1.55±0.08	1.33±0.04

Chitinase activity was estimated using *O*-phosphoric acid (88% w/v) swollen chitin

A 3.7 Downstream processing of chitinase and other enzymes

The chitinase and other enzymes of *M. verrucaria* were concentrated using different methods such as membrane concentration (Hollow fiber membrane, 5 KD), freeze drying, spray drying and ammonium sulphate (90%) precipitation. Membrane concentration and freeze-drying gave higher yield of enzymes compared to spray drying and ammonium sulphate precipitation. The recovery of all enzymes using membrane concentration and freeze drying were 80-90%, while with spray drying and ammonium sulphate (90%) precipitation it was 33-50% (**Fig**. **3.6**). Lower recovery of enzymes by spray drying can be attributed to less yield of protein during the process.

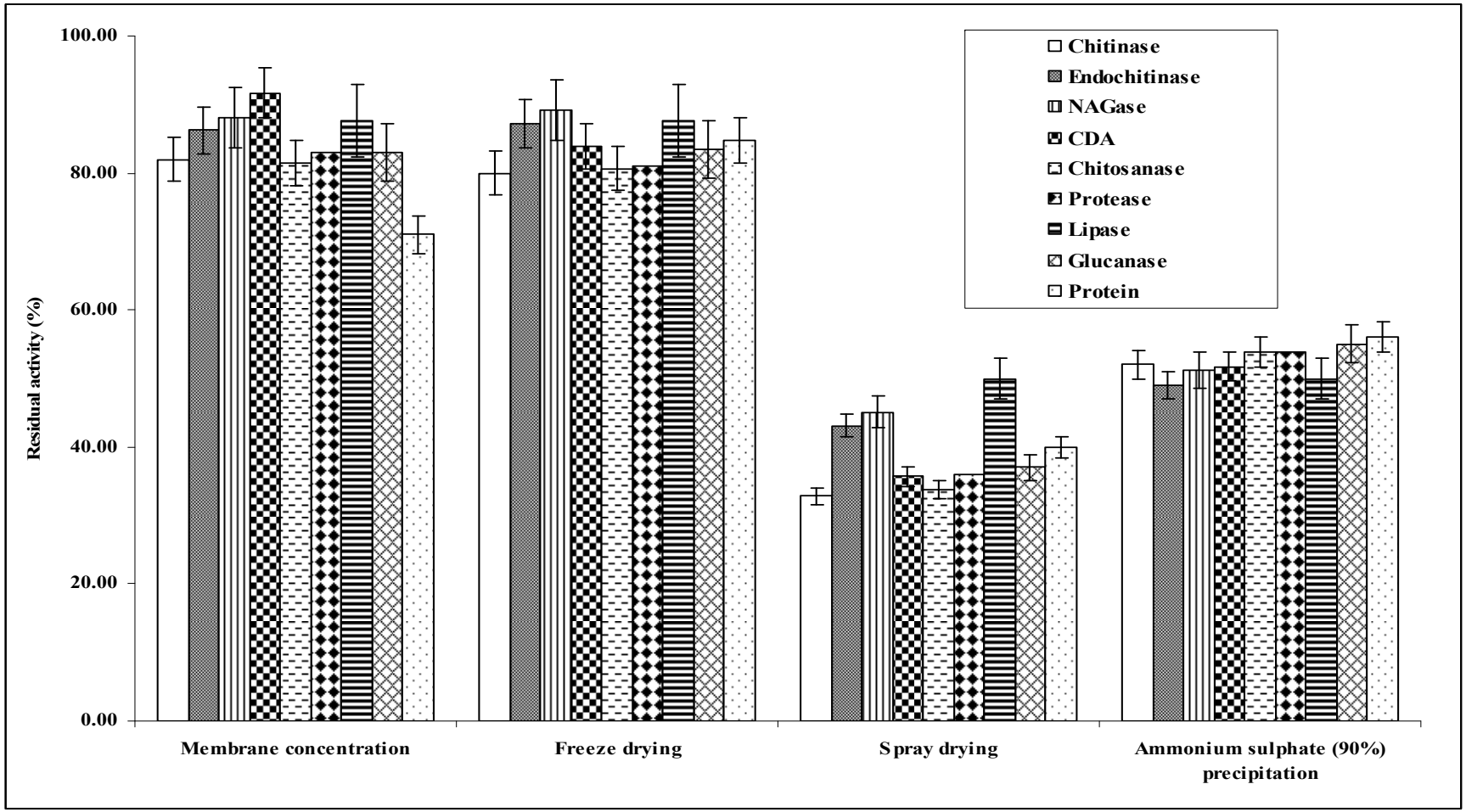

Fig 3.6 Downstream processing of enzymes using different methods such as membrane concentration, freeze drying, spray drying and ammonium sulphate (90%) precipitation

B 3 Optimization of solid state fermentation

B 3.1 Introduction

Since high costs of commercial chitinase production restrict large-scale applications, in recent years, there has been a lot of interest in enhancing the production of chitinase using fermentation techniques. Both submerged fermentation (SmF) and solid state fermentation (SSF) were employed for microbial chitinase production. SSF has recently gained importance for the production of microbial enzymes due to several economic advantages over conventional submerged fermentation (Viniegra-Gonzalez et al 2003; Lonsane et al 1985). SSF offers a better alternative for cost effective production of enzymes, since it can be carried out using cheaply available agro-industrial residues such as wheat bran, rice husk, sugar cane bagasse, etc (Binod et al 2005). Different types of substrates, which contain chitin, have also been tried for the production of chitinase, which included fungal cell walls, crab and shrimp shells (Tagawa and Okazaki, 1991; Wang and Chang, 1997; Casio et al 1982).

Among the various groups of microorganisms used in SSF, filamentous fungi are most widely exploited because of their ability to grow on complex solid substrates and production of wide range of extracellular enzymes (Lekha and Lonsane, 1994). SSF has been reported to be an economical alternative to SmF for the production of high activity of proteases by *Streptomyces rimosus* (Yang and Wang, 1999). Few reports are available on production of chitinases under SSF conditions, while voluminous literature exists on the production of chitinases by fungi in submerged fermentation (Patidar et al 2005). Earlier production of chitinolytic enzymes by *M. verrucaria* has been reported under shake flask conditions (Vyas and Deshpande, 1989). The present study reports the optimization of biophysical and nutritional conditions for the production of chitinase and other enzymes by *M. verrucaria* under SSF conditions.

B 3.2 Results and Discussion

B 3.2.1 Effect of different substrates

In the present studies SSF was carried out using different substrates (such as sorghum, bajra, rice, wheat, wheat bran and rice bran) mixed with crude chitin (1%). The fermentation was carried out in 250 ml flask containing 25 g substrate at 28°C for 7 d. Maximum enzyme production by *M. verrucaria* was supported by sorghum and chitin mixture (chitinase 0.115 ±0.005 U/g). However, less enzyme production was observed using rice bran and chitin (chitinase 0.053 ± 0.002 U/g) after 7 d (**Fig. 3.7**).

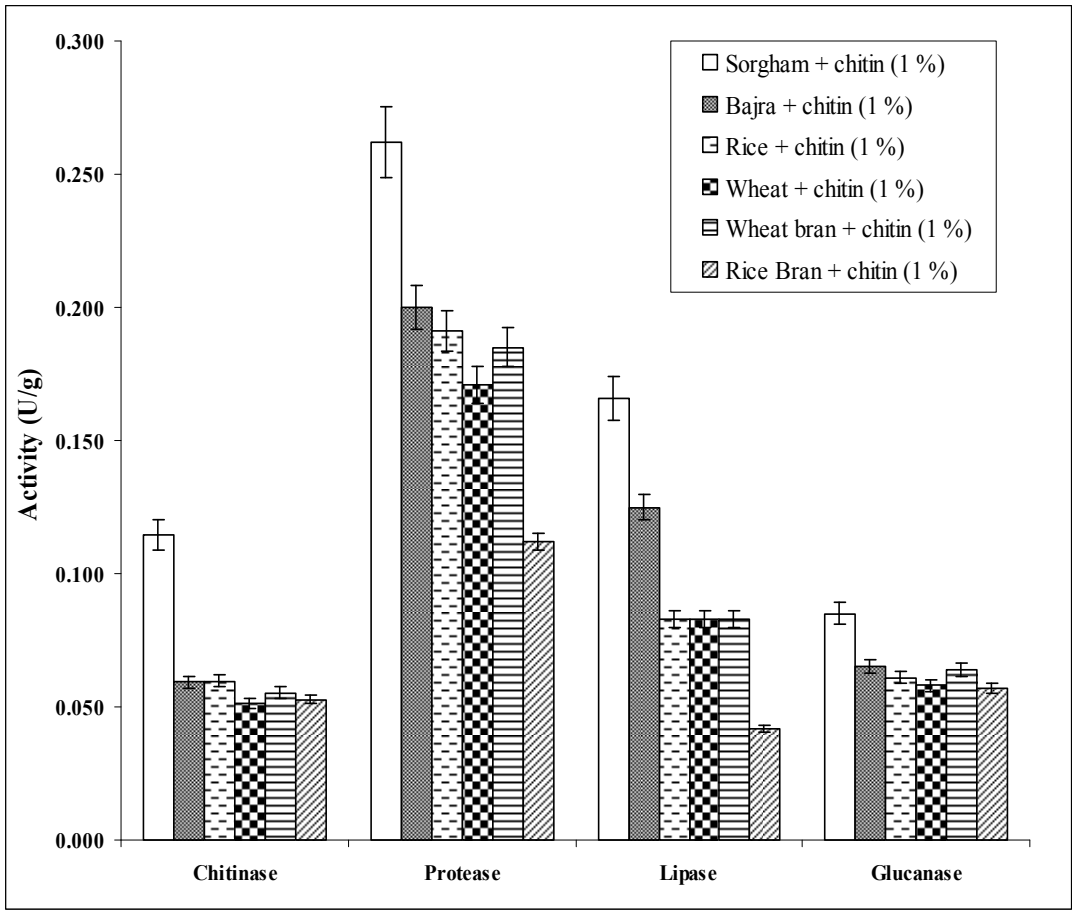

Fig. 3.7 Effect of different substrates with chitin (1%) on chitinase and other enzyme production by *Myrothecium verrucaria*

B 3.2.2 Effect of chitin concentration

The solid state fermentation was carried out using sorghum and different concentration of crude chitin (no addition, 1, 5, 10, 25, 50, 75 % and only chitin)**.** The maximum enzyme production (chitinase; 0.803 ± 0.025 U/g) was achieved using 25 % chitin at 28°C after 7 d. Above 25% chitin the chitinase and other enzyme production was low (**Fig 3.8**).

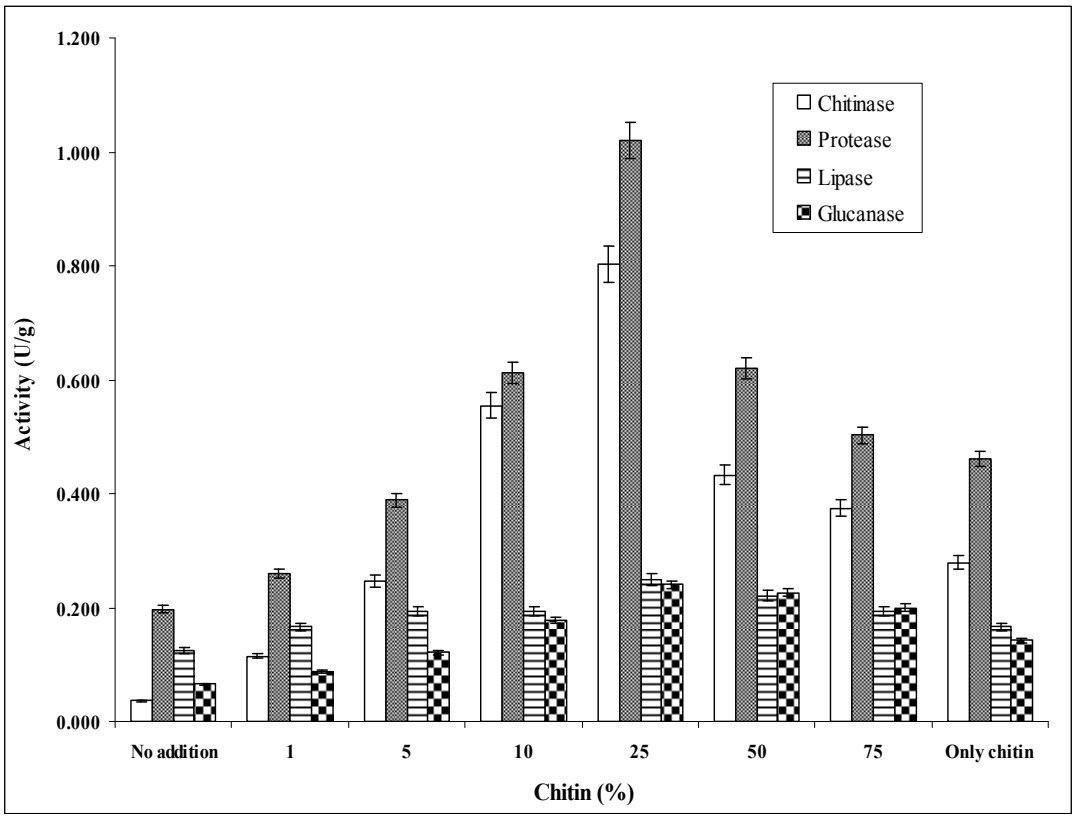

Fig 3.8 Effect of crude chitin concentration (w/w) with sorgham on enzyme production under solid state fermentation by *Myrothecium verrucaria*

B 3.2.3 Effect of different chitin

Effect of different chitin (crude chitin, practical grade chitin and purified chitin) mixed with sorgham on chitinase and other enzyme production was also studied. The chitinase production was found to be higher using sorgham mixed with purified chitin (1.23 ± 0.05 U/g) than practical grade chitin (1.07 ± 0.07 U/g) and crude chitin (0.80 ±

0.03 U/g). However the protease production was found to be higher using crude chitin as compared to other chitin. The lipase and β-1,3-glucanase production was almost similer for all chitin **(Table 3.7)**.

Table 3.7 Effect of different chitin on enzyme production by *Myrothecium verrucaria*

	Activity (U/ml)			
Substrate	**Chitinase**	**Protease**	**Lipase**	**β-1,3-glucanase**
Sorgham	0.03±0.003	0.20±0.01	0.13±0.04	0.06±0.01
Sorgham + crude chitin	0.80±0.03	1.00±0.06	0.25±0.04	0.24±0.01
Sorgham + practical grade chitin	1.07±0.07	0.60±0.05	0.33±0.04	0.26±0.01
Sorgham + purified chitin	1.23±0.05	0.32±0.03	0.33±0.04	0.27±0.01

The fermentation was carried out using 25% chitin at 28°C with 70-80 RH for 7 d. After fermentation the enzymes were extracted with acetate buffer (pH 5.0, 50 mM).

B 3.2.4 Effect of initial moisture content

The initial moisture content has been shown to affect the production of hydrolytic enzymes under SSF conditions by influencing the growth of the organism (Patidar et al 2005). SSF was carried out with substrates having different levels of initial moistures. The fermentation was carried at 28°C for 7 d. Maximum enzyme activities (chitinase 0.803±0.03 U/g; protease 1.02±0.08 U/g; lipase 0.249±0.04 U/g and β-1,3-glucanase 0.24±0.01 U/g) were obtained when 12.5 ml distilled water was added to 25 g substrate (**Fig. 3.9**). The initial moisture content of 65% was found to be suitable for maximum production of *N*-acetyl-β-D-glucosaminidase when grown on wheat bran and chitin as carbon sources in solid-state fermentation by *F. oxysporum* (Gkargkas et al 2004). However, in the present studies the moisture content of 33% (12.5 ml water in 25 g substrate) was suitable for maximum production of chitinase and other enzymes by *M.*

verrucaria when grown on sorghum and chitin mixture (3:1). The initial moisture content was calculated using following formula [(weight of liquid phase/total weight of liquid and solid phase) × 100]. The difference in optimum moisture content in the above examples can be attributed to soaking capacity of the substrate.

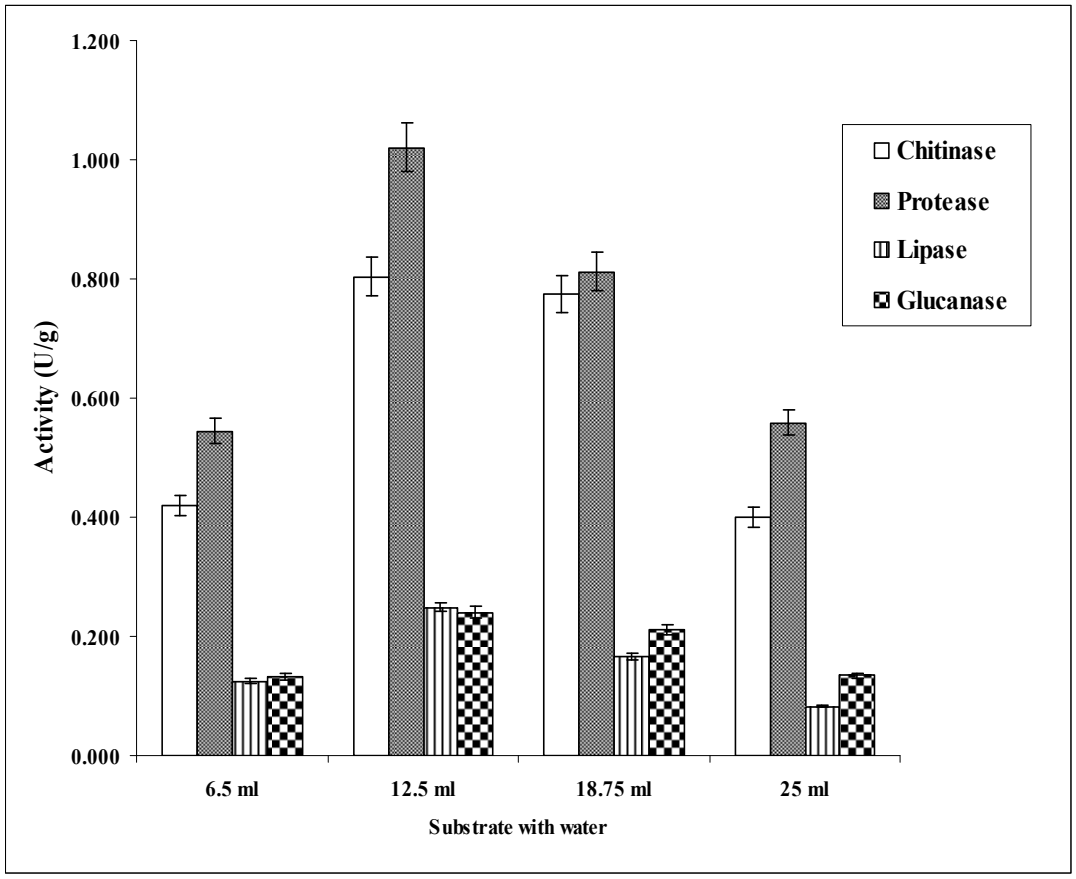

Fig 3.9 Effect of initial water content of the substrate (sorgham with 25% chitin) on enzyme production by *Myrothecium verrucaria*

The decrease in the enzyme activity with increase in substrate moisture might be attributed to the phenomenon of flooding of inter particle space of the substrate. Increased moisture level causes decreased porosity, alteration in substrate particle structure, lowers oxygen transfer and enhanced formation of aerial mycelia (Pandey, 1994; Raimboult and Alazard, 1980). Fungi would prefer unbound moisture for survival in such a way that it may not hamper with its metabolic pathways. Similarly, moisture level lower than optimum leads to higher water tension, lower degree of swelling and

reduced solubility of the nutrients of the solid substrate. A related criterion with this is the water activity of the medium, which is considered as the fundamental parameter for mass transfer of water and isolates across the cell membrane. When water is made available in a lower or higher quantity than that is optimally required, the productivity of the process is significantly affected. Moisture optimization can be used to regulate and to modify the metabolic activity of the micro-organism (Pandey et al 2000).

B 3.2.5 Effect of initial pH of the medium

Initial pH of the substrate (Sorgham + chitin) was adjusted by adding 12.5 ml distilled water having different pH. The fermentation was carried out at 28°C with 70-80 RH for 7 d. Higher chitinase and other enzyme production was observed at pH 6.0 (chitinase 1.22±0.02 U/g; protease 1.20±0.05 U/g; lipase 0.33±0.04 U/g and β-1,3-glucanase 0.42±0.02 U/g) (**Table 3.8**). After fermentation the pH of the medium was 7.2±0.1. The initial pH of the medium strongly affects the growth and activity of the microorganisms. Fungal strains are noted for their best performance for enzyme production in the initial pH range of 3.5-6.0 and low pH avoids contamination by other microbes (Nampoothiri et al 2004).

Table 3.8 Effect of initial pH of medium on the production of chitinase and other enzymes by *Myrothecium verrucaria*

Initial pH of medium	Activity (U/ml)			
	Chitinase	**Protease**	**Lipase**	**β-1,3-glucanase**
5.0	0.96±0.02	0.91±0.02	0.25±0.04	0.37±0.02
6.0	1.22±0.02	1.20±0.05	0.33±0.04	0.42±0.02
7.0	0.80±0.02	0.92±0.02	0.17±0.04	0.32±0.02
8.0	0.80±0.01	0.85±0.03	0.17±0.04	0.35±0.01
9.0	0.426±0.01	0.75± 0.01	0.17±0.04	0.15±0.01

The pH of substrate was adjusted using 1 N HCl/NaOH.

B 3.2.6 Effect of different extractant for the recovery of enzymes

Different solvents/extractants were tried for the recovery of enzymes from substrate such as, Triton X-100, glycerol 3% (w/v) + ethanol 10% (v/v), acetate buffer (pH 5.0, 50 mM), 1% sodium chloride, distilled water and Tween 80 (0.1%). Maximum recovery of enzymes (Chitinase 1.52±0.07 U/g, protease 1.42±0.02 U/g, lipase 0.33±0.08 U/g and glucanase 0.43±0.02 U/g) was obtained using 1% sodium chloride from the fermented substrate (**Table 3.9**). Patidar et al (2005) also reported maximum recovery of chitinase from mouldy bran using 1.0% sodium chloride solution. The maximum recovery of proteases with 1.0% sodium chloride solution from mouldy bran of *Mucor bacilliformis* (Fernendez-Lahore et al 1998) and *Rhizopus oligosporus* (Ikasari and Mitchell, 1996) was also reported previously. On the contrary, an aqueous mixture of 3% glycerol with 10% ethanol extracted proteases maximally from mouldy bran of *Rhizopus oryzae* (Tunga et al 1999). However, in the present studies lower recovery of all enzymes were observed using 3% glycerol and 10% ethanol.

Table 3.9 Effect of different extractants for the recovery of chitinase and other enzymes from substrate

	Activity (U/g)			
Extractant	**Chitinase**	**Protease**	**Lipase**	**β-1,3-Glucanase**
Triton X-100	1.12±0.07	1.14±0.05	0.25±0.08	0.31±0.04
Sodium chloride (1%)	1.52±0.07	1.42±0.02	0.33±0.08	0.43±0.02
Glycerol 3% + ethanol 10%	0.73±0.02	0.81±0.01	0.17±0.08	0.26±0.01
Distilled water	0.96±0.02	0.92±0.01	0.25±0.08	0.38±0.02
Acetate buffer (pH 5.0, 50 mM)	1.22±0.02	1.20±0.10	0.33±0.04	0.42±0.01
Tween 80 (0.1%)	1.33±0.03	1.28±0.10	0.25±0.04	0.40±0.01

The enzymes were extracted from the substrate (25 g) using 125 ml extractntant at 28°C under shaking (180 rpm) for 30 min as described under material and methods.

From the above studies it can be seen that the enzyme production by *M. verrucaria* was supported by sorghum and chitin mixture (3:1). Initial pH 6.0 with 33% moisture content of the medium was suitable for higher enzyme production, while maximum recovery of enzymes from fermented substrate were obtained using 1% sodium chloride. In the optimized medium the production of other chitinolytic enzymes (endochitinase, NAGase, chitin deacetylase and chitosanase) were also studied (**Table 3.10)**.

Table 3.10 Chitinases and other enzyme production in optimized medium by *Myrothecium verrucaria* under solid state fermentation

Enzyme (s)	**Activity (U/g)**
Chitinase	1.52±0.07
Endo-chitinase	0.44±0.02
Endo-chitinase (a)	0.26±0.01
NAGase	5.4±0.2
NAGase (a)	1.25±0.06
CDA	$0.9 \pm 0.2 \times 10^{-3}$
Chitosanase	1.21±0.1
Lipase	0.33±0.08
Protease	1.42±0.02
β-1,3-glucanase	0.43±0.02

Fermentation was carried out at 28°C with 70-80% RH for 7 d as described under material and method. Endochitinase (a) and Endochitinase (b) were estimated using ethylene glycol chitin and 4-MU-$(GlcNAc)_3$ as a substrate respectively. NAGase (a) and NAGase (b) were estimated using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide and (4-MU-GlcNAc) as a substrate respectively.

Effect of olive oil in optimized medium on chitinase and other enzyme production was also studied using *M. verrucaria*. Chitinase production was increased from 1.52 ±

0.1 U/g to 1.62 ± 0.2 U/g with the addition of olive oil (2% wt/wt) in the optimized medium. While the lipase activity was significantly increased from 0.33 ± 0.04 U/g to 0.50±0.04 U/g after 7 d. Comparative evaluation of chitinase and other enzyme production under submerged and solid state fermentation by *M. verrucaria* was carried out (**Table 3.11).**

Table 3.11 Comparative evaluation of enzyme production under submerged and solid state fermentation by *Myrothecium verrucaria*

Fermentation	Submerged fermentation (100 L)	Solid state fermentation (25 g extracted with 125 ml 1% NaCl)
Chitinase activity	1 U/ml	1.5 U/g
Enzyme mixture	Low protease (Chitinase: protease, 3:1)	High protease (Chitinase: protease, 1:1)
Medium	Salt solution plus chitin	Sorgham: Chitin (3:1)
Enzyme recovery	Centrifugation, membrane filtration	If necessory, membrane filtration
Cost analysis		
Chitinase (1000 U)	Rs. 80/-	Rs. 14/-
Issues/barriers	Operational cost and media	High protease, chances of contamination, manpower

The cost analysis of the data generated in the laboratory on submerged and solid state fermentation revealed the usefulness of solid state fermentation. However, the high protease activity, chances of contamination and requirement of more manpower for solid state fermentation could be the major issues.

Chapter 4

Biochemical characteristics and stability of chitinolytic enzyme complex of *Myrothecium verrucaria*

4.1 Introduction

Nowadays, some of the mechanisms of enzyme stabilization are better understood and a rational screening of different stabilizing agents for a given enzyme can be carried out successfully. Different alternatives are available for enzyme stabilisation such as genetic engineering, chemical modification and immobilization. However, from an industrial perspective, the simplest and cheapest method to achieve enhanced enzyme stability is by the addition of stabilising agents (Alloue et al 2008). Polyhydric alcohols are additives that are commonly employed for enzyme stabilization and formulation (Noel et al 2005). The polyols have been reported for their stabilizing effect on various enzymes (Kristjansson and Kinsella, 1991; Carpenter et al 1990).

Chitinases hydrolyze chitin, a linear polysaccharide consisting of β-l, 4-*N*-acetylgucosamine (GlcNAc), which is an abundant biopolymer. These enzymes are essential to chitin containing organisms (fungi, insects, crustaceans) and used by many bacteria to utilise chitin as a source of carbon and energy (Gohel and Naseby, 2007). Chitinases of various origins possess versatile enzymological properties and are usually constituents of complex chitinolytic enzyme systems (Fukamizo, 2000). Furthermore, chitinases together with proteases, β-1,3-glucanases and lipases are frequently considered critical in the biocontrol of phytopathogenic fungi and insect (Shaikh and Deshpande, 1993; Patil et al 2000). Chitinases therefore play vital role in agricultural industries.

The process of biocontrol agents face a problem in developing biopesticides for the control of pests in agriculture because crops are grown under a multiplicity of climatic and environmental conditions which include temperature, rainfall, soil type, crop variety which change from farm to farm or even within one field (Gohel et al 2006). The enzyme stability that needs to be addressed depends on the mode of application. For instance, in case of foliar application the effect of temperature, pH and sunlight (UV)

exposure is important. In case of soil application, the adsorption of enzyme mixture on organic matter, degradation by the soil flora adds to instability of the enzyme mixture.

M. verrucaria produces chitinase and other enzymes when grown on purified chitin as a carbon source. In the present investigations, the effect of different temperatures, pH, light (UV) and soil conditions on the stability of chitinase and other enzymes was studied. The effect of various polyols (ethylene glycol, glycerol, xylitol and sorbitol) on the thermostability of enzymes has also been studied.

4.2 Results and Discussion

4.2.1 Optimum temperature and pH of enzyme complex of *M. verrucaria*

To determine the effect of temperature on chitinase and other enzymes from *M. verrucaria*, enzyme activities was estimated over the temperature range of 30-70°C at pH 5.0. The optimum temperature for chitinase (acid swollen chitin degrading activity), endochitinase, NAGase and β-1,3-glucanase was 50°C, while lipase and protease showed maximum activity at 37°C **(Table 4.1)**. The optimum temperature of 50°C was reported for chitinase from crude and purified preparation of *Penicillium aculeatum* (Binod et al 2005). Similar results were observed for chitinase from *T. harzianum* and *B. pabuli* (De La et al 1992; Frandberg and Schnurer, 1994). The optimum temperature for the protease activity from *Bacillus cereus* was 60°C in the presence of Ca^{2+} and 50°C in the absence of Ca^{2+} (Ghorbel et al 2003). Lipase activity (measured on olive oil emulsion) from *Yarrowia lipolytica* was determined from 25 to 60°C. The optimum temperature of the purified lipase was 40°C. The activity dropped sharply above 45°C with no activity remaining at 60°C (Yu et al 2007). The optimum temperature for extracellular β-1,3-glucanases activity (measured on 0.25% laminarin) from *Trichoderma asperellum* was found to be 55°C (Bara et al 2003).

Table 4.1 Effect of different temperatures on the activity of chitinase and other enzymes of *Myrothecium verrucaria*

	Activity U/ml					
Temperature (°C)	**Chitinase**	**Endochitinase**	**NAGase**	**Lipase**	**Protease**	**β-1,3-Glucanase**
30	3.27±0.11	0.84±0.04	10.71±0.35	3.40±0.17	0.17±0.008	0.37±0.03
37	3.99±0.17	1.02±0.04	13.16±0.52	4.32±0.08	0.30±0.010	0.45±0.01
45	4.70±0.10	1.20±0.04	15.17±0.22	3.90±0.08	0.28±0.011	0.58±0.01
50	5.00±0.20	1.29±0.03	15.88±0.62	3.49±0.08	0.26±0.007	0.60±0.04
55	4.46±0.12	1.17±0.04	14.10±0.41	3.24±0.04	0.24±0.011	0.54±0.01
60	3.72±0.10	1.00±0.01	12.30±0.32	2.82±0.08	0.20±0.006	0.46±0.03
70	2.56±0.07	0.64±0.01	7.84±0.22	2.32±0.04	0.14±0.006	0.31±0.03

The optimum temperature for chitinases and other enzymes from crude culture filtrate was determined by estimating the enzyme activities at different temperatures

The optimum pH of chitinase and other enzymes from *M. verrucaria* was determined by measuring their activities in the buffers of different pH values. The following buffer systems were used: acetate buffer (pH 4.0-5.0, 50 mM), phosphate buffer (pH 6.0-7.0, 50 mM), Tris-HCl buffer (pH 8, 50 mM) and carbonate bicarbonate buffer (pH 9.0-10.0, 50 mM). Chitinase, endochitinase, NAGase and β-1,3-glucanase had optimum pH 5.0. Lipase showed maximum activity at pH 7.0 while protease at pH 10.0 **(Table 4.2)**. The optimum pH for the chitinase (measured on colloidal chitin) produced by *Bacillus* and *Pseudomonas aeruginosa* strains were nearly neutral (Yuli et al 2004; Wang and Chang, 1997). The chitinase from *Bacillus circulans* and *Aeromonas* sp. exhibited higher activities in acidic conditions (Sakai et al 1998; Ueda and Arai, 1992), while chitinase from *B. bassiana* was reported to be active at pH 9.2 (Suresh and Chandrasekaran, 1999). The optimum pH for protease and chitinase from *B. cereus* were at pH 9 and pH 5, respectively (Nilegaonkar et al 2007; Wang et al 2009). The optimum pH for protease activity from *B. cereus* was 8.0 (Ghorbel et al 2003). Crude lipase from *Penicillium aurantiogriseum* showed maximal activity at pH 8.0 (Lima et al 2004). The lipase from *Y. lipolytica* showed activity in a pH range of 5.5-9.0. Maximal activity was observed at pH 8.0 and the activity decreased rapidly at pH above 8.0 with only about 20% of the activity remaining at pH 9.0 (Yu et al 2007). β-1,3-Glucanase from *Trichoderma asperellum* showed optimum pH of 5.1 (Bara et al 2003). The optimal activities of fungal β-1,3-glucanases are usually in the range of 4.0 and 6.0 (Pitson et al 1993)

Table 4.2 Effect of different pH on the activity of chitinase and other enzymes of *Myrothecium verrucaria*

	Enzyme activity (U/ml)					
pH	**Chitinase**	**Endochitinase**	**NAGase**	**Lipase**	**Protease**	**β-1,3-Glucanase**
4	3.92±0.13	1.04±0.03	13.04±0.48	0.74±0.06	0.02±0.003	0.54±0.03
5	5.00±0.14	1.29±0.03	15.90±0.20	3.70±0.14	0.16±0.008	0.60±0.02
6	4.72±0.06	1.16±0.02	14.33±0.56	3.88±0.16	0.19±0.004	0.50±0.02
7	4.56±0.15	1.07±0.03	13.36±0.43	4.32±0.06	0.21±0.010	0.45±0.02
8	3.36±0.08	0.88±0.06	10.63±0.32	3.75±0.23	0.24±0.015	0.29±0.01
9	1.81±0.09	0.54±0.04	6.12±0.16	1.81±0.21	0.27±0.010	0.13±0.01
10	0.61±0.05	0.17±0.03	2.58±0.32	0.97±0.04	0.30±0.023	0.08±0.01

The following buffer systems were used for the estimation of enzyme activities: Acetate buffer (pH 4.0-5.0, 50 mM), phosphate buffer (pH 6.0-7.0, 50 mM), Tris-HCl buffer (pH 8, 50 mM) and carbonate bicarbonate buffer (pH 9.0-10.0, 50 mM).

4.2.2 Temperature stability of chitinase and other enzymes of *M. verrucaria*

4.2.2.1 Effect of polyols on the temperature stability

Stability of *M. verrucaria* enzyme mixture was carried out at 4°C, 25°C and 40°C. Initially the enzyme stability was studied at 40°C with and without polyols such as ethylene glycol, glycerol, xylitol and sorbitol (1-5M). Glycerol (5M) showed maximum protection for chitinases, β-1,3-glucanase and lipase while maximum stability of protease was observed in the presence of sorbitol (5M). In the presence of glycerol 5M chitinase, endochitinase, NAGase retained 50-52% of initial activity at 40°C after 3 h. While lipase and β-1,3-glucanase retained 56-60% of initial activities (**Fig. 4.1**). The influence of various polyols such as ethylene glycol, glycerol, xylitol and sorbitol on the thermostability of lipase from *Candida cylindracea* was also examined. All polyols stabilized lipase. However, maximum protection of lipase was observed in the presence of sorbitol (Matsumoto et al 1997). In the present studies, protease retained 58% activity in presence of sorbitol (5M) and 51% in the presence of glycerol (5M) after 3 h at 40°C (**Fig. 4.1**). Ghorbel et al (2003) also studied the influence of various polyols (10%) such as glycerol, mannitol, sorbitol and xylitol, on the thermostability of protease from *B. cereus* at 60°C. Most polyols used improved protease stability. However, maximum stability was observed with sorbitol. The half-life of protease activity in the presence of sorbitol at 60°C was 180 min while in the absence of any additive the half-life was 45 min. In the present studies xylitol was also found to be effective for the stabilization of endochitinase and lipase next to glycerol, and also found better for protease stabilization next to sorbitol. Ethylene glycol was also found to be second best polyol for the thermostability of chitinase (acid swollen chitin degrading activity) and β-1,3-glucanase (**Fig. 4.1**).

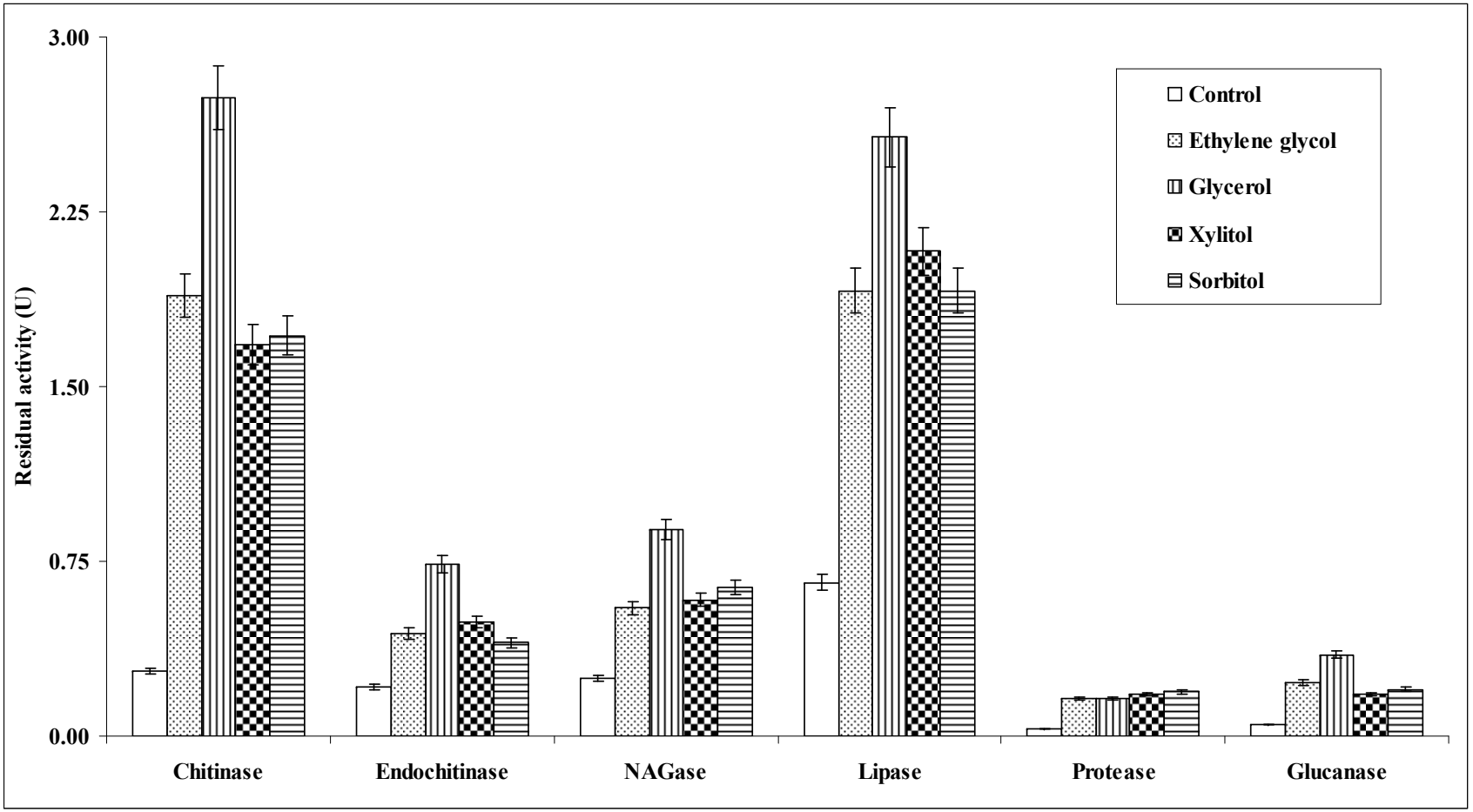

Fig. 4.1 Effect of polyols on the temperature stability of chitinase and other enzymes of *Myrothecium verrucaria*: Enzyme activities after 3h of incubation at 40°C with and without polyols. Control was maintained without polyol. Initial enzyme activities were chitinase 5.36 U/ml, endochitinase 1.43 U/ml, *N*-acetylglucosaminidase 1.77 U/0.1 ml, lipase 4.23 U/ml, protease 0.32 U/ml and β-1,3-glucanase 0.63 U/ml

Effect of different concentration of glycerol (1M-5M) on chitinase and other enzymes of *M. verrucaria* was also studied. As the concentration of glycerol increased the protective effect was also increased for all the enzymes (**Fig. 4.2**). The effect of different concentration of sorbitol (1-5M) on protease activity from *B. cereus* was studied and protective effect increased with increasing sorbitol concentration (Ghorbel et al 2003). Similarly, increased concentration of sorbitol showed increased protective effect for lipase from *C. cylindracea* (Matsumoto et al 1997). According to Hahn-Hagerdal (1986) addition of polyols to the enzyme solutions strengthens the hydrophobic interactions among the non-polar amino acid residues making the protein more resistant to unfolding and thermal denaturation.

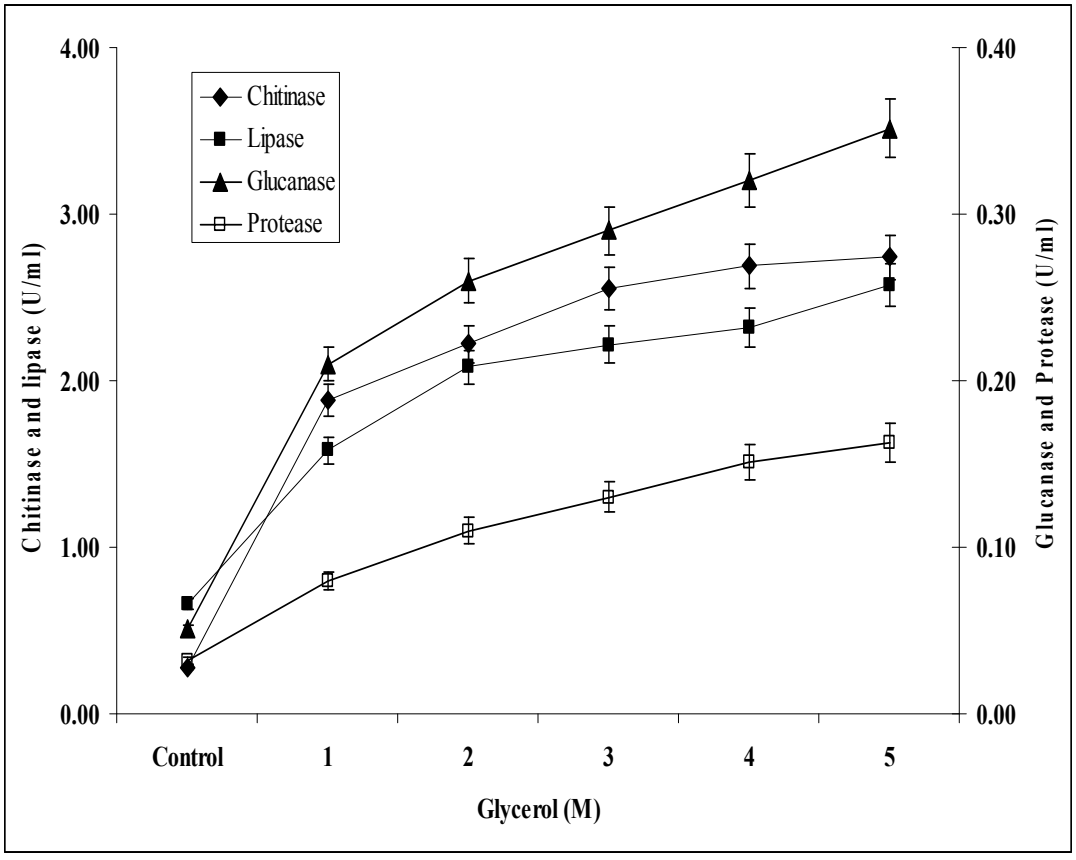

Fig. 4.2 Effect of different concentration of glycerol (1M-5M) on the temperature stability of chitinase and other enzymes of *Myrothecium verrucaria* at 40°C after 3h. Control was maintained without glycerol. Initial enzyme activities were chitinase 5.36 U/ml, lipase 4.23 U/ml and protease 0.32 U/ml, β-1,3-glucanase 0.63 U/ml

An alternative explanation has been proposed by Graber and Combos (1989) for the thermostability of amylase from *A. oryzae.* They have reported that polyols acted as competitive inhibitors and the interaction between the polyols and the active site of the enzyme contributed to the thermostability of the enzyme.

In the present study, glycerol was found to be suitable polyol for protection of chitinases and other enzymes of *M. verrucaria* at 40°C. Thus the enzyme stability in liquid conditions and in lyophilized powder form was carried out with and with out glycerol at 4°C, 25°C and 40°C. In presence of glycerol (5M) under liquid conditions all enzymes retained 60-70% activity at 4°C after 6 months. In absence of glycerol activity of these enzymes was 15-22%. In presence of glycerol (5M) all enzymes showed 48-60% activity at 25°C after 3 weeks while in absence of glycerol all enzymes retained <15% activity **(Fig. 4.3; Table 4.3)**. Glycerol was also found to stabilize chitinase from *Bacillus* sp. In presence of glycerol it retained almost 100 % activity at 25°C after 4 weeks while >75 % activity after 8 weeks (Bhushan, 2000). In lyophilized powder 70-75% enzyme activity was observed at 4°C after 1 year, while > 90% enzyme activity was obtained in lyophilized powder mixed with glycerol (enzyme powder 1g: glycerol 5g). The protective effect of glycerol was more promising at 25°C and 40°C **(Table 4.3).**

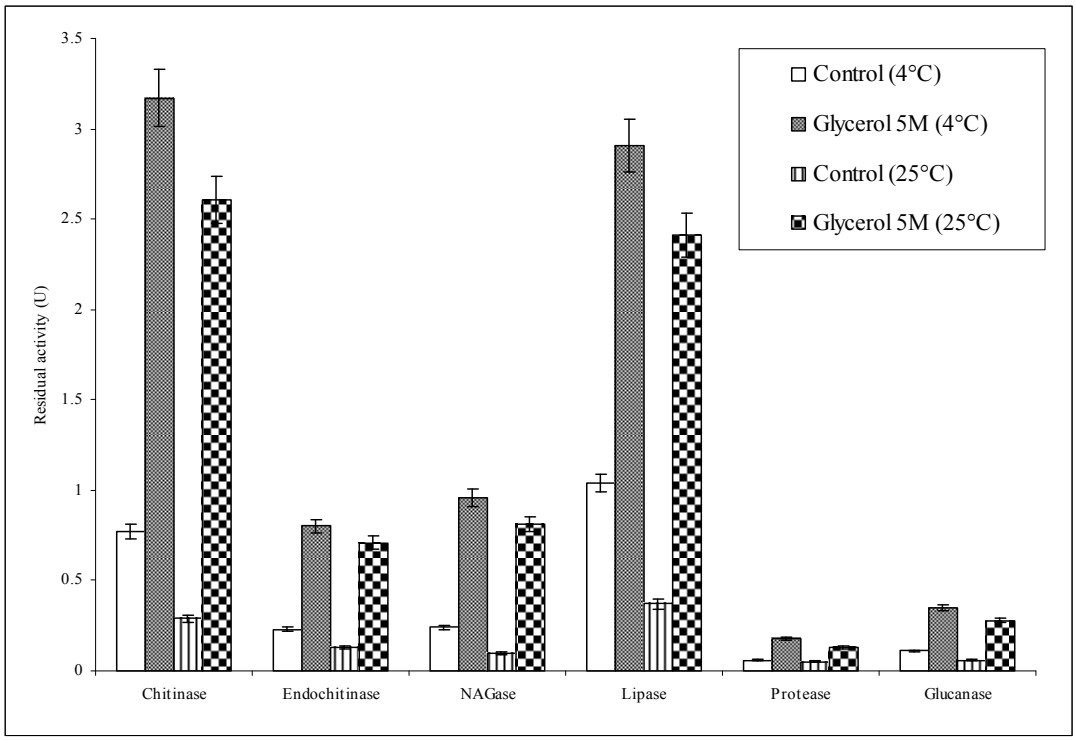

Fig. 4.3 Effect of glycerol on the temperature stability of chitinase and other enzymes of *Myrothecium verrucaria* at 4°C after 6 months and at 25°C after 3 weeks*:* The initial enzyme activities were chitinase 5.10 U/ml; endochitinase 1.34 U/ml; *N*-acetylglucosaminidase 1.62 U/0.1 ml; lipase 4.07 U/ml; protease 0.27 U/ml and β-1,3-glucanase 0.55 U/ml

Table 4.3 Effect of glycerol on the temperature stability of chitinase and other enzymes of *Myrothecium verrucaria*

Temperature (°C)	Residual chitinase and other enzyme activities (%)		
	Enzyme with out glycerol	Enzyme with glycerol *	Time
Liquid form			
4	15-22	60-70	6 months
25	<15	48-60	3 weeks
40	<15	51-61	3 h
Powder form			
4	70-75	>90	1 year
25	60-65	80-85	2 months
40	40-50	57-65	1 week

*Under liquid conditions enzymes with 5M glycerol was used while for powder form, 1 g lyophilized enzyme powder was mixed with 5 g glycerol

4.2.3 pH stability of chitinase and other enzymes of *M. verrucaria*

The pH stability of chitinase and other enzymes were carried out by incubating the enzyme preparation at different pH (4 to 10) at 25°C. Chitinase, endochitinase and NAGase were stable in the pH range (5 to 7), with 59-72% residual activities at 25°C after 7 d. These enzymes also retained 50% of initial activities at pH 4 and 8 at 25°C after 7 d. *Streptomyces thermoviolaceus* chitinase showed residual activity to be more than 80% in the range of pH 4 to 12 at 60°C, it also retained its original activity during incubation in 50 mM Tris-HCl, pH 7.0, for 14 days at 50°C (Tsujibo et al 1993). The protease from *M. verrucaria* was more stability in the pH range of 6-10, with 71-79% residual activities at 25°C after 7 d. It also exhibited 60% residual activity at pH 5. The pH stability profile of protease and chitinase activity from *B. cereus* were determined by the measurement of the residual activity at pH 7.0 after incubation at various pH values at 37°C for 60 min. The protease and chitinase had broader pH stability range (pH 3-11) (Wang et al 2009). Lipase from *M. verrucaria* was stable in the pH range 5-7, with 65-74% residual activities at 25°C after 7 d. It also exhibited 45% and 43% residual activities at pH 4 and 8, respectively. The lipase activity of the crude extract from *P. aurantiogriseum* remained stable in the pH range of 5.0-9.0 with 80% residual activity at 28°C after 1 h. However, fungal lipases are, in general, quite stable in the pH range from 4 to 7 and unstable in alkaline pH values, as has been observed for the lipases of *Penicillium* and *Mucor* sp (Lima et al 2004). β-1,3-Glucanase from *M. verrucaria* was stable in the pH range 4-6, with 70-79% residual activities at 25°C after 7 d. It also exhibited 65% and 48% residual activity at pH 7 and 8. (**Fig. 4.4**). The purified endo-β-1,3-glucanase (measured on laminarin) from *Agaricus brasiliensis* showed high pH stability within the range of pH 3.5-6.0 (Shu et al 2006).

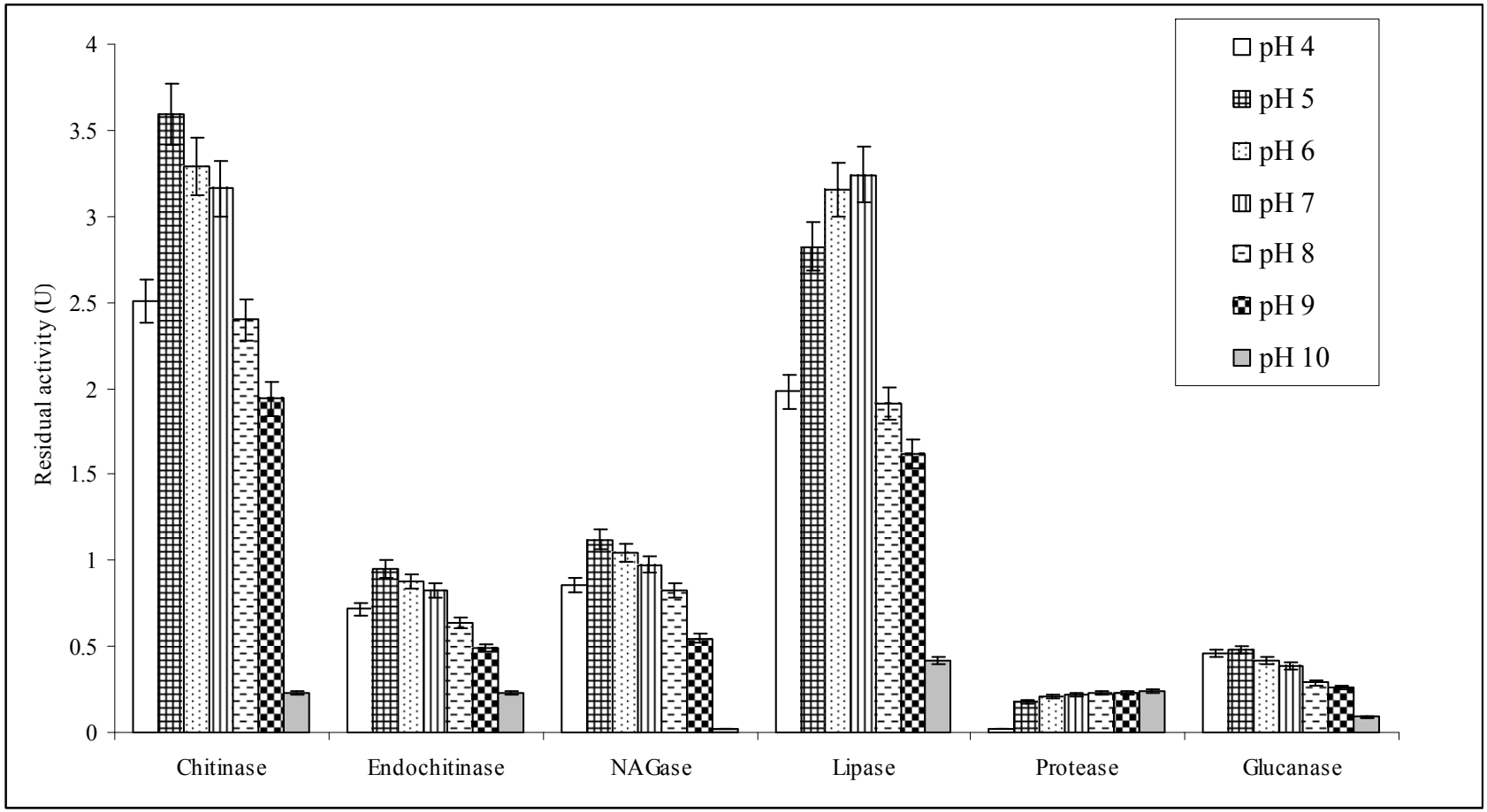

Fig. 4.4 pH stability of chitinase and other enzymes of *Myrothecium verrucaria* at 25°C after 7 d. The initial enzyme activities were chitinase 5 U/ml; endochitinase 1.36 U/ml; NAGase (*N*-acetylglucosaminidase) 1.65 U/0.1ml; lipase 4.32 U/ml; protease 0.3 U/ml and β-1,3-glucanase 0.6 U/ml. The following buffer systems were used for the pH stability study: acetate buffer (pH 4.0-5.0, 50 mM), phosphate buffer (pH 6.0-7.0, 50 mM), Tris-HCl buffer (pH 8, 50 mM) and carbonate bicarbonate buffer (pH 9.0-10.0, 50 mM).

4.2.4 Light (UV) stability of chitinase and other enzymes of *M. verrucaria*

Due to strong absorption of aromatic amino acids (tyrosine, tryptophan, phenyl alanine) at about 280 nm, proteins are known to be highly susceptible to UV-B radiation, either by photodestruction of respective molecules or splitting of disulfide bridges between two cystine residues. Since these bonds are important for the tertiary structure of protein, its function can be strongly influenced (Bischof et al 2000). During the aerial spray, the ultraviolet radiation can affect the enzyme preparation of *M. verrucaria*. Thus, the effect of ultraviolet radiation on chitinase and other enzymes such as lipase, protease and glucanase was studied. In the presence of ultraviolet radiation the chitinase and other enzymes retained 85-90% residual activities at 25°C after exposure for 5h (**Fig. 4.5**).

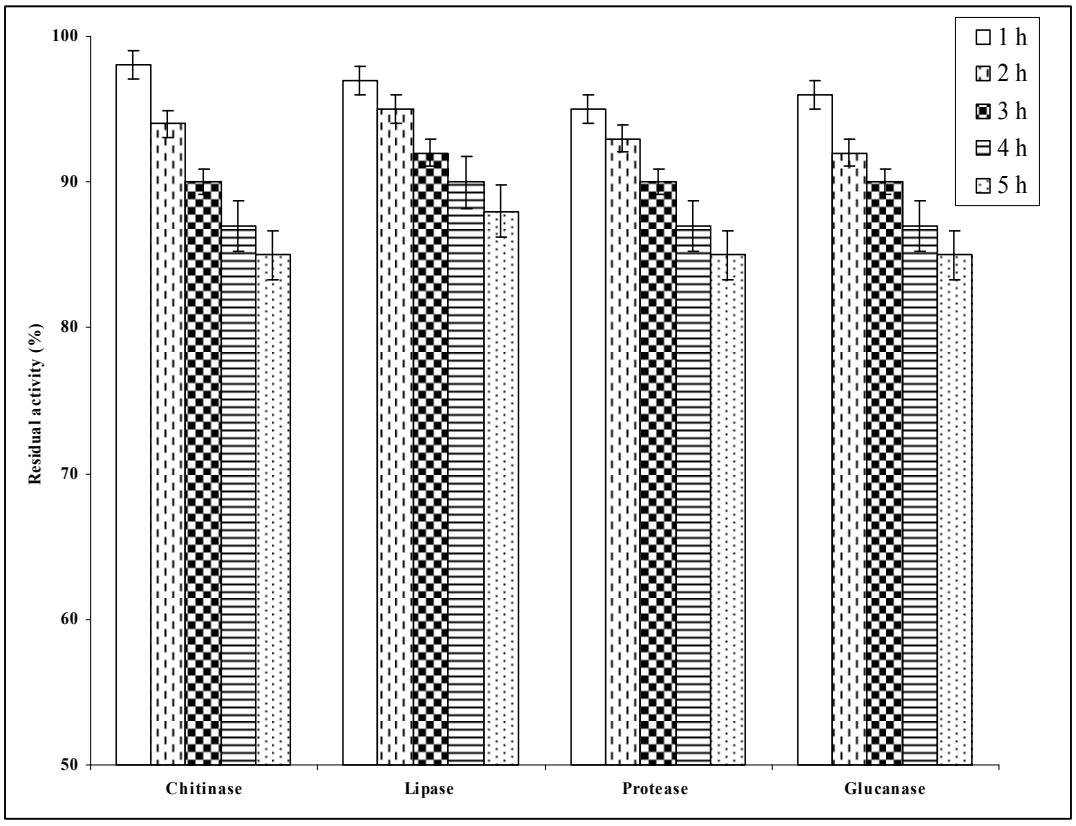

Fig. 4.5 Effect of ultraviolet (UV) radiations on enzymes of *Myrothecium verrucaria.* The crude culture filtrate containing chitinases and other enzymes (25 ml preparation) in glass petriplate was irradiated with 12-W electric UV lamp from 20 cm distance at 25°C for 5 h.

The cumulitive effect of sunlight and temperature on the stability of chitinase and other enzymes of *M. verrucaria* was also studied in presence and absence of glycerol (1M) for 7 d. The enzyme samples incubated in dark at RT and at 4°C were served as controls. Chitinase and other enzymes retained 41-46% activity in presence of sunlight after 5 d. While in presence of glycerol under sunlight all enzymes showed 60-65% activity after 5 d. The enzymes incubated in dark retained 51-55% activity at RT after 5 d and at 4°C, 88-92% activities were observed (**Fig. 4.6**). From above results it can be seen that slight reduction (15-20%) in the enzyme activities of *M. verrucaria* were observed in presence of sunlight. Protective effect of glycerol for enzymes in presence of sunlight was also observed. The reduction in enzyme activities can mainly be attributed to temperature. Natural sunlight especially the UV portion of the spectrum UV-B (280-310 nm) and UV-A (320-400 nm) was responsible for inactivation of granulosis virus of *Pieris brassicae* (David, 1969). The short persistence of *B. thuringensis* on crops following exposure to direct sunlight has been reported many times. *B. thuringensis* applied to spruce trees lost 50% of the insecticidal activity in 8 d in shade, compared with 50% loss in 2 d in sunlight (Ragaei, 1999). Ignoffo and Garcia (1992) studied the influence of conidial colour on the inactivation of several fungi (Hyphomycetes) by simulated sunlight. The black conidia of *A. niger* were significantly more stable (14.8±2.7 h) to sunlight than the lighter pigmented conidia of all the other isolates. The half-life of yellow conidia of *N. rileyi* was 1.1 ± 0.1 h. In addition, dry conidia of *N. rileyi* were more stable to sunlight than wet conidia. In the present investigation the significant effect of direct sunlight and artificial UV radiation on *M. verrucaria* enzyme preparation was not observed. This can be attributed to yellow to orange colour of the enzyme preparation.

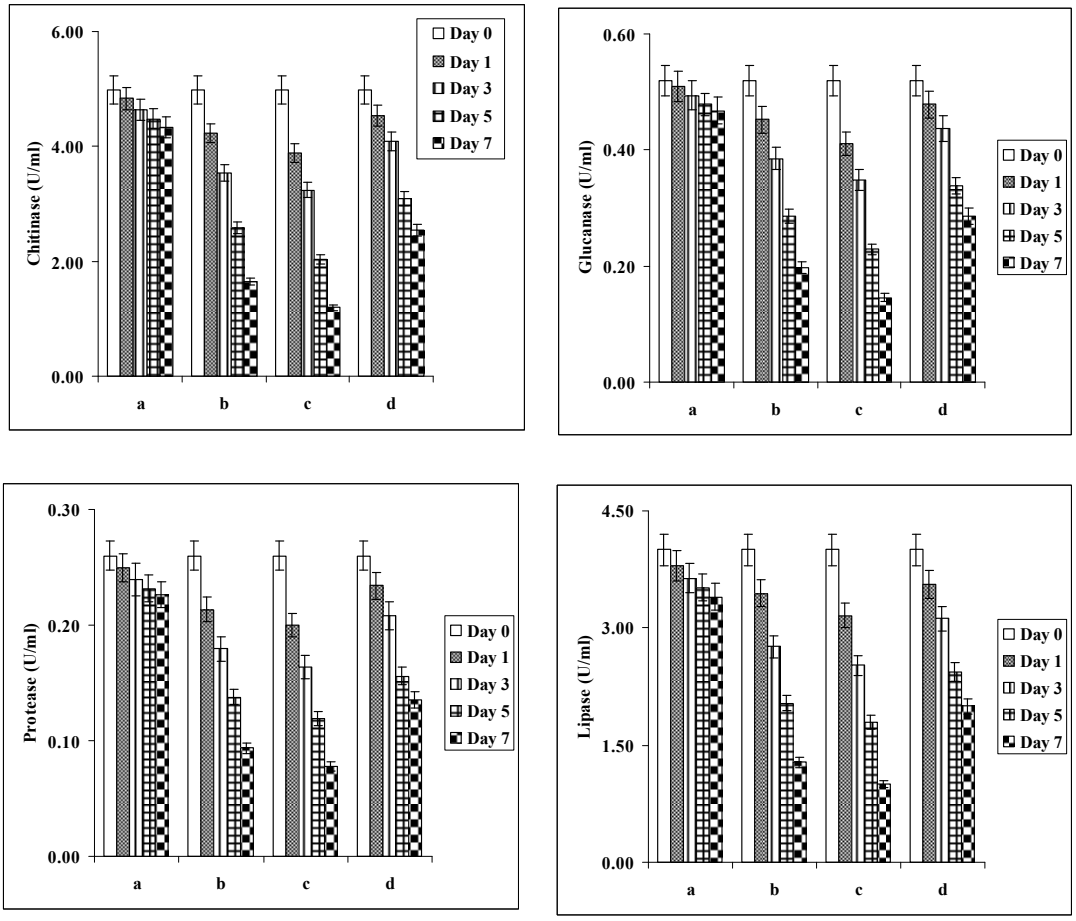

Fig. 4.6 Effect of sunlight on the stability of enzymes of *Myrothecium verrucaria*. **a:** enzymes were incubated at 4°C; **b:** enzymes were incubated in dark at RT; **c:** enzymes were incubated in presence of sunlight at RT; **d**: enzymes with 1M glycerol were incubated in presence of sunlight at RT. The temperature during daytime was 22-30°C while during night the temperature was 15-22°C.

4.2.5 Adsorption and stability of enzymes of *M. verrucaria* in presence of soil constituents

During the pot experiment studies, a pot containing soil and peanut seeds (2 no.) was drenched with *M. verrucaria* enzyme mixture. The 100 ml enzyme preparation (containing chitinase, glucanase and protease) was drenched/pot and the adsorption of enzymes and protein on soil organic matter was determined. Maximum enzyme activities were found to be adsorbed at top portion (50-52%) of the pot, while adsorption in the middle portion was 20-23% and at the bottom 10-17% adsorption was detected. Similar

pattern for adsorption of protein was also observed. Total recovery of enzyme was 83-91% while total protein recovery was 92% (**Table 4.4**). Adsorption and binding of proteins to soil and the stability of enzyme soil complex to microbial attack have been extensively studied (Stotzky, 1986; Boyed and Mortland, 1990; Nannipieri et al 1996). According to Stotzky (1986), a protein can be released from protein clay complex by water washing.

Table 4.4 Adsorption of enzymes of *Myrothecium verrucaria* with soil constituents

Portion of pot	Chitinase (U)	β-1,3-Glucanase (U)	Protease (U)	Protein (mg)
Top	25.9±1.0	3.7±0.20	1.3±0.20	9.7±0.2
Middle	10.8±0.8	1.6±0.20	0.5±0.03	4.2±0.2
Bottom	8.3±0.6	1.0±0.05	0.2±0.02	2.7±0.1
Total activity recovered	45.0±2.0	6.3±0.30	2.0±0.10	16.6±0.5
Initial activity	49.7±2.3	7.4±0.26	2.4±0.21	18.0±2.0

The soil samples (10g) were taken from different portions of the pot (top 1-3 cm, middle 4-6 cm and bottom 7-9 cm). The enzyme activities in the samples were estimated by repeated washing (2-3 times) of soil with acetate buffer (pH 5.0, 50 mM).

The enzyme stability in presence of soil constituents was also studied. The residual enzyme activities in the pots were estimated every day. Chitinase, β-1,3-glucanase and protease were stable in the soil with 51-57% residual activity at 25°C after 5 d (**Table 4.5**).

Table 4.5 Stability of enzymes of *Myrothecium verrucaria* in presence of soil constituents

Day	Enzyme activities (U)		
	Chitinase	**β-1,3-Glucanase**	**Protease**
0	50±2	7.0±0.2	2.0±0.20
1	42±3	6.1±0.3	1.8±0.05
3	35±1	5.0±0.4	1.5±0.05
5	26±3	3.8±0.2	1.1±0.04
7	19±1	3.0±0.2	0.9±0.02

For stability study 10 g soil was taken every day from top, middle and bottom portion of the pot. The residual enzyme activities in the samples were estimated by repeated washing (2-3 times) of soil with acetate buffer (pH 5.0, 50 mM).

The stability of enzyme preparation of *M. verrucaria* in presence of soil can be attributed to adsorption of enzymes in soil. It was also reported that free enzymes are less stable as compared to adsorbed/immobilized enzymes (Burns, 1982).

Chapter 5

Proof of concept

Field performance studies of chitinase and other enzymes of *Myrothecium verrucaria* either singly or in combination for the biocontrol of insect pests as well as plant pathogenic fungi

5.1 Introduction

There are several insect and fungal pests in India which cause severe loss in different field crops. Sugarcane woolly aphid (*C. lanigera*) has become a serious pest in Maharashtra. The woolly aphids protective cover comprises of extensive woolly mass, especially containing wax (lipids) followed by the other insect cuticle components such as chitin and lipoproteins. The woolly aphid multiplies profusely and desaps the foliage heavily and excretes honey dew like substances on the leaves which favour the multiplication of black sooty mould (*Capnodium* sp.), resulting in drying of leaves and ultimately decrease in yield and sugar recovery (Joshi and Viraktamath, 2004). Commonly used chemical control includes malathion and dimethoate sprays. However, penetration of chemicals up to insect body is sometimes difficult due to waxy coating. As a result, woolly aphids reappear after 10-15 days and again multiply profusely on sugarcane leaves.

Another important insect pest is *H. armigera,* a highly polyphagous agricultural pest. Host species for *H. armigera* include important agricultural crops such as cotton, maize, chickpea, pigeon pea, sorghum, sunflower, soyabean and groundnuts (Cunningham et al 1999). It is one of the most important insect pests in the world due to its mobility, high polyphagy, short life cycle and high reproductive rate (Lawo et al 2008). Recently, field populations of *Helicoverpa* from the main tobacco-growing regions of China showed their resistance to the conventional insecticidal chemicals fenvalerate (pyrethroid), phoxim (organophosphate), and methomyl (carbamate) under laboratory conditions (Xia et al 2009). Diseases caused by soil pathogenic fungi are a limiting factor in peanut production, and they are one of the main causes of the reduction of the planting area. *F. solani, S. rolfsii* and *R. solani* causes root, stalk, crown and fruit

decay in peanut. The disease causes the death of plants, with the consequent economic losses (Rojo et al 2007).

Earlier, *M. anisopliae* has been used for control of *H. armigera* infestation on pulses (Nahar et al 2003; Nahar et al 2004b; Kulkarni et al 2008). It was reported that in fungus-insect interactions chitinases, lipase and protease play a significant role in pathogenesis (Krieger et al 2003; El-Sayed et al 1989; St. Leger et al 1986b; 1991; Nahar et al 2008; Kulkarni et al 2008). *B. thuringiensis* is also most widely used microbial control agent. Bt products are primarily used for the control of lepidopteran pests, such as cotton bollworm, *H. armigera*, Other species of bacteria such as *B. sphaericus* is now commercially produced for the control of mosquito (Lacey and Undeen, 1986; Charles et al 1996; Nicolas et al 1994).

M. verrucaria produces extracellularly chitinase and other enzymes mainly lipase, β-1,3-glucanase and protease (Vyas and Deshpande, 1989; Deshpande, 1986; Shaikh and Deshpande, 1993; Patil et al 2000). To control insect pests such as *C. lanigera*, *H. armigera,* and fungal pathogens like *F. oxysporum* and *S. rolfsii*, an attempt was made to develop an enzyme based biocontrol agent i.e. crude culture filtrate of *M. verrucaria* containing chitinase and other enzymes. Also significance of lipase and other enzymes in the biocontrol of *C. lanigera,* along with entomopathogen *M. anisopliae* and *Bacillus* sp. B1 was evaluated.

5.2 Results and Discussion

5.2.1 Significance of cuticle degrading enzymes with special reference to lipase in the biocontrol of sugarcane woolly aphids

5.2.1.1 Enzyme production

The **Table 5.1** describes the extracellular enzyme activities produced by *M. verrucaria, M. anisopliae* and *Bacillus* sp. All the enzyme activities were found to be

higher in *M. verrucaria* however the productivities (U/d) for protease were higher for *M. anisopliae* and *Bacillus* sp.

Table 5.1 Extracellular enzyme activities produced by *Myrothecium verrucaria*, *Metarhizium anisopliae* and *Bacillus* sp.

Enzymes	Activity (U/ml)		
	M. verrucaria	***M. anisopliae***	***Bacillus* sp.**
Chitinase	1.80±0.2	3.95×10^{-3}±0.11	6.0×10^{-3}±0.5
Endo-chitinase	0.50±0.03	1.13×10^{-3}±0.07	1.1×10^{-3}±0.09
NAGase	6.00± 0.4	1.77×10^{-3}±0.03	1.9×10^{-3}±0.08
CDA	1×10^{-3} ±0.1	1.30×10^{-3}±0.19	0.1×10^{-3}±0.01
Chitosanase	1.60±0.1	32.39×10^{-3}±1.12	4×10^{-3}±0.07
Protease	0.012±0.002	3.38±0.15	0.27±0.02
β-1,3-Glucanase	0.190±0.01	0.05±0.001	0.08±0.006
Lipase	1.58±0.08	0.996±0.04	0.125±0.01

All the enzyme activities were reported as mean ± standard deviation;
NAGase- N-acetylglucosaminidase; CDA- Chitin deacetylase

5.2.1.2 Waxy mass analysis of *C. lanigera*

The population of *C. lanigera* (woolly aphid) is female dominated and males are rare. Females produce viable progenies with out males and this condition is called parthenogenesis. They are viviparous, that is, give birth to young ones directly instead of laying eggs. One female can give birth to 217 young ones in its life span of 20 days. New born ones (first instar) do not have the woolly coating. Waxy coating increase gradually from second instar. Third instar shows clear inter segmental lines while fully grown aphid gives totally woolly appearance. Usually the adult is also wingless with a woolly coating. But when the population is more the nymphs transform into winged adults called

alates. Alates are black in colour without woolly coating over their body. The different stages of woolly aphids are shown in **Fig. 5.1**. Different insect pests such as aphids, white flies, and mealy bugs secrete waxy material for the protection (Smith, 1999). Cuticle lipids from whiteflies, *Bemisia argentifolii*, were found to be inhibitory to the germination of *B. bassiana* and *P. fumosoroseus* (James et al 2003).

Fig. 5.1 Different stages of *Ceratovacuna lanigera* (woolly aphids) of sugarcane; **a**, New born (first instar) aphids with out waxy mass; **b**, second to third instar aphids with growing waxy mass; **c**, fully grown aphid gives totally woolly appearance; **d,** winged adults called alates, Alates are black in colour with out the woolly coating over their body

The hydrolysis of waxy mass and cuticular surface lipids of *C. lanigera* was carried out using *M. verrucaria* enzyme preparation. The hydrolyzed waxy mass and cuticular surface lipids were analyzed using gas chromatography. It was observed that the wax particles and cuticular surface lipids of woolly aphids are composed of a mixture of long-chain aldehydes and long chain alcohols, the major chain lengths being C24, C26 and C28 (**Fig. 5.2**). Nelson et al (2003) reported the analysis of the waxy particles of

white flies and dusty wing *Semidalis flinti* Meinander (Neuroptera: Coniopterygidae), a predator of white flies and aphids. The whitefly waxy particles consist of a mixture of a long-chain aldehyde and a long-chain alcohol with major components of C30, C32 or C34. Nelson et al (1997) also reported the composition of the wax particles and surface wax of adult whiteflies. The wax particles composed of a mixture of long-chain aldehydes and long-chain alcohols with the major chain lengths being C30, C32, or C34. The cuticular surface wax composed of C40 and C42. The wax layer in locusts comprises 80% hydrocarbons, and small amounts of esters, free fatty acids, free primary alcohols, and possibly some triacyglycerols (Jarrold et al 2007).

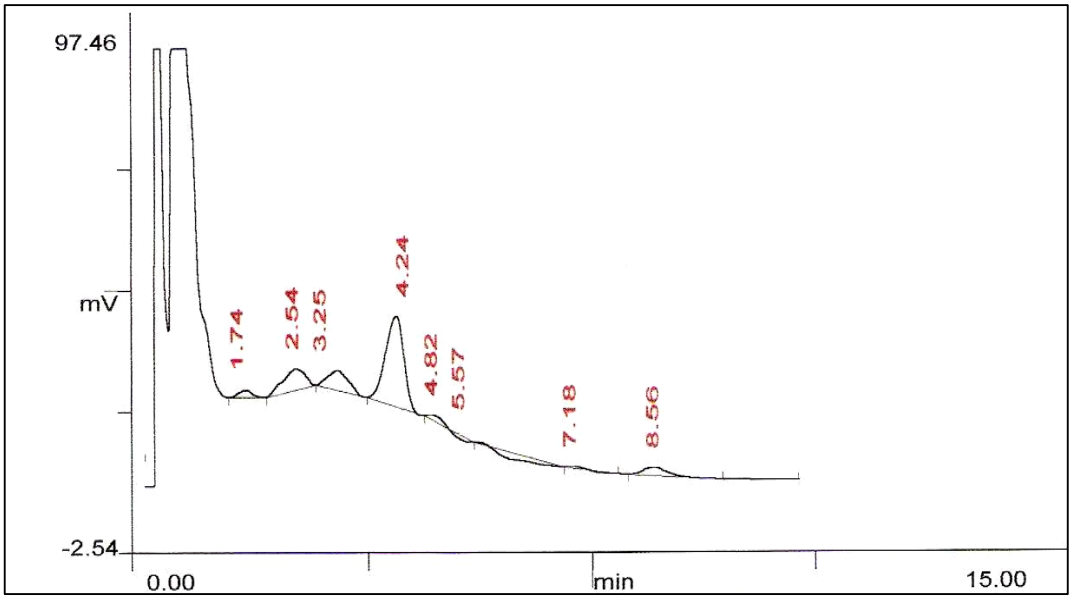

Fig. 5.2 Hydrolysis of woolly mass of *Ceratovacuna lanigera* using enzyme preparation of *Myrothecium verrucaria* and its analysis by gas chromatography**.** The hyrolysis was carried out for 24h at 25°C. The peak 2.54, 3.25 and 4.24 represents long chain aldehydes and alcohol with major chain length being C24, C26 and C28.

5.2.1.3 Bioassay with *C. lanigera*

The enzyme mixtures produced by *M. verrucaria*, *M. anisopliae* and *Bacillus* sp. B1 were used for the bioassay. To study the effect of lipase activity, *per se*, the lyophilized powder of *M. verrucaria* crude culture filtrate was diluted to obtain lipase

activities 1-5 U/ml. The other activities (Chitinases and protease) were proportionately varying in the preparations. The mortality of *C. lanigera* was 32% with commercial lipase (Sigma). The mortality of *C. lanigera* was found to increase as the lipase and other enzyme activities of *M. verrucaria* increased. It can be seen from the **Table 5.2** that the lipase activity significantly contributed in the mortality of the aphids. However, all other cuticle degrading enzyme activities were indeed necessary to hydrolyse the aphid cuticle. The mortality was 73-88% with *M. verrucaria* enzyme preparation measured as 1-5 U/ml lipase activity. The *C. lanigera* mortality with enzyme preparation (lipase 1 U/ml) from *M. anisopliae* and *Bacillus* sp. B1 was 59 and 54% respectively. Effect of *M. anisopliae* conidia singly and in combination with *M. verrucaria* enzyme preparation was also studied. The mortality of *C. lanigera* was 66±4% using *M. anisopliae* conidia, however the mortality increased to 96±3% using combined treatment. *Bacillus* sp. B1 liquid culture grown in chitin medium (enzymes + cells) showed 59% mortality of *C. lanigera* (**Table 5.2**). In the biocontrol of insect pests enzymic and non-enzymic killing components were involved (Krieger et al 2003; Nahar et al 2004a; St. Leger et al 1986a). The contribution of chitinases, lipase and protease were highlighted earlier during the pathogenesis of *M. anisopliae* against *H. armigera* (Kulkarni et al 2008). In the present investivagations enzymes like lipase, chitinases and protease were found to be promising singly as well as in combination with microorganisms. However, microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic bacteria, viruses, fungi and microbial metabolites (Kramer and Muthukrishnan, 1997). Synergistic effects among chitinolytic enzymes and microbial insecticides have been known to occur since the early 1970s. Larvae of the spruce budworm, *Choristoneura fumiferana*, died more rapidly when exposed to chitinase-Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff, 1973; 1974; Lysenko, 1976; Morris, 1976).

The larvicidal activity of a nuclear polyhedrovirus towards gypsy moth larvae was increased about five fold when it was co-administered with a bacterial chitinase (Shapiro et al 1987). An enhanced toxic effect towards *S. littoralis* also resulted when Bt toxin and bacterial endochitinase were incorporated into a semisynthetic insect diet (Regev et al 1996).

Table 5.2 Correlation between lipase activities of *Myrothecium verrucaria*, *Metarhizium anisopliae* and of *Bacillus* sp. with mortality of *Ceratovacuna lanigera* of sugarcane

Organism	**Lipase (U/ml)**	**Mortality of *C. lanigera* (%)** Mean±SD
M. verrucaria	1.0	73±3
	2.0	77±2
	3.0	82±3
	4.0	83±4
	5.0	88±2
Bacillus sp. enzyme	1.0	54±4
M. anisopliae enzyme	1.0	59±2
Commercial lipase	2.0	32± 3
M.v. enzyme + *M.a.* conidia	1.0	96±3
M. anisopliae conidia	-	66±4
Bacillus sp. liquid culture	0.1	59±4

The bioassay was carried out under laboratory conditions at 28°C with 70-80% RH for 96 h. Mortality was corrected over control by Abbott's (1925) formula; phosphate buffer (pH 6.8, 50 mM) was used as a control for enzyme preparation while distilled water containing 0.1% Tween 80 was used as control for *M. anisopliae* conidia and *Bacillus* sp. liquid culture (cells+enzyme); SD- Standard Deviation; Commercial lipase (from *Candida rugosa*) was procured from Sigma Chemical Co, USA

5.2.1.4 Germination of conidia of *Capnodium* sp.

The *C. lanigera* multiplies profusely and desaps the foliage heavily and excretes honey dew on the leaves which favours the multiplication of black sooty mould (*Capnodium* sp.). Sooty mould is the name used for any saprophytic fungus which forms a dark brown-black mycelium over leaves, fruits and other plant parts. Sooty moulds can often be peeled off the leaves or fruits. In India rice starch was sprayed onto plants to control sooty moulds. Another solution is to control the insects that produce the honey dew droppings (Fox, 1997). In the present studies, the effect of enzyme preparations of *M. verrucaia, M. anisopliae* and *Bacillus* sp. B1 on conidial germination of *Capnodium* sp. was studied. The percent germination of *Capnodium* sp. reduced by 25-35% after 24 h due to the enzyme treatments of *M. verrucaia, M. anisopliae* and *Bacillus* sp. B1. From the above results it can be seen that all the above enzyme preparations can control *C. lanigera* as well as sooty mould of sugarcane. The β-1,3-glucanase activity of enzyme preparations may be contributing significantly in the mycolytic activity.

5.2.1.5 Field experiments

Performances of crude enzyme preparation of *M. verrucaria,* liquid culture of *Bacillus* sp. B1, *M. anisopliae* conidia, singly and in combination with *M. verrucaria* crude enzyme preparation were evaluated against *C. lanigera* in sugarcane (variety CO86032) fields. The metasystox treatment and plots treated with water containing 0.1% Tween 80 were used as controls. Under field conditions, the efficacy of *M. verrucaria* enzyme preparation measured as lipase 5U/ml was 61±21%, which was attributed to the spraying efficiency, stability in the field and other parameters, if any. The % efficacy for *Bacillus* sp. B1 was 49±20% (**Table 5.3**). The importance of bacteria, fungi and fungal enzymes in insect pathogenesis has been shown earlier (Chavan et al 2008). Soybean oil-degrading bacterial cultures (*Pseudomonas* sp. and *Rhodococcus* sp.) showed potential

for control of green peach aphids. Approximately 60% mortality of aphids was observed when the cultures were applied alone onto aphids (Kim et al 2007). Importance of chitinases, lipase and protease from *M. anisopliae* for the mortality of *H. armigera* has been reported earlier (Kulkarni et al 2008; Nahar et al 2008; Orlando et al 2005). *Fusarium moniliforme* was found to be a promising biocontrol agent for the control of woolly aphids of sugarcane. Reductions of aphid populations up to 60% were observed when the fungus was applied as two sprays at a week interval in the field (Mehetre et al 2008). In the present investigation, similar control of woolly aphid (54±21%) was observed using *M. anisopliae* conidia. The efficacy of *M. anisopliae* conidia to control *C. lanigera* was increased to 77±19% with the prior spray of *M. verrucaria* enzyme (Lipase 1U/ml). This can be attributed to the better fungus-insect interaction after the successful removal of the woolly mass by the enzyme treatment. The use of enzyme treatment before entomopathogenic fungus *V. lecanii*, for the control of pests like green peach aphids and beetles was successfully tried (Higuchi et al 1998).

Table 5.3 Biocontrol of *Ceratovacuna lanigera* using enzyme preparation of *Myrothecium verrucaria*, *Metarhizium anisopliae* conidia and *Bacillus* sp.

Different treatments	Efficacy (%) Mean±SD
M. v. enzymes (lipase 5U/ml)	61±21
M. anisopliae. (5×10^{12} conidia/ha)	54±21
M. v. enzymes (lipase 1U/ml) + *M. anisopliae.* (5×10^{12} conidia/ha)	77±19
Bacillus sp. liquid culture (5×10^{12} cfu/ha)	49±20
Metasystox	57±21

Efficacy (%) was calculated using Henderson and Tilton (1955) formula
SD- Standard Deviation; cfu- colony forming unit

The effects of different treatments on *C. lanigera* from present investigations are shown in **Fig. 5.3.**

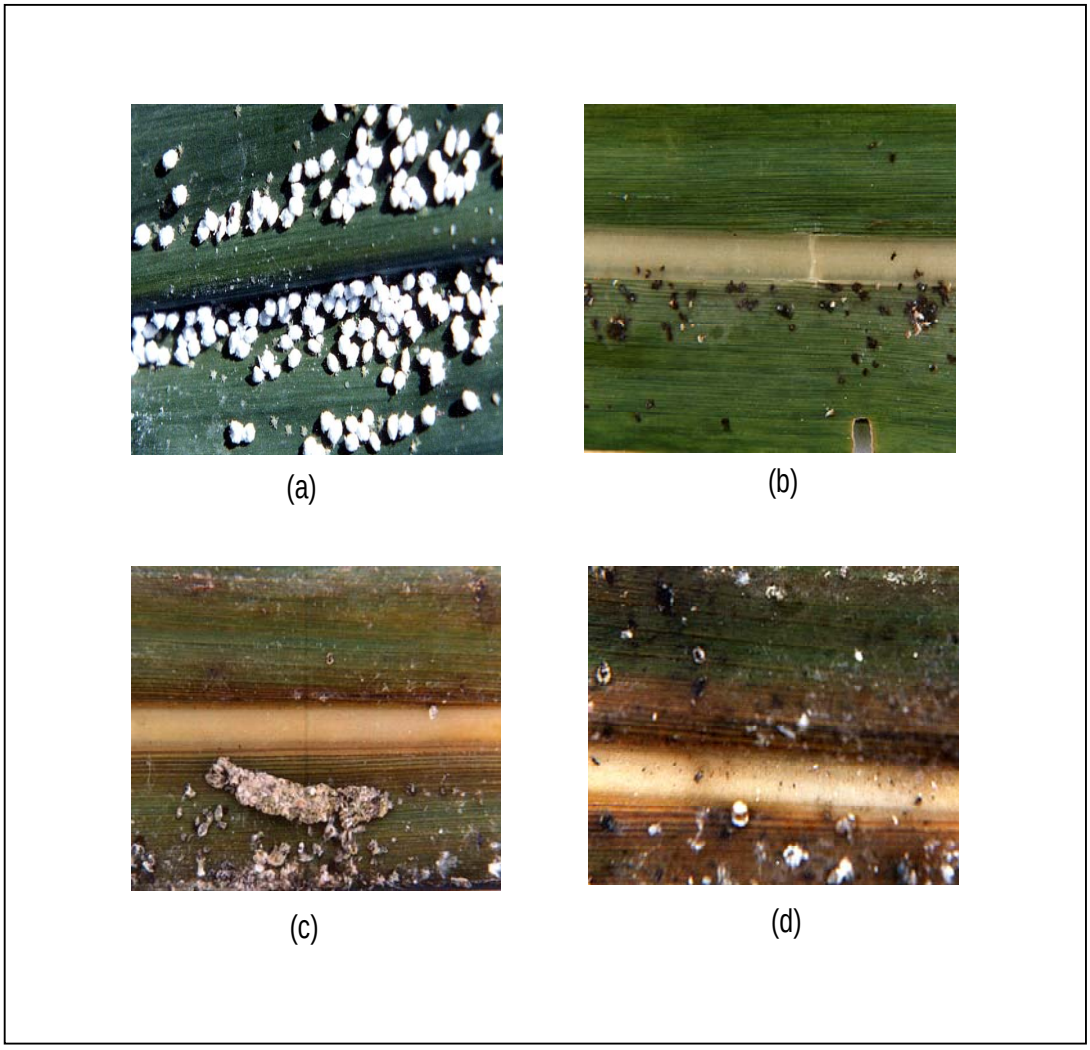

Fig 5.3 Effect of different treatments on *Ceratovacuna lanigera* of sugarcane (a) Aphids treated with water containing 0.1% Tween 80 (b) Aphids treated with *Myrothecium verrucaria* enzyme preparation (c) Aphids treated with *Metarhizium anisopliae* conidia (d) Aphids treated with a chemical insecticide, Metasystox

Differences in the live *C. lanigera* population on sugarcane leaf before and after different treatments were recorded upto 28 d (**Fig. 5.4**).

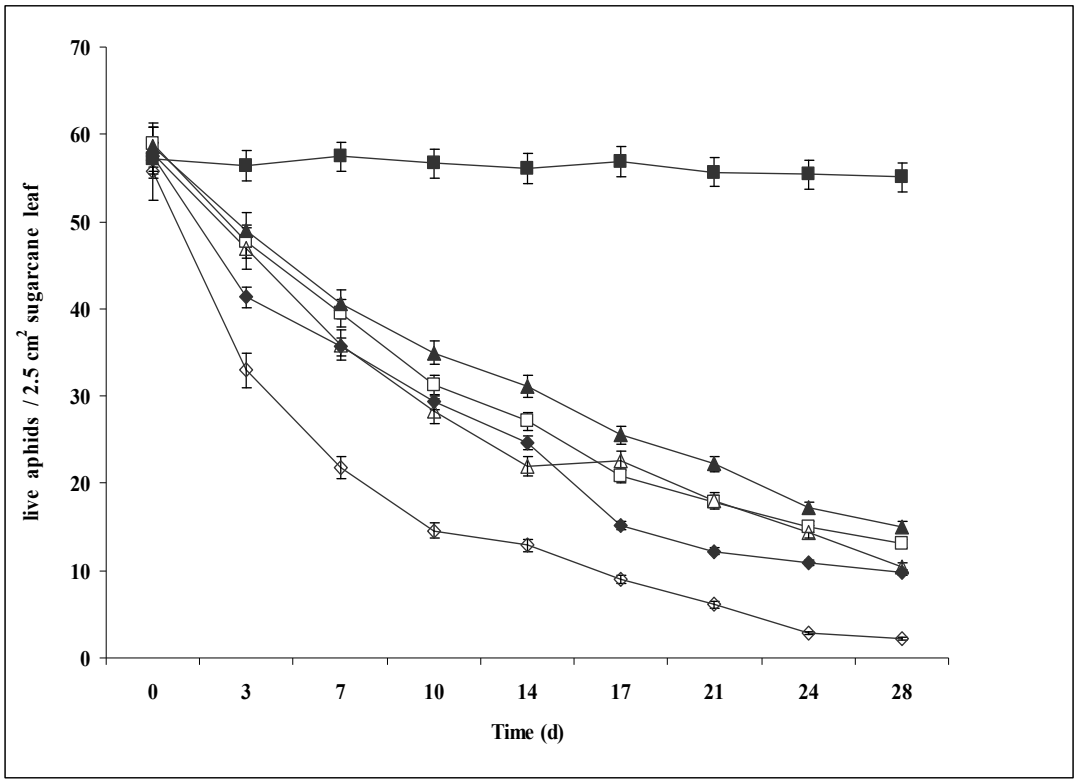

Fig. 5.4 Surviving population of *Ceratovacuna lanigera* on sugarcane leaf after different insecticidal treatments at different time intervals. ♦-♦, *Myrothecium verrucaria* enzyme (lipase 5U/ml); □-□, *Metarhizium anisopliae* (5×10^{12} conidia/ha); ▲-▲, *Bacillus* sp. liquid culture (5×10^{12} cfu/ha); ◇-◇, *M. verrucaria* enzyme (lipase 1U/ml) + *M. anisopliae* (5×10^{12} conidia/ha); Δ-Δ, Metasystox; ■-■, control pots treated with water containing 0.1% Tween 80

In demonstration trial performances of *M. anisopliae* conidia, singly and in combination with *M. verrucaria* enzyme were evaluated against *C. lanigera* in sugarcane (variety CO86032) fields. The combination of enzyme preparation with *M. anisopliae* conidia showed better performance (68±19% efficacy) compared to farmer's practice (40±18% efficacy) (**Table 5.4**). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* towards diamondback moth larvae (Wiwat et al 1996). Similarly the prior spray of *M. verrucaria* crude enzyme mixture to *M. anisopliae* conidia increased the efficacy of combined preparation against woolly aphids of sugarcane (**Table 5.3 and Table 5.4**).

Table 5.4 Comparative evaluation of different strategies used in the biocontrol of *Ceratovacuna lanigera* on the farmer's sugarcane field

Different Treatments	Efficacy (%) Mean±SD
M. anisopliae (5×10^{12} conidia/ha)	55±20
M. verrucaria enzyme (lipase 1U/ml) + *M. anisopliae* (5×10^{12} conidia/ha)	68±19
Farmer's practice[a]	40±18

[a]The farmer's practice included first spray of neem seed kernel extract (5%) in water and a second spray of Metasytox 2ml/L. The dose for all the above treatments was 500 L/ha, SD- Standard Deviation

These treatments can further be tested singly or in IPM module sequentially against *C. lanigera.* Furthermore, similar strategy can also be used for the control of mealy bugs (which also constitute waxy mass as a protective cover) in grapes as well as in cotton.

5.2.2 Biocontrol of *H. armigera* using chitinolytic enzymes of *M. verrucaria*

5.2.2.1 Bioassay with *H. armigera*

In case of *H. armigera* bioassay, the enzyme mixtures produced by *M. verrucaria* and *M. anisopliae* were used. To study the effect of chitinase activity, *per se*, *M. verrucaria* enzyme preparation was diluted to obtain chitinase activity 1 to 5 U/ml. The other enzyme activities changed proportionately in the preparations. Chitinase activity significantly contributed to the mortality of *H. armigera*. The mortality of *H. armigera* (30-89%) increased as the enzyme activity increased (measured as chitinase 1U-5U). Incorporation of glycerol (1M) to *M. verrucaria* enzyme preparation showed increased mortality of *H. armigera*. The mortality of *H. armigera* increased from 53±3% to 71±4% due to the addition of glycerol (1M) to *M. verrucaria* enzyme preparation. The *M.*

anisopliae enzyme preparation also showed 54±3% mortality of *H. armigera.* Earlier, St. Leger et al (1986a; 1996) reported that chitinases and proteases were virulence factors for entomopathogenicity. Fang et al (2005) reported that the endochitinase from *M. anisopliae* showed only marginal correlation with virulence towards the aphid *Myzus persicae* while overproduction of endochitinase increased virulence of *B. bassiana* towards aphids. This variation could be attributed to the multiplicity of chitinases (Patil et al 2000). Hegedus and Khachatourians (1995) suggested that the significance of any enzyme(s) was dependent upon the cuticle characteristics and physiological state of the insect as well as the mechanism of invasion by the fungus. For instance, the lipase activity contributed to the initial phase of infection when the germinating conidium had to break the epicuticle layer to gain entry into the insect. Earlier Nahar et al (2004a) showed importance of chitin deacetylase and chitosanase from *M. anisopliae,* in *H. armigera* cuticle softening and pathogenesis. Recently importance of chitinolytic enzymes (chitinase, endochitinase, NAGase, CDA and chitosanase) and others such as protease and lipase have been shown in pathogenesis of *H. armigera* (Nahar et al 2008; Kulkarni et al 2008). In the present investigations, as the chitinolytic enzyme activities of *M. verrucaria* increased, the mortality of *H. armigera* increased. Addition of glycerol to the enzyme preparation of *M. verrucaria* showed higher mortality of *H. armigera* as compared to enzyme without glycerol (**Fig. 5.5**). This can be attributed to the increased stability of the chitinolytic enzymes of *M. verrucaria* due to glycerol. However, all other enzyme activities were indeed necessary to hydrolyze other cuticle components.

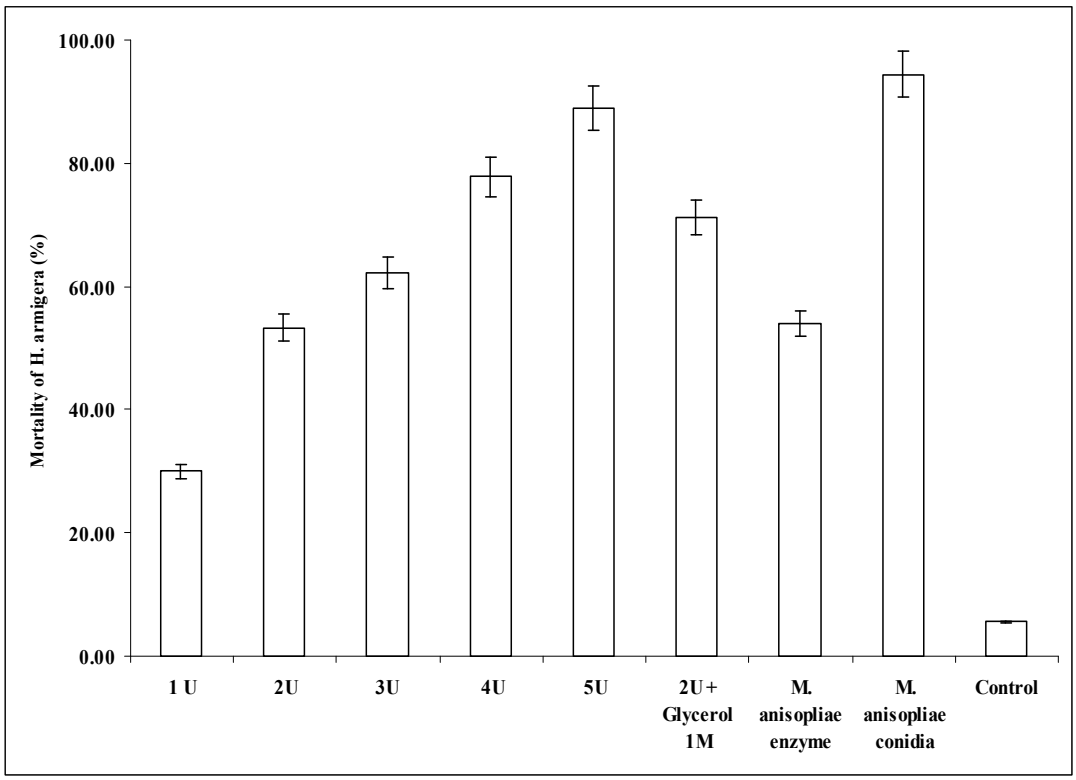

Fig. 5.5 Mortality (%) of *Helicoverpa armigera* using enzyme preparations from *Myrothecium verrucaria*, *Metarhizium anisopliae* and *M. anisopliae* conidia

5.2.2.2 Field experiments

Based on the laboratory performance the *M. verrucaria* enzyme preparations were used for the control of *H. armigera* in chickpea field. *M. anisopliae* conidia and a chemical insecticide endosulfan were also used for the control of *H. armigera* in chickpea. Differences in *H. armigera* population due to different treatments were recorded up to 42 d (**Fig. 5.6**).

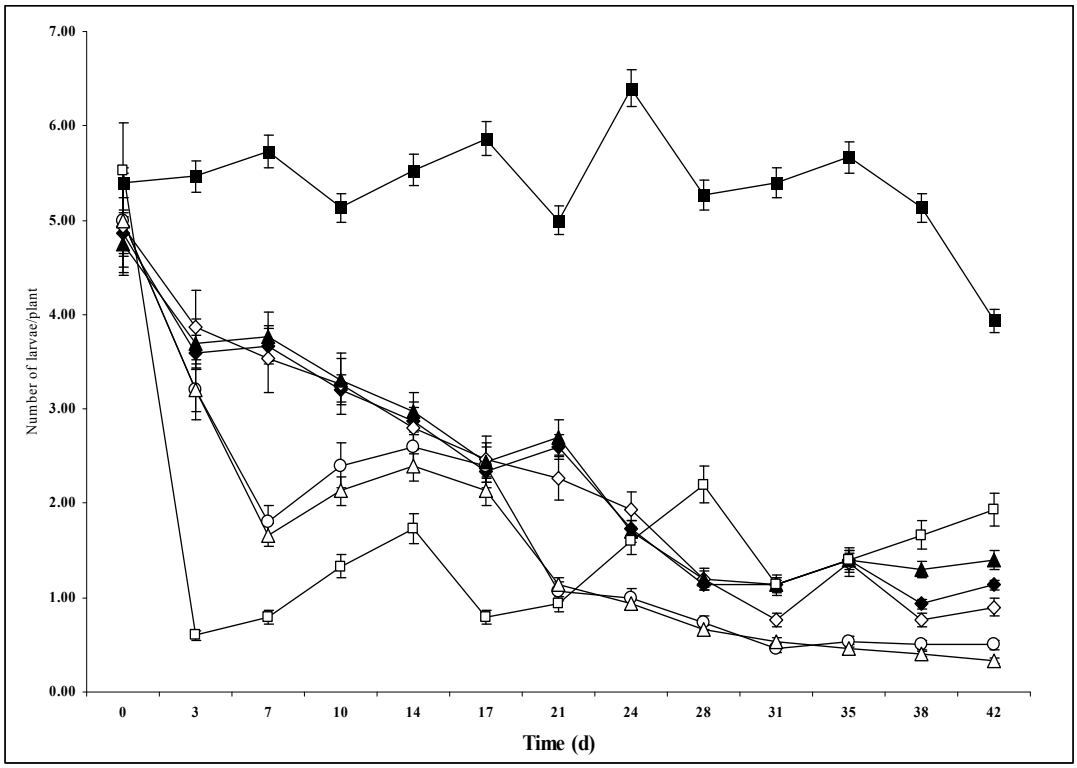

Fig. 5.6 Surviving population of *Helicoverpa armigera* on chickpea after different insecticidal treatments at different time intervals. ♦-♦, *Myrothecium verrucaria* enzymes (SmF preparation; chitinase 2U/ml) ; o-o, *M. verrucaria* enzymes (SmF preparation; chitinase 2U/ml) with glycerol 1M; ◊-◊, *M. verrucaria* enzymes (SSF preparation; chitinase 2U/ml); □-□, Endosulfan (2 ml/L); ▲-▲, *Metarhizium anisopliae* enzyme preparation (chitin deacetylase 2.26×10^{-3} U/ml); Δ-Δ, *M. anisopliae* conidia (5×10^{12} /ha); ■-■, control plots treated with water containing 0.1% Tween 80

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Under field conditions, the efficacies of *M. verrucaria* enzyme preparations (SmF and SSF) measured as chitinase 2 U/ml were 55±23% and 58±20% respectively. The incorporation of glycerol (1M) in enzyme preparation (SmF, chitinase 2 U/ml) of *M. verrucaria* showed increased efficacy (70±19%) against *H. armigera.* The *M. anisopliae* enzyme preparation also showed 52±23% efficacy against *H. armigera*. Foliar application of microbial chitinases was found to be effective for the control of raspberry midge blight in Siberia (Shternshis et al 2002a). Recently, Shternshis (2005) reported that % mortality of *P. sticticalis* doubled when *B. thuringiensis* was applied in combination with bacterial chitinase preparation. The insecticidal activity of two granulosis viruses

and NPV was significantly increased by the addition of bacterial chitinase preparation (Duzhak et al 1991). The addition of a very low amount of exogenous chitinase was reported to reduce the doses of baculovirus. The addition of exogenous chitinase favoured their penetration through chitinous peritrophic membrane in the gut resulting in improvement in biocontrol efficacy. *M. anisopliae* conidia and endosulfan also showed 72-74% efficacy. The percent pod damage of chickpea in the control plots was 40 ± 8% while in all the treated plots it was 16-28 %. The grain yield of chickpea in control plot was 10±0.1 q/ha while in all the treated plots it was 15-20 q/ha (**Table 5.5**).

Table 5.5 The evaluation of enzyme preparations of *Myrothecium verrucaria* and *Metarhizium anisopliae* against *Helicoverpa armigera* infestation on chickpea under field conditions

Treatments	Efficacy* (%) mean±SD	Pod damage (%) mean±SD	Grain yield (q/ha) mean±SD
M. verrucaria enzymes			
SmF preparation (chitinase 2U/ml)	55±23	27±5	15.37±1.1
SmF preparation (chitinase 2U/ml) + glycerol (1M)	70±19	19±4	19.67±0.7
SSF preparation (chitinase 2U/ml)	58±20	25±3	17.28±1.1
M. anisopliae			
Enzyme preparation (**CDA 2.26×10^{-3} U/ml)	52±23	28±5	15.00±1.0
Conidia (5×10^{12} /ha) in water with 0.1% Tween 80	72±19	18±3	19.88±0.9
Endosulfan (2ml/L)	74±12	16±7	20.00±1.0
Control (Water with 0.1% Tween 80)	-	40±8	10.00±1.0

*After Henderson and Tilton (1955); **CDA - Chitin deacetylase; SD-Standard Deviation; All the treatments were sprayed using knapsack sprayer with dose of 500 L water/ha; SmF: Submerged fermentation; SSF: Solid State Fermentation

5.2.3 Biocontrol of *F. oxysporum* and *S. rolfsii* using chitinolytic enzymes of *M. verrucaria*

5.2.3.1 Pot experiment

The performance of enzymes (100 ml preparation/pot, chitinase 50 U, β-1,3-glucanase 7 U and protease 2 U) of *M. verrucaria* and *Bacillus* sp. B1 (chitinase 1 U, β-1,3-glucanase 8 U and protease 27 U) was tested against *S. rolfsii* and *F. oxysporum*. Each treatment contained 10 pots with 2 seeds per pot. The germination of peanut seeds was 70±5% treated with enzyme mixture of *M. verrucaria* in *F. oxysporum* infested pots, while 40±5% germination was observed due to the treatment with enzyme mixture of *Bacillus* sp. B1 (**Fig. 5.7**). Biocontrol of soil born pathogen involves antibiosis, competition or hyperparasitism. Among them, hyperparasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi (Chet et al 1990). Chitinolytic enzymes have been considered important in the biological control of soilborne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin (Bartnicki-Garcia, 1968; Chet, 1987). Chitinase and β-1,3-glucanase when acting alone or synergistically, were shown to inhibit the growth of pathogenic fungi by degradation or lysis of fungal cell walls (Mauch et al 1988; Wang et al 1999). Thus, it was assumed that mycelial lysis and release of *N*-acetylglucosamine from *R. solani* were mainly associated with the endochitinase activity of *Paenibacillus illinoisensis*. The crude culture filtrate of *P. illinoisensis* also significantly suppressed damping-off incidence by *R. solani* in cucumber seedlings. The importance of chitinase and β-1,3-glucanase in biocontrol of *S. sclerotiorum* by *Pseudomonas* and *Bacillus* species has been reported (Fernando et al 2007). Thus, in the present investigations control of *F. oxysporum* and *S. rolfsii* can be attributed to chitinase and β-1,3-glucanase and protease activity of *M. verrucaria* enzyme preparation.

In the control treatment (pots containing sterile soil irrigated with distilled water) 90±5% germination was seen in while only 10±5% germination was observed in *F. oxysporum* infested pots drenched with sterile distilled water. The pots treated with carzim showed 60±10% germination of peanut seeds in *F. oxysporum* infested soil. The similar results were observed in case of *S. rolfsii* using above treatments (**Fig. 5.7**).

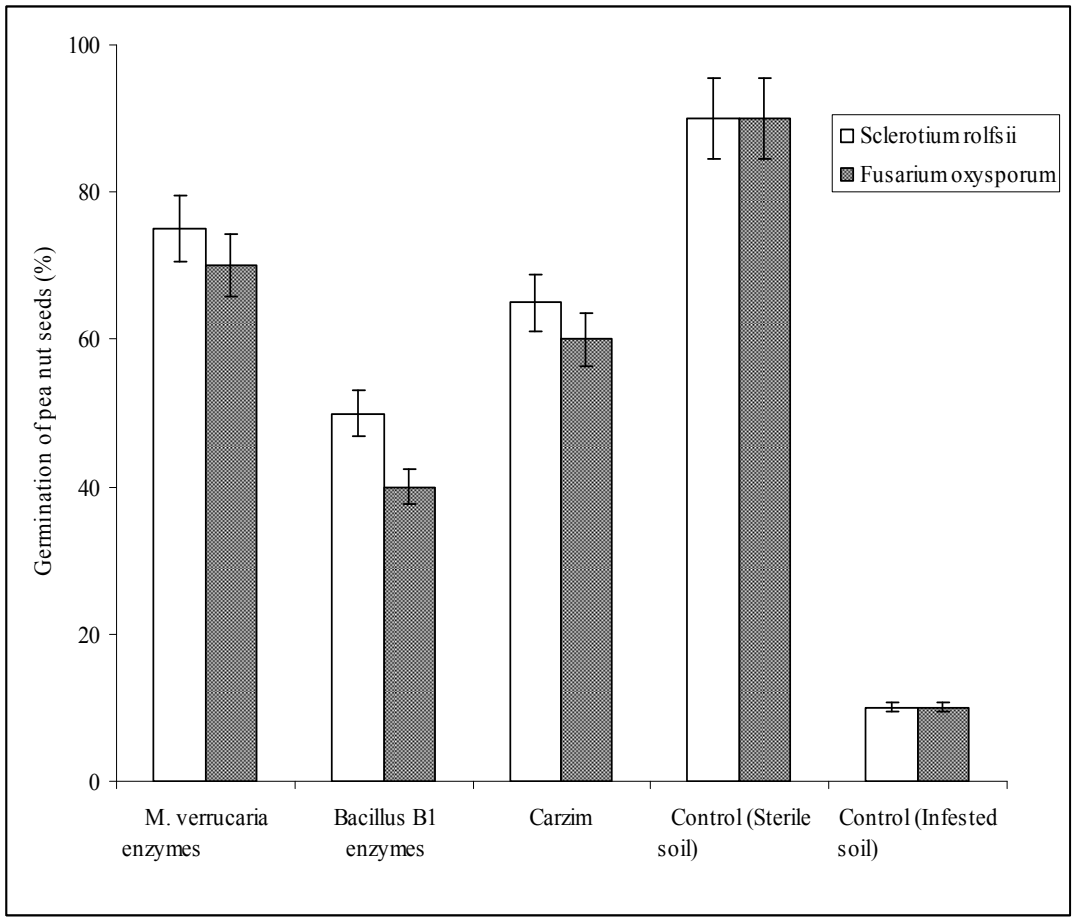

Fig. 5.7 Biocontrol of *Fusarium oxysporum* and *Sclerotium rolfsii* using enzyme preparations from *Myrothecium verrucaria* and *Bacillus* sp.

From the above studies it can be seen that the enzyme preparation from *M. verrucaria* showed better control of insect pests (*C. lanigera* in sugarcane and *H. armigera* in chickpea) as well as plant pathogenic fungi (*F. oxysporum* and *S. rolfsii* in peanut). This enzyme preparation will further be tested for the control of insect pest and fungal pathogens singly or in IPM module in different crops in agriculture.

Chapter 6

Conclusion

In many plant species, local invasion of the pathogen induces production of PR-proteins like chitinases, β-1,3-glucanases, proteinases, proteinase inhibitors, etc. As pathogenic fungi and insects contain chitin in their protective covers, induction of chitinases in plants is the main defense response. In fact, there is no other better proof for the contribution of plant chitinases in self defense than the formation of chitosan in the cell wall of a bean rust fungus, *Uromyces viciae-fabae* to combat with the chitinase activity (Patil et al 2000). Number of soil bacteria also produce chitinolytic enzymes. Roberts and Selitrennikoff (1988) studied plant and bacterial chitinases for anti-fungal activity and enzyme specificity. According to them, chitinases isolated from the grains of wheat, barley, and maize functioned as endochitinases and inhibited hyphal elongation of test fungi. In contrast, bacterial chitinases from *S. marcescens, Streptomyces griseus,* and *Pseudomonas stutzeri* act as exo-enzymes and had no effect on hyphal extension of test fungi like *T. reesei* and *Phycomyces blackesleeanus.* However, Ordentlich et al (1988) attributed slower disease development by *S. rolfsii* on bean seeds in presence of *S. marcescens* to its high chitinase activity. Furthermore, Shapira et al (1989) cloned *S. marcescens* chitinase gene in *E. coli* and the chitinase preparation obtained was found to be effective in reducing disease incidence caused by *S. rolfsii* in beans and *R. solani* in cotton under greenhouse conditions. This contradiction in the observations made by Roberts and Selitrennikoff (1988) versus Ordentlich et al (1988) and particularly, Shapira et al (1989) may be attributed to the difference in the test fungi used and other factors that contribute in the killing of pathogenic fungus. Several species of fungi are very potent biocontrol agents of plant pathogenic fungi and insects. The mycoparasitic and entomopathogenic fungi produce chitinases for invasion and as one of the host killing components. A *F. chlamydosporum* strain, a mycoparasite of groundnut rust, *P. arachidis* produces an endo-chitinase that inhibits germination of uredospores of the rust fungus.

This indicates the significant contribution of chitinase in the biocontrol of groundnut rust (Mathivanan et al 1998). Chitinolytic enzymes of *T. harzianum,* a most studied mycoparasitic fungus, were found to be inhibitory to a wider range of deleterious fungi than similar enzymes from other sources (Lorito et al 1993). No other chitinases have been reported with a broad range of activity, synergy between different enzymes, or synergy with a number of other biological and chemical control agents. *M. verrucaria* produced high levels of extracellular mycolytic enzymes, viz. chitinase, β-1,3-glucanase and proteinase. The crude mycolytic preparation was found to be effective against *S. rolfsii*, a root infecting fungus of groundnut. To control pests such as longhorn beetles and aphids, the enzymic treatment before or simultaneously along with the entomopathogenic fungus, itself was successfully tried (Higuchi et al 1998). The insect-pathogenic fungi, *B. bassiana, B. brongniartii,* and *V. lecanii* produced cuticle degrading enzymes when grown on chitin containing medium. The pretreatment of insects with the enzyme solution was reported to be useful.

The cuticle degrading enzymes (CDEs) mainly include proteases, lipases and chitinases, and enzymes such as chitinases, proteases and glucanases are the main components of the mycolytic enzyme (ME) complex (Deshpande, 1999). The composition of CDE and ME is not drastically different. It has been suggested that among these, chitinases contribute significantly in the degradation of protective covers. Therefore, most of the studies for the production of CDEs and MEs and their use in the field are centered around chitinases. In the present work, in addition to chitinases the role of lipases in the effective control of woolly aphids has also been investigated. Furthermore, the attempts have been made to understand the barriers and issues in converting "proof of concept" research in to possible commercial venture. Different ways for the (apparent) cost-effective production/use of chitinolytic enzymes include: increased shelf life, solid state

fermentation, enzymes with different mechanism of action (Exo-, Endo-, Exo-endo or Endo-exo action), wide range of applications, multiple substrates in addition to chitin, and change in priority (e.g.organic farming).

One of the advantages of *Myrothecium* preparation is a range of enzymes produced when chitin is used as a sole carbon source. All these enzymes collectively named as CDE or ME play significant role in biocontrol of plant pathogenic fungi and insects. The optimization of submerged fermentation up to 100 L scale using crude chitin did not increase the fold production of the enzymes but the time required was reduced to a certain extent. However, in addition to the expected shelf life of 18 months at room temperature, the environment stability of the biocontrol preparation used for either soil application or for foliage spray is a major concern. The addition of polyols either glycerol (5M), xylitol (5M) or sorbitol (5M) protected the enzyme mixture by retaining 50-60% activities for 3 h at 40°C. In a lyophilized powder for all enzyme activities were found to be stable at 25°C for 2 months. Indeed it is a long way to reach the mark of 18 months. The cost analysis of the data generated in the laboratory on submerged and solid state fermentation revealed the usefulness of solid state fermentation. However, the high protease activity, chances of contamination and requirement of more manpower for solid state fermentation could be the major issues.

Two insect pests, *C. lanigera* on sugarcane and *H. armigera* on pulses and two fungal pathogens on peanuts, *S. rolfsii* and *F. oxysporum* were identified for the "proof of concept" studies. The lipase activity of the *M. verrucaria* preparation contributed significantly in the control of woolly aphids in sugarcane by hydrolyzing waxy mass. While the chitinolytic and proteolytic activities further contributed in the killing process. Additionally β-1,3-glucanase activity affected the growth of a sooty mould, *Capnodium*. It will be interesting to increase the scope of the enzyme mixture singly or in combination

with other lipases for the control of mealy bugs on grapes and also on cotton having waxy coating to make the high volume low value product for the market. Similarly the combination of *Metarhizium* spores with *Myrothecium* enzyme mixture was found to be effective against *C. lanigera* in sugarcane. The soil application of the enzyme mixture for the control of root infecting pathogens was found to be effective in the laboratory. However, the enzyme stability in soil, and economic threshold level of the infection are two important parameters need consideration. *M. verrucaria* chitinolytic enzymes, in addition to the agriculture, can be used for the hydrolysis of chitinous waste for single cell protein production, alcohol fermentation, chitooligosaccharide production, etc. All these applications can make the chitinolytic enzymes commercially viable as high volume low cost product.

Chapter 7

References

Literature cited

Abbott WS (1925) J Econ Entomol 18: 265-267.

Alloue WAM, Destain J, Ongena M, Blecker C and Thonart P (2008) Preparative Biochem Biotechnol 38: 217-228.

Amiri-Besheli B, Khambay B, Cameron S, Deadman MI and Butt TM (2000) Mycol Res 104: 447-452.

Anon (1997) Organophosphate insecticides - an Indian analysis. Pestic World 1: 8-9.

Araki Y and Ito E (1975) Euro J Biochem 55: 71-78.

Arora DK and Saikia R (2005) In: Proceedings of ICAR-CABI Workshop on biopesticide formulations and application, Eds. Rabindra RJ, Hussaini SS, and Ramanujam B, Precision Fototype Services, PDBC Bangalore, India, pp. 13-26.

Auld BA (1992) In: Biological control of locusts and grasshoppers CAB International, Wallmgford, UK, pp. 219-229.

Babujee L and Gananamanickam SS (2000) Curr Sci 78: 248-257.

Baptiwale H (2004) In: Proceedings of The international workshop on entomopathogenic fungi a valuable alternative to fight against insect pests, Ed. Deshpande MV, National Chemical Laboratory, Pune, India, pp. 185-206.

Bara MTF, Lima AL and Ulhoa CJ (2003) FEMS Microbiol Lett 219: 81-85.

Barlow F (1985) In: Pesticide application: principles and practice, Ed. Huskel PT, Claredon press, Oxford UK, pp 1-34.

Bartnicki-Garcia S (1968) Annu Rev Microbiol 22: 87-108.

Basha Saleem and Ulaganathan Kandasamy (2002) Curr Sci 82: 1457-1463.

Bejarano-alcazar J, Blanco-Lopez MA, Melero-Vara JM and Jimenez-Diaz RM (1997) Plant Pathol 46: 168-178.

Berg B and Petterson G (1977) J Appl Bacterol 42: 65-75.

Bertagnolli BL, Dal Soglio FK and Sinclair JB (1996) Physiol Mol Plant Pathol 48: 145-160.

Bhushan B (2000) J Appl Microbiol 88: 800-808.

Bidochka MJ and Khachatourians GG (1988) Appl Environ Microbiol 54: 2699-2704.

Binod P, Pusztahelyi T, Nagyc V, Sandhya C, Szakacs G, Pocsi I and Pandey A (2005) Enzyme Microb Technol 36: 880-887.

Binod P, Sandhya C, Suma P, Szakacs G and Pandey A (2007a) Bioresour Technol 98: 2742-2748.

Binod P, Sukumaran RK, Shirke SV, Rajput JC and Pandey A (2007b) J Appl Microbiol 103: 1845-1852.

Bischof K, Hanelt D and Wiencke (2000) Planta 211: 555-562.

Boer De M, Bom P, Kindt F, Keurentjes JB, Van der Sluis I, Van Loon LC and Bakker PAMH (2003) Phytopathol 93: 626-632.

Boruah HP Deka and Dileep Kumar BS (2003) Indian J Exp Biol 41: 627-631.

Boyd SA and Mortland MM (1990) In: Soil biochemistry, Eds. Bollag JM, Stotzky G and Marcel Dekker, New York NY, 6: 1-28.

Brar SK, Verma M, Tyagi RD and Valero JR (2006) Process Biochem 41: 323-342.

Brar SK, Verma M, Tyagi RD, Valéro JR and Surampalli RY (2008) Environ Technol 29: 161-170.

Brimmer TA and Boland GJ (2003) Agric Ecosyst Environ 100: 3-16.

Bryant JE (1994) Agric Ecosys Environ 49: 31-35.

Budi SW, Tuinen Dvan, Arnould C, Dumas-Gaudot E, Gianinazzi- Pearson V and Gianinazzi S (2000) Appl Soil Ecol 15: 191-199.

Burns RG (1982) Soil Biol Biochem 14: 423-427.

Butt TM, Jackson CW and Magan N (2000) In: Fungi as biocontrol agents: Progress, Problems and Potential, Eds. Butt TM, Jackson CW and Magan N, CABI Publishing Co. UK, pp. 1-8.

Carpenter JF, Crowe JH and Arakawa T (1990) J Dairy Sci 73: 3627-3636.

Casio IG, Fischer RA and Carroad T (1982) J Food Sci 47: 901-11.

Chahal DS (1985) Appl Environ Microbiol 499: 205-10.

Charles JF, Nielsen-Leroux C and Delecluse C (1996) Annu Rev Entomol 41: 451-472.

Charnley AK (1989) In: Biotechnology of fungi for improving plant growth, Eds. Whipps JA and Lumsden RD, Cambridge University Press UK, pp. 85-117.

Chavan S, Ghormade V, Nahar P and Deshpande MV (2006) In: Plant protection in new millennium Vol II, Eds: Gadewar AV and Singh BP, Satish Serial Publishing House, New Delhi, pp. 227-243.

Chavan S, Kulkarni M and Deshpande MV (2008) In Rev Plant Pathol, Eds. Reddy SM and Gour HN, Scientific Publishers, Jodhpur, India, 4: 393-420.

Chet I (1987) In: Innovative approaches to plant disease control, Ed. Chet I, Wiley, New York, pp. 137-160.

Chet I, Ordentlich A, Shapira R and Oppenheim A (1990) Plant Soil 129: 85-92.

Cook RJ, Bruckart WL, Coulson JR, Goettel MS, Humber RA, Lumsden RD, Maddox JV, McManus ML, Moore L, Meyer SF, Quimby PC, Stack JP and Vaughn JL (1996) Biol Control 7: 333-351.

Cory JS and Bishop DHL (1995) In: Methods in Molecular Biology, Vol 39: Baculovirus Expression Protocols (Richardson, C D Ed.), Humana, Totowa NJ, pp. 277-294.

Couch TL and Ignoffo CM (1981) In: Microbial control of pests and plant diseases 1970-1980, Ed. Burgs HD, Academic Press London, pp. 621-634.

Cross JV and Polonenko DR (1996) Can J Plant Pathol 18: 446-454.

Cunningham JP, Zalucki MP and West SA (1999) Bull Entomol Res 89: 201-207.

Dahiya N, Tewari R and Hoondal GS (2006) Appl Microbiol Biotechnol 71: 773-782.

Dass C and Teyegaga A (1996) Aust J Bot 44: 705-712.

David WAL (1969) J Invertebr Pathol 14: 336-342.

De La Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T and Pinto-Toro J (1992) Eur J Biochem 206: 859-67.

De La Cruz J, Pintor-Toro JA, Benitez T and Llobell A (1995) J Bacteriol 177: 1864-1871

De Waard MA, Georgopoulos SG, Hollomon DW, Ishii H, Leroux P, Ragsdale NN and Schwinn (1993) Annu Rev Phytopathol 31: 403-421.

Delp CJ (1995) In: Modern selective fungicides, Ed. Lyr H Gustav and Fisher Verlag, Jena, Germany pp. 291-303.

Deshpande MV (1986) J Sci Ind Res 45: 273-81.

Deshpande MV (1998) Novel Biopesticides, CSIR Golden Jubilee Series, CSIR, New Delhi.

Deshpande MV (1999) Crit Rev Microbiol 25: 229-243.

Deshpande MV (2005) In: Proceedings of ICAR-CABI workshop on biopesticide formulations and application, Eds. Rabindra RJ, Hussaini SS and Ramanujam B, Precision Fototype Services, PDBC Bangalore, India, pp. 150-158.

Diekman MA and Green ML (1992) J Anim Sci 70: 1615-1627.

Domnas AJ and Warner SA (1991) Crit Rev Microbiol 18: 1-13.

Donzelli BG, Siebert KJ and Harman GE (2005) Enzyme Microb Technol 37: 82-92.

Duzhak AB, Salganik RI, Shternshis MV, Ermakova NI, Tsvetkova VP, Andreeva IV and Kushnikova TA (1991) Siberian J Ecol 5: 457-461.

Elad Y, Barak R and Chet I (1984) Soil Biol Biochem 16: 381-386.

Elkinton JS, Hajek AE, Boettner GH and Simons EE (1991) Environ Entomol 20: 1601-1605.

El-Sayed GN, Coudron TA and Ignoffo CM (1989) J Invertebr Pathol 54: 394-403.

El-Sayed GN, Ignoffo CM, Leathers TD and Gupta SC (1993) Mycopathologia 122:79-87.

Fadare TA and Amusa NA (2003) African J Biotechnol 2: 425-428.

Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, Feng J, Yang X, Zhang Y and Pei Y (2005) Appl Environ Microbiol 71: 363-370.

Faria MR and Wraight SP (2007) Biol Control 43: 237-256.

Felse P and Panda T (1999) Process Biochem 34: 563-566.

Felse P and Panda T (2000a) Biochem Eng J 4: 115-120.

Felse P and Panda T (2000b) Bioprocess Eng 23: 127-34.

Fenice M, Leuba JL and Fedrici F (1998a) J Ferment Bioeng 86: 620-623.

Fenice M, Selbmann L, Giambattista RD and Federici F (1998b) Res Microbiol 149: 289-300.

Fernando WGD, Nakkeeran S, Zhang Y and Savchuk S (2007) Crop Prot 26: 100-107.

Fernendez-Lahore HM, Fraile ER and Cascone O (1998) J Biotechnol 62: 83-93.

Fitt GP (1989) Ann Rev Entomol 34: 17-52.

Fox RTV (1997) Mycologist 11: 88

Frandberg E and Schnurer J (1994) J Appl Microbiol 76: 361-367.
Fukamizo T (2000) Curr Protein Pept Sci 1: 105-124.

Furlong Michael J and Pell Judith K (2001) Biol Control 22**:** 288-299.

Gao K, Liu X, Kang Z and Mendgen K (2005) J Phytopathol 153: 280-290.

Gaugler R (1997) Phytoparasitica 25: 179-182.

Ghorbel Basma, Sellami-Kamoun Alya and Nasri Moncef (2003) Enzyme Microb Technol 32: 513-518.

Ghormade V, Lachake SA and Deshpande MV (2000) Folia Microbiol 45: 231-238.

Gill SS, Cowles EA and Pietrantonio PV (1992) Annu Rev Entomol 37: 615-636.

Gkargkas Konstantinos, Mamma Diomi, Nedev Georgi, Topakas Evangelos, Christakopoulos Paul, Kekos Dimitris and Macris Basil J (2004) Process Biochem 39: 1599-1605.

Gohel V, Maisuria V and Chhatpar HS (2007) Enzyme Microb Technol 40: 1608-1614.

Gohel V and Naseby DC (2007) Biochem Eng J 35: 150-157.

Gohel V, Singh A, Maisuria V, Phadnis A and Chhatpar HS (2006) African J Biotechnol 5: 54-72.

Goldman MHS and Goldman GH (1998) Genet Mol Biol 21: ISSN 1415-1457.

Good TA and Bessman SP (1964) Anal Biochem 9: 253-262.

Graber M and Combes D (1989) Enzyme Microb Technol 11: 673-677.

Graeme-Cook KA and Faull JL (1991) Can J Microbiol 37: 659-664.

Green AT, Healy MG and Healy A (2005) J Chem Technol Biotechnol 80: 28-34.

Groner A (1990) In: Safety of microbial insecticides, Eds Laird M and Lacey LA and Davidson EW, CRC Press, Boca Raton, FL, pp. 135-147.

Grover A and Pental D (2003) Curr Sci 84: 310-320.

Gupta SC, Leathers TD, El-Sayed GN and Ignoffo CM (1992) Exp Mycol 16: 132-137.

Gupta R, Saxena RK, Chaturvedi P and Virdi JS (1995) J Appl Bacteriol 78: 378-383.

Hadwiger LA and Beckman JM (1980) Plant Physiol 66: 205-211.

Hahn-Hagerdal B (1986) Enzyme Microb Technol 8: 322-327.

Handelsman J and Stabb E (1996) Plant Cell 8: 1855-1869.

Hegedus DD and Khachatourians GG (1995) Biotechnol Adv 13: 455-490.

Henderson CF and Tilton EW (1955) J Econ Entomol 48: 158-161.

Herms S, Seehaus K, Koehle H and Conrath U (2003) Plant Physiol 130: 120-127.

Higuchi T, Okubo K, Saiga T and Senda S (1998) Jpn Kokai Tokkyo Koho JP 10067613 A2 10 (From CA 128: Patent 254077, 1998).

Hofte H and Whiteley HR (1989) Microbiol Rev 53: 242-255.

Horinouchia H, Muslimb A, Suzukic T and Hyakumachi M (2007) Crop Prot 26: 1514-1523.

Hostetter DL and Puttler B (1991) Environ Entomol 20: 1480-1488.

Huang Da-Fang, Zhang Jie, Song Fu-Ping and Lang Zhi-Hong (2007) J Invertebr Pathol 95: 175-180.

Huber J (1986) In: The biology of *Baculoviruses,* Eds. Granados RR and Federici BA, CRC Press, Boca Raton, FL. Vol. II, pp. 181-202.

Hundal BS, Anand and Ramandeep (2006) Icfai J Manage Econ 4: 32-37.

Hynes Russell K and Boyetchko Susan M (2006) Soil Biol Biochem 38: 845-849.

Ignoffo CM and Garcia C (1992) Environ Entomol 21: 913-917.

Ignoffo CM, Futtler B, Marston NL, Hostetter DL and Dickerson WA (1975) J Invertebr Pathol 25: 135-137.

Ikasari L and Mitchell DA (1996) Enzyme Microb Technol 19: 171-175.

James RR, Buckner JS and Freeman TP (2003) J Invertebr Pathol 84: 67-74.

Jassim HK, Foster HA and Fairhurst CP (1990) Ann Appl Biol 117: 187-196.

Jeffries P (1997) In: The Mycota IV, Environmental and microbial relationships, Ed. Soderstrom W, Springer-Verlag , Berlin, Heidelberg pp. 149-163.

Jarrold Samantha L, Moore David, Potter Ursula and Charnley A Keith (2007) Mycol Res 111: 240-249.

Joshi S and Viraktamath CA (2004) Curr Sci 87: 307-316.

Jsten P, Paul GC, Nienow AW and Thomas CR (1996) Biotechnol Bioeng 52: 672-684.

Jun Y, Bridge PD and Evans HC (1991) J Gen Microbiol 137: 1437-1444.

Kang SC, Park S and Lee DG (1998) FEMS Microbiol Lett 165: 267-271.

Kao Po-Min, Chena Chih-I, Huang Shu-Chen, Chang Yung-Chi, Tsai Po-Jen and Liu Yung-Chuan (2007) Biochem Eng J 34: 172-178.

Kauss H and Bausch B (1988) In: Methods in Enzymology, Eds. Woods WA and Kellogg ST, San Diego: Academic press, 161: 518-523.

Kawchuk LM, Holley JD, Lynch DR and Clear RM (1994) American J Potato Res 71: 185-192.

Khoury C, Minier M, van Huynch N and Le Goffic F (1997) Biotech lett 19: 1143-1146.

Kim Seul Ki, Seo Ri Kim, Min Seok Choi, Chang Eon Park, Young Cheo Kim, Kil Yong Kim, Kyung Sook Whang, Kyung-taek oh and In Seon Kim (2007) J Microbiol Biotechnol 17: 1700-1703.

Klein MG and Kaya HK (1995) Mem Inst Oswaldo Cruz 90: 87-95.

Knowles BH (1994) Adv Insect Physiol 24: 275-308.

Kombrink E and Somssich IE (1995) Adv Bot Res 21: 2-34.

Kouvelis VN, Zare R, Bridge PD and Typas MA (1999) Lett Appl Microbiol 28: 263-268.

Kramer Karl J and Muthukrishnan Subbaratnam (1997) Insect Biochem Molec Biol 27: 887-900.

Kranthi KR, Jadhav DR, Kranthia S, Wanjaria RR, Alia SS and Russellc DA (2002) Crop Prot 21: 449-460.

Kranthi KR, Jadhav DR, Wanjari RR, Ali S Shakir and Russell D (2001) Bull Entomol Res 91: 37-46.

Krieger de Moraes, Schrank A and Vainstein M (2003) Curr Microbiol 46: 205-210.

Kristjansson MM and Kinsella JE (1991) Adv Food Nutr Res 35: 237-258.

Kulkarni SA, Ghormade V, Kulkarni G, Kapoor M, Chavan SB, Rajendran A, Patil SK, Shouche Y and Deshpande MV (2008) Biocontrol Sci Technol 18: 809-828.

Kumar A (2005) Spice India 18: 28-31.
Lacey LA and Undeen AH (1986) Annu Rev Entomol 31: 265-296.

Lacey LA, Frutos R, Kaya HK and Vail P (2001) Biol Control 21: 230-248.

Lawo NC, Mahon RJ, Milner RJ, Sarmah BK, Higgins TJV and Romeis J (2008) Appl Environ Microbiol 74: 4381-4389.

Lekha PK and Lonsane BK (1994) Process Biochem 29: 497-503.

Leroux P, Chapeland F, Desbrosses D and Gredt M (1999) Crop Prot 18: 687-697.

Li GQ, Huang HC, Kokko E and Acharya SN (2002) Bot Bull Acad Sin 43: 211-218.

Lima V, Krieger N, Mitchell D and Fontana J (2004) Biochem Eng J 18: 65-71.

Litovitz TL, Smilkstein M and Felberg L (1996) Am J Emerg Med 15: 447-500**.**

Liu BL, Kao PM, Tzeng YM and Feng KC (2003) Enzyme Microb Technol 33: 410-415.

Lonsane BK, Ghildyal NP, Budiatman S and Ramakrishna SV (1985) Enzyme Microb Technol 7: 258-65.

Lord JC and Howard RW (2004) Mycopathologia 158: 211-217.

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL and Di Pietro A (1993) Phytopathol 83: 302-307.

Lowry OH, Rosenbough NJ, Farr AL and Rancall RJ (1951) J Biol Chem 193: 256-257.

Lysenko O (1976) J Invertebr Pathol 27: 385-386.

Mahadevan B and Crawford DL (1997) Enzyme Microb Technol 20: 489-493.

Mallikarjuna N, Kranthi KR, Jadhav DR, Kranthi S and Chandra S (2004) J Appl Entomol 128: 321-328.

Mathivanan N, Kabilan V and Murugesan K (1998) Can J Microbiol 44: 646-651.

Matsumoto M, Kida K and Kondo K (1997) J Chem Technol Biotechnol 70: 188-192.

Matsumoto Y, Saucedo-Castaneda G, Revah S and Shirai K (2004) Process Biochem 39: 665-671.

Mauch F, Mauch-Mani B and Boller T (1988) Plant Physiol 88: 936-942.

Ma Zhonghua and Michailides Themis J (2005) Crop Prot 24: 853-863.

McCormack J, Hackett T, Tuohy MG and Coughlan MP (1991) Biotech Lett 13: 677-682.
Mehetre S, Mukherjee P and Kale S (2008) Biocontrol Sci Technol 18: 215-220.

Mendonsa ES, Vartak PH, Rao JU and Deshpande MV (1996) Biotech Lett 18: 373-376.

Montesinos Emilio (2003) Int Microbiol 6: 245-252.

Morris ON (1976) Can Entomol 108: 3225-3233.

Moscardi F and Sosa-Gomez DR (2000) In: Field manual of techniques in invertebrate pathology: Application and evaluation of pathogens for control of insects and other invertebrate pests, Eds. Lacey LA and Kaya HK, Kluwer Academic, Dordrecht pp. 447-466.

Nahar PB, Ghormade V and Deshpande MV (2004a) J Invertebr Pathol 85: 80-88.

Nahar PB, Kulkarni SA, Kulye MS, Chavan SB, Kulkarni G, Rajendran A, Yadav PD, Shouche Y and Deshpande MV (2008) Biocontrol Sci Technol 18: 337-355.

Nahar PB, Kulye MS, Yadav PD, Hassani M, Tuor U, Keller S and Deshpande MV (2003) J Mycol Plant Pathol 33: 372-377.

Nahar PB, Yadav PD, Kulye MS, Hadapad A, Hassani M, Tuor U, Keller S, Chandele A, Thomas B and Deshpande MV (2004b) J Biol Control 18: 1-7.

Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G and Pandey A (2004) Process Biochem 39: 1583-1590.

Nannipieri P, Sastre I, Landi L, Lobo MC and Pietramellara (1996) Soil Biol Biochem 28: 107-112.

Nelson DR, Walker GP, Buckner JS and Fatland CL (1997) Comp Biochem Physiol 117B: 241-251.

Nelson DR, Freeman TP, Buckner JS, Hoelmer KA, Jackson CG and Hagler JR (2003) Comp Biochem Physiol 136: 343-356.

Nelson PE, Desjardins A and Plattner RD (1993) Annu Rev Phytopathol 31: 233-252.

Nicolas L, Regis LN and Rios EM (1994) FEMS Microbiol Lett 124: 271-276.

Nielsen P and Sorensen J (1997) FEMS Microbiol Ecol 22: 183-192.

Nilegaonkar SS, Zambare VP, Kanekar PP, Dhakephalkar PK and Sarnaik SS (2007) Bioresour Technol 98: 1238-1245.

Noel M, Lozano P and Combes D (2005) Bioprocess Biosys Eng 27: 375-380.

Ohishi K, Yamagishi M, Ohata T, Suzuki M, Izumida H, Sano H, Nishijama M and Miwa T (1996) J Ferment Bioeng 82: 598-600.

Omura S, Arai N, Yamaguchi Y, Masuma R, Iwai Y, Namikashi M, Turberg A, Kolbl H and Shiomi K (2000) J Antibiot (Tokyo) 53: 603-608.

Ordentlich A, Elad Y and Chat I (1987) Soil Biol Biochem 19: 747-751.

Ordentlich A, Elad Y and Chet I (1988) Phytopathol 78: 84-88.

Orlando Beys Silva Walter, Mitidieri Sydnei, Schrank Augusto and Henning Vainstein Marilene (2005) Process Biochem 40: 321-326.

Pandey A (1994) In: Solid state fermentation, Ed. Pandey A, Wiley Eastern Ltd., New Delhi, pp. 3-10.

Pandey A, Selvakumar P, Soccol CR and Nigam P (1999) Curr Sci 77: 149-162.

Pandey A, Soccol CR and Mitchell DA (2000) Process Biochem 35: 1153-69.

Patibanda AK, Upadhyay JP and Mukhopadhyay AN (2002) J Biol Control 16: 57-63.

Patidar P, Agrawal D, Banerjee T and Patil S (2005) Process Biochem 40: 2962-2967.

Patil RS, Deshpande AM, Natu AA, Nahar PB, Chitnis M, Ghormade V, Laxman RS,

Rokade S and Deshpande MV (2001) J Biol Control 15: 157-164.

Patil RS, Ghormade V and Deshpande MV (2000) Enzyme Microb Technol 26: 473-483.

Pawar CS (1998) Pestol 22: 51-59.

Payne CC (1982) Parasitol 84: 35-77.

Pignede G, Wang H, Fudalej F, Gaillardin C, Seman M and Nicaud JM (2000) J Bacteriol 182: 2802-2810.

Pitson SM, Seviour RJ and McDougall BM (1993) Enzyme Microb Technol 15: 178-192.

Podile AR and Dube HC (1988) Curr Sci 57: 183-186.

Podile AR and Prakash AP (1996) Can J Microbiol 42: 533-538.

Prasad R (1993) Pestic Sci 38: 273-275.

Prasad R (2004) In: Proceedings of The international workshop on entomopathogenic fungi a valuable alternative to fight against insect pests. Ed. Deshpande, MV, National Chemical Laboratory, Pune, India, pp. 135-138.

Purdue Mark P, Hoppin Jane A, Blair Aaron, Dosemeci Mustafa and Alavanja Michael CR (2007) Int J Cancer 120: 642-649.

Qiu D (2005) In: Biological Control in China, Towards to the 21st Century, Ed. Yang H, The Press of Chinese Agricultural Science and Technology, Beijing, pp. 21-26 (in Chinese).

Quesada-Moraga E and Vey A (2003) Biocontrol Sci Technol 13: 323-340.

Rabindra (2005) In: Proceedings of ICAR-CABI workshop on biopesticide formulations and application, Eds. Rabindra RJ, Hussaini SS, and Ramanujam B, precision fototype services, PDBC Bangalore, India, pp. 1-12.

Ragaei M (1999) J Appl Entomol 123: 381-384.

Raimboult M and Alazard D (1980) Eur J Appl Microbiol 9: 199-209.

Rajamohanan PR, Ganapathy S, Vyas PR, Ravikumar A and Deshpande MV (1996) J Biochem Biophys Methods 31: 151-163.

Rangeshwaran R, Prasad RD and Anuroop CP (2001) J Biol Control 15: 165-170.

Reddy KVS and Zehr UB (2004) In: Proceedings for the 4th international crop science congress, Eds. Fischer T, Turner N, Angus J, McIntyre L, Robertson M, Borrell A and Lloyd D, The Regional Institute Ltd, Gosford, Australia, pp 1-8.

Regev A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz-Kalman Z, Koncz C, Schell J and Zilberstein A (1996) Appl Environ Microbiol 62: 3581-3586.

Reissig JL, Strominger JL and Leloir LFA (1955) J Biol Chem 217: 959-966.

Ridout CJ, Coleysmith J and Lynch JM (1988) Enzyme Microb Technol 10: 180-187.

Roberts WA and Selitrennikoff CP (1988) J Gen Microbiol 134: 169-176.

Rodham DK, Wang Y, Cantwell JB, Winn PD and Foundling J (1999) Pestic Sci 55: 340-2.

Rogalski J, Krasowaska B, Glowiak G, Wojcik W and Targonski Z (1997) Acta Microbiol Pol 46: 363-375.

Rojo Federico G, Reynoso Maria M, Ferez Marcela, Chulze1 Sofia N and Torres Adriana M (2007) Crop Prot 26: 549-555.

Rombke J and Moltmann JF (1966) In: Applied Ecotoxicology pp. 282.

Rosell Gloria, Quero Carmen, Coll Josep and Guerrero Angel (2008) J Pestic Sci 33: 103-121.

Ryder MH, Yan Zhi N, Terrace TE, Rovira AD, Tang Wen H and Correll RL (1999) Soil Biol Biochem 31: 19-29.

Sakai K, Yakota A, Kurokawa H, Wakayama M and Moriguchi M (1998) Appl Environ Microbiol 64: 3340-97.

Samuels RI and Paterson IC (1995) Comp Biochem Physiol 110B: 661-669.

Sanchez PA and Swaminathan MS (2005) Science 307: 357-359.

Sandhya C, Adapa LK, Nampoothiri KM, Binod P, Szakacs G and Pandey A (2004) J Basic Microbiol 44: 49-58.

Sandhya C, Binod P, Nampoothiri KM, Szakacs G and Pandey A (2005a) Appl Biochem Biotechnol 127: 1-16.

Sandhya C, Sumantha A, Szakacs G and Pandey A (2005b) Process Biochem 40: 2689-2694.

Saraji Mohammad and Esteki Nafise (2008) Anal Bioanal Chem 391:1091-1100.

Sathiah N, Rabindra RJ, Palaniswami S, Jayanth KP, Subramanian S, Senthilkumar CM, Rajasekaran B and Kennedy JS (2005) In: Proceedings of ICAR-CABI workshop on biopesticide formulations and application, Eds. Rabindra RJ, Hussaini SS, and Ramanujam B, precision fototype services, PDBC Bangalore, India, pp. 49-88.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Ziegler DR and Dean DH (1998) Microbiol Mol Biol Rev 62: 775-806.

Seifert KA, Hamilton WE, Breuil C and Best M (1987) Can J Microbiol 33: 1102-1107.

Shaikh SA and Deshpande MV (1993) World J Microbiol Biotechnol 9: 468-475.

Shakeri J and Foster H (2007) Enzyme Microb Technol 40: 961-968.

Shapira R, Ordentlich A, Chet I and Oppenheim AB (1989) Phytopathol 79: 1246-1249.

Shapiro M, Preisler HK and Robertson JL (1987) J Econ Entomol 80: 1113-1116.

Shetty PK, Murugan M and Sreeja KG (2008) Curr Sci 95: 457-464.

Shieh TR (1995) In: Proceedings of American Chemical Society, Eds. Ragsdale NN, Kearney PC and Plimmer JR, Eighth international congress of pesticide chemistry options 2000, Washmgton, DC, pp. 104-l14.

Shternshis MV (2005) J Agric Technol 1: 1-18.

Shternshis MV, Beljaev AA, shpatova TV, Bokova JV and Duzhak AB (2002a) Biocontrol 47: 696-706.

Shternshis MV, Ovchinnikova LA, Duzhak AB and Tomilova OG (2002b) Arch Phytopathol Plant Prot 35: 161-165.

Shu Chin-Hang, Xu Chun-Jun and Lin En-Shu (2006) Process Biochem 41: 1229-1233.

Sim T and Oh JCS (1990) J Ind Microbiol 5:153-8.

Singh Kunwar P, Malik Amrita and Sinha Sarita (2007) Environ Monit Assess 125: 147-155.

Smirnoff WA (1973) J Invertebr Pathol 21: 116-118.

Smirnoff WA (1974) J Invertebr Pathol 24: 344-348.

Smith RG (1999) J Nat History 33: 513-530.

Somogyi M (1952) J Biol Chem 195: 19-23.

Sosa-Gomez DR, Boucias DG and Nation JL (1997) J Invertebr Pathol 69: 31-39.

Steinke WE and Akesson NB (1993) In: Pesticide formulations and application systems, Eds. Devlsetty BN, Chasm DG and Berger PD, ASM STP 1146, American society for testing and materials, Philadelphia, Vol 12: 257-271.

St. Leger RJ, Cooper RM and Charnley AK (1986a) J Gen Microbiol 132: 1509-1517.

St. Leger RJ, Cooper RM and Charnley AK (1986b) J Invertebr Pathol 47: 167-177.

St. Leger RJ, Goettel M, Roberts DW and Staples RC (1991) J Invertebr Pathol 58: 168-179.

St. Leger RJ, Joshi L, Bidochka MJ, Rizzo NW and Roberts DW (1996) Appl Environ Microbiol 62: 907-912.

Stotzky G (1986) In: Interactions of soil minerals and natural organics and microbes. Eds Huang PM and Schnitzer M, Soil Science Society of America, Madison, WI, pp: 305-428.

Strasser H, Vey A and Butt TM (2000) Biocontrol Sci Technol 10: 717-735.

Sudisha J, Amruthesh KN, Deepak SA, Shetty NP, Sarosh BR and Shekar Shetty H (2005) Pestic Biochem Physiol 81: 188-197.

Suresh PV and Chandrasekaran M (1999) Process Biochem 34: 257-267.

Tagawa K and Okazaki K (1991) J ferment Bioeng 71: 230-236.

Takayanagi T, Ajisaka K, Takiguchi Y and Shimhara K (1991) Biochem Biophy Acta 1078: 404-410.

Tanaka H, Ogasawara N, Nakajima T and Tamari K (1970) J Gen Appl Microbial 16: 39-60.

Thakore Yatin (2006) Fall industrial biotechnology, Gen publishing inc, a Mary Ann Liebert inc company 2: 194-208.

Thamthiankul S, Suan-Ngay S, Tantimavanich S and Panbangred W (2001) Appl Microbiol Biotechnol 56: 395-401.

Trejo-Estrada SR, Paszczynski A and Crawford DL (1998) J Ind Microbiol Biotechnol 21: 81-90.

Tripathi P, Dubey N and Shukla A (2008) World J Microbiol Biotechnol 24: 39-46.

Tsujibo Hiroshi, Minoura Katsuhiko, Miyamoto Katsushiro, Endo Hiroshi, Moriwaki Masafumi and Inamori Yoshihiko (1993) Appl Environ Microbiol 59: 620-622.

Tunga R, Banerjee R and Bhattacharyya BC (1999) Bioprocess Eng 20: 485-9.

Tweddell RJ, Jabaji-Hare SH and Charest PM (1994) Appl Environ Microbiol 60: 489-498.

Ueda M and Arai M (1992) Biosci Biotechnol Biochem 56: 460-464.

Uma Devi K, Murali Mohan CH, Padmavathi J and Ramesh K (2003) Biocontrol Sci Technol 13: 367-371.

Uri ND (1999) In: Methods in biotechnology, Eds. Hall FR and Menn JJ, Humana Press, New York, Vol 5: 55-73.

Usui T, Hayashi Y, Nanjo F and Ishido Y (1984) Biochem Biophy Acta 923: 302-339.

Utkhede RS and Smith EM (1992) Can J Microbiol 38: 1270-1273.

Vail PV, Hostetter DL and Hoffmann DF (1999) Int Pest Manage Rev 4: 231-257.

Van de Velde K and Kiekens P (2004) Carbohydr Polym 58: 409-416.

Vey A, Hoagland RE and Butt TM (2001) In: Fungi as biocontrol agents: progress, problems and potential, Eds. Butt TM, Jackson CW and Magan N, CAB Publishing Co., UK, pp. 311-346.

Viniegra-Gonzalez G, Favela-Torres E, Aguilar CN, Romero-Gomez SJ and Dıaz-Godınz Augur C (2003) Biochem Eng 13: 157-67.

Vyas PR and Deshpande MV (1989) J Gen Appl Microbiol 35: 343-350.

Wada AC, Joshua SD, Ndarubu AA and Usman A (2008) Arch Phytopathol Plant Prot 1: 1-10.

Wahab S (2004) In: Proceedings of The international workshop on entomopathogenic fungi a valuable alternative to fight against insect pests, Ed. Deshpande MV, National Chemical Laboratory, Pune, India. pp. 185-206.

Wang San-Lang and Chang WT (1997) Appl Environ Microbiol 63: 380-386.

Wang San-Lang, Chao Chia-Hsing, Liang Tzu-Wen and Chen Chung-Chih (2009) Marine Biotechnol DOI 10.1007/s10126-008-9149-y.

Wang San-Lang, Yieh TC and Shih IL (1999) Enzyme Microb Technol 25: 142-148.

Weller DM, Zhang BX and Cook RJ (1985) Plant Dis 69: 710-713.

Wiwat C, Lertcanawanichakul M, Siwayapram P, Pantuwatana S and Bhumiratana A (1996) Gene 179: 119-126.

Xia Xiao Ming, Wang Kai Yun and Wang Hong Yan (2009) Crop Prot 28: 162-167.

Yabuki M, Uchiyama A, Suzuki K, Ando A and Fuji TJ (1988) J Gen Appl Microbiol 34: 255-270.

Yang SS and Wang JY (1999) Bot Bull Acad Sinica 40: 259-65.

Yasmin Ahmad, hameed A and Ghaffar A (2006) Pak J Bot 38: 1305-1316.

Young ME, Bell RL and Carroad PA (1985a) Biotechnol Bioeng 27: 769-775.

Young ME, Bell RL and Carroad PA (1985b) Biotechnol Bioeng 27: 776-780.

Yuli Purwani E, Suhartono Maggy T, Rukayadi Yaya, Hwang Jae K and Pyun Yu Ryang (2004) Enzyme Microb Technol 35: 147-153.

Yu Mingrui, Qin Shaowei and Tan Tianwei (2007) Process Biochem 42: 384-391.

Zalucki MP, Daglish G, Firempong S and Twine PH (1986) Australian J Zool 34: 779-814.

Zang KB, Wang XG and Huang MZ (2001) In: Proceedings of the III Asia pacific crop protection conference, Ed. Suchak's consultancy services, pp. 2-7, New Delhi, India.

Zhang Wenming, Wolf Thomas M, Bailey Karen L, Mortensen Knud and Boyetchko Susan M (2003) Biol Control 26: 95-108.

List of Research Papers/ Reviews / Patents/ Presentations

1. Kulkarni S, Ghormade V, Kulkarni G, Kapoor M, **Chavan S**, Rajendran A, Patil S, Shouche Y and Deshpande MV (2008) Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. Biocontrol Sci Technol 18: 809-828.
2. **Chavan S**, Kulkarni M and Deshpande MV (2008) Status of microbial pesticides in India. Rev Plant Pathol 4: 393-420, Editors Reddy SM and Gour HN, Scientific Publishers, Jodhpur, India.
3. Nahar P, Kulkarni S, Kulye M, **Chavan S**, Kulkarni G, Rajendran A, Yadav P, Shouche Y, Deshpande MV (2008) Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: Noctuidae). Biocontrol Sci Technol 18: 337-355.
4. Ghormade V, Jossi W, **Chavan S**, Rajendran A, Ghondhalekar A, Widmer F, Keller S, Enkerli J (2007) Evaluation of *Metarhizium anisopliae* isolates for biocontrol of *Agriotes* based on genetic, biochemical and virulence characters. IOBC/WPRS Bulletin 30: 77-82.
5. **Chavan S**, Ghormade V, Nahar P, Deshpande MV (2006) Entomopathogenic fungi: A valuable tool to fight against insect pests. In: Plant Protection in New Millennium, Eds. Gadewar AV and Singh BP, Satish Serial Publishing House, New Delhi, pp. 227-243.

Patent

1. Deshpande MV, **Chavan S**, Kulkarni M (2004) A composition for the control of woolly aphid infestation and a method of treatment thereof (File No. 414 NF/2004).

Presentations

1. **Chavan S,** Kolomiets E, Kuptsov, Mandrik M, Kulkarni M and Deshpande MV (2008) Significance of cuticle degrading enzymes with special reference to lipase in the biocontrol of sugarcane woolly aphids. The paper has been presented in 30th Annual Conference and Symposium on Advances in Biotechnology for Plant Protection at University of Mysore, Karnataka, India on November 17-19.
2. **Chavan S**, Patil S, Kadam M, Rajendran A, Jamdagni BM and Deshpande MV (2007) Biocontrol of *Helicoverpa armigera* (Hub.) in chickpea using cuticle degrading enzyme preparations of *Myrothecium verrucaria*. The poster has been presented in National Symposium on Legumes for Ecological Sustainability: Emerging Challenges and Opportunities at Indian Institute of Pulses Research, Kanpur on November 3-5.
3. Kadam M, **Chavan S**, Rajendran A and Deshpande MV (2007) *Myrothecium verrucaria*: Solid state fermentation for the production of cuticle degrading and mycolytic enzyme complex. The poster has been presented in International conference on Biology of Yeast and Filamentous Fungi, at National Chemical Laboratory, Pune, Maharashtra, India on Feb 15 -17.
4. Kulkarni S, Patil S, **Chavan S**, Rajendran A, Kulkarni M, Shinde G, Ingle M and Deshpande MV (2006) Studies on bioefficacy of fungi and fungal products against *Helicoverpa armigera* (Hub.) on cotton. The poster has been presented in National Symposium of Indian Society for Mycology and Plant Pathology and Annual Meet at Department of Plant Pathology College of Agriculture, GB Pant University, Pantnagar, Uttaranchal, India on Nov 7-9.